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VARIATION OF FRUITS MORPHOMETRIC PARAMETERS AND BIOACTIVE COMPOUNDS OF *ASIMINA TRILOBA* (L.) DUNAL GERMPLASM COLLECTION

Ján Brindza, Olga Grygorieva, Svitlana Klymenko, Olena Vergun, Ján Mareček, Eva Ivanišová

ABSTRACT

The objective of this study was to evaluate the morphological parameters and bioactive compounds (antioxidant activity, total polyphenol, flavonoid, and phenolic acid content) of 6 genotypes of dry *Asimina triloba* (L.) fruit from Slovak University of Agriculture in Nitra (Slovakia). Genotypes were obtained from the seeds that were sown in the year 2000. Their morphometric parameters were following: fruit weight from 59.00 to 241.19 g, fruit length from 50.14 to 140.11 mm, fruit diameter from 37.55 to 64.67 mm, number of fruits per cluster from 2 to 8, seed weight from 0.06 to 1.80 g, seed length from 16.33 to 29.11 mm, seed width from 9.56 to 18.33 mm, seed thickness from 4.98 to 9.75 mm, number of seeds in fruit from 4 to 16. The shape indexes of fruits were found ranging from 1.53 to 2.16. The variability of important is the average seeds weight from 7.40 to 35.61%, fruit weight from 14.84 to 32.95%, number of fruits per cluster from 18.21 to 32.54% and a number of seeds in fruit from 13.49 to 27.72%. The other characteristics are more or less stable. Total polyphenol content ranged from 22.13 to 37.36 mg GAE per g, total flavonoid content from 15.10 to 32.03 mg QE per g and phenolic acids content from 0.23 to 0.76 mg CAE per g. All tested samples exhibited DPPH radical scavenging activities with values from 2.84 to 7.04 mg TEAC per g. Antioxidant activity by molybdenum reducing antioxidant power method ranged from 97.25 to 275.41 mg TEAC per g of dry matter. Differences between the genotypes were significant in all observed parameters. This species is potential for propagation and practice used in the Slovak Republic.

Keywords: *Asimina triloba*; Dunal; fruits; seeds; variability; bioactive compounds

INTRODUCTION

The searching of new plant species, which are the valuable source of biologically active compounds, is an actual branch of modern biological science in the last time. The introduction and selection of these plants, cultivation technology and extraction ways of biologically active compounds complex and creation on its basis the new generation of nutritional supplements one of the most important scientific directions **(Brindza et al., 2007; Grygorieva et al., 2014; Monka et al., 2014; Ivanišová et al., 2017; Vinogradova et al., 2017; Horčinová Sedláčková et al., 2018)**. *Asimina triloba* (L.) Dunal. relates to plants that are a source of polysaccharides, free amino acids, mineral compounds, flavonoids, etc.

Species of the genus *Asimina* Adans. belong to the family *Annonaceae* Juss. *Asimina triloba* (pawpaw, paw paw, paw-paw, common pawpaw) is deciduous tree native to eastern North America and Canada **(Layne, 1996)**.

Asimina triloba fruits are rich in nutritive components such as vitamins and minerals **(Templeton et al., 2003; Pomper and Layne 2005)**, a good source of potassium (3000 – 3800 $mg.kg^{-1}$) and several essential amino acids (mean value: 40 $mg.kg^{-1}$ of protein), and they contain significant amounts of riboflavin (0.06 – 0.15 $mg.kg^{-1}$), niacin (10 – 12 $mg.kg^{-1}$), calcium (500 – 800 $mg.kg^{-1}$), phosphorus (400 – 500 $mg.kg^{-1}$) and zinc (10 – 12 $mg.kg^{-1}$) **(Galli et al., 2007)**, to have a high polyphenolic content **(Harris and Brannan, 2009; Brannan et al., 2014; Brannan, 2016)**.

Pawpaws can be used as an alternative to bananas fruits in most recipes **(Jones and Layne, 1995)**. Fruits of pawpaw are very fragrant and resemble a combination of aromas of banana and mango, and may be used commercially in cosmetics and skin products **(Layne, 1996; Brannan and Holben, 2012)**.

Biologically active compounds are not only in fruits but in different parts of the plant: roots, bark, twigs, leaves, flowers, and seeds **(Hui et al., 1989; Zhao et al., 1992, 1993, 1994; Alali et al., 1999; Goodrich et al., 2006; Cuendet et al., 2008; Farag, 2009; Pande and Akoh, 2010)**. The roots, twigs, flowers, and seeds of pawpaw contain acetogenins, which are strong inhibitors of cancer cells **(Ratnayake et al., 1992; McLaughlin and Hui, 1993; Woo et al., 1995; Ko et al., 2011; Sica et al., 2016)**. Pawpaw leaf essential oil has strong activity against cancer cell lines **(Alali et al., 1999; Farag, 2009)**.

Asimina triloba fruit, leaf, bark, and twig extract may be an effective insect feeding deterrent **(Rupprecht et al., 1986; Ratnayake et al., 1992; Zhao et al., 1994; Gu et al., 1999; Sedlacek et al., 2010)**.

Scientific hypothesis

Evaluating of fruit quality formation by their qualitative parameters in the adaptation process of *Asimina triloba* at the agroecological conditions of the experimental base in Nitra.

MATERIAL AND METHODOLOGY

Biological material

The observations of the collection genotypes of *Asimina triloba* in the period 2017 were performed during mass fruiting. We have described 6 genotypes (referred as AzT-01 to AzT-06) of *Asimina triloba*.

Morphometric characteristics

The ripened fruits were picked from trees in maturity stage. Pomological characteristics were conducted with four replications on a total of 30 fruits per genotypes. In the study only one plant was used per genotype. The following measurements were taken: fruit weight, in g, fruit length, in mm, fruit diameter, in mm, and seed weight, in g, seed length, in mm, seed diameter, in mm, number of fruits per cluster, and a number of seeds in fruit.

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and CentralChem (Slovakia).

Preparation of sample extracts

The dry *Asimina triloba* pulp was used for detection of total phenolic content and total flavonoid content. An amount of 0.25 g of each sample was extracted with 20 mL of 80% ethanol for 24 h. Then, the sample in 80% ethanol was centrifuged at 4000 rpm (Rotofix 32 A, Hettich, Germany) for 10 min and the supernatant was used for measurement with the DPPH and molybdenum reducing antioxidant power methods.

Determination of antioxidant activity

Free radical scavenging activity

Free radical scavenging activity of samples was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sanches-Moreno et al., 1998)**. An amount of 0.4mL of the sample was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL methanol). The absorbance of the reaction mixture was determined with the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $mg.L^{-1}$; $R^2 = 0.989$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Molybdenum reducing antioxidant power

Molybdenum reducing antioxidant power of samples was determined by the method of **Prieto et al. (1999)** with slight modifications. The mixture of the sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90°C for 120 min, then rapidly cooled. The absorbance at 700 nm was detected with the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10 – 1000 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Determination of total polyphenol content

The total polyphenol content was measured by the method of **Singleton and Rossi (1965)** using the Folin-Ciocalteu reagent. A quantity of 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured with the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 300 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard. The results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Determination of total flavonoid content

The total flavonoid content was determined by the modified method described by **Shafii et al. (2017)**. An aliquot of 0.5 mL of the sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (1 – 400 $mg.L^{-1}$; $R^2 = 0.9977$) was used as the standard. The results were expressed in $mg.g^{-1}$ quercetin equivalents.

Determination of phenolic acids

Total phenolic acids content was determined using the method of Farmakopea Polska (1999). A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 $mg.L^{-1}$, $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ caffeic acid equivalents.

Statistic analysis

Basic statistical analyses were performed using PAST 2.17. Data were analysed with ANOVA test and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). Variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by **Stehlíková (1998)**.

RESULTS AND DISCUSSION

Morphometric measurements of fruits and seeds of selected plants are very important studying parameters because of some reasons such as origin and conditions of growth. It is also necessary indicators at the new conditions of introduction in a new area.

Figure 1 Fruits of *Asimina triloba* (L.) Dunal.

Table 1 The variability of some morphometric parameters of fruits of *Asimina triloba* (L.) Dunal genotypes.

Genotypes	min	max	mean	V%	Genotypes	min	max	mean	V%
Fruit weight (g)									
AzT-01	60.80	211.90	130.83	28.73	**AzY-04**	80.88	241.19	154.63	32.95
AzT-02	67.56	120.05	92.17	15.16	**AzY-05**	59.00	190.45	114.27	23.57
AzT-03	111.23	184.21	149.31	14.84	**AzY-06**	77.19	106.67	106.67	17.81
Fruit length (mm)									
AzT-01	50.14	105.02	84.16	17.59	**AzY-04**	72.19	140.11	108.83	17.11
AzT-02	67.80	91.14	80.77	9.44	**AzY-05**	76.50	112.01	100.15	10.03
AzT-03	90.55	114.68	103.07	7.47	**AzY-06**	64.18	89.96	78.16	10.65
Fruit diameter (mm)									
AzT-01	41.78	64.67	53.03	10.09	**AzY-04**	41.66	59.12	50.33	10.57
AzT-02	37.55	49.69	44.80	6.86	**AzY-05**	41.00	55.06	47.82	7.76
AzT-03	48.82	60.04	55.06	6.00	**AzY-06**	40.69	61.15	50.84	12.77
Number of fruits per cluster									
AzT-01	3	5	3.68	18.21	**AzY-04**	3	6	4.07	29,12
AzT-02	3	5	3.60	22.79	**AzY-05**	3	5	4.00	23.57
AzT-03	2	6	4.07	26.32	**AzY-06**	3	8	4.61	32.54
Seed weight (g)									
AzT-01	0.10	1.11	0.81	35.71	**AzY-04**	0. 60	1.68	1.13	20.57
AzT-02	0.60	1.98	1.21	20.03	**AzY-05**	0.70	1.40	1.15	14.98
AzT-03	1.20	1.60	1.35	7.40	**AzY-06**	0.60	1.50	1.08	24.58
Seed length (mm)									
AzT-01	21.34	29.05	25.73	8.82	**AzY-04**	18,11	29.00	25.52	9.76
AzT-02	18.61	27.11	23.58	9.99	**AzY-05**	16.33	23.81	20.43	10.24
AzT-03	21.10	29.11	26.03	9.94	**AzY-06**	18.25	28.77	23.89	14.46
Seed width (mm)									
AzT-01	9.75	12.78	11.60	7.06	**AzY-04**	10.15	14.90	13.05	7.67
AzT-02	10.29	14.88	12.97	7.51	**AzY-05**	10.78	16.00	13.76	7.98
AzT-03	11.89	18.33	14.86	9.81	**AzY-06**	9.56	12.00	10.88	8.12
Seed thickness (mm)									
AzT-01	5.56	7.34	6.52	7.57	**AzY-04**	5.12	9.39	6.76	12.71
AzT-02	4.98	8.11	6.72	9.91	**AzY-05**	6.16	8.98	7.71	9.02
AzT-03	5.22	9.75	6.96	16.49	**AzY-06**	5.40	8.06	6.26	14.01
Number of seeds in the fruit									
AzT-01	**7**	10	8.50	15.92	**AzY-04**	12	16	13.36	13.49
AzT-02	8	12	9.81	13.52	**AzY-05**	4	12	8.53	27.72
AzT-03	8	14	10.91	17.66	**AzY-06**	7	12	9.90	16.80

Note: n – the number of measurements; min, max – minimal and maximal measured values; mean – the arithmetic mean; V – coefficient of variation (%).

Morphological parameters of *Asimina triloba* fruits and seeds

In our study, the weight of *Asimina triloba* fruits (Figure 1) was in the range from 92.17 (AzT-02) to 149.31 g (AzT-03) (Table 1). **Crabtree (2004)** determined the fruit weight from 88.10 to 141.80 g**, Donno et al. (2014)** determined the fruit weight from 39.40 to 238.70 g. Investigations of **Klymenko et al. (2017)** established the range of fruits weight of variety from 39.40 to 238.70 g. In our experiments, the fruit length and diameter were determined in the range from 78.16 (AzT-06) to 108.83 mm (AzT-04) and from 44.80 (AzT-02) to 55.06 mm (AzT-03), respectively. The length and diameter of fruits was determined in the range from 61.98 - 91.16 mm and from 41.27 – 49.65 mm, respectively, by **Donno et al. (2014)**, from 14.0 to 46.0 mm and from 7.0 to 20.0 mm by **Szilagyi et al. (2016)**, from 49.25 to 135.00 mm and from 37.55 to 63.80 mm, respectively, by **Klymenko et al. (2017).** A number of fruits per cluster were identified in the range from 2 (AzT-03) to 8 (AzT-06).

Crabtree (2004) determined the average number of fruits from 1.8 to 3.0, **Klymenko et al. (2017)** determined the number of fruits per cluster from 2 to 7.

Morphological parameters of ranged seeds weight from 0.81 (AzT-01) to 1.35 g (AzT-03), seed length from 20.43 (AzT-05) to 26.03 mm (AzT-03), seed width from 10.88 (AzT-06) to 14.86 mm (AzT-03), seed thickness from 6.26 (AzT-06) to 7.71 mm (AzT-05).

Investigations of **Klymenko et al. (2017)** established the range of seeds weight, length, width and thickness of genotypes from 0.60 to 1.80 g, from 17.10 to 28.80 mm, from 10.86 to 16.46 mm, from 5.54 to 9.15 mm, respectively. **Szilagyi et al. (2016)** determined the weight, length, and width of the seeds in the range from 1.30 to 1.65 g, from 2.51 to 2.69 mm, from 1.30 to 1.36 mm, respectively. A number of seeds in fruit were identified in the range from 4 (Az-05) to 16 (Az-04). The number of seeds in the fruit was determined in the range from 5 to 12 by **Klymenko et al. (2017)**.

The analysis of coefficient of variation showed the difference of variability of morphological signs between *Asimina triloba* samples. Data showed that the most variable important selection signs are the seeds weight from 7.40 to 35.71%, fruit weight from 14.84 to 32.95%, a number of fruits per cluster from 18.21 to 32.54% and a number of seeds in fruit 13.49 to 27.72%.

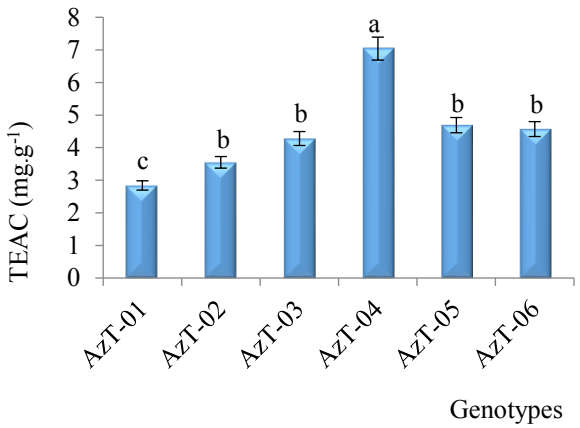

Figure 2 Antioxidant activity of the *Asimina triloba* (L.) Dunal genotypes evaluated by the DPPH method (different superscripts in each column indicate the significant differences in the mean at p <0.05).

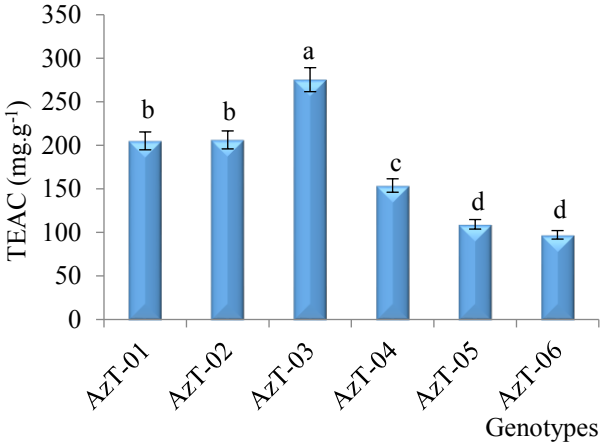

Figure 3 Antioxidant activity of *Asimina triloba* (L.) Dunal genotypes evaluated by the molybdenum reducing antioxidant power (different superscripts in each column indicates the significant differences in the mean at p <0.05).

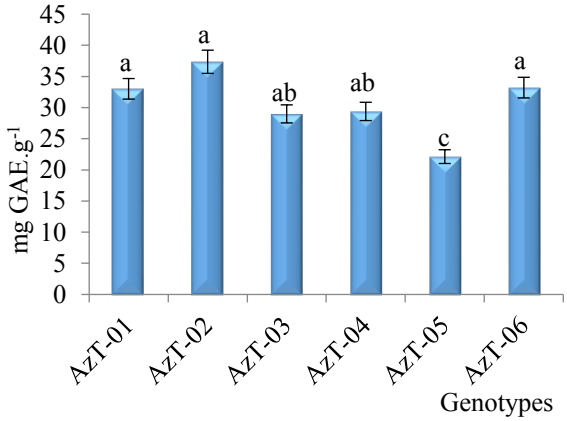

Figure 4 Total polyphenol content in *Asimina triloba* (L.) Dunal genotypes (different superscripts in each column indicate the significant differences in the mean at p <0.05).

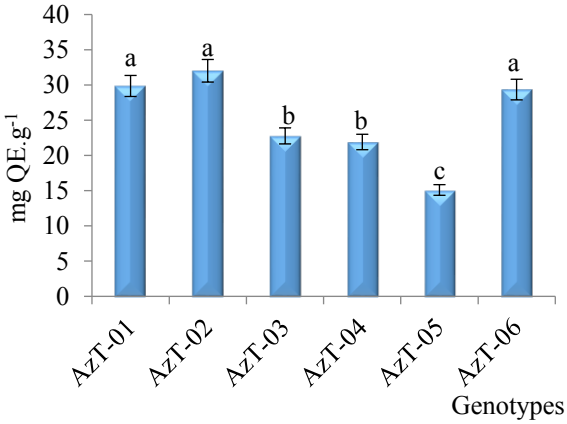

Figure 5 Total flavonoid content in *Asimina triloba* (L.) Dunal genotypes (different superscripts in each column indicate the significant differences in the mean at p <0.05).

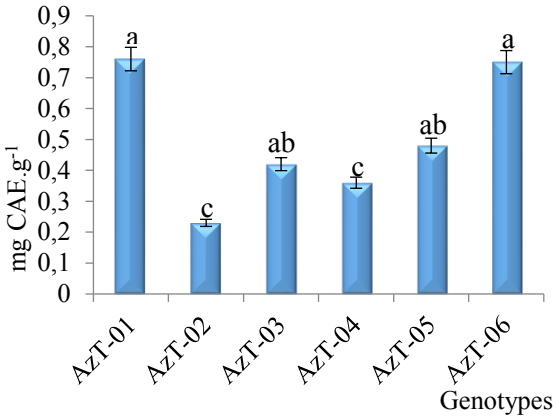

Figure 6 Total phenolic acid content in *Asimina triloba* (L.) Dunal genotypes (different superscripts in each column indicate the significant differences in the mean at $p < 0.05$).

Table 2 The correlation coefficients of a linear relationship between the biological activities of tested *Asimina triloba* (L.) Dunal genotypes.

Parameter	Polyphenols	Phenolic acids	Flavonoids	DPPH method
Phenolic acids	-0.013*			
Flavonoids	0.976*	0.184*		
DPPH method	-0.394	-0.326	-0.518	
MRP method	0.286*	-0.329	0.240*	-0.359

Note: Significant according to the *t*-test ($p < 0.05$).

These results indicate the promise of breeding in this way of investigations. The stable signs are seed width from 7.06 to 9.81%.

Antioxidant activity of *Asimina triloba* measured with the DPPH and molybdenum reducing antioxidant power methods

The antioxidant activity of *Asimina triloba* genotypes evaluated by the DPPH method (Figure 2) ranged from 2.84 (AzT-01) to 7.04 mg TEAC.g^{-1} (AzT-04). The degree of mean variability of parameters was confirmed by the variation coefficient (31.77%) in all the genotypes tested.

The antioxidant activity evaluated by the molybdenum reducing antioxidant power (Figure 3) varied from 97.25 (AzT-06) to 275.41 mg TEAC.g^{-1} (AzT-03). The degree of mean variability of parameters was confirmed by the variation coefficient (35.07%) in tested genotypes.

Nam et al. (2017) from methanolic extracts fruits obtained with DPPH method found the antioxidant activity to be 4.18 mg.mL^{-1}. **Donno et al. (2014)** found the total antioxidant activity from 889.6 to 1519.6 mg.kg^{-1}. **Kobayashi et al. (2008)** in ripe fruits from 15.57 to 17.04 mmol TE.g^{-1} FW. Antioxidant activity of *Asimina triloba* fruits could be influenced by the degree of maturity and cultivars **(Kobayashi et al., 2008; Donno et al., 2014)**. Since there are differences in genotypes of *Asimina triloba* cultivars grown under different geographic and agroecological conditions, our results are difficult to compare with the previously reported.

Total polyphenol, flavonoid, and phenolic acid content

The total polyphenol content (Figure 4) in *Asimina triloba* genotypes ranged from 22.13 (AzT-05) to 37.36 mg GAE.g^{-1} (AzT-02). The variation coefficient (16.87%) confirms the high variability of the parameter.

In our mind, the differences between the present and previously conducted studies may be attributable to the plant geographical origin as well as the different methods of extraction.

The total flavonoid content (Figure 5) varied from 15.10 (AzT-05) to 32.02 mg.g^{-1} QE (AzT-02). The variation coefficient (25.39%) supported the observations on high variability of this parameter.

The total phenolic acid content was found to vary significantly among the various *Asimina triloba* genotypes (Figure 6), which may be due to their different botanical and regional origins. The mean total phenolic acid of the studied fruits genotypes was 25.16 mg.g^{-1} CAE, with the highest phenolic acid recorded by genotypes AzT-02 at 32.02 mg.g^{-1} CAE, indicating its superior antioxidant potential. The variation coefficient (25.39%) supported the observations on high variability of this parameter. **Kobayashi et al. (2008)** found in ripe fruits the total phenolic content from 64.11 to 98.42 mg.g^{-1} GAE.

Correlation analysis

Correlation analysis was used to explore the relationships between the individual polyphenols, phenolics, flavonoids compounds and antioxidant capacities (DPPH and MRP methods) measured for all fruit extracts from six *Asimina triloba* genotypes (Table 2). It was observed a strong linear correlation between the polyphenol and flavonoid contents ($r = 0.976$).

CONCLUSION

Asimina triloba is a new introductive species in the European conditions. Originally it was used for decorative purposes. Nowadays it uses more widespread in many European countries as promising fruit plant. Fruits have specific taste, aroma, energy, therapeutic and pharmaceutics properties and uses. Presented results of this study showed

that *Asimina triloba* was well adapted in the conditions of Nitra in the Slovak Republic. All genotypes produce fruits comparable with foreign cultivars by morphological parameters and also by the content of biologically active compounds. Thus, this species is potential for propagation and practice used in the Slovak Republic.

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CONSUMERS´ AWARENESS OF FOOD SAFETY

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ABSTRACT
Eating food is one of the most important needs of every person, so their safety and quality should be crucial for everyone. People expect, that food they eat is hygienically and health safe. Unfortunately, people usually start to focus on food safety only when various food scandals are exposed and it is too late. Mass consumption of food is the cause of a high risk to human health, but only in the case of harmful food. Food-borne diseases are a common and widespread phenomenon in all parts of the world, regardless of the economic development of the country. Protection of human, animal and plant health is one of the main economic priorities of each country. The political objective of the European Union is therefore to ensure that European Union citizens have access to safe and nutritious foods, so it must meet strict safety standards. In ensuring food safety, it is necessary to take into account all aspects of the food production chain as a whole, because each subject can have a potential impact on food safety. This paper deals with the issues of food safety and food quality. The main objective was to find out how consumers perceive higher quality food and whether they read information included on the food packaging. Primary data were obtained from a survey that was conducted on the sample of 478 respondents living in Slovakia. For a deeper analysis, several assumptions, which were verified by Friedman Test, Chi-Square Test of Independence, Wilcoxon Signed-Rank Test, were formulated. The survey has found out that 84% of respondents buy higher quality food and 60% purchase them because of health-related reasons. More than half of respondents search for the information about food safety on the Internet and the same percentage considere government as the most reliable source of information about food safety. Unfortunately, just more than one quarter of them read the information on food label and for 34% is this information unsufficient.

Keywords: Food; Product Packaging; Food Safety; Consumer; National Mark

INTRODUCTION
Food safety is undoubtedly one of the major global concerns that human beings have to confront and are continuously fighting for **(Lv et al., 2018)**. The world will need to feed around 9 billion people by the year 2050 and to do so through safe sustainable food chains **(Godfrai et al., 2010)**. Nowadays billions of people in the world are at risk of unsafe food. Many millions become sick while hundreds of thousands die every year because they consume unsafe food. Therefore, safe food saves lives and it also enhances individual and population health **(Fung et al., 2018)**.

Originally, the term "food safety" was used to describe whether a country had access to enough food to meet dietary energy requirements **(Pinstrup-Andersen, 2009)**. Now food safety is defined as the degree of confidence that food will not cause harm or sickness to the consumer when it is prepared, served and eaten according to its intended use **(WHO, 2003)**. Causes of food contamination, that can cause adverse effects in humans if consumed, could be classified into one of the three following groups – biological hazards (e.g. bacteria, viruses), chemical hazards (e.g. veterinary drug residues, disinfectants), or physical hazards (e.g. plastic, metal, bone) **(FSA, 2009)**. Certain groups of people are susceptible to foodborne disease more than others. Especially vulnerable groups are children or people who suffer chronic illnesses **(Socas-Rodríguez et al., 2017)**. Most of the population will surely get over soon, but these groups can have a long-term effect which can be serious in some cases and lead to a final death of the consumer, especially with certain bacteria infections (**U.S. Department of Health & Human Services, 2018**). The most foodborne disease outbreaks occur at home, restaurants, and/or at social functions, so food safety awareness and education should be emphasized and encouraged among citizens **(Stratev et al., 2017)**. The global food sector operates in an environment where policies, standards, regulations, guidelines, education and advice relating to food, including those related to the safety of food, are continuously being either developed or updated **(King et al., 2017)**. Scientific risk assessment is the basis for legislative development. Based on the Regulation no.178/2002 of the European Parliament and of

the European Council, the European Food Safety Authority (EFSA) was established **(Bírošová and Kačenová, 2010)**. EFSA is an independent agency of the European Union. It´s main role as stated in its founding regulations is to provide independent scientific advice and perform risk assessment and communication on food and feed safety topics to support risk managers at EU level, including EC, European Parliament, and EU Member States **(Regulation (EC) No 178/2002)**. Governments in many countries are fighting for safe food too. They have established new institutions, standards, and methods for regulating food safety and have increased their investments in hazard control systems (e.g. good agricultural practices (GAP), good manufacturing practices (GMP), good hygiene practice (GHP), hazard analysis and critical control point (HACCP) facilities **(Liu et al., 2015; Kecskes-Nagy et al., 2016; Korzenszky et al., 2013)**. Animal welfare is an important condition for obtaining high-quality and safe food in many states too **(Adámková et al., 2017)**.

Food safety is the responsibility of every person involved in the food supply chain from farm to the end users in assuring the safety and quality of the food **(New et al., 2017)**. Detection of food contaminants has been attracting remarkable attention in last decade, which ensures the governments and customers to recognize whether the food is safe **(Cocolin et al., 2011)**. As a result, regulators, producers, and retailers alike are trying to regain consumers' confidence by redesigning legislation and quality-assurance programs. These efforts can only succeed in restoring consumers' confidence if new standards of process and product attributes are successfully communicated. Product labeling is one way to accomplish such communication **(Roosen, 2003).** In Slovak Republic, Značka kvality SK is the guarantee of the highest quality of agricultural and food products. It informs consumers that every product labelled with the logo of the Značka kvality SK, has fulfilled the requirements placed on the granting thereof, complied declared technological process and higher quality parameters. Therefore, these products are clearly different from competing products **(MP SR, 2009)**. When it comes to food, nonfood, specific products or services, they can carry prestigious logo Slovak Gold, which in a simple visual form informs that the quality of the product has been professionally verified and confirmed by independent, non-state authority, thereby it protects consumers from problematic production of all sorts of origins.

Scientific hypothesis

Hypothesis No. 1: We assume that most respondents do not buy high quality food.

Hypothesis No. 2: We expect that most consumers search for the information about the food safety on the Internet.

Hypothesis No. 3: We assume that more women than men know the national label for quality agricultural products "Značka kvality SK".

Hypothesis no. 4: We assume that fewer men than women know the product certification system "Slovak gold".

MATERIAL AND METHODOLOGY

The main aim of the present paper is to examine consumers´ awareness of food safety and the relationship between consumers and food quality. In order to meet the stated objective, a questionnaire survey was conducted in the territory of the Slovak Republic in the months of January – March 2018, involving 478 respondents of different age categories. Secondary informations were obtained from the information available to the public as well as from scientific and professional publications of domestic and foreign authors dealing with the given issues. Questions in the questionnaire were divided into two groups - nine classification questions and 16 questions related to the food quality. Potential respondents have received the questionnaire in a paper form. After completing, all correctly filled questionnaires were transformed into the Google Forms internet application.

Statisic analysis

Primarily, the information obtained through the questionnaire survey was processed out by the statistical methods of Friedman´s test, which is a non-parametric alternative to the repeated measures ANOVA where the assumption of normality is not acceptable. Usually it is used in case of ordinal dependent variable. This occurs especially in case of questioner survey, when each respondent assesses more than two products using the same scale. In case of Friedman´s test applications should be met following conditions: One group that is measured on three or more different occasions - group is a random sample from the population, dependent variable should be measured at the ordinal or continuous level and samples do not need to be normally distributed. The non-parametric post-hoc test called Nemenyi test which is based on the Kruskal-Wallis method of ranking in a one-way classification and Chi-Square test of Independence to investigate relationship between categorical variables.

The established hypotheses were verified by appropriate mathematical-statistical methods that enabled the hypothesis to be confirmed or rejected.

The probability level is determined on the base of alpha ($\alpha = 0.05$), which is compared with the significance level (p-value). Based on alpha (α), we can evaluate the hypothesis with the p-value comparison. If p-value is lower than alpha (α), H_0 will be rejected. If p-value is higher than alpha (α), H_0 will be accepted.

RESULTS AND DISCUSSION

The majority of totaly 478 respondents was represented by women (58%). Most of the respondents (46%) were aged from 21 to 30 years, followed by the interval from 41 to 50 years (17%) and further from 31 to 40 years (16%).

Group with completed higher education represented 46.8%, secondary education 41.8%, apprenticeship 8.1% and basic education only 3.3% of respondents.In terms of their permanent residence, respondents came from all 8 regions of the Slovak Republic, namely 47.2% from the rural areas and 52.8% from the urban areas. The monthly family income of questioned respondents ranged from the category "up to" 330 € to the category "more than 831 €" (Figure 1). The obtained results were anticipated because

Table 1: Characteristics of Respondents.

Gender	**%**
Female	59
Male	41
Place of Residence	**%**
Village	53
City	47
Age Structure	**%**
Less than 20 years old	6
21 – 30 years old	46
31 – 4 0 years old	16
41 – 50 years old	17
51 – 60 years old	10
61 years old and more	5
Net Family Income	**%**
Up to 330 €	8
331 – 500 €	9
501 – 660 €	21
661 – 830 €	22
831 € and more	40

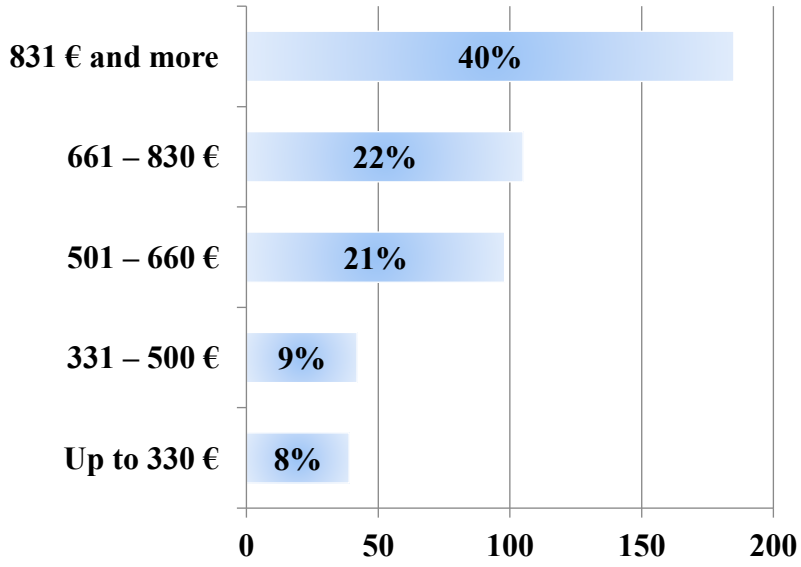

Figure 1 Monthly family income.

the survey was aimed at the general public and all age groups. As it could be seen from the Figure 1, the most respondents reported a monthly family income 831 € and more. Second most-ranked group was represented by an income from 661 € to 830 € (22%). The smallest income ranges (up to 330 €) was marked only by 8% of the respondents.

The first factual question asked in the questionnaire survey was whether the respondent purchases higher quality food. Up to 84% of respondents have claimed that they buy higher quality food on regular basis. This percentage could be so high because of the fact that more than 80% of respondents earn more than 500 € per month. On the other hand, 16% answered this question negatively. Figure 2 reports that 85% of women and 81% of men responded positively. These results were expected, because women are more interested in healthy lifestyle **(Fiala and Brázdová, 2000)** and they are also more likely to have higher quality diet than men **(Hiza et. al, 2013)**.

In relation to this question we formulated the following hypothesis:

H_0: Most consumers do not buy high quality food.

H_1: Most consumers buy high quality food.

To verify the hypothesis, the Wilcoxon signed rank test and significance level were used:

$$p = 0.0742 > \alpha = 0.05$$

Based on the results, we accept the zero hypotheses. Within 5% level of significance, we can claim that consumers do not buy high quality foods.

Most respondents (62%) claimed that they purchase higher-quality food because of health reasons – they believe that these foods can prevent illnesses or in some cases even cure them. The second reason was that these foods are controlled better, so they must fulfil higher requirements (18%), the third was that some people see quality food as pleasure or possibility of trying something unusual and 14% of the sample do so, since they earn enough money to afford them. Healthy foods have many advantages and a lot of customers buy them for various reasons, therefore retailers should focus on creating healthy corners where quality, fresh and especially health beneficial foods are sold. Priority should be to suppress sales of unhealthy products like tobacco, alcohol and various frozen semi-finished products. Based on the **Minkler et al. (2018)** research, in such stores customers buy up to 35% less unhealthy and harmful food.

The following question was dedicated to the factors which could lead to higher frequency of purchasing high quality food even when they are more expensive. Most respondents (60%) said they would be willing to buy more of these food if they had higher living standard. Similar survey was done by **Asif et al. (2018)** who found out that 55% of respondents would be more likely to buy higher quality foods if their economic situation changed to better. This fact can be caused by todays consumption time when people want to earn more money to buy more products. Over 20% of our respondents would purchase them only in the case of their illness and 24% of them would do so if they had more information about them. Consumers´ awareness about food quality and food safety is the key, because if customers do not know about these products, they simply can not search for them in stores and the probability of purchasing them is low. 4% of our respondents would welcome more propagation in electronic media.

On the other hand, 59.2% of respondents do not buy food of higher quality because they believe that more expensive does not always mean better. Another reason was according to 41% their price and 3.6% thought that they simply do not need quality food.

The main objective of the next question was to find out whether and how often respondents perceive information on the food packaging (Figure 3).

The above mentioned figure shows that 164 respondents (34.5%) usually seek for the information on the product packaging when purchasing food. Other 29.8% of respondents do so sometimes and 27.1% read product labels every time they shop. For 41 respondents, these informations are totally irrelevant and not important. According to several studies, packaging plays a very important role in the process of selling food **(Ahmed et al., 2005)**. Nowadays, packaging has become an integrated marketing tool and acts as the most important promotional tool **(Jerzyk, 2016)**. Another study has confirmed that consumers emphasize the freshness of the food, which is mostly stated on the wrapper **(Heide and Olsen, 2017).** There is a strong link between food packaging and consumer behavior, as shopping becomes a habit in which consumers choose individual foods based on their packaging **(Srey et al., 2013)**.

For two-thirds of respondents (66%) the information on food packaging is usually sufficient. Surprisingly, 23% of respondents reported mostly insufficient information and 2.9% always insufficient. Therefore, food producers should be more responsible and maybe consider adding more information than it is required by legislation.

Information on the packaging of products corresponds to the reality according to experience of 82.5% our respondents. Negative experience with discrepancy had only 17.5% of them. Because of many food scandals some customers are sceptic and do not trust companies even if the producers are periodically controled. Legislation states that packaging of packaged foods must include the mandatory data which can also be supplemented by voluntary data. Regulations of the European Parliament and the Council of the EU states that mandatory information must be easily accessible, indelible and big enough to be read. The only exception is represented only by the cathegory of non-packaged food. Mandatory data can not be masked, overlaid, or interrupted by other text or images. Pictograms or symbols can only supplement these data **(Regulation (EU) No 1169/2011)**.

In relation to the previous question we were interested also in the fact, whether information on the packaging of products is understandable. For 294 respondents (61.5%) these informations are clear. However, 21.5% of respondents staited that informations are incomprehensible mostly and for 4.2% always (Figure 4). These results may be caused mostly by the fact, that the above mentioned legislation is in many cases by companies not fulfilled.

Product labels also include information about the country of origin (where every single food comes from, or where it was produced). The following question was therefore addressed to the given issue. In particular, we focused on whether consumers consider it necessary or unnecessary to indicate the country of origin and the manufacturer of the food item. Most respondents, namely 333 (69.8%), consider placing the name of producer and the country of origin directly on the food packaging to be decisive and for one quarter it isimportant. The rest of the respondents (7.5%) consider this information to be unimportant. Even nowadays some consumers do not care where the food they are purchasing and subsequently eating was produced and factors like price, brand or taste and aroma are more important **(Golian et al., 2018)**.

Another pair of questions was focused on knowing how quality food are labeled in Slovakia. The first one was dedicated to the lable "Slovak Gold". Out of 478, 273 respondents said that they are familiar with the mentioned label. Other 42% answered this question negatively. The main reason for the lack of recognition can be the nowadays absence of promotion of this label in television, printed media and on the Internet.

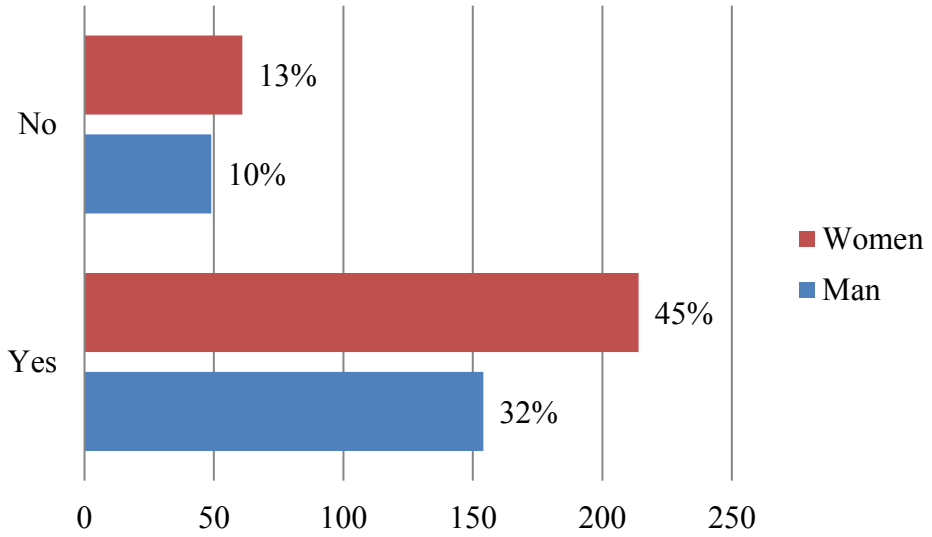

Figure 2 Dependence of buying higher quality food on respondent's gender.

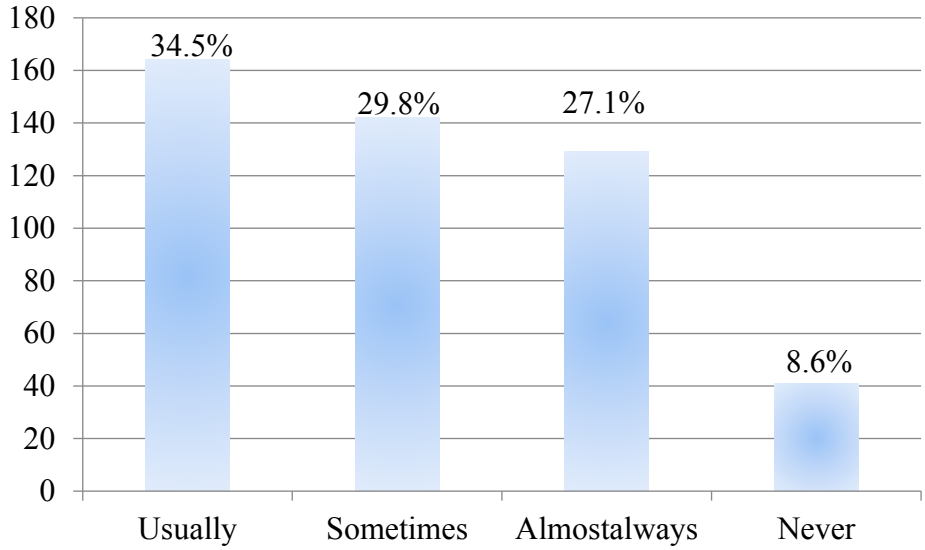

Figure 3 Frequency of reading information on food packaging.

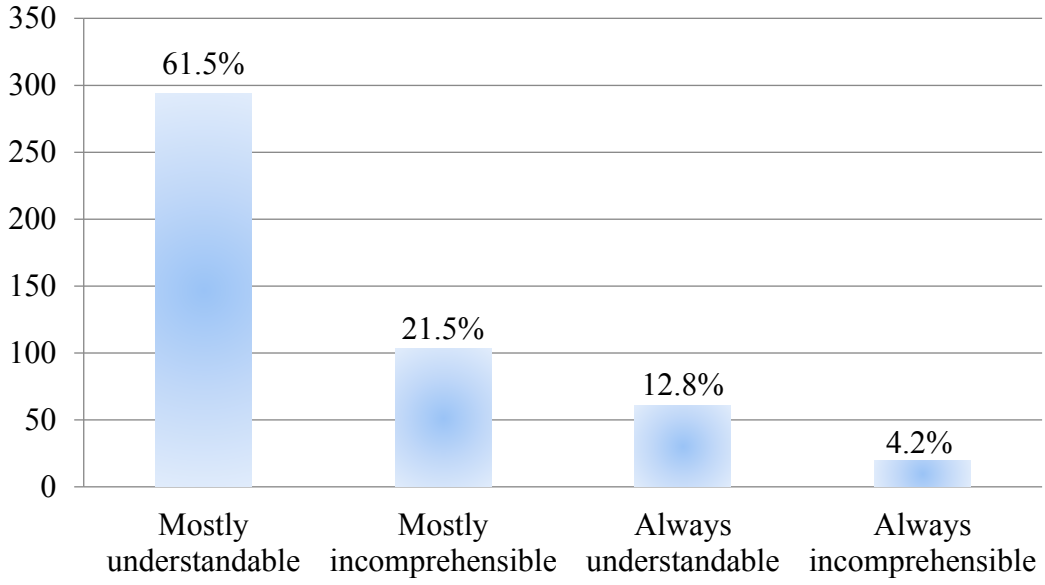

Figure 4 Clarity of information on food packaging.

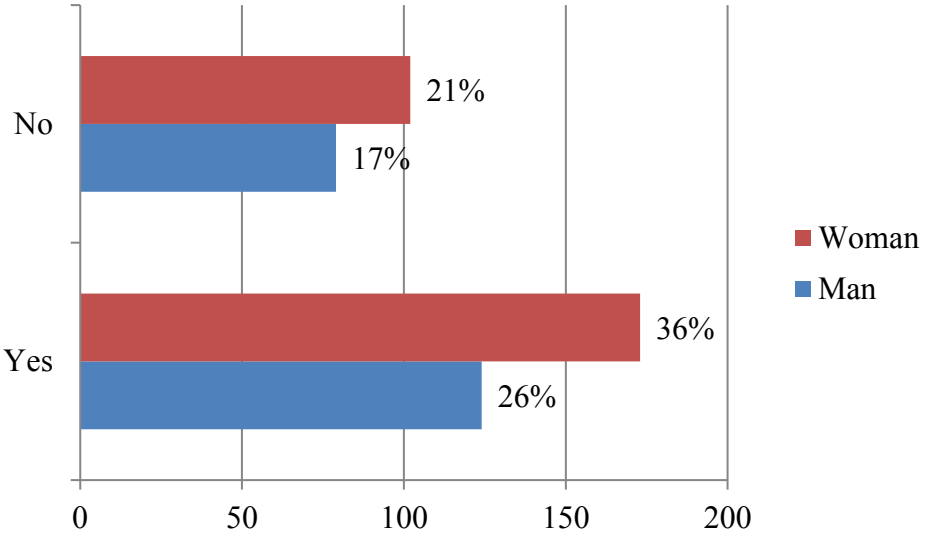

Figure 5 Dependence of knowing "Značkakvality SK" on respondent's gender.

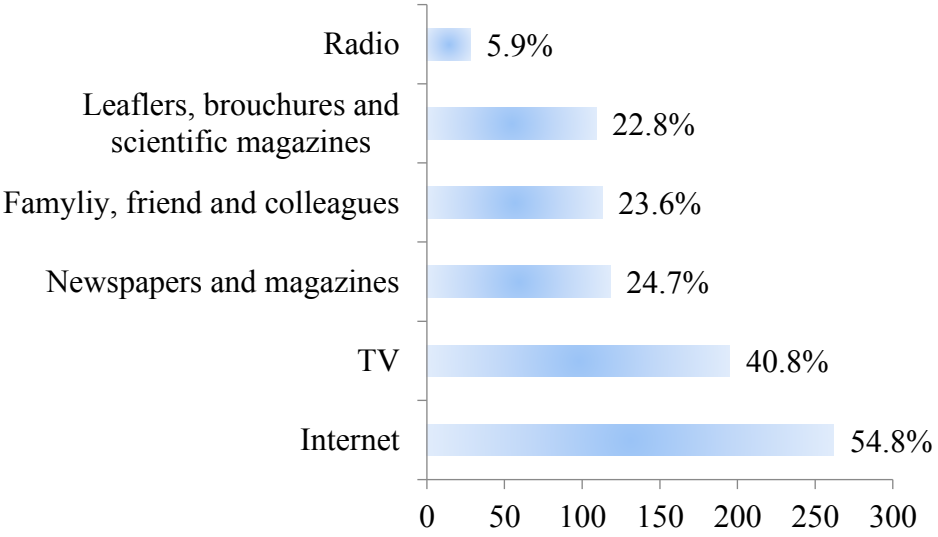

Figure 6 Source of information about food safety.

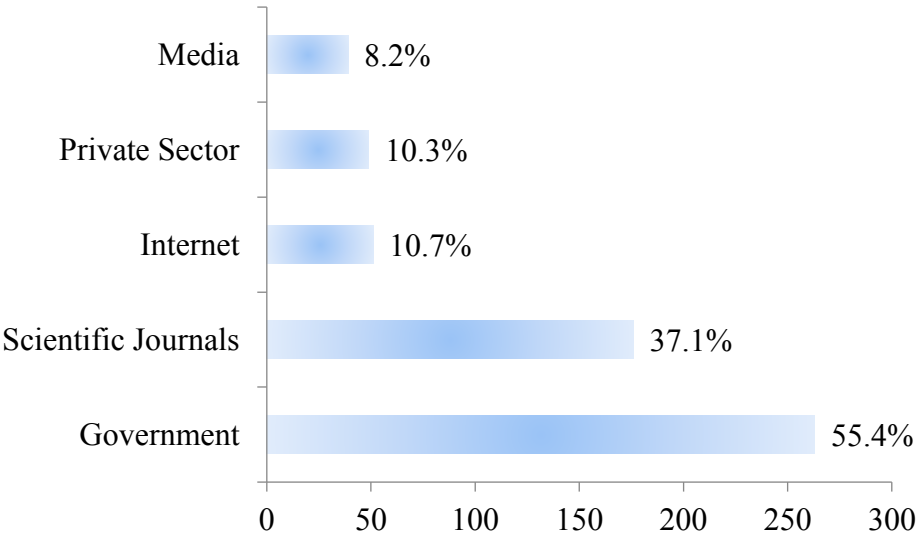

Figure 7 The most reliable sources of information about food safety.

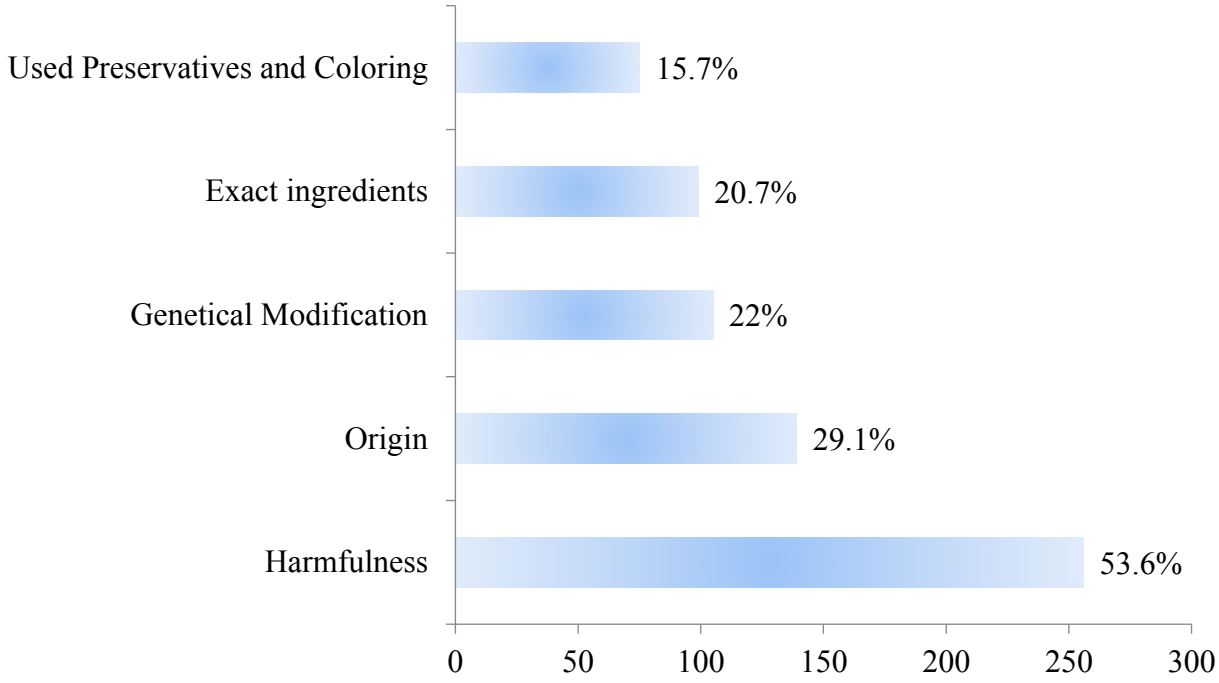

Figure 8 Types of information that are missed by consumers.

H_0: Fewer men than women do not know the lable "Slovak Gold".
H_1: Fewer men than women know the lable "Slovak Gold".
To verify hypothesis no. 4, the Chi-square test of independence and the level of significance were used:

$$p = 0.0001 < \alpha = 0.05$$

To test the hypotheses, calculated *p*-value was compared to the estimation risk alpha. The null hypothesis was rejected and therefore the alternative hypothesis H_1 was accepted. So, with 95% of probability, it is possible to claim that fewer men than women do not know the lable "Slovak Gold".

The second question (Figure 5) was focused on the national brand of high-quality agricultural products „Značka kvality SK“. The brand itself was created in the year 2004 and its main objective was to inform Slovak consumers about the quality of domestic food, which should support the consumption of domestic food products **(MP SR, 2009)**.

The graph shows that more than 50% of respondents know the logo of Značka kvality SK. Very similar results were achieved by **Nadányiová (2015)**, resp. **Košičiarová et al. (2016)**. More women (36%) than men. The larger unknown national brand of high-quality raw materials is visible in women's eyes, and up to 21%. For men it is 17%. This result, the greater lack of recognition of national brands of quality raw materials for women, surprised us. Research is supposed to confirm that women go shopping more often. This fact was also confirmed in the survey above **(Bhaskar et al., 2018)**. Of course, they often go shopping for the unborn, even to observe the information given on the clouds, and therefore do not have to label individual brands.

H_0: More women than men do not know the national label for quality of agricultural products “Značka kvality SK”.
H_1: More women than men know the national label for quality of agricultural products “Značka kvality SK”.

Statistical testing was performed by using the Wilcoxon signed rank test and significance levels:

$$p = 0.0125 < \alpha = 0.05$$

After comparing the *p*-value and the alpha value, we reject the null hypothesis. After accepting the alternative hypothesis, we can conclude that more women than men know the national label for quality of agricultural products “Značka kvality SK”.

The last five questions were devoted to consumers´ awareness of food safety. The largest number of respondents (40.5%) has rather enough information and 14% of them has definitely enough information about the food safety. But it is striking that 45.5% of our respondents were lacking this type of information. Based on the mentioned results, government and producers should teach consumers how to choose safe food in stores, store them at home and prepare them so they can eat nutritious health beneficial food. Even **Zan´s (2017)** survey has found out that food safety education has a positive impact on customers, because up to 37.4% of respondents have showed improved behavior when it comes to food.

In the next question, respondents were asked to identify 3 of the 6 sources they obtain information about the food safety (Figure 6).

Most respondents search for these information on the Internet (54.8%), second mostly used source was TV (40.8%) and third were newspapers and magazines (24.7%).

H_0: Most consumers search for least information about the food safety on the Internet.
H_1: Most consumers search for the most information about the food safety on the Internet.

To test the hypothesis No. 2, the Friedman´s test and the level of significance were used:

$$p = 0.0021 < \alpha = 0.05$$

The last null hypothesis was rejected because the level of significance alpha had a lower value than *p*-value. Qualitative statistics confirmed that most consumers search for the most information about the food safety on the Internet.

Furthermore, the questionnaire survey examined which source of information about the food safety is according to consumers the most trustworthy (Figure 7). When answering this question, respondents could choose more than one answer. The most trustworthy source is according to the survey the government (55.4%). Almost 40% of respondents thought that reliable information can be found in scientific journals dedicated to this issue. Nearly the same percentage marked options the Internet (10.7%) and the private sector (10.3%). Just 8.2% of respondents absolutely rely on information obtained from media. People should choose source they draw information from more carefully, because information coming from media or the Internet can be sometimes distorted. The most reliable are official documents of Ministry of Agriculture and Rural Development of the Slovak Republic (it is the highest organ responsible for safety of food in Slovak Republic) and European Union (its main function is to ensure safe and nutritious food and feed, high level of animal health, animal welfare, plant protection and clear and understandable information about the origin, content and use of food) **(EU, 2014)**.

The last question looked closer to the issue of type of the information which is missed by respondents. Respondents were asked to fill in up to 3 answers (Figure 8). Figure 8 shows that 53.6% of respondents were lacking the information about potential harmfulness of food. Second biggest group (29,1%) want to know the country of origin of the bought food. They are not satisfied with the lable „made in EU "they want the exact country to be identified. 22% of respondents had filled in the option „genetical modifications "and 20,7% the option „exact ingredients ". Customers are interested in ingredients, but moreover they want to know whether they are natural or artificially created in the laboratory.

CONCLUSION

Based on the results of this research we can conclude that 39% of respondents had insufficient information about the safety of purchased foods. One reason for being uninformed may be that they simply do not care about food quality. When it comes to source of information about the food safety, consumers usually search for them on the Internet (54.8%), in TV (40.8%) or in newspapers and magazines (24,7%). Paradoxically, for the most trustworthy source providing food safety information respondents (55.4%) marked the government and just 10,7% trust information found online.

The survey also found out that up to 84% of respondents buy higher quality food on daily basis. Most of them (62%) claimed that they do so for health reasons and to maintain healthy lifestyle. 48% of questioned said they do not recognize the lable "Slovak Gold". This may be due to the consumer's failure to read basic information on the food packaging or to insufficient advertising. Therefore, the Ministry of Agriculture and Rural Development should create informational campaign to support selling domestic food.

For 66% of respondents the information on the food packaging is sufficient. Interestingly, 23% of respondents reported a lack of information, especially about potential harmfullness of food, the state where the food was produced and the origin of used ingredients.

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CONSUMER BEHAVIOUR OF YOUNG GENERATION IN SLOVAKIA TOWARDS COCOA-ENRICHED HONEY

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ABSTRACT
The new trend of healthy lifestyle increases consumers´ attention towards superfoods or functional food. Due to this fact, honey enriched with various healthy foods such as cocoa, cinnamon, ginger or dried fruits has started to appear on the European market. The aim of this research paper was to investigate consumer´s perception and preferences for cocoa-enriched honey. Consumer research was based on questionnaire survey extended by product testing. This survey was conducted in 2018 (February and March) and in total 257 young Slovak consumers between 18 - 30 years participated. Each respondent tested and evaluated sensory attributes of the product (taste, aroma, colour and texture) using a 5-point scale. Statistical analyses included Friedman test, Mann-Whitney U test, Fisher's Exact Test, Pearson Chi-square test and Cramer'V coefficient. Results showed that the cocoa-enriched honey was evaluated as tasty, aromatic, gently, delicious, special, with ideal sweetness and amount of cocoa. All sensory attributes were evaluated positively (2 - good). Females were more interested in the purchase of this product. Moreover, the product would be purchased mostly by respondents who consider it a healthier alternative to commercial chocolate spreads or by those who consider their eating habits healthy. Laboratory tests revealed that the antioxidant activity of the product was higher in comparison to normal honey. In conclusion, the obtained information could be used in product positioning, promotion and designing appropriate marketing strategy.

Keywords: honey; cocoa; product testing; questionnaire survey; Slovakia

INTRODUCTION
Honey is considered to be a complex food due to its rich nutritional value and biological variability. In general, honey is perceived as a natural sweetener and widely accepted by consumers as a healthy alternative to sugar. Moreover, it contains a wide spectrum of vitamins, minerals, enzymes, amino acids as well as antioxidant compounds such as phenolics acids and flavonoids **(Pyrzynska and Biesaga, 2009; Weston, 2000; Kačániová et al., 2015)**. Besides nutritional value, honey has many antimicrobial and healing properties. In medicine, honey is used for curing various diseases including skin wounds, colds or diseases of gastrointestinal character. Nowadays, honey is frequently used in apitherapy which is an important part of the complementary and alternative medicine. Honey and other bee products are commonly used in pharmacy as diet supplements, prophylactic agents or drug components **(Gašic et al., 2014; Lusby et al., 2005; Al-Mamary et al., 2002)**.

Cocoa powder is a plant-based product, which is obtained by milling roasted cocoa nibs and contains more than 300 constituents including minerals, polyphenol acids and flavonoids. Many studies have proven its antioxidant, anti-inflammatory and cardio-protective properties. Approximately 30% of polyphenol content is made by flavonoids (procyanidin, catechin and epicatechin) therefore this product has a significantly higher antioxidant activity than for example pomegranate, blueberries or cranberries. There is no doubt that cocoa powder has potential health benefits and is perceived by many consumers as a functional food **(Todorovic et al., 2017; Araujo et al., 2013; Lapčík et al., 2017; Godočíková et al., 2016; Kozelová et al., 2014).**

Due to the increasing attention of consumers towards healthy products and functional food, new honey-based products appeared on the European market. Usually, these products are a combination of honey and other healthy foods such as nuts, dried fruits including cherries, prunes, apricots or even various spices (cinnamon, ginger, chilli or cocoa powder). Sometimes, honey is mixed with other bee products such as propolis, pollen, royal jelly or bee bread. The final product (enriched honey) usually has higher antioxidant properties as well as nutritional value **(Kowalski and Makarewicz, 2017; Ćetković et al., 2014; Tumbas et al., 2012; Vulić et al., 2015; Wilczyńska et al., 2017).**

Scientific hypothesis
The aim of this research paper is to study the perception of young Slovak consumers and their preferences towards cocoa-enriched honey.

Several hypothesis were formulated:
Hypothesis 1: - There exist differences in evaluation of sensory attributes (taste, aroma, colour, texture).
Hypothesis 2: - There exist differences in evaluation of semantic differential between genders.
Hypothesis 3: - There exists dependence between purchase intentions and gender.
Hypothesis 4: - There exists dependence between purchase intentions and one´s perception of healthy eating habits.
Hypothesis 5: - There exists dependence between purchase intentions and product perception as a healthier alternative to commercial spreads.
Hypothesis 6: - The antioxidant activity of cocoa-enriched honey will be higher in comparison to normal honey.

MATERIAL AND METHODOLOGY

Consumer research

The primary data were based on questionnaire survey and product testing both conducted in the spring 2018 (February - March). The tested product was rapeseed honey in creamed consistency enriched with cocoa powder (see Table 1). Respondents evaluated both sensory and abstract attributes using a 5-point scale. Afterwards each respondent answered the questions regarding consumer and purchasing behaviour. Target group were 257 young consumers between 18 – 30 years. According to socio-demographic characteristics, the research sample comprises women (56.03%) with secondary education (73.15%) and living in the city (52.53%). The majority of respondents were students (54.09%) or employed (45.91%) with a monthly income up to 600 € (67.32%).

Figure 1 Cocoa-enriched honey.

Statisic analysis

Statistical analyses were conducted in SPSS version 25 (IBM) and following non-parametric tests were applied:

- Friedman test
- Mann-Whitney U test
- Fisher's Exact Test,
- Pearson Chi-square test
- Cramer'V coefficient

Laboratory testing

Sample preparation

An amount of 0.25 g of sample was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids, phenolic acids). Extraction was carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations

Determination of antioxidant activity

The radical scavenging activity of extracts was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sánchéz-Moreno et al., 1998)**. The sample (0.4 mL) was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL methanol). The absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 - 100 $mg.L^{-1}$; $R^2 = 0.989$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents. All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Determination of polyphenol content

Total polyphenol content extracts was measured by the method of **Singleton and Rossi (1965)**, using Folin-Ciocalteu reagent. 0.1 mL of each sample was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 300 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Determination of flavonoid content

Total flavonoids were determined using the modified method of **Willett (2002)**. 0.5 mL of sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M potassium acetate and 4.3 mL of distilled water. After 30 min in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.5 – 20 $mg.L^{-1}$; $R^2 = 0.989$) was used as the standard and the results were expressed in $mg.g^{-1}$ quercetin equivalents.

Determination of phenolic acid content

Total phenolic acid content was determined using method of **Farmakopea Polska (1999)**. A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. The absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 $mg.L^{-1}$, $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ caffeic acid equivalents.

RESULTS AND DISCUSSION

Respondents firstly evaluated product´s taste, aroma, colour and texture using a 5-point scale (1 – very good and 5 – very bad). By applying the Friedman test, the differences between these sensory attributes were tested. Based on the results, the first hypothesis was confirmed (p-value = 0.014). In average, all attributtes were evalueted as good. The best evaluation obtained texture (2.29) followedby taste (2.32), colour (2.33) and aroma (2.46). Moreover, Figure 2 illustrates sensory evaluations according to gender. Based on the Mann-Whitney U test, there do not exist significant differences in evaluations between males and females. However, it could be stated that females evaluated slightly better. Similar sensory evaluation was conducted by **Šedík et al. (2018a)**.

Next, respondents evaluated selected characteristics of the product displayed by a 5-point scale using modified semantic differential. In general, results showed that respondents perceived product positively. On average, product was evaluated as tasty (2.3), with ideal sweetness (1.6), aromatic (2.3), gentle (2.6) delicious (2.3) special (2.4) with adequate amount of cocoa (2.4). The product was perceived neither irresistible nor average (3.2), and as for the question whether it has more honey or cocoa taste, respondents felt slightly more the taste of honey (2.7). Furthermore, Figure 2 illustrates evaluations of semantic differential by gender. It could be observed that females evaluated all selected characteristics more positively. Nevertheless, the only statistically significant differences were proven and hypothesis 2 was confirmed (see Table 1) in terms of tasty/disgusting (p-value = 0.044), delicious/tasteless (p-value = 0.039), special/ordinary (p-value = 0.037) adequate/too much amount of cocoa (p-value = 0.030) and honey taste/cocoa taste (p-value = 0.003). Females felt the taste of honey more intensively than males. The third hypothesis assumed differences between males and females in purchase intention of the product. Application of Fisher's Exact Test proved that there exist statistically significant differences. Females are more interested in product purchase than males (Figure 4). The main motives of purchase were taste, followed by health aspect while the frequent reasons of not purchasing the product were too intensive sweet and honey taste, or simply preference of normal honey. Besides purchase motives and barriers, the study investigated the impact of healthy eating habits on purchase intentions. By applying Pearson Chi-square test, hypothesis 4 was confirmed and there exists statistically significant dependency (p-value = 0.000). Based on the results, it could be concluded that the majority of respondents who do not consider their eating habits healthy at all, would not purchase cocoa-enriched honey (Figure 5).

Hypothesis 5 assumed dependency between product perception as a healthier alternative to commercial spreads and purchase intention. Based on the result of Pearson Chi-square test, this hypothesis was confirmed. The majority of respondents who perceive this product as a healthier alternative would purchase it (Figure 6).

In addition, respondents representing the young generation consume honey mostly occasionally. This situation could be improved by health promotion campaigns even in the framework of school catering programs **(Bittsánszky et al., 2015)**. According to **Guziy et al. (2017)**, honey consumers in Slovakia mostly consider the country of origin, type and price before purchasing a product. The size and design of packaging play the least important role, which was proven by applying an eye-tracking experiment as well **(Hazuchová et al., 2018).**

Table 1 Gender differences in product evaluation.

Product characteristic	Sig.
taste	.246
aroma	.059
colour	.341
texture	.302
tasty/disgusting	.044**
ideal/insufficient sweetness	.309
aromatic/without aroma	.051
gentle/strong	.115
delicious/tasteless	.039**
special/ordinary	.037**
irresistible/average	.201
adequate/too much amount of cocoa	.030**
honey taste/cocoa taste	.003**

Note: **Statistically significant for p <0.05 Mann-Whitney U test.

In order to study if enrichment of honey by cocoa powder would increase antioxidant activity and prove the hypothesis, the product was analysed in laboratory conditions.

Antioxidant activity

The antioxidant activity of rapeseed honey enriched with cocoa powder determined with the DPPH method was 6.73 mg TEAC.g^{-1} (Table 3). Pure rapeseed honey is rich in compounds with antioxidant activity, especially flavonoids and phenolic acids. Honey enriched with herbs, spices, bee products, fruits as well as coffee and cocoa beans is very popular nowadays. Some studies reported that phenolic compounds found in cocoa beans may present different properties such as antioxidant, anticarcinogenic, and antiradical activities. Polyphenols are the main antioxidant-active constituents of cocoa. Flavanols and procyanidins have previously been identified as the active antioxidant agents of cocoa **(Bauer et al., 2016)**. Cocoa beans as well as cocoa powder are very attractive for consumer also for organoleptic properties. Bitter and chocolate taste of cocoa powder is very good with the combination of sweet honey taste. Adding cocoa powder to honey can also increase antioxidant activity. **Jaafar et al. (2017)** tested the antioxidant activity of monofloral honey (regarding rapeseed honey) and determined values from 0.08 to 0.51 AEAC.g^{-1} (ascorbic acid equivalent antioxidant capacity). In our study the activity was several times higher probably due to the cocoa powder addition.

Total polyphenol, flavonoid and phenol acid content

The results of total polyphenol, flavonoid and phenolic acid content are summarized in Table 3. Pure rapeseed honey is rich in phenolic substances. **Wen et al. (2017)** determined in this honey presence of gallic, protocatechuic, p-hydroxybenzoic, caffeic, ferulic, p-coumaric and benzoic acid.

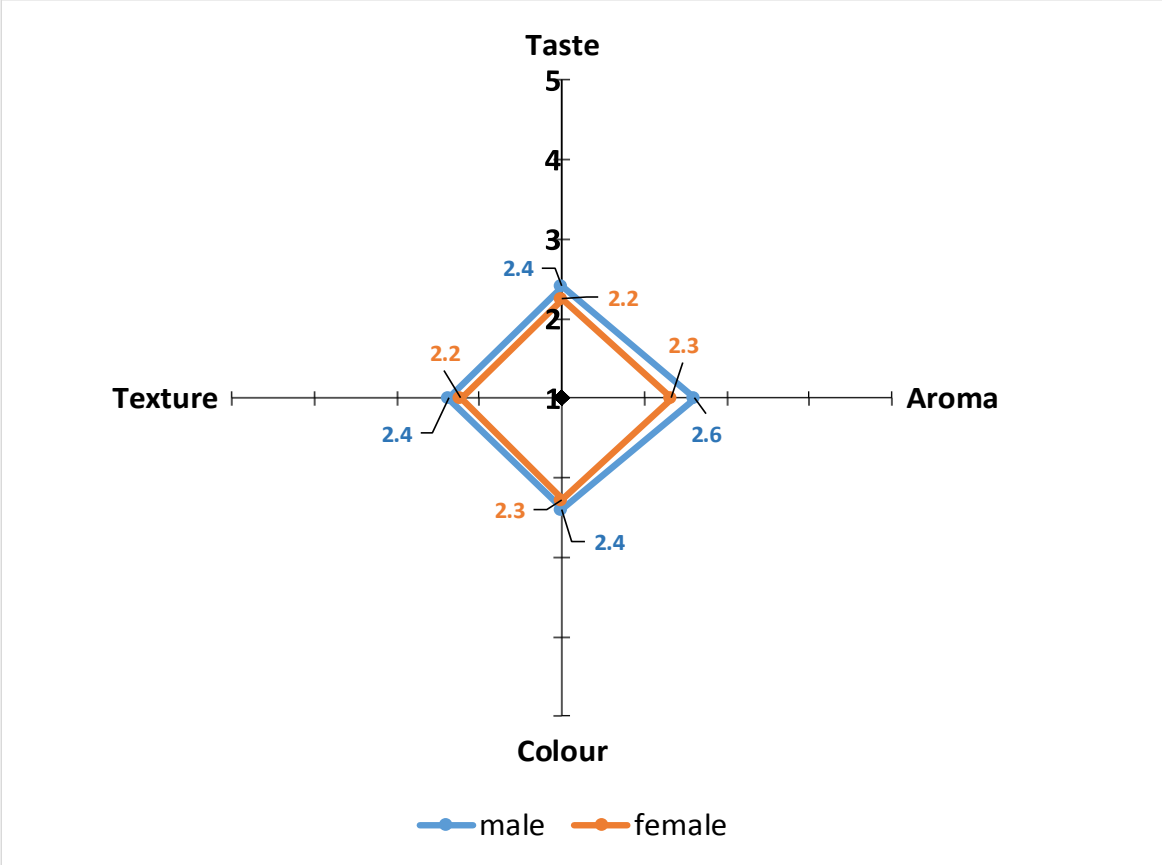

Figure 2 Sensory evaluation of cocoa-enriched honey by gender.

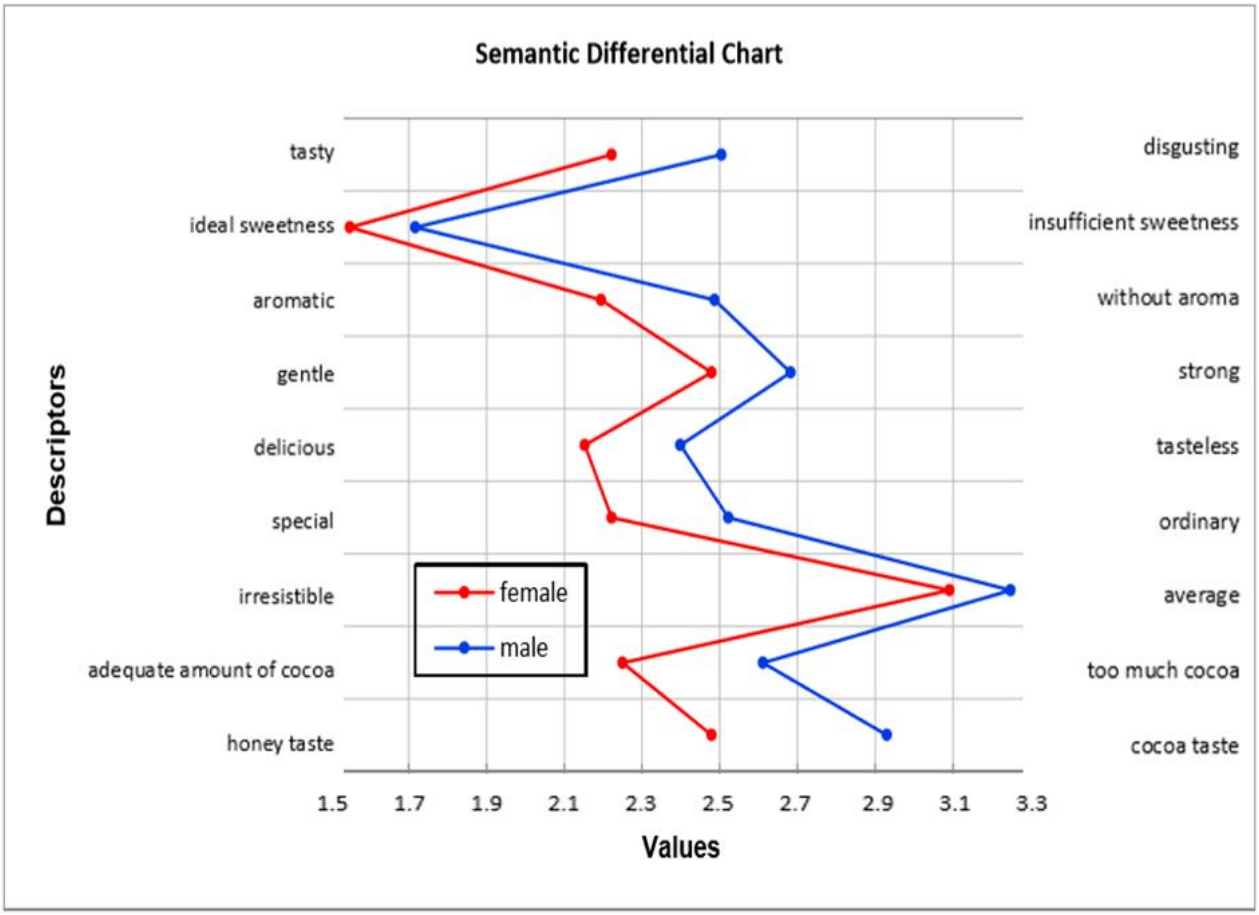

Figure 3 Semantic differential: product evaluation of selected characteristics.

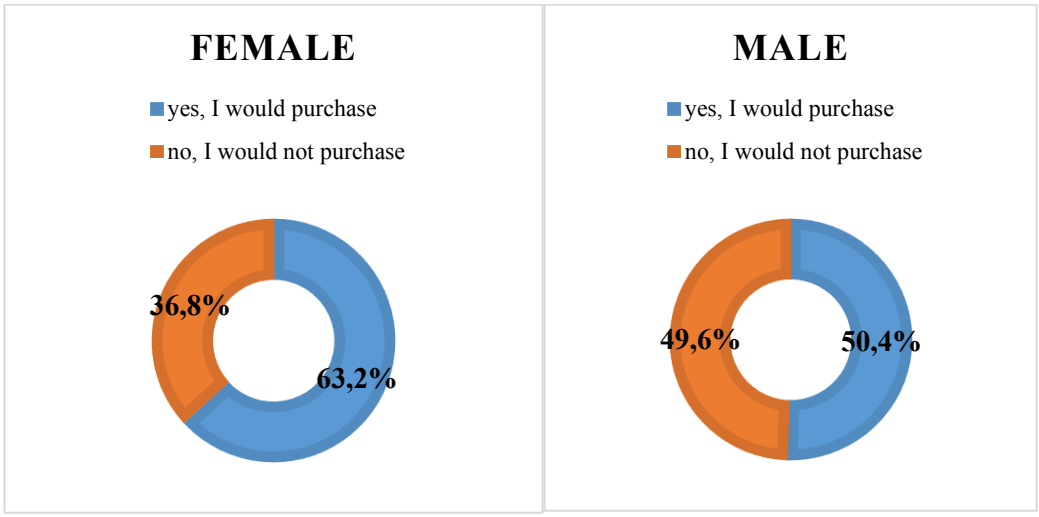

Figure 4 Purchase intentions of cocoa-enriched honey by gender.

Table 2 Impact of selected factors on purchase intentions.

	Sig.	Cramer´s V coefficient
Purchase intentions and gender	.043**	.128
Purchase intentions and healthy eating habits	.000**	.313
Purchase intentions and product perception	.000**	.526

Note: **Statistically significant for p <0.05 Pearson Chi-square test and Fisher´s Exact Test.

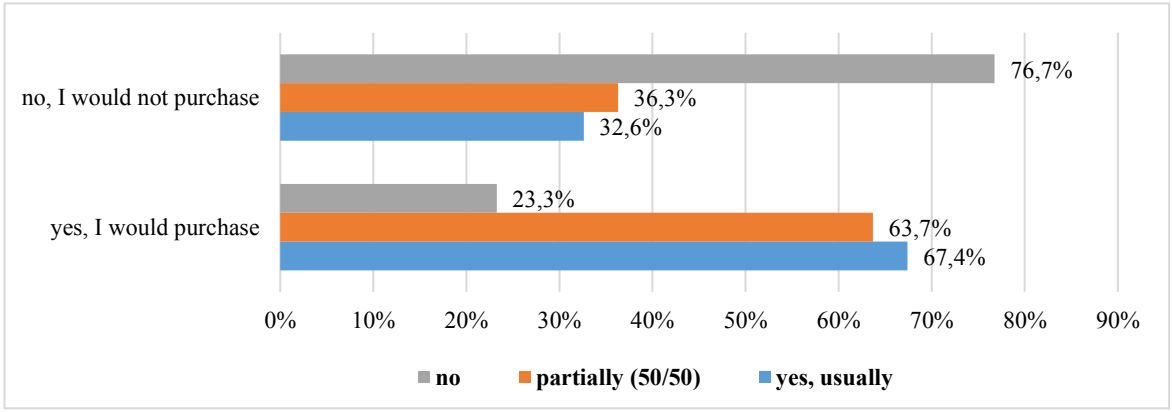

Figure 5 Respondents' purchase intentions and perception of their healthy eating habits.

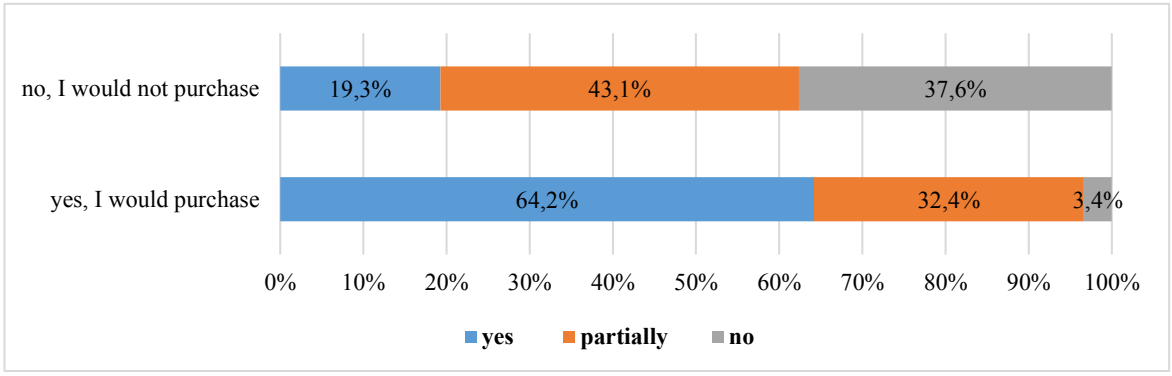

Figure 6 Respondents' purchase intentions and product perception as healthier alternative to commercial chocolate spreads.

While gallic, ferulic and protocatechuic acids were determined only in small amount, benzoic acid (18.11 mg.kg^{-1}) and p-hydroxybenzoic (1.22 mg.kg^{-1}) were dominant, detected in higher amount. **Özkök et al. (2010)** determined total phenolic acid content in Turkish monofloral honey and their results ranged from 0.035 to 0.36 mg GAE.g^{-1} (gallic acid equivalent). Total phenolic acid content in our sample of monofloral honey is higher due to the cocoa powder addition. **Ali et al. (2015)** determined in cocoa powder gallic acid, protocatechuic and chlorogenic. Protocatechuic acid was dominant with amount 18.8 mg.g^{-1}. **Wen et al. (2017)** also determined the flavonoid composition in rapeseed honey and found the presence of rutin, myricetin, morin, quercetin, kaempferol,

galangin, apigenin and chrysin. Morin (11.12 mg.kg^{-1}) and quercetin (5.18 mg.kg^{-1}) were dominant in this type of honey. According to **Ayoub et al. (2009)** the flavonoid concentration in honey is approximately 0.02 mg.g^{-1}. In our sample enriched with cocoa powder the amount of flavonoids was higher. Cocoa powder is a very good source of phenolic compounds especially flavonoids. Flavonoids present in cocoa include flavanols, anthocyanins, flavonols, and flavones. Flavanols, the most abundant flavonoids in cocoa, comprise the monomeric flavanols (+)-catechin and (-)-epicatechin, and their oligomeric and polymeric forms (procyanidins). (-)-Epicatechin has been reported as the major monomeric flavanol in cocoa, representing ca. 35% of the total phenolic content **(Andres-Lacueva et al., 2008)**. Apart from polyphenol content, cocoa is also rich in methylxanthine, namely caffeine and theobromine. These substances have stimulating effect on the human body, so we can recognize that consumption of honey enriched with cocoa powder can have not only antioxidant but also stimulating effect on the organism.

For testing the last hypothesis, the results of antioxidant activity, total polyphenol, flavonoid and phenolic acid content (Table 3) were comapred with similar laboratory tests of normal honey, where resutls were as follows: DPPH [mg TEAC.g^{-1}] 0.313 ±0.018, TPC [mg GAE.g^{-1}] 1.212 ±0.011, TFC [mg QE.g^{-1}] 0.033 ±0.013 and TPAC [mg CAE.g^{-1}] 0.084 ±0.022 **(Šedík et al., 2018b)**. It could be concluded that the antioxidant activity of cocoa-enriched honey was few times higher.

Table 3 Antioxidant activity, total polyphenol, flavonoid and phenolic acid content in analyzed sample.

Parameter	Cocoa-enriched honey
DPPH [mg TEAC.g^{-1}]	6.73±0.98
TPC [mg GAE.g^{-1}]	4.84±0.67
TFC [mg QE.g^{-1}]	0.25±0.03
TPAC[mg CAE.g^{-1}]	2.71±0.16

Note: DPPH - radical scavenging activity; TEAC - trolox equivalent antioxidant capacity; GAE - gallic acid equivalent; QE - quercetin equivalent; CAE - caffeic acid equivalent; mean (n = 3) ± standard deviation.

CONCLUSION

Consumer research showed that cocoa-enriched honey obtained positive evaluations among young consumers in Slovakia. Taste, aroma, colour and texture was evaluated as good. Furthermore, the product was described as tasty, aromatic, gentle, delicious, special, with ideal sweetness and adequate amount of cocoa. The product scored as neither irresistible nor average and the dominant taste of the product was neither honey nor cocoa. However, females felt the honey taste more than males. In some characteristics, females evaluated this product more positively.

In general, the majority of respondents would purchase the product. However, female consumers were more interested in product purchase than male consumers. Moreover, respondents who considered their eating habits healthy and those who perceived this product as a healthier alternative to commercial chocolate spreads had higher willingness to purchase it.

Regarding the antioxidant activity, the tested products obtained a few times higher results than normal honey, therefore, it is assumed that this combination of two ingredients increases the overall benefits for health of final products. In fact, this observation could be used in tailoring a suitable marketing strategy and product positioning as a functional food. The target group should be female consumers who are interested in healthy lifestyle.

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ECOTOXICOLOGICAL STUDIES OF AKMOLA REGION LAKES

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ABSTRACT
The research object is water, bottom sediments in Akmola region lakes located in the intensive agriculture area. The territory of Akmola region is subjected to intensive human impacts, including the inevitable pollution with agricultural pesticides, which are ecotoxicants. The work has carried out hydrochemical studies in technogenic polluted lakes: general hydrochemical indicators, persistent organic pollutant content. The POPs in the samples were determined on the gas chromatograph Clarus 580 (PerkinElmer) with a mass spectrometer detector Clarus-SQ 8. According to the analysis results, the general hydrochemical pollution is classified as an average. The MPC excess indicators in the Akmola region lakes are observed for salt ammonium (up to 0.002 MPC), magnesium (up to 1.15 MPC), nitrites (up to 1.12 MPC), petroleum products (up to 1.98 MPC), iron (up to 2.0 MPC), SSAS (up to 3.8 MPC). High concentration indicators for the sulphate (3.5 MPC), copper (4.3 MPC), magnesium (1.125 MPC). Mainly the lakes are dominated by sulfates, ion chloride. But in general among 21 investigated POPs 8 substances are accumulated in bottom sediments of the investigated Zhalauly, Tastykol lakes, Unnamed Lake to the south of Akkol village, Itemgen lake, Zhalanash lake near Malinovka village (near Astana city), Kokay, Yesey, Bolshoe Chebachie.

Keywords: ecotoxicants; persistent organic pollutants; monitoring; accumulation; lake

INTRODUCTION
In Kazakhstan there is still no universal systematic monitoring of persistent organic pollutants **(Kholubek et al., 2012)**. Upon that, a number of pilot projects of initial assistance to the Republic of Kazakhstan on obligations under the Stockholm Convention on Persistent Organic Pollutants has given reasoned conclusion that the problem is urgent in the republic **(Kholubek et al., 2012; Zhanadilov et al., 2015.; Shabanova et al., 2010)**. This primarily relates to areas exposed to intense pollution by agricultural pesticides.

Ecological problems of Akmola region is largely determined by agro-industrial specialization of the region. The main branch of the region specialization is the production and processing of agricultural products (76%), including grain production – 56.8%. Akmola region has the highest percentage of rural population – 54.8% **(Investment opportunities of the region, 2017; Sydykov et al., 2004; Seitkasymova, 2015)**.

The industry of this region is represented by the gold ore, uranium extraction, pharmaceutical and chemical industry, machine building and production of building materials **(Sydykov et al., 2004; Press service of the Prime Minister of the Republic of Kazakhstan, 2018)**.

The area is rich in land resources. The total area of agricultural land is 13,791.7 thousand ha, areas of natural pastures are wide and occupy 6592.5 thousand hectares, the arable land is about 7,100 thousand ha. Almost all arable lands use some insecticides, fungicides - substances that are long-persistent in the environment, accumulated in the soil, in bottom sediments of stagnant water **(Ljunggren et al., 2014; Jepson and Law, 2016)**.

In addition, the territory of the region under study still keep in some places some pesticides in abandoned warehouses without recycling, that were applied since the middle of last century. Names, respectively, contents of some pesticides are no longer possible to determine without analytical tests.

Thus, the territory of Akmola region is subjected to intensive human impacts, including the inevitable pollution with agricultural pesticides, which are ecotoxicants.

Detecting most POPs directly in water is not recommended **(Kholubek et al., 2012; Ministry of Health of the USSR, 1987)** because many of them are insoluble in water, and soluble POPs can be eliminated through bioaccumulation in living organism tissues. Thus, in our opinion, monitoring surface water for the POP content should begin with bottom sediments. In this case,

hydrochemical indicators should be analyzed as the accompanying background or synergistic factor increasing violations in biocenosis and pathomorphology of aquatic organisms **(Yemelyanova and Lobchenko, 2002.; WHO, 2011; Kukeyeva et al., 2015; Grancová-Bielková and Sokol, 2010; Akbayeva et al., 2014)**.

The research aim: Study the content of ecotoxicants in the Akmola region lakes.

Scientific hypothesis

In Kazakhstan, by virtue of years of the uncontrolled POPs use and production of industrial toxins, violations of their operation and storage rules, a unique dangerous xenobiotic profile could emerge, which practically has not been studied **(Akbayeva et al., 2016)**.

In the Republic of Kazakhstan, an average of up to 0.57 kg of pesticides was applied per 1 ha of arable land **(Shabanova et al., 2010)**. Considering that pesticides were used from the 50s, about 475 tons of pesticides were used on the territory of the Akmola region with an area of 14,621.9 hectares until 2008, including POPs pesticides. In this regard, pesticides from contaminated soil should ultimately accumulate at the bottom of water bodies. Thus, lakes are the ultimate storage of POPs.

The research results should be informative and methodological rationale for more extensive researches in this area.

MATERIAL AND METHODOLOGY

In 2016 some of the chemical components was studied in the water and bottom sediments of the Akmola region lakes: Zhalauly and Tastykol lakes, the unnamed lake to the south of the district center of Akkol village and unnamed lake 40 km south from Astrakhanka village, Itemgen lake at Buland village, Zhalanash lake at Malinovka village (near Astana city). These lakes are located on the territory of the agricultural pesticides intensive use or places of POP substances storage. Lakes of the Korgalzhyn lake system: Kokay, Yesey, Sultankeldy are located in the south of the Akmola region territory in the lower part of the Nura River valley, which runs a large farmland. There have also been studied lakes of Shchuchinsk - Borov resort zone Borovoye and Bolshoe Chebachie that are under intensive anthropogenic impact.

In the lakes, hydrochemical samples of water and bottom sediment were selected in the summer months of July - August 2016.

These analyzes have determined the Hydrochemical water pollution index (WPI) **(Sibagatullina and Mazurkin, 2009)**.

Determining POPs in bottom sediments

It has been carried out in accordance with the methodological guidelines for determining HCH and DDT in silt sulfide medical muds with gas-liquid chromatography **(Ministry of Health of the USSR, 1987)**. The samples were selected with a bottom sampler from 10 – 70 cm depth from the surface. Samples were taken at five points by the "envelope" principle. Selected from all the mud was thoroughly mixed, and an average sample was selected in a glass jar of 500 mL. For further analysis, grounds were air dried.

The POPs in the samples were determined on the gas chromatograph Clarus 580 (PerkinElmer) with a mass spectrometer detector Clarus-SQ 8.

Chromatographic conditions: capillary column RestekRxi®-1 ms 0.25 mm x 30m x 0.25 mm, sample volume: 1.0 mcL, the carrier gas, the carrier gas velocity: 1 $ml.min^{-1}$, flow division of 1:25, t columns: 40 °C, the rise of 2 °C.min^{-1} to 280 °C, t evaporator – 280 °C, mass spectrometric detector: t – 240 °C, EI +=70 eB, the scanning time – 4 to 120 minutes, the ion scan mode – 39 – 500 $m.z^{-1}$.

Statisic analysis

All data were expressed as mean. The percentage components content was automatically calculated based on the peak areas of total ion chromatograms. The components were identified by mass spectra and retention times, using a NIST library.

RESULTS AND DISCUSSION

Indicators of the MPC excess in the Akmola region lakes for salt ammonium (up to 0.002 MPC), magnesium (up to 1.15 MPC), nitrites (up to 1.12 MPC), petroleum products (up to 1.98 MPC), iron (up to 2.0 MPC), SSAS (up to 3.8 MPC). High concentration indicators for the sulphate (3.5 MPC), copper (4.3 MPC), magnesium (1.125 MPC). Mainly the lakes are dominated by sulfates, ion chloride (Table 1).

Lakes the most salty and with a hard water are Zhalauly and Zhalanash lakes - the water hardness of 7.5 $mg.dm^{-3}$ and 8.37 $mg.dm^{-3}$, respectively.

According to the water pollution index (WPI) the first class (relatively clean) may only include Sultankeldy, Yesey, Kokay waters; the second class (slightly dirty) - Zhalauly, Itemgen, Unnamed Lake (Akkol village) Zhalanash, Borovoe, Bolshoe Chebachie; the third class (polluted) - Tastykol, Unnamed Lake (Astrakhanka village) **(Sibagatullina and Mazurkin, 2009)**.

Water is not recommended as a key matrix for lipophilic and non-Arctic initial twelve POPs, so the analysis of surface water is not recommended: for aldrin, chlordane, DDT, dieldrin, endrin, HCB, heptachlor, mirex, PCB, PCDP/PCDF, toxaphene. Water-soluble POPs: chlordecone, α-HCH, β-HCH, γ-HCH, PFOS (perfluorooctanesulfonate). Besides, the POP concentration in water may vary seasonally due to the seasonal activity of phytoplankton and certain organic substances, and other impacting factors, such as rainfall, flow volume, etc. Based on this, we have not detected POPs in the lake waters, and immediately began to define in the bottom sediments, where they can sediment as lipophilic compounds on the adsorbent composed of dead biota, benthic microorganisms.

Almost in all the studied lakes (Table 2), certain POPs have been found, except for the Unnamed Lake (at Astrakhanka village), Borovoye and Sultankeldy. Among the pesticides investigated by us, no sample has such compounds detected as: alpha-hexachlorocyclohexane, beta-hexachlorocyclohexane, chlordecone, hexabromobiphenyl, hexabromodiphenyl ether, lindane, hexachloran, mirex, pentachlorobenzene (in quintozene), perfluorooctane sulfonate and its salts, endosulfan and

Table 1 Contents of the chemical components in the Akmola region lakes.

Component name	MPC	Akmola region lakes										
		Zhalauly Lake	Tastykol Lake	Itemgen Lake	Unnamed lake (Akkol village	Unnamed lake (40 km south from Astrakhanka village)	Zhalanash Lake	Borovoe Lake	Bolshoe Chebachie Lake	Kokay Lake	Yesey Lake	Sultankeldy Lake
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
Temperature, °C		14	18	18.8	23	19	18.9	22	20	16	22	24
pH value		8.25	6.7	8.2	6.6	7.6	8.1	7.8	7.9	7.6	7.7	7.0
BOD 5 mg.dm^{-3}	3	1.28	3.15	1.48	2.2	3.5	1.89	1.63	2.15	2.35	1.78	2.6
Calcium, mg.dm^{-3}	180	76.6	10.23	84.2	21.63	63	90.8	68.5	78.3	65.3	55.2	45
Hardness, mEq.dm^{-3}	10.0	7.5	1.7	7.1	1.49	5.19	8.37	3.7	4.37	3.7	3.4	2.65
Nitrite nitrogen, mg.dm^{-3}	0.02	0.002	0.0012	0.009	0.001	0.002	0.001	0.002	0.013	0.004	0.003	0.001
Nitrite nitrogen, mg.dm^{-3}	9.1	4.47	8.3	0.67	4.36	9.06	0.54	2.4	3.8	4.31	8.0	3.16
Sulfate ions, mg.dm^{-3}	100	212	46.52	249	59.18	126.31	305	65.9	78.8	225	125	47.3
Chloride ions, mg.dm^{-3}	300	277	222	268	196	276	291	150	79	147	198	146
Salt ammonium, mg.dm^{-3}	0.5	0.98	0.42	0.02	0/07	0.61	0.06	0.33	0.47	0.014	0.24	0.003
Phosphates, mg.dm^{-3}	3.5	0.26	1.34	0.035	0.078	2.83	0.05	1.02	0.76	0.47	0.35	1.34
Total ferrum, mg.dm^{-3}	0.1	0.065	0.03	0.072	0.04	0.16	0.049	0.08	0.1	0.03	0.02	0.02
SSAS, mg.dm^{-3}	0.1	0.02	0.87	0.05	0.008	1.0	0.03	0.21	0.15	0	0	0.005
Magnesium, mg.dm^{-3}	40	45.5	14.63	35.3	5.07	25	48.6	3.92	5.77	5.41	8.12	5.47
Copper, mg.dm^{-3}	0.001	0.001	0.32	0.003	0.002	0.005	0.004	0.002	0.02	0	0.0002	0.0001
Petroleum products, mg.dm^{-3}	0.05	0.033	0.003	0.120	0.054	0.06	0.134	0.05	0.046	0.0007	0.0007	0.006
WPI	-	1.32	3.8	1.25	1.55	4.08	3.1	1.14	1.75	0.6	0.71	0.38

isomers, tiodan, paraquat. Thus, among 21 tested substances, 12 substances are not accumulated in the bottom sediments of lakes studied.

Lake, where studied chlorinated pesticides are found, are located in areas of the most intensive agriculture, in particular, Zhalauly lake – where 5 pesticides are found (endrin, heptachlor, hexachlorobenzene, DDT, polychlorinated biphenyl), in 3 lakes 3 pesticides are found: Tastykol (aldrin, hexachlorobenzene, DDT), Itemgen (heptachlor, DDT, polychlorinated biphenyl) and Zhalanash (dieldrin, hexachlorobenzene, DDT). One pesticide – (hexa hexachlorobenzene), Bolshoe Chebachie (Aldrin) and Yesey (Aldrin). 2 pesticides were found in Kokay lake (chlordane, hexachlorobenzene).

The quantitative pesticides distribution in lakes is shown in Table 3: aldrin has been found in 3 samples of Tastykol lake (0.06 x 10^{-6} mg.kg^{-1}), Yesey (0.3 x 10^{-6} mg.kg^{-1}), Bolshoe Chebachie (1.8 x 10^{-6} mg.kg^{-1}), chlordane - in 1 Kokay lake (1.3 x 10^{-6} mg.kg^{-1}), dieldrin in 1 Zhalanash lake (0.04 x 10^{-6} mg.kg^{-1}), endrin - in 1 Zhalauly lake (0.6 x 10^{-6} mg.kg^{-1}), heptachlor - in 2 Zhalauly lakes (0.22 x 10^{-6} mg.kg^{-1}) and Itemgen (0.04 x 10^{-6} mg.kg^{-1}), hexachlorobenzene was found in 5 Zhalauly lakes (0.07 x 10^{-6} mg.kg^{-1}), Tastykol (0.47 x 10^{-6} mg.kg^{-1}),

Table 2a POPs content in the bottom sediments of the Akmola region lakes.

Lake name	Aldrin (mg.kg^{-1})	Alpha hexachlorocyclohexane (mg.kg^{-1})	Beta hexachlorocyclohexane (mg.kg^{-1})	Chlordane (mg.kg^{-1})	Chlordecone (mg.kg^{-1})	Dieldrin (mg.kg^{-1})	Endrin (mg.kg^{-1})	Heptachlor (mg.kg^{-1})	Hexabromobiphenyl (mg.kg^{-1})	Hexabromodiphenyl ether (mg.kg^{-1})
Zhalauly	-	-	-	-	-	-	$0.6\cdot10^{-6}$	$0.22\cdot10^{-6}$	-	-
Tastykol	$0.06\cdot10^{-6}$	-	-	-	-	-	-	-	-	-
Itemgen	-	-	-	-	-	-	-	$0.04\cdot10^{-6}$	-	-
Unnamed Lake in Akkol village	-	-	-	-	-	-	-	-	-	-
Unnamed Lake in Astrakhanka village	-	-	-	-	-	-	-	-	-	-
Zhalanash	-	-	-	-	-	$0.04\cdot10^{-6}$	-	-	-	-
Borovoe	-	-	-	-	-	-	-	-	-	-
Bolshoe Chebachie	$1.8\cdot10^{-6}$	-	-	-	-	-	-	-	-	-
Kokay	-	-	-	$1.3\cdot10^{-6}$	-	-	-	-	-	-
Yesey	$0.3\cdot10^{-6}$	-	-	-	-	-	-	-	-	-
Sultankeldy	-	-	-	-	-	-	-	-	-	-
Total amount of POPs accumulated in the bottom sediments of lakes	$2.16\cdot10^{-6}$			$1.3\cdot10^{-6}$		$0.04\cdot10^{-6}$	$0.6\cdot10^{-6}$	$0.26\cdot10^{-6}$		

unnamed lake in Akkol village (0.066 x 10^{-6} mg.kg^{-1}), Zhalanash (0.5 x 10^{-6} mg.kg^{-1}), Kokay (0.24 x 10^{-6} mg.kg^{-1}), DDT - in 4 Zhalauly lakes (2.3 x 10^{-6} mg.kg^{-1}), Tastykol (1.57 x 10^{-6} mg.kg^{-1}), Itemgen (3.05 x 10^{-6} mg.kg^{-1}), Zhalanash (2.34 x 10^{-6} mg.kg^{-1}), polychlorinated biphenyl - in 2 Zhalauly lakes (0.31 x 10^{-6} mg.kg^{-1}), Itemgen (1.87 x 10^{-6} mg.kg^{-1}).

For all studied Akmola region lakes, the POPs content was summed and their specific content identified in the bottom sediments in the region. It is possible to draw up the next POPs decreasing row: 54% (DDT) >12.71% (polychlorinated biphenyl) >12.59% (aldrin) >7.85% (hexachlorobenzene) >7.58% (chlordane) >3.49% (endrin) >1.51% (heptachlor) >0.23% (dieldrin).

The proposed POPs row in the Akmola region may be represented as a diagram (Fig.1).

Thus, the studied waters cannot be classified as toxic as the POPs content in the bottom sediments is of background character, even without exceeding the maximum allowable concentrations of these substances in the water. However, the presence of these substances in the water may indicate the threat growing as environmentally long persistent toxicants will continue to be accumulated by different migration routes, as they are in the water **(Gopal et al., 2016)**. As a result, this can be a serious obstacle to the production of organic products **(Kádeková et al., 2017)**.

Table 2b POPs content in the bottom sediments of the Akmola region lakes.

Lake name	Hexachlorobenzene (mg.kg^{-1})	Lindane, Hexachlorane (mg.kg^{-1})	Mirex (mg.kg^{-1})	Pentachlorobenzene (in quintozene (mg.kg^{-1})	Toxaphene (mg.kg^{-1})	DDT (1,1,1-trichlor-2,2-di (*n* -chlorophenyl) ethane (mg.kg^{-1})	Perfluorooctane sulfonate and its salts (mg.kg^{-1})	Endosulfan and isomers, tiodan (mg/kg)	Paraquat (mg/kg)	Polychlorinated biphenyl (mg/kg)
Zhalauly	$0.07 \cdot 10^{-6}$	-	-	-	-	$2.3 \cdot 10^{-6}$	-	-	-	$0.31 \cdot 10^{-6}$
Tastykol	$0.47 \cdot 10^{-6}$	-	-	-	-	$1.57 \cdot 10^{-6}$	-	-	-	-
Itemgen	-	-	-	-	-	$3.05 \cdot 10^{-6}$	-	-	-	$1.87 \cdot 10^{-6}$
Unnamed Lake in Akkol village	$0.066 \cdot 10^{-6}$	-	-	-	-	-	-	-	-	-
Unnamed Lake in Astrakhanka village	-	-	-	-	-	-	-	-	-	-
Zhalanash	$0.5 \cdot 10^{-6}$	-	-	-	-	$2.34 \cdot 10^{-6}$	-	-	-	-
Borovoe	-	-	-	-	-	-	-	-	-	-
Bolshoe Chebachie	-	-	-	-	-	-	-	-	-	-
Kokay	$0.24 \cdot 10^{-6}$	-	-	-	-	-	-	-	-	-
Yesey	-	-	-	-	-	-	-	-	-	-
Sultankeldy	-	-	-	-	-	-	-	-	-	-
Total amount of POPs accumulated in the bottom sediments of lakes	$1.346 \cdot 10^{-6}$					$9.26 \cdot 10^{-6}$				$2.18 \cdot 10^{-6}$

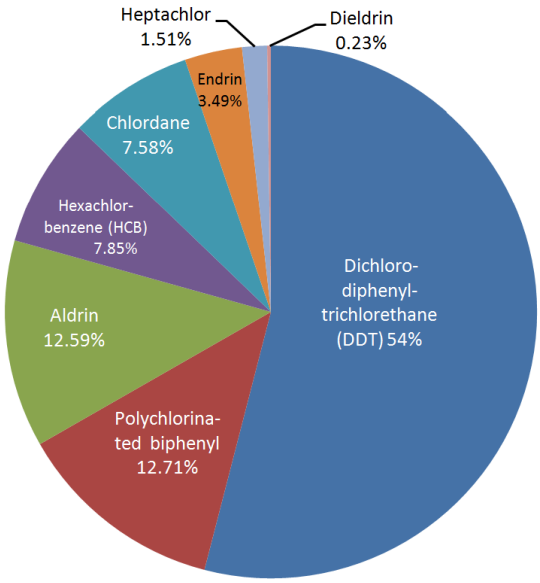

Figure 1 Generalized POPs share in bottom sediments of the Akmola region.

CONCLUSION

The obtained results of the work performed for 2016 allow for the following conclusions:

1. The MPC excess indicators in the Akmola region lakes are observed for salt ammonium (up to 0.002 MPC), magnesium (up to 1.15 MPC), nitrites (up to 1.12 MPC), petroleum products (up to 1.98 MPC), iron (up to 2.0 MPC), SSAS (up to 3.8 MPC). High concentration indicators for the sulphate (3.5 MPC), copper (4.3 MPC), magnesium (1.125 MPC). Mainly the lakes are dominated by sulfates, ion chloride. Lakes the most salty and with a hard water are Zhalauly and Zhalanash lakes - the water hardness of 7.5 $mg.dm^{-3}$ and 8.37 $mg.dm^{-3}$, respectively. For the studied water pollution index, the I class (relatively clean) can include Sultankeldy, Esey, Kokay waters; the II class (slightly dirty) - Zhalauly, Borovoye, Bolshoe Chebachie.

2. Thus, accordance with sanitary requirements, the studied waters cannot be classified as toxic as the POPs content in the bottom sediments is of background character, even without exceeding the maximum allowable concentrations of these substances in the water. However, the presence of these substances in the water may indicate the threat growing as environmentally long persistent toxicants will continue to be accumulated by different migration routes, as they are in the water.

3. But in general among 21 investigated POPs 8 substances are accumulated in bottom sediments of the investigated Zhalauly, Tastykol lakes, Unnamed Lake to the south of Akkol village, Itemgen lake, Zhalanash lake near Malinovka village (near Astana city), Kokay, Yesey, Bolshoe Chebachie.

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THE COMPOSITION AND CONTENT OF PHENOLIC COMPOUNDS IN TEA, GROWN IN HUMID SUBTROPICS OF RUSSIA

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ABSTRACT
The article presents the results of studies on the dynamics, qualitative and quantitative composition of phenolic compounds of tea raw materials and tea of new forms of selection of the Institute. The regularity in their synthesis by months is determined, which affects the quality indicators of the end product and necessitates blending to the repaired tea. The accumulation of tannins in tea raw materials depends on hydrothermal conditions, in particular, the amount of precipitation. The content of tannin increases from May to June, then there is some decline in their content, due to hydrothermal stress, slowing the synthesis of tannins in the tea leaf. The content of the water-soluble fraction gradually increases from May to July, and then there is a slight decline. It is shown that the accumulation of phenolic compounds in tea raw materials varies during the collection season. The contents of theaflavins increased from the beginning of the collecting sheet to its completion. The content of thearubigins showed peaks: the lowest rate in June, the highest in August. It was revealed that a sharp drop in the synthesis of thearubigins in June is associated with the onset of the summer dormancy of growth and synthetic processes. The synthesis of both indicators depends on meteorological conditions. The comparative analysis of the samples of tea raw materials collected from experimental plants is carried out. It is shown that the content of tannin and extractive substances in the raw materials of the studied varieties and mutant forms is high. In terms of the ratio of theaflavins and thearubigins, tea made from experienced raw materials meets international requirements. Determination of the qualitative composition of the catechin group of green tea, produced from raw materials of new forms, showed a high level of accumulation of the main groups of catechins.

Keywords: *Camellia sinensis*; cultivar; black tea; green tea; hydrothermal condition; tannin; extractive compounds; flavonoid; catechin

INTRODUCTION
Tea plant (*Camellia sinensis* (L.) O. Kuntze) is the most ancient crop. As a cultivated plant, tea has been cultivated in China since ancient times, since the XVIII century – in India and Sri Lanka, since the XVII century – in Russia, and since the XIX century – on vast areas in various parts of the globe. But now the only European tea producer is Russia, and the only place in Russia where tea is grown on an industrial scale is the humid subtropics of the Krasnodar region **(Ryndin et al., 2017; Belous and Platonova, 2018)**.

The mass distribution of tea is due not only to the fact that it is a pleasant, thirst-quenching drink, but to a large extent, and its effect on the human body. Active substances of tea are polyphenols, flavonoids, aromatic substances, vitamins, plant pigments, amino acids, mineral salts, etc. **(Willson, 1975; Gramza et al., 2005; Khan and Mukhtar, 2007; Belous, 2013)**. The tea contains more than 600 chemicals involved not only in the creation of specific properties of tea-aroma, taste and color of the infusion, but also the healing properties of the product **(Vorontsov, 1946; Mgaloblishvili and Tsutsunava, 1979; Salah et al., 1995; Khvedelidze and Gvinianidze, 2004)**. In the last decade, the interest in tea has increased, due to the content of polyphenolic compounds with antioxidant effect **(Salah et al., 1995; Wright, 2005; Platonova, Belous and Ostadalova, 2017; Platonova and Belous, 2018)**.

Industrial plantations of Krasnodar region are a mixture of different morphological groups, as laid seed populations of Georgian and Chinese origin. In this regard, plants differ in yield, growth ability, and most importantly, biochemical parameters that determine the quality of the finished product. Improving the varietal composition of tea plantations is one of the urgent tasks of tea growing. On the basis of the all-Russian research Institute of floriculture and subtropical crops for a long period of work on the culture of tea created varieties characterized by high yield and product quality (cv. Sochi, Karatum, Matsestinsky, Vano, etc.) **(Prokopenko and Tuov, 1994)**. But work in this direction continues. The presence at the Institute of

highly productive varieties, as well as promising clones and hybrids, are a reliable basis for the creation of modern tea plantations. This article is devoted to the study of the features of the composition and changes of the catechin complex of new forms of tea, selected by scientists of the Institute. We have determined the composition of the catechin complex in green tea, made from raw materials grown on the plantation of the Institute.

Scientific hypothesis

All over the world works on studying of a green tea leaf and transformations which proceed in it at technological processing are conducted, biochemical researches of raw materials and a finished product are carried out. The study of the content, accumulation and transformation of substances during growth, development of green leaf, it's processing, is necessary to improve the quality of raw materials and finished tea. The main purposes in tea biochemistry are the transformation and accumulation of phenolic compounds, as they provide the most valuable properties of tea. The compositions of phenolic compounds of tea are change and depending from season and the technology of tea processing.

MATERIAL AND METHODOLOGY

Objects of study were samples of green tea made from 2 – 3 leaf sprouts following promising forms: form No. 3823, No. 582, No. 855, No. 2264, grown for an experienced collector-uterine site, founded in 1984 – 1985 in the village of Uch-Dere (Sochi, Lazarevsky region). Control – cultivar Colchida.

Colchida is a clone of large-leaved Chinese tea, high-crop productivity, in 1995 entered in the State register of selection achievements. Form No. 3823 – source population Kimini, high gustatory qualities and yield. Form No 582 – the initial population of Colchida, the variety productivity is high, has a specific flavor infusion. Form No. 855 – the original Georgian population of the No. 8, the average crop productivity, specific aroma with a light rose fragrance. Form No. 2264 – the original Georgian population of the No. 15, crop productivity and quality indicators are above average **(Gvasaliya, 2018; Prokopenko and Tuov, 1994)**.

Green tea was produced in the laboratory of biotechnology, physiology and biochemistry of plants (BPhBP) of the Russian research Institute of floriculture and subtropical crops.

The study was performed on the chromatograph MiLiChrom A-02 (Institute of Chromatography "EcoNova", Novosibirsk, Russia), 2 × 75 mm column with a sorbent ProntoSIL 120-5-C18 AQ (Bischoff Analysentechnik und Geräte, Germany). Chromatograms were processed using a computer program AlphaChrom (Institute of Chromatography "EcoNova", Novosibirsk, Russia). Aqueous 0.2 M LiClO4 in 0.005 M HClO4 was used as eluent A, and acetonitrile (NPK Cryochrome, St. Petersburg, Russia) was used as eluent B. The following catechins were used as standards: catechin, epicatechin, gallocatechin, gallocatechin gallate, epigallocatechin, epicatechin gallate, epigallocatechin gallate, gallic acid, caffeine. All reagents were chemically pure or analytical grade and contained more than 98% of the main substance. Extraction method was based on well-known method **(ISO 14502-2:2005)** with some modifications. The dried tea leaf was homogenized in a mortar, sieved through a 0.5 mm sieve; a portion of 20 mg was placed in a 1.5 mL tube and extracted with 1 mL of ethanol 70% for 30 minutes at 70 °C. After extraction, the sample was filtered through a 0.45 μm filter and used for HPLC analysis.

The conditions of chromatographic analysis are: column temperature 40 °C, flow 200 $\mu L.min^{-1}$, detection 210, 220, 230, 240, 250, 260, 280, 300 nm. Elution was made in gradient mode from 0% to 40% B for 2800 μL. All measurements were performed in triplicate.

Flavonoids were determined on a PE – 5400 wi spectrophotometer at a wavelength of 665 nm (theaflavins) and 825.5 nm (thearubigins) **(AOAC International, 2009)**.

The tannin content was determined by the method of Levental with a conversion factor of 5.82 according to **Dzhemuhadze (1946)**, extractive substances – by the gravimetric method according to **Vorontsov (1946)**.

Statisic analysis

Statistical processing of the experimental data was carried out using the ANOVA package in STATGRAPHICS Centurion XV (version 15.1.02, StatPoint Technologies) and MS Excel 2007. Statistical analysis included univariate analysis of variance (method of comparing averages using variance analysis, *t*-test) and variance analysis (ANOVA). The significance of difference between the means of the least significant difference (LSD) results with $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate and the values were expressed as mean ±SD. The differences between the samples were assessed using unpaired *t*-test. Correlation analysis with calculation of pair correlation coefficient, for establish the dependence of parameters on abiotic factors was used.

RESULTS AND DISCUSSION

One of the main places among the substances that make up the tea leaves is a complex of tannins. All the basic properties of the finished product – its color, taste and aroma – are to varying degrees related to their transformations in the tea leaf. Tannins are the most mobile and active compounds, so they are primarily subject to certain changes under different growing conditions or processing of tea.

Studying the content of tannins in tea raw materials in the dynamics, we identified the main patterns in their synthesis by months, which significantly affects the quality indicators of the finished product and necessitates the blending of finished tea (technological method of mixing the semi-finished product to obtain a quality brand). We noted that the total content of tannins in the three-leaf flush varies during the tea leaf collection season (Figure 1).

As can be seen from Figure 1, the quantitative content of tannin in Russia's humid subtropics (unlike other tea-producing countries) increases from May to June, then there is some decline in their content, which is connected, in our opinion, with temperature and arid stress slowing down the synthesis of tannins in the tea leaf.

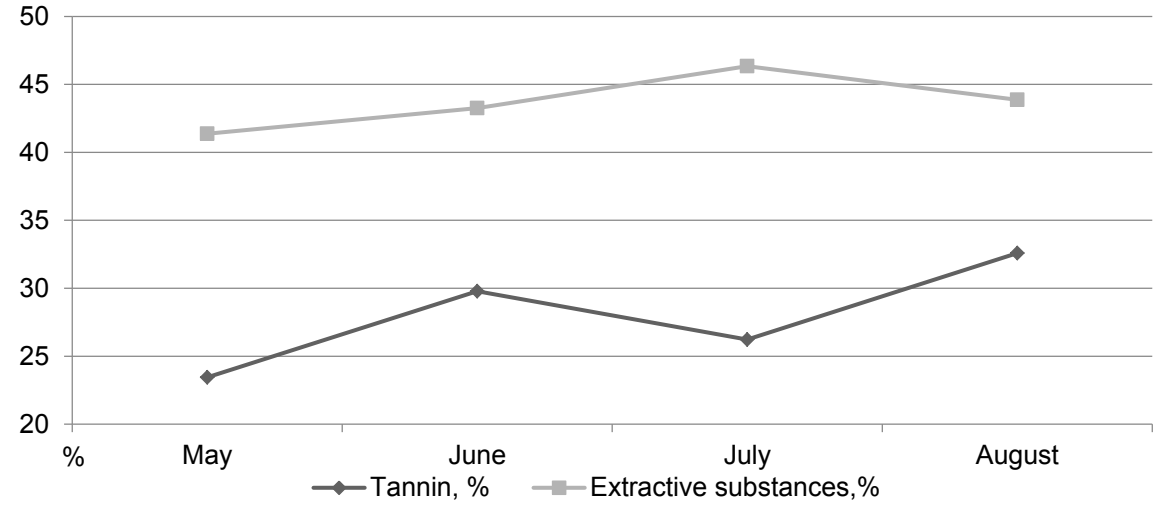

Figure 1 Seasonal changes in the content of tannins and extractive substances in tea raw materials, average over 11 years.

Table 1 Pair correlation coefficients between quality indicators of tea raw materials and hydrothermal factors, average over 11 years.

Parameter	Precipitation, mm	T, °C
Tannin, %	-0.88	-0.45
Extractive substances, %	-0.74	-0.35
Caffeine, %	-0.86	-0.54

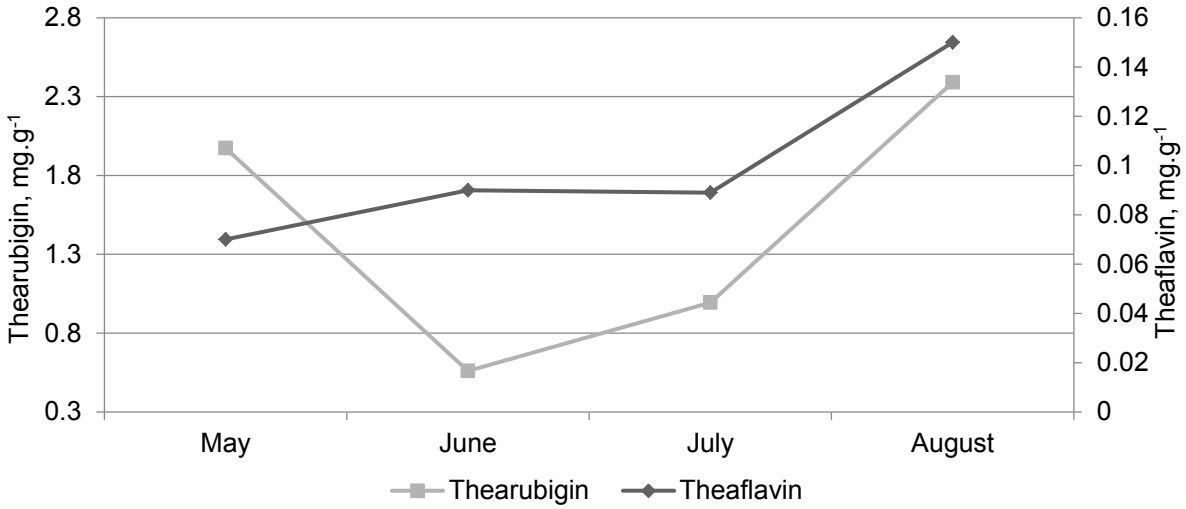

Figure 2 The content of flavonoids in the samples of finished tea made from plants of the variety Colhida, average over 2 years.

Table 2 Pair correlation coefficients between hydrothermal factors and flavonoid content ($p \leq 0.05$).

Parameters	**Theaflavins, mg.g^{-1}**	**Thearubigins, mg.g^{-1}**
Thearubigins, mg.g^{-1}	0.928	-
Temperature, ºC	0.860	0.184
Precipitation, mm	-0.776	-0.562

As the hydrothermal conditions reach their optimum, the tannin content is rapidly increasing, reaching an average of 30.29 – 32.58% in August.

Other dynamics were noted in the accumulation of extractive substances in the tea leaf (Figure 1). From May to July there is a gradual increase in the content of the water-soluble fraction, followed by a slight gradual decline.

Statistical processing showed that the accumulation of tannins in tea raw materials directly depends on hydrothermal conditions, and to a greater extent, not on temperature conditions of vegetation, but on the amount of precipitation (Table 1). Moreover, the relationship between quality indicators and climatic factors is opposite, as the strength of the factor increases, the content of tannins decreases significantly ($p \leq 0.05$).

Also, we were able to trace the dynamics of the formation of thearubigins and theaflavins in finished tea by months and their dependence on hydrothermal factors. It was established that the content of theaflavins increased from the beginning of the collection of the leaf (in May 0.07 $mg.g^{-1}$) to its completion (in August 0.15 $mg.g^{-1}$). Peaks were observed in the content of thearubigins: the lowest value in June was 0.56 $mg.g^{-1}$, the highest – in August, 2.39 $mg.g^{-1}$ (Figure 2).

The sharp decline in the synthesis of thearubigins in June may be due to the onset of a period of summer dormancy of growth and synthetic processes, especially since during the same period the accumulation of theaflavins reached a plateau - from June to July there were no changes in their content (Figure 2).

To establish the influence of hydrothermal factors on the accumulation of flavonoids, the relationship between these indicators was calculated (Table 2).

As can be seen from the given data presented in Table 2, the synthesis of both indicators is quite closely related, which is a well-known fact: it is precisely during oxidation that theaflavins quickly turn into thearubigins. At the same time, the accumulation of these flavonoids by the tea plant directly depends on meteorological conditions: as the air temperature rises and the amount of precipitation decreases, the content of both flavonoid groups increases. This may be due to a high concentration of cell sap and, as a consequence, activation of the antioxidant system of plants. Dispersion analysis indicates that it is theaflavins that closely correlate with hydrothermal factors, while thearubigins, being secondary metabolites relative to theaflavins, react poorly to the temperature factor. Rainfall are affecting the functional state of the plant itself **(Belous, 2008)**, it was provided an optimal water balance of cells and the synthesis of flavonoids. Flavonoids in the manufacture of tea, during oxidative processes, can indirectly affect the synthesis of thearubigins.

In addition to identifying dependencies and establishing the dynamics in the accumulation of tannins, we conducted a comparative analysis of samples of tea raw materials collected from experimental plants (Table 3). As can be seen from Table 3, the content of tannin and extractive substances in the raw materials of the studied varieties and mutant forms are high.

Tea produced from plants of mutant forms No. 582 and No. 2264 in terms of the content of theaflavins showed the highest values, while the highest content of thearubigins was observed in tea obtained from raw materials of the Colkhida variety and form No. 2264 (Table 3). Since theaflavins are unstable compounds and easily oxidize to thearubigins during oxidation, at present there is no single standard for their content in the finished product. But, according to international rules, any blend of tea should have a ratio of theaflavins and thearubigins not lower than 1:16, and in super tea 1:10. According to this indicator, all the tea produced in the laboratory of the institute`s laboratory from raw materials harvested from experimental plants complies with international requirements.

Determination of the qualitative composition of the catechin group in green tea, using the example of the most promising form No. 582 (Figure 3), showed that the largest amount of epigallocatechin gallate, which is characterized by the highest antioxidant activity of the main catechins (epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate), the prevalence of this group in selected forms of teas reflects their high value.

A comparison of tea in terms of the quantitative content of the catechol complex showed that tea of new forms of selection of the institute is characterized by a high level of accumulation of various groups of catechins (Table 4).

The variety Colchida, being a large leaf tea, is characterized by a higher content of EGKG, which is also confirmed by literature data. **(Haslam, 1989; Gzhidzhiechvili et al., 1984)**. At the same time, all selected forms contain a lower caffeine value. Despite the fact that the chemical connection between caffeine tea and tannins neutralizes its effect on the human body (compared to pure caffeine coffee), the lower content of theine in new forms of tea makes them more attractive against the background of a rich caffeine variety Colchida.

Table 3 Phenolic compounds of black tea and tea leaves (raw materials), average over 5 years.

Varieties, variety forms	Tannin, %	Extractive substances, %	Theaflavins $mg.g^{-1}$ *	Thearubigins, $mg.g^{-1}$ *
Camellia sinensis cv. Colchida	28.7 ±1.7	42.40 ±1.0	0.15 ±0.02	2.39 ±0.44
Camellia sinensis mf. № 3823	28.3 ±1.6	43.41 ±1.3	0.11 ±0.01	1.13 ±0.16
Camellia sinensis mf. № 582	30.1 ±2.1	43.92 ±1.9	0.16 ±0.01	2.04 ±0.49
Camellia sinensis mf. № 855	29.8 ±1.7	43.90 ±1.3	0.12 ±0.01	1.24 ±0.15
Camellia sinensis mf. № 2264	28.2 ±1.3	42.45 ±1.4	0.16 ±0.01	2.26 ±0.26
LSD ($p \leq 0.05$)	1.01	0.89	0.03	0.09

Note: * data over two years.

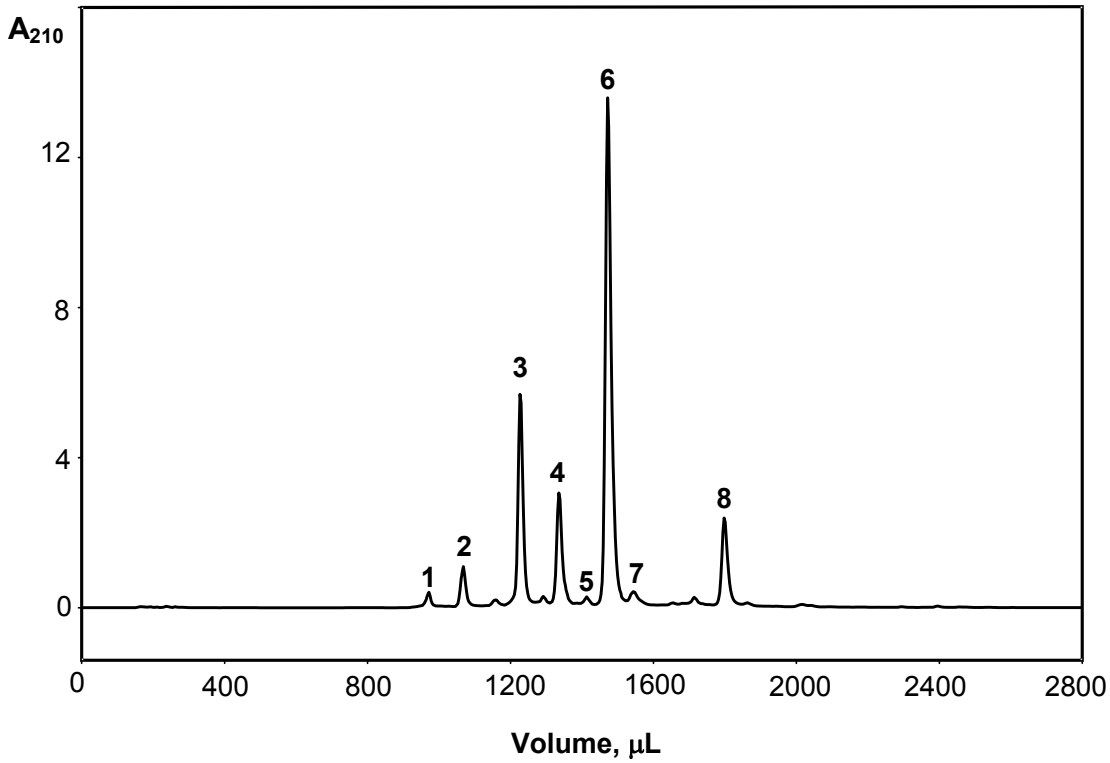

Figure 3 Chromatogram of green tea extract from sample No. 582. Note: the peak numbers correspond to: (1) gallic acid, (2) hallocatechin, (3) epigallocatechin, (4) caffeine, (5) epicatechin, (6) epigallocatechin gallate, (7) gallocatechin gallate and (8) epicatechin gallate.

Table 4 Pair correlation coefficients between quality indicators of tea raw materials and hydrothermal factors, average over 11 years.

Verity	Caffeine, %	GAK, %	EGKG, %	EKG, %	GKG, %	GK, %	EK, %	EGK, %
Camellia sinensis cv. Colchida	3.10 ±0.12	0.13 ±0.007	7.03 ±0.42	1.23 ±0.04	0.40 ±0.04	0.37 ±0.07	0.033 ±0.004	1.95 ±0.10
Camellia sinensis mf. № 3823	1.96 ±0.10	0.13 ±0.010	5.64 ±0.40	0.76 ±0.04	0.23 ±0.04	0.40 ±0.03	0.024 ±0.006	2.80 ±0.14
Camellia sinensis mf. № 582	2.62 ±0.10	0.17 ±0.009	7.90 ±0.50	1.24 ±0.07	0.28 ±0.02	0.50 ±0.07	0.026 ±0.006	3.33 ±0.15
Camellia sinensis mf. № 855	2.60 ±0.08	0.15 ±0.008	5.95 ±0.33	0.99 ±0.05	0.12 ±0.01	0.38 ±0.05	0.040 ±0.005	2.91 ±0.18
Camellia sinensis mf. № 2264	1.57 ±0.08	0.08 ±0.010	5.98 ±0.37	0.93 ±0.05	0.39 ±0.05	0.45 ±0.01	0.031 ±0.005	2.97 ±0.19

The closest to the control variety is green tea, produced from raw materials of mold No. 582. The low content of catechin group substances is characteristic of forms No. 3823, 855 and 2264.

CONCLUSION

Thus, we have studied the dynamics, composition and content of phenolic compounds in tea raw materials and tea of new forms of plant selection. It is shown that the total content of tannins in the three-leaf flush varies during the harvest season and the accumulation of phenolic compounds in tea raw materials directly depends on the hydrothermal conditions, in particular, on the amount of precipitation during the growing season. The dynamics of the formation of thearubigins and theaflavins in finished tea by months is traced; it has been established that the accumulation of flavonoids directly depends on meteorological conditions: as the air temperature rises and the amount of precipitation decreases, their content increases. A comparative analysis of black tea samples was carried out, which showed that, based on the ratio of theaflavins and thearubigins, tea collected from experimental plants complies with international

requirements. Determination of the qualitative composition of the catechin group in green tea showed that the greatest amount falls on epigallocatechin gallate and tea of new forms of selection of the institute characterized by a high level of accumulation of identified groups of catechins.

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NEUROMARKETING AND THE DECISION-MAKING PROCESS OF THE GENERATION Y WINE CONSUMERS IN THE SLOVAK REPUBLIC

Jana Němcová, Jakub Berčík

ABSTRACT

In recent years, interest in scholarly research on buying behaviour of Generation Y has grown. However, studies are mainly realized abroad and many of them deal with this issue in general. The purpose of this paper is to identify the factors influencing the decision-making process of the Generation Y customers in the selection of wine in the Slovak Republic. A total of 21 respondents participated in the survey. Eye-tracking and a questionnaire were selected for research. For processing and evaluating the eye-tracking research, the Gazepoint Analysis UX Edition software and Microsoft Excel were used. For statistical data analysis, the Kruskal-Wallis test and Spearman's non-parametric test were performed. Based on the results of the questionnaire and the testing, a label was the most important factor. Differences were noted at the moment of examining the information on a label. The most important factor determined by the questionnaire survey was variety, or vintage year, but by using measurement, the most important factor was label design. With regard to bottle shape, the most preferred was the Bordeaux type of bottle. Testing was carried out in laboratory conditions that only simulated the real selection of wine. This could have caused the difference between conscious decision-making and unconscious visual attention. Therefore, in the future, it is recommended to carry out similar research using a mobile eye camera to realize the test with real wine bottles. It is also assumed to involve other methods to obtain information about real attention of the tested probands. The presented research provides information for winegrowers and merchants who can improve their products and communicate effectively with customers. Findings are particularly beneficial because this research is among the first carried out in this area and it was not based only on conscious participation of respondents, but also on unconscious perception, because a deeper understanding of unconscious influences, that shape consumer's decision, helps to better understand consumer's behaviour.

Keywords: consumer behaviour; decision-making process; Generation Y; wine factor; Slovak Republic

INTRODUCTION

Many marketing specialists have been dealing with consumer behaviour for a long time to get the best possible placement of their products on the market. **Schiffman and Wisenblit (2015)** describe consumer behaviour as a set of consumer activities while searching for, purchasing, using, evaluating, and disposing of products or services that are expected to meet their needs. Businesses need to know the answers to questions such as how and why consumers buy and consume in order to develop their products and effectively communicate with customers **(Szmigin and Piacentini, 2015)**. The key point of marketing is the focus on the customer and his decision-making. Understanding the processes relating to these decisions is crucial to establishing a policy **(Sethna and Blythe, 2016)**. The consumer decision-making process consists of many theoretical constructs and is often complicated, as stated by **Roe and Bruwer (2017)**. According to **Tomek and Vávrová (2011)**, no company on the market knows in advance when and whether buyers will demand its products at all. In order to be successful sellers on the market, they must ensure that their activities meet the wishes and needs of target customers. Based on this, they must understand the mechanisms and determinants that influence the purchasing decisions of customers. Understanding the forecast of consumers' and purchasing behaviour is one of the main goals of marketing specialists.

Today's market has changed significantly. Sale is more difficult, in part due to more sophisticated buyers who have more information, more intense competition, a longer sales cycle, and increased resistance to traditional techniques. However, a modern approach using neuromarketing helps to create effective marketing strategies, thereby increasing motivation to purchase, the number of concluded contracts and the multiplication of

revenue **(Berčík et al., 2016)**. Neuromarketing is a newly emerging discipline discovered in 2002 that connects consumer behaviour with neuroscience. It provides modern methods for direct mind detection without the need for demanding cognitive or conscious participation **(Morin, 2011)**. Currently, neuromarketing is used for the commercial exploitation of neuroscience knowledge and tools which help companies to better understand consumer reactions to the various communication efforts connected with brands, products or services **(Ramsøy, 2015)**.

There is a large amount of wine products for retail and it is very difficult for the consumer to decide what to buy. However, there is no united approach or theory of wine consumer decision-making that describes consumer characteristics, product involvement, etc. **Lockshin and Corsi (2012)** add that a large number of published articles show growing popularity of wine and interest in consumer behaviour research, and provide a detailed analysis of articles divided by theme in their publication. According to **Babin and Harris (2016)**, consumers make decisions for the purpose of getting something of value and quality to improve their lives. Companies then try to offer consumers products and services, including experience, that will bring them such value.

Anchor and Lacinová (2015) mention that, for the consumer behaviour research on the wine market, it is important to know the motives for wine drinking. Identifying the importance of wine in the consumers' lifestyle and recognizing the situations in which the consume of wines by customers facilitate the understanding of the issue of consumer purchasing decisions. According to **Marinelli et al. (2014)**, understanding the approach of purchasing wine is an important factor for the winemaking industry. Understanding these attitudes, particularly among the younger generation, can be useful for solving the long-standing traditions of the wine region. At present, many marketers deal precisely with the needs of young people aged between 18 and 34, called Generation Y or Millennials **(Schiffman and Wisenblit, 2015)**. In recent years, this generation has also been intensively studied in relation to the wine market **(Mueller et al., 2011)**. In general, Generation Y is defined as the age cohort born in the 1980s and 1990s. Some authors define this segment as people who were born slightly later in time, for example **Sethna and Blythe (2016)** describe this generation as a generation of people born between 1981 – 2001 (aged between 17 – 37 in 2018). This generation cohort has an increasing interest in wine, it is willing to pay for wine and its consumption is different from previous generations **(Hall et al., 2004; Thach and Olsen, 2006)**. It is a very promising wine consuming segment, which will become more and more important in the future as wine consumers **(de Magistris et al., 2011; Charters et al., 2011)**. **Chrysochou et al. (2012)** are inclined to think that young Generation Y is quickly adopting wine as its preferred drink and is therefore a consumer segment with the potential to increase the growth of wine consumption in the near future. Taking into account the differences among age groups will bring new opportunities for winegrowers and wine merchants. Also **Fountain and Lamb (2011)** highlight the need to promote an interest in wine among the younger generation. This results from the fact that the Baby Boomer generation, for a long time perceived as the leading wine consumers, is ageing and, therefore, it is now necessary to pay more attention to young consumers in the wine industry. Generation Y is currently considered one of the largest demographic groups. However, there are also differences within generations, especially among these young people who behave very differently and therefore cannot even generalize this cohort **(Bergh and Behrer, 2016)**. Therefore, only a narrower part of this generation has been selected for consumer behaviour research on the wine market in Slovakia, namely young people aged between 18 – 25.

There are many foreign studies in which authors deal with the purchasing behaviour of Generation Y in relation to wine. As far as the importance of attributes is concerned, this issue was most often explored by **de Magistris et al. (2011); Elliot and Barth (2012); Dlačić and Kadić-Maglajlić (2013); Stanciu and Neagu (2014); Hristov and Kuhar (2015)** and **Lategan et al. (2017)**. In the Czech Republic, the area of consumer behaviour and Generation Y is solved by **Veselá and Zich (2015)** who describe the effect connected to the country of origin and its influence on the consumer behaviour of the Czech and Slovak young consumers, and **Velčovská (2018)** who examines perception of product origin and its labelling in the context of food quality and safety among Czech, Slovak and Polish Generation Y. However, there is a lack of research applied to Generation Y and wine market. Scientific literature on wine Generation Y is very rare also in Slovakia. This study should therefore contribute to the expansion of literature on this issue.

Wine is one of the most consumed beverages and has been known in human civilization for many millennia. Besides its economic importance, it can also positively influence human health. In some European countries, a glass of wine is intrinsically linked to good food **(Tariba, 2011; Brunner and Siegrist, 2011)**. There are many studies that confirm the usefulness of wine when consumed in low quantities. The moderate consumption of this traditional drink may have positive effects, for example, in cardiovascular diseases, diabetes, osteoporosis or neurological diseases **(Artero et al., 2015; Šamánek and Urbanová, 2013)**.

The wine market has made significant progress over the last two decades. Both the legislation and the level of technical equipment for wine production have improved. According to The International Organisation of Vine and Wine **(OIV, 2017)**, world wine consumption has a relatively stable character over the long term. Although there was a slight decline in wine consumption in France last year, other traditionally wine consuming countries such as Spain and Portugal notified a stable consumption. On the contrary, there was a significant increase in wine consumption in Italy. The USA also showed a growing domestic consumption and once again confirmed its excellent position on the global wine market.

The Slovak Republic is not part of the world's significant producers or consumers of wine. However, the wine-growing tradition of this country has deep roots and vine growing on the territory of today's Slovakia is connected with an interesting history. **Regulation No 313/2009** divides the Slovak wine-growing region into 6 areas, namely Malokarpatská, Južnoslovenská, Stredoslovenská,

Nitrianska, Východoslovenská and the Tokaj wine-growing area. The total area of vineyards currently stands at 10,800 ha, of which the cultivated area of vineyards represents 8,872 ha. In 2015, the total wine consumption was 80,210 hL, which was 14.8 hl per capita. In 2016, there was a slight decrease and the consumption of wine per capita fell to 13.9 hL. The production of wine reached 310,000 hL for the period 2016/17, but in the following year the production of wine is expected to increase to 340,000 hL. The countries with the largest import of grapes and wine to Slovakia include Hungary, Italy and Germany. On the other hand, Slovakia most often exports grapes and wines to the Czech Republic and Hungary **(Meravá, 2017)**.

Scientific hypothesis

As mentioned above, many authors are concerned with consumer behaviour on the wine market. However, these surveys are aimed at all age groups, and thus there are few publications describing behaviour of the young generation. Therefore, this article is concentrated on the still often marginalized Generation Y and its decision-making process in the selection of wine in the Slovak Republic. The main objective of the article is to reveal the factors that have a crucial influence on decision-making of the Generation Y customer when choosing wine in Slovakia. The purpose of research is to identify the wine attributes by which young customers choose wines, and thus to understand their purchasing decision-making process. In relation to the hypothesis, the *p*-value $p = 0.05$ was used, where we tested whether there is difference in tracking wines based on location in the image stimulus (whether it is located on the right, left or centre). Using the *p*-value $p = 0.05$, we verified the dependence between first tracking and time spent.

MATERIAL AND METHODOLOGY

For this research, a neuromarketing method called eye-tracking was chosen, which explores unconscious visual attention of the respondents. Specifically, the Gazepoint GP3 eye-tracker (Gazepoint Canada) with data collection frequency of 60 Hz was selected – the respondent was sitting in front of the computer screen and was watching the images presented. Eye-tracking testing was then completed by a questionnaire that was conducted electronically using a cloud storage service called Google Drive. For the purposes of this research, which ran from April to May 2017, the Laboratory of Consumer Studies at the Faculty of Economics and Management of Slovak University of Agriculture in Nitra was used.

Testing was attended by a total of 21 respondents who subsequently completed a questionnaire containing questions that resulted from eye-tracking testing. The aim of the questionnaire was to find information about wine preferences given consciously by the respondents. Subsequently, it was examined whether there are differences between conscious (questionnaire) and unconscious (eye-tracking) perceptions of marketing stimuli.

This research was based on the theoretical background and consultation offered by the specialist staff of the wine shop in Zlín in the Czech Republic. Before the research itself, different combinations of wine bottles had to be created, which were then professionally photographed and adjusted. Each photo contained 3 wine bottles. Variants were drawn up in combinations by brand, variety and discount, then by brand, variety and quality class, by bottle shape and by bottle that was awarded medal. Measurement included 5 photos with white wine. Prepared photos were then uploaded into the software program and were projected to the respondents in the eye-tracking testing. The factors influencing customers' decision-making in the wine selection included: colour of wine, label design, the information on a label (brand/producer, variety, vintage year, quality class of wine, wine-growing region, residual sugar content, actual alcoholic strength and nominal volume), bottle shape, bottle colour, medals, price and discounts.

Three research questions were defined:

RQ1: What are the key factors that influence conscious decision-making of Generation Y on the wine market?

RQ2: What are the key factors that influence unconscious decision-making of Generation Y on the wine market?

RQ3: Is there a difference between conscious and unconscious perceptions of marketing aspects when choosing wine among Generation Y?

The Sample of the Respondents

Eye-tracking was attended by 21 respondents who filled out a questionnaire immediately after the testing. The purpose of the questioning was to find the conscious answers to the questions. These were the respondents aged 18 – 25 who had drunk or at least bought wine for the last 12 months. According to **Noble et al. (2009)**, precisely this age category is an important segment in the lucrative college market. Eye-tracking was conducted using a stationary eye camera, and following filing of a questionnaire was carried out electronically using a cloud storage service called Google Drive.

As shown in Table 1 below, 11 women (52%) and

Table 1 Sample characteristics.

Sample characteristics		Number	%
Gender	Female	11	52
	Male	10	48
Age	19 years	1	5
	20 years	5	24
	21 years	11	52
	23 years	1	5
	25 years	3	14
Social status	Student	21	81
	Employee – part time job	5	19
	Employee – full time job	0	0
	Self-employed	0	0
	Unemployee	0	0
Region	Bratislavský	0	0
	Trnavský	2	10
	Nitriansky	14	67
	Trenčiansky	2	9
	Žilinský	2	9
	Banskobystrický	1	5
	Prešovský	0	0
	Košický	0	0

10 men (48%) were among the total sample of the respondents. Regarding the age of the participants, 5% were 19 years old, 24% were 20 years old, 52% were at the age of 21. 5% at the age of 23, and 14% were 25 years old. Most of the participants were students (81%) and then 19% (5 respondents) employed in part-time. The majority of the respondents (67%) came from the Nitriansky region, then Trnavský (10%), Trenčiansky (9%) and the Žilinský region (9%) and only 1 respondent (5%) lived in the Banskobystrický region.

Statistic analysis

For processing and evaluating eye-tracking research, the Gazepoint Analysis UX Edition software – version 3.1.0 (Gazepoint Canada) and Microsoft Excel were used. Timing for switching individual slides was set to 15 seconds. For the statistical data analysis, inductive statistics was selected, namely the Kruskal-Wallis test and Spearman's non-parametric test. The statistical processing was carried out in R version 3.5.1 (R Core Team (2017)). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria **(R Foundation, 2017)**.

RESULTS AND DISCUSSION

As the testing was conducted in laboratory conditions through graphic visuals on which 3 wines were displayed at the same time, the detection of the actual perception of individual attributes was preceded by a test, which verified whether it depends where the product (wine) is located in the picture (right, centre, left) in order to find out the influence of the respondents. Based on the data derived from an eye camera (Time to first view in (ms)), the Kruskal-Wallis test, which is a non-parametric one-way anova, it was discovered that there is no difference ($p = 0.44$) in tracking wines based on location in the image stimulus (whether it is located on the right, left or centre).

The correlation between all dates of individual wines viewing time, measured using the Spearman's non-parametric coefficient, represents a value (-0.77), which is a strong negative dependence, which means that the more people watch the wines on the left, the more time raises the other two wines (centre and right), respectively, when they look at the first wine on the left, other wines have a longer viewed time (Viewed Time in (s)).

In the questionnaire, a Likert's five-point scale, ranging from 'completely agree' (1) to 'completely disagree' (5) with influence of factor, was used for the identification of the key factors influencing conscious decision-making of Generation Y on the wine market. Based on the answers of the Generation Y respondents recorded in the questionnaire and the relative calculated score, label and data associated with it (e.g. brand, variety, quality class etc.) appear to be the most important factor influencing the wine selection. According to the answers and the score achieved, bottle shape (16) and bottle colour (-8) are the least important factors, as can be seen in Figure 1.

The finding that the label is the most important factor when choosing white wine is also confirmed by the measuring through an eye camera. Based on the eye-tracking curves (fixation points), it is possible to see in Figure 2 that the primary views led precisely to the label. This fact is also confirmed by the areas of interest in Table 2, where the lowest value (1.69 ms) is based on elapsed time to the first fixation (view) and the calculated median.

Similarly, in the case of the length of time spent (Table 3), it can be stated that the longest visual attention based on the measured median values happened in the case of a label (3.19 s). Contrary to the answers of the questionnaire, the fact is that the probands were more interested in bottle shape/colour compared to 'price', or 'discount'. The median of elapsed time when they were tracking bottle shape/colour is 1.94 ms, while for prices it is up to 5.36 ms. In terms of time spent, bottle shape/colour even represented the longest viewed attribute (3.63 s), as can be seen in Table 3.

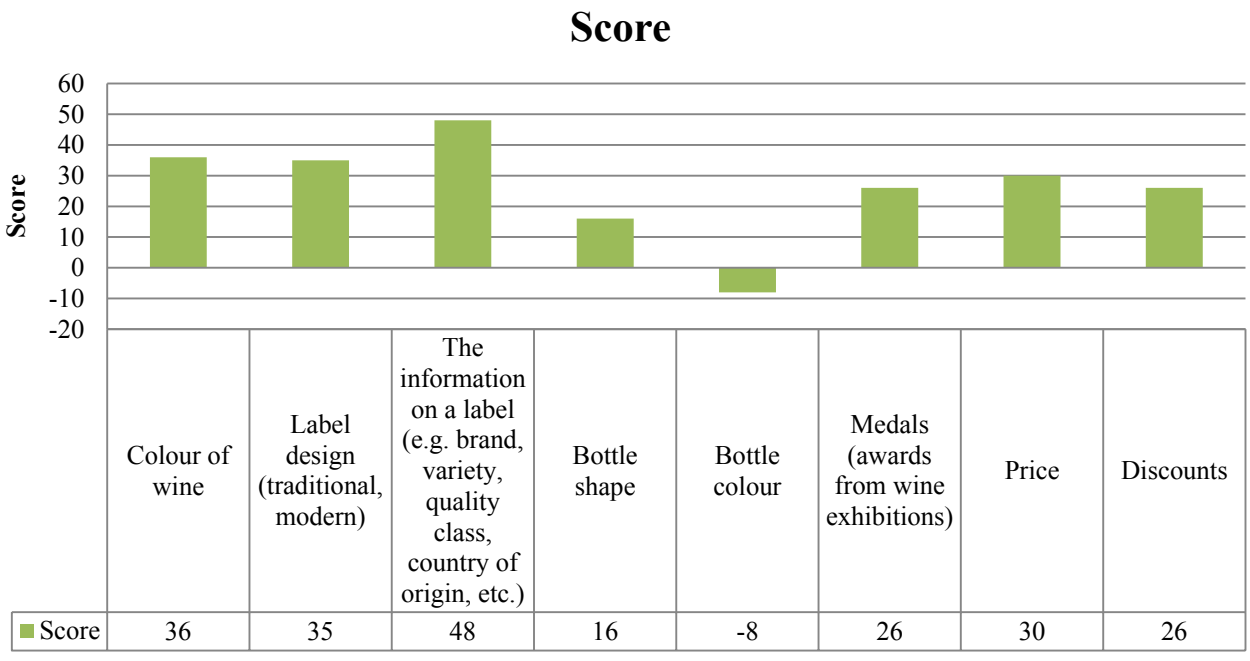

Figure 1 Factors Influencing the Bottled Wine Selection of Generation Y.

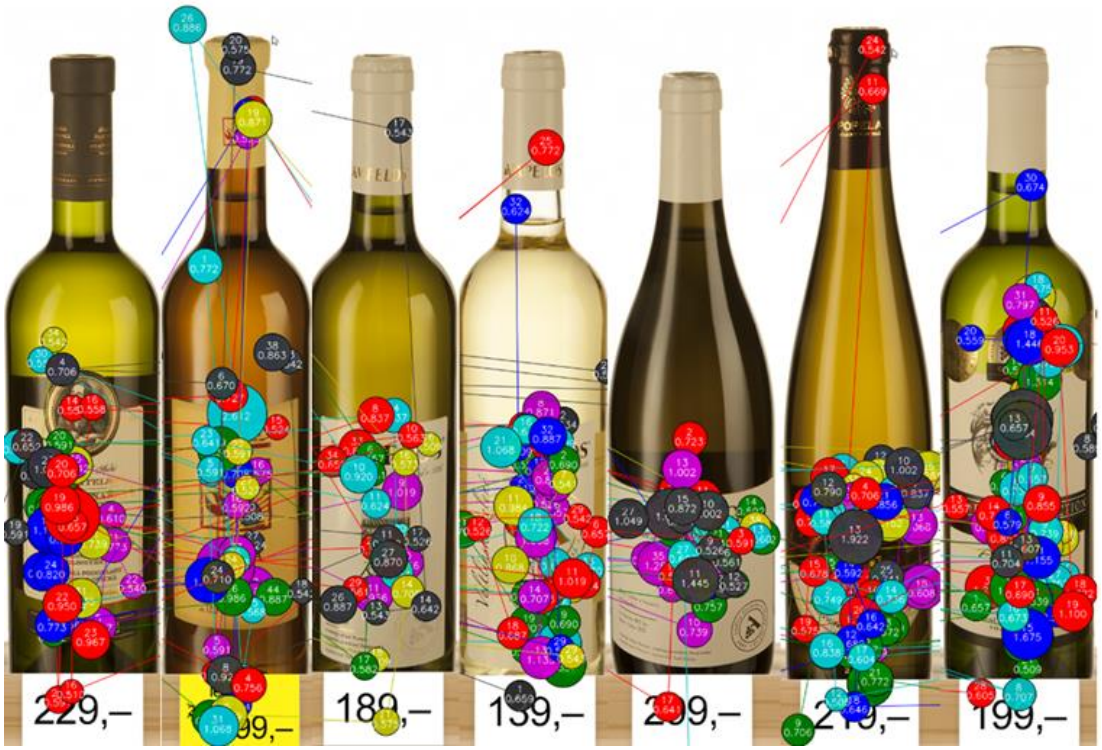

Figure 2 Eye-tracking Curves of Tested White Wine Visuals.

Table 2 Comparison of Perception of Individual Attributes Based on Areas of Interest (AOI) – Time to First Fixation (ms).

Time to first view			
Product	**Label**	**Bottle shape/colour**	**Price/Discount**
Šlechtitelská stanice vinařská Velké Pavlovice	2.77	2.69	5.36
Vinařství Gréger	0.87	0.36	4.10
Vinařství Ampelos (green bottle, classic)	3.69	3.12	6.61
Vinařství Ampelos (white bottle, classic)	0.90	0.69	4.39
Vinařství Ampelos (gray-green bottle, modern)	1.44	1.10	5.57
Vinařství Popela	1.69	2.18	4.15
Vinex	2.23	1.94	7.71
Average	**1.94**	**1.73**	**5.41**
Median	**1.69**	**1.94**	**5.36**

Table 3 Comparison of Perception of Individual Attributes Based on Areas of Interest (AOI) – Viewed Time (s).

Viewed Time (s)			
Product	**Label**	**Bottle shape/colour**	**Price/Discount**
Šlechtitelská stanice vinařská Velké Pavlovice	2.81	3.39	0.33
Vinařství Gréger	2.42	3.63	0.61
Vinařství Ampelos (green bottle, classic)	1.53	2.20	0.25
Vinařství Ampelos (white bottle, classic)	3.74	4.21	0.39
Vinařství Ampelos (gray-green bottle, modern)	3.19	3.53	0.39
Vinařství Popela	3.55	4.14	0.58
Vinex	5.11	5.58	0.27
Average	**3.19**	**3.81**	**0.40**
Median	**3.19**	**3.63**	**0.39**

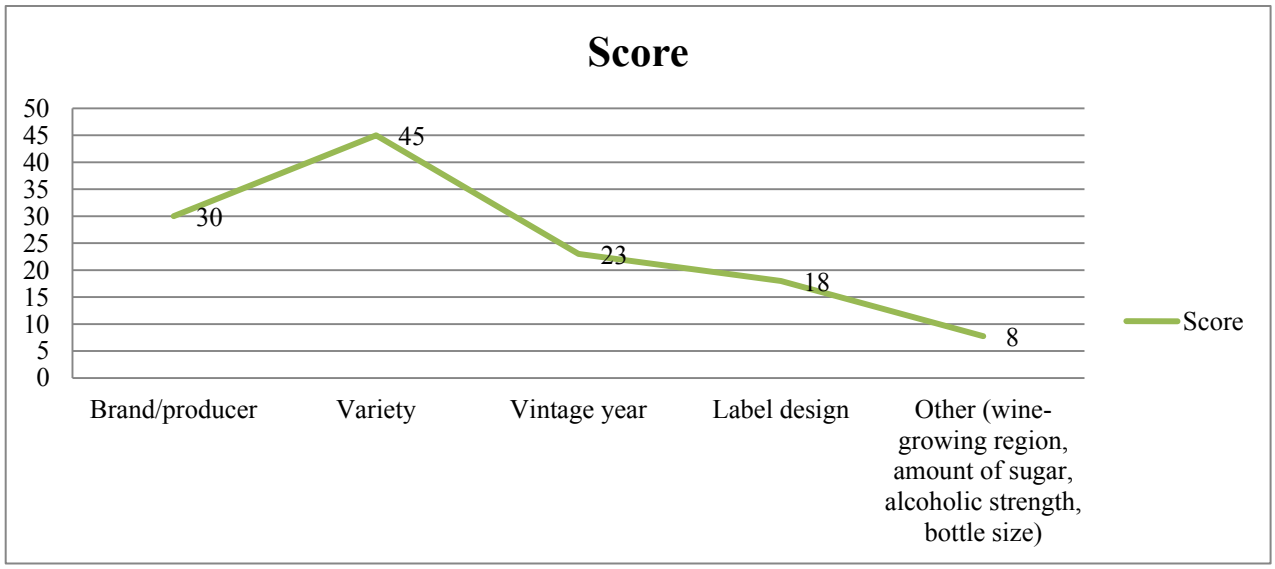

Figure 3 Importance of the Information on a Label Based on the Questionnaire Survey.

Based on conscious feedback (Figure 3) and the calculated score (45), indication of variety (e.g. Grüner Veltliner) is the most important information on a wine label. On the other hand, according to the respondents' answers, data such as a wine-growing region, the amount of sugar, alcohol, and bottle size (the 'other' option) are the least important.

However, in terms of visual attention and overall time spent, they were interested in the whole label design, in which they spent up to 3.585 s based on the measured median values. In this context, up to 76% of the respondents say that they prefer the traditional look of the label (motives of grapes, the grapevine or other wine theme) compared to the modern ones (geometric patterns, spirals, and non-traditional colours). Variety and vintage year interested the respondents only 1.140 s based on the median, which is 0.435 s less than brand. Based on the time spent, it can be said that consumer was most interested in total label design, brand and then variety or vintage year (Table 4). This fact and the difference over conscious feedback (variety was the most important) could also be caused by the fact that it was not a real choice and consumers saw the stimuli only in visual form.

Regarding bottle shape, the Generation Y respondents reported the most Bordeaux type of a bottle (62%) in the questionnaire (see Figure 4), which is often used primarily for red wine. A minor part of the respondents (5%) preferred Burgunder type (a bottle which is smaller and a little dumpy with dent), which is used all over the world for white and red wine. Based on the measurement of

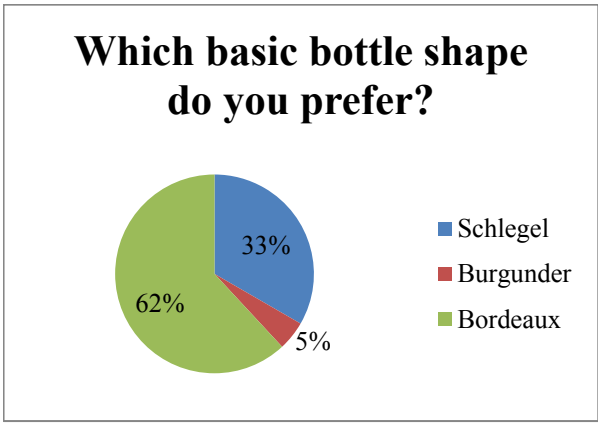

Figure 4 Wine Bottle Preference Based on the Questionnaire Survey.

Table 4 Comparison of Perception of the Information on a Label through Areas of Interest (AOI) – Viewed Time (s).

Product/ attribute	Šlechtitelská stanice vinařská Velké Pavlovice	Vinařství Gréger	Vinařství Ampelos (green bottle, classic)	Vinařství Ampelos (white bottle, classic)	Vinařství Ampelos (gray-green bottle, modern)	Vinařství Popela	Vinex	Median
Variety and vintage year	1.21	0.99	0.53	1.07	1.22	1.91	1.49	**1.140**
Brand	1.68	1.33	1.25	1.47	2.21	1.94	2.66	**1.575**
Price	0.63	0.23	0.29	0.39	0.38	0.40	0.25	**0.385**
Label design	3.33	3.59	1.97	4.32	3.60	4.25	5.60	**3.595**
Bottle cap	0.27	0.57	0.28	0.49	0.35	0.40	0.26	**0.375**

visual attention through an eye camera, it is possible to say that wines from brands such as Vinařství Ampelos (Time to first fixation 0.53 ms), Šlechtitelská stanice vinařská Velké Pavlovice (Time to first fixation 0.63 ms), Vinařství Gréger (Time to first fixation 0.99 ms), which are filled in Bordeaux type of bottles, attracted the majority of attendants' attention.

CONCLUSION

The submitted paper is focused on the examination of the factors influencing the selection of white wines, by means of a questionnaire survey and biometric method – eye-tracking. The most important factor influencing decision-making of Generation Y can be considered a wine label based on the results of the performed test. In the real selection, it is possible to assume that the most important factor would be price, or discount, that was greatly ignored in this case, because it was not a real scenario. In the case of a detailed examination of the information on a label, differences between the respondents' answers and real visual attention were noted. While the participants considered variety, or vintage year as the most important factors, on the other hand, on the basis of the answers of the questionnaire, they were most concentrated on label design and subsequently on the brand of the wine on the basis of visual attention. In the case of bottle preferences in which wine is stored, the respondents' answers also coincided with their real attention in terms of primary attracting. Despite some weaknesses, this test shows that the use of an eye camera when examining the factors influencing consumer choice has a great potential because it can provide a new view of the decision-making process based on real data.

In the future, it is planned to carry out similar research using a mobile eye camera to realize the test with real wine bottles. It is also assumed that engaging of other methods will be possible (e.g. biometric methods of recognizing micro-emotions based on facial expressions – Facereading) in order to obtain information about real attention of the tested probands due to the individual attributes that influence their selection. Based on the statistical tests, it was found out that wine to which the respondents look at first has the least viewed time compared to the other two wines, and so it would be good to use a balancing block, such as Latin squares, to prevent this effect, which should be a subject for further experiments. Latin squares are one of the CRBD systems (Complete Randomized Block Design). The first evaluator gets the ABC order, the second BCA, the third CAB (Williams Latin square).

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RHEOLOGICAL AND FUNCTIONAL PROPERTIES OF ROSELLE (*HIBISCUS SABDARIFFA*) LEAVES PUREE

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ABSTRACT
Pureed form of leaves (*Hibiscus sabdariffa* L. (Roselle)) was taken for physicochemical and rheological analysis at temperatures and TSS range of 278 K – 318 K and 3 – 5 °Brix respectively. The steady-state rheological analysis was performed with a shear rate of 1 – 100 $s^{-1.}$ Different rheological models are tried; Power-law was best fitted with the experimental data (R^2 ≥0.98). Temperature dependence of viscosity was found out using an Arrhenius-type relationship at a shear rate of 10, 50, 100 s^{-1} IR analysis was done to know the influence of functional groups on rheological properties of purees. Consistency index (K) of puree increases with increase in TSS content but at a fixed TSS, there is a decrease in K with an increase in temperatures but the opposite was observed for flow behavior index (n). Puree showed a shear thinning behavior with an increment in temperature level and puree having 5 °Brix (8.37) has higher activation energy ($kJ.mol^{-1}$) than 3 °Brix (6.32).

Keywords: Roselle leaves; puree; TSS; temperature

INTRODUCTION
Roselle (*Hibiscus sabdariffa*) locally known as gongura, belongs to the *Malvaceae* family and are cultivated in large quantities in the tropical regions of Asia, Africa and Central and South America. It is an annual or perennial herb with leaves is 8 – 15 cm long and profoundly 3 to 5 lobed and arranged alternately on the stem (Figure 1). These leaves are most commonly used by the people as curries, potherbs and mixed green salad **(Adegunloye et al., 1996)**. Roselle consumed throughout the southern part of India, is one of the unique dishes in many eateries, hotels, restaurants and food joints, and often called as the king of all Andhra food. The leaves are used for making pickles and chutney and are often named as gongura pickle, ambadi, and chutney. It is a very good source of folic acid and iron **(Sutton, 2004)**. The high iron content imparts the leave sourness and bitterness. It is a rich source of minerals, organic acids, vitamins, and antioxidants, which have distinctive medical benefits such as antiscorbutic, diuretic impacts, emollient, narcotic and many more uses **(Ramakrishna et al., 2008)**.

Fresh Roselle leaves are perishable in nature and the quality of the leaves deteriorates due to microbial and physiological activities during the period of storage and transportation and hence requires immediate processing and preservation **(Singh et al., 2014)**. Minimally processed state in form of puree has been very attractive in processing, handling, storing and selling. These are easily consumable and hence, there is a need for methods to preserve and maintain the quality of purees within fresh form until consumed by the customer. As puree is a mixture of soft particles in serum or viscous gel and finds uses in as topping, seasoning and as recipes for the fast-food industry **(Colin-Henrion et al., 2007)**.

Flow properties of purees product are of extensive interest for the development of new product items for manufacturing, quality control, and engineering applications **(Bayod, 2008; Alvarado and Romero, 1989; Rao et al., 1981)**. Also, there is a need to characterize the effect of rheological properties during food handling operations and storage period. With increasing demand, and its commercial importance of puree in our day to day activity, the present research was conducted: a) to assess the physicochemical properties of the puree, b) to assess the change in the rheological behavior of the purees and try to decide a best rheological model to the concerned flow curves obtained during rheological analysis, c) study the effect of various parameters such as TSS (3 – 5 °Brix), pH (1.3 – 5.3), and temperature (278 K – 318 K) on the rheological behavior of puree concentrate.

Scientific hypothesis
Temperatures, TSS and pH are the important parameters, which affect the rheological properties of Roselle leaves puree.

MATERIAL AND METHODOLOGY

Preparation of blanched puree

Roselle leaves were purchased from the local market of Raipur (Chhattisgarh, India), washed thoroughly with running water, and then destemmed. Leaves were blanched at 363 K for 5 min. Efficacy and duration of blanching were determined by peroxidase inactivity test. Chilled leaves after removing excess amount of water were grounded using a mixer grounder (Bajaj, India) at 298 K for 15 min with the addition of distilled water. The grounded puree was filtered through filter paper (Whatman, 125 mm). Uniform consistency of the puree was maintained by passing through a 12-mesh screen. The lab size rotary evaporator (Cole-Parmer, India) prepared the puree concentrates of different TSS at 323 K and 160 mm Hg pressure. Samples of the desired range of TSS (3, 4.35, 5 °Brix) were obtained by diluting with appropriate amounts of distilled water.

Physico-chemical analysis

Moisture content was determined as per **Garcia-Segovia et al. (2010)** at 333 K on a wet basis. Ash content was calculated as per the standard method of **AOAC (1984)**. Chlorophyll content was analyzed using the method described by **Arnon (1949)**. The ascorbic acid content of the puree was found out by titration method **(Ranganna, 1986)**. Titratable acidity was calculated in terms of citric acid equivalent as per the process proposed by **Wang et al. (1995)**. TSS (°Brix) and pH was measured using digital refractometer (Atago, Japan) and pH meter (Handy) respectively. The acidic pH (1.3 – 5.3) of purees was maintained by using a buffer for physicochemical and rheological analysis.

FTIR analysis of Roselle puree

The spectra of puree are used for analysis by FTIR instrument (Bruker, Germany) in the frequency ranging from 500 cm^{-1} to 4000 cm^{-1}. The functional groups present in the puree are identified using the spectral data obtained with the reference.

Rheological measurement

The rheological test was conducted in a modular compact Rheometer MCR 102 (Anton Paar, Germany), mounted with ST22-4V-40 (four-bladed vane geometry). The temperature of the cup was varied from 278 K – 318 K, for various experimental runs. The experiment was performed within shear rate (1 – 100 s^{-1}). The rheological estimations were investigated utilizing the Rheoplus software package of Anton Paar.

Rheological models

Numerous factors influence the selection of a rheological model to describe the flow behavior of a particular fluid. In this work, the experimental values of shear rate and shear stress were fitted to the various non-Newtonian models as listed in Eq. (1 – 6).

Power Law: $$\tau = K(\gamma)^n \quad (1)$$

Herschel-Buckley: $$\tau^{0.5} = \tau_0 + K(\gamma)^n \quad (2)$$

Casson : $$\tau^{1/2} = \tau_0^{1/2} + K\gamma^{1/2} \quad (3)$$

Heinz-Casson: $$\tau^n = \tau_0 + K\gamma^n \quad (4)$$

Vocadlo: $$\tau^{1/n} = \tau_0^{1/n} + K\gamma \quad (5)$$

Mizrahi- Berk: $$\tau^{1/2} = \tau_0^{1/2} + K\gamma^n \quad (6)$$

where, τ , γ , K, n, τ_0 is shear Stress (Pa), shear rate (s^{-1}), consistency index ($Pa.s^{-1}$), behavior index (dimensionless), yield stress (Pa), respectively.

Statisic analysis

The selection of most suitable model among these, for prediction of the flow behavior of puree, was investigated taking account of the statistical parameters, root means square error (RMSE) (Eq. (7)) and coefficient of determination (R^2) (Eq. (8)). The fitted data, which showed the lowest values for RMSE and highest values for the R^2, was considered as the best-fit model. ANOVA and t-test were used to determine the mean differences. The significant difference was defined at p <0.05 with 95%

Figure 1 Photo of Hibiscus Sabdariffa leaves attached with stem part.

level of confidence.

The average value of all the experimental data was analyzed using statistical parameters including Root mean square error (RMSE) and coefficient of determination (R^2) were determined and were defined as,

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N}(EV - MV)^2}{N}} \quad (7)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{N}(MV - EV)^2}{\sum_{i=1}^{N}(EV - EV_{avg})^2} \quad (8)$$

Where EV is the experimental value, MV is the modeled value EV_{avg} is the average of the experimental value and N is the number of observations.

Effect of temperature on apparent viscosity

Temperature dependency on its viscosity was determined using Arrhenius model (Eq. (9)) **(Sengül et al. 2005)**.

$$\eta = \eta_0 \cdot \exp\left(\frac{E_a}{RT}\right) \quad (9)$$

Where, η_0 is the proportionality constant, E_a the activation energy (kJ.mol^{-1}), R the universal law gas constant (J.mol^{-1} K^{-1}), and T the absolute temperature (K).

Effect of TSS on activation energy

Two models, namely, the power law Eq. (10) and exponential model Eq. (11) were used to describe the variation of the activation energy with the TSS, as given below.

$$E_a = a(C)^b \quad (10)$$

$$E_a = a \exp(bC) \quad (11)$$

Where, a (Empirical constant (kJ.mol^{-1})), C (TSS (°Brix)) and b (Constant).

The combined effect of temperature and TSS content on apparent viscosity

The combined effect of temperature and TSS on apparent viscosity was evaluated by Power law type Eq. (12), first-order exponential model Eq. (13) and second-order exponential model Eq. (14) **(Juszczak and Fortuna 2004; Manjunatha et al. 2012)**. The estimates and fits of the different parameters were found at 5% significant level (p <0.05). The suitability of the model was choose based on RMSE values and correlation coefficient (r).

$$\eta = a(C)^C Exp(\frac{E_a}{RT}) \quad (12)$$

$$\eta = aExp(\frac{E_a}{RT} + cC) \quad (13)$$

$$\eta = aExp(\frac{E_a}{RT} + cC + dC^2) \quad (14)$$

Where, η, E_a, R, C, T is apparent viscosity (Pa.s^{-1}) at shear rates (10, 50 and 100 s^{-1}), flow activation energy (kJ.mol^{-1}), gas constant, TSS (°Brix), absolute temperature (K), respectively, a is pre-exponential constant (mPa.s^{-1}), b=Ea/R, c is constant (°Brix^1) and d is constant (°Brix^2).

Effect of temperature on K and n

The effect of temperature on flow behavior index (n) and consistency coefficient (K) was evaluated using a modified Turian approach through a regression analysis **(Turian, 1964)** (Eqs. (15) and (16)):

$$\log k = \log k_0 - A_1 T \quad (15)$$

$$n = n_0 + A_2 T \quad (16)$$

Where A_1 and A_2 are the slopes for Turian models. Higher A_1 and A_2 represent more dependency of n and k to the temperature.

Effect of pH on viscosity of puree

Changes in viscosities of puree with TSS (3 °Brix) having different pH value were assessed. The pH range was varied from 1.3 to 5.3 (increment of 2 was adjusted using 0.2 M Na_2HPO_4 and 0.1 M citric acid) at shear rates (10, 50 and 100 s^{-1}) and fixed temperature of 303 K.

RESULTS AND DISCUSSION

Physico-chemical measurements

The physicochemical characteristics of purees are shown in Table 1. The purees having 3 °Brix shows the high moisture content, due to the presence of higher concentration of water. Purees having higher TSS showed the highest value of acidity, compared to that of lower TSS value, probably due to the presence of the highest amount of total organic acid (like ascorbic acid) in the leaf (57.2 mg.100g^{-1}).

The amounts of ascorbic acid reported in the present

Table 1 Physicochemical composition of Roselle leaves puree concentrates used for experiments.

Parameter		Quantity		
		3 °Brix	4.35 °Brix	5 °Brix
Moisture (%)		84.25 ±1.81	81.19 ±1.11	77.55 ±0.81
pH		1.4 ±0.011	2.3 ±0.014	5.8 ±0.023
Density		1.052 ±0.04	1.066 ±0.05	1.069 ±0.082
Ash content (%)		4.52 ±0.11	4.64 ±0.19	4.72 ±0.17
Dry matter content (%)		38.29 ±0.032	39.5 ±0.092	40.1 ±0.019
Chlorophyll (mg.mL^{-1})	a	2.19 ±0.13	2.2 ±0.16	2.30 ±0.18
	b	0.31 ±0.03	0.25 ±0.08	0.36 ±0.02
	Total	2.50 ±0.16	2.54 ±0.24	2.66 ±0.20
Ascorbic acid (mg.100g^{-1})		54 ±0.18	55.6 ±0.34	57.2 ±0.21
Titrable acidity (%)		0.62 ±0.012	0.65 ±0.022	0.69 ±0.015

Note: The results were expressed as mean values ±SD (n = 2).

work were found higher than the ones previously reported in the literature **(Nnam and Onyeke, 2003)**.

The differences observed might be due to different varieties, harvest conditions, genetics, environment, and ecology. The different TSS of purees showed different values of chlorophyll content due to the difference in the soluble solid present in the puree of all TSS. Ash content and density of puree increase slightly with an increase in TSS value, as TSS value represent its solid content.

The FTIR spectra confirmed the presence of –OH stretching (have a similarity to 3672 – 29,345 cm^{-1}), –CH stretching (have a similarity to 2856 cm^{-1}), bending aromatic compounds overtone (have a close similarity to 1792 and 1734.8), C=N stretching imine/oxime or C=O stretching (have a similarity to 1659.1 cm^{-1}), conjugated ketone or alkenes (have a similarity to 1635.1 cm^{-1}), and OH bending phenol (have a similarity to 1368 cm^{-1}).The frequency of absorbance at 2856 cm^{-1} confirmed the presence of –CH stretching due to the formation of H-bond. At 1659.1, broad-shaped bands with medium intensities were observed which indicates the C=O stretching of COOH and Acetyl groups in the sample **(Femenia et al. 2003)**. It was noted that with the increase in TSS, the intensity of the peaks also increased, this might be due to the reason that during the concentration process there is a conversion in functional groups. The presence of the biologically active compound is indicated by the presence of acetyl group, which helps to develop cross-molecular barrier in the cell **(Chang et al. 2011)**. In the case of Aloe Vera powder, **Kim et al. (2009)** reported similar band formation. The previous studies of strong bands on Aloe Vera depict the presence of monosaccharide units in the branched regions such as glucan and galactose in the range of 1156 – 1230 cm^{-1} **(Gentilini et al.2014; Ray and Aswatha, 2013)**.

Rheological analysis of Roselle puree

Figure (1-3) exhibits the effect of temperature on steady shear properties of the purees with a TSS of 3, 4.35 and 5 °Brix.

The obtained data were fitted to six non-Newtonian models (Eqs. (1-6)). The best numerical models for characterizing the flow characteristics of purees was chosen based on the coefficient of determination (R^2), RMSEs, the presence of yield stress and overall bias factors acquired during the fitting (Table 3 – 8). Although all models fitted well with the test data, at 5 °Brix esteem, Herschel-Buckley, Heinz-Casson, Vocadlo, and Mizrahi-Berk fitted model demonstrated negative values, which are having no physical importance. Hence RMSE values were not calculated for the rest of these unfitted models. Power law and Casson model fit satisfactorily at all temperature ranges examined and the R^2 value was found to be greater than 0.97 and hence were more appropriate to describe the rheological flow behavior of the puree.

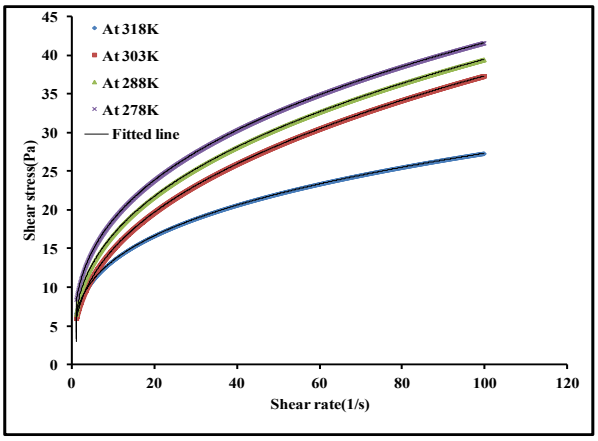


Figure 1 Shear rate vs. shear stress for the 3 °Brix of Roselle leaves puree at various temperatures (T=278 K – 318 K).

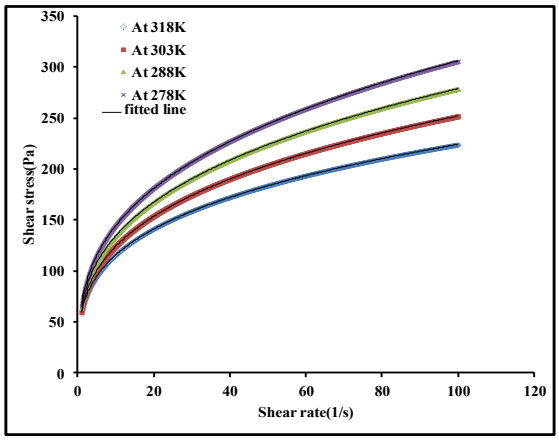


Figure 2 Shear rate vs. shear stress for the 4.35 °Brix of Roselle leaves puree at various temperatures (T = 278 K – 318 K).

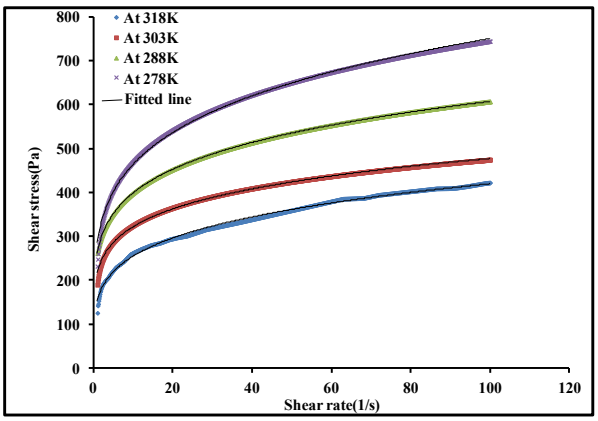


Figure 3 Shear rate vs. shear stress for the 5 °Brix of Roselle leaves puree at various temperatures (T = 278 K – 318 K).

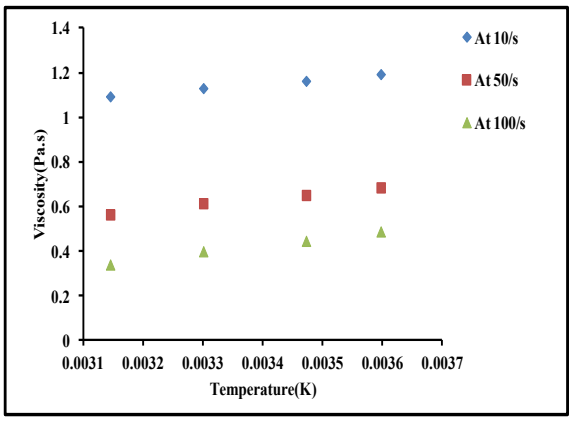


Figure 4 Shear rate vs. viscosity for the 3 °Brix of Roselle leaves puree at various temperatures (T = 278 K– 318 K).

Table 2 Functional groups present in Roselle leaves puree of 5 °Brix by FTIR analysis.

Functional groups	Wave number
Unknown	3750.3
Unknown	3708
Free alcohol O-H Stretching	3672
Intermolecular bonded alcohol O-H Stretching	3408
Intermolecular bonded alcohol O-H Stretching	2934.5
C-H stretching alkane	2856
C-H bending aromatic compounds overtone	1792 1734.8
C = N stretching imines/ oxime or C = O stretching	1659.1
Conjugated ketone or alkenes	1635.1
O-H bending phenol	1368
C-N stretching amine	1230 1156

Table 3 Parameters of the Ostwald-de-Waelle (power law) model fitted to the data of Roselle leaves puree.

TSS	T (K)	*n*	K	R^2	η_{50}	RMSE
3	278	0.396	8.477	1.00	0.642	3.360
	288	0.345	7.854	0.98	0.592	3.394
	303	0.343	6.010	1.00	0.543	4.125
	318	0.307	6.645	0.98	0.449	2.412
4.35	278	0.323	68.915	0.98	4.850	4.105
	288	0.310	65.340	0.99	4.485	4.013
	303	0.304	61.810	1.00	4.120	3.394
	318	0.285	60.170	0.97	3.670	2.351
5	278	0.193	304.940	0.98	12.600	4.324
	288	0.182	261.738	0.98	10.280	4.054
	303	0.158	350.280	1.00	9.250	3.387
	318	0.068	228.240	0.98	7.960	3.312

Table 4 Parameters of the Heschel-Buckley model fitted to the data of Roselle leaves puree.

TSS	T (K)	τ_0	*n*	K	R^2	RMSE
3	278	6.319	0.442	4.640	0.97	2.541
	288	6.673	0.461	3.816	0.97	3.145
	303	6.491	0.471	3.277	0.98	4.051
	318	5.699	0.440	2.896	0.89	3.360
4.35	278	59.199	0.431	33.945	0.97	3.394
	288	57.799	0.430	30.658	0.95	4.125
	303	56.399	0.428	27.301	0.94	2.111
	318	49.214	0.396	28.364	0.97	1.854
5	278	401.122	0.533	30.064	0.98	1.594
	288	365.004	0.507	23.418	0.96	1.141
	303	389.834	4.865	20.100	0.94	1.845
	318	-25528.300	0.001	25.240	0.84	1.098

Effect of K and n on the temperature at different TSS

The variation of K value with temperature (278 – 318 K) of puree is given in Table 3 for power law fitted model. K varied from 6.01 to 350.28 $Pa.s^{-1}$ in puree with different TSS range. The lower values for K were observed at a higher temperature (318 K). The variation was more noticeable on puree having lower TSS value (3 °Brix). Higher TSS purees were found to have higher K value in magnitude. The increments in K were found with an increase in TSS value at a fixed temperature probably due to increment in the particle-to-particle contact **(Chin et al., 2009)**. A similar result was obtained for another vegetable **(Ahmed et al., 2013; Karababa and Develi Isikli, 2005)**. The impact of factors was discovered significant (p <0.05) for all TSS (Table 3). The dependence of K on the temperature variation was also performed using Turian model (Eq. (15)) for Puree. Highest flow behavior dependency was found for 5 °Brix at varying temperatures

(Table 9). Increments in the temperature from 278 K to 318 K significantly affect the consistency index.

The *n* value of the puree ranged from 0.068 to 0.396 (Table 3) for various TSS studied, signifying gently non-Newtonian pseudoplastic shear thinning behavior of puree **(Muller 1974, Steffe, 1996)**. Turian models (Eq. (16)) were used to monitor the dependence of *n* on its temperature change and the model parameters are shown in Table 9. The variation of *n* value with temperature was lower (0.307 – 0.396) in lower TSS value as compared with the higher TSS value (0.068 – 0.193) in all temperature range. This may be because of the increase in soluble solid content. The decrement in n value of puree showed the steady loss of pseudo-elasticity **(Sikora et al., 2007)**. The sample having different temperature demonstrated different flow behavior values for different TSS sample. When the temperature varied from 278 K to 318 K, the n values decrease with increment in temperature. Comparative patterns for the n were accounted by **Rao et al. (1981)** for tomato juice concentrate. Besides TSS, the impact of every single other variable was found significant (p <0.05) (Table 3).

Effect of temperature on viscosity at a different shear rate

Effect of temperature on viscosity is important in determining the rates of heat exchange, energy utilization, and flow rates so it is feasible to assure persistent product flow **(Nindo, 2005)**. **Karwoski (2013)** reported that TSS and temperature influence physical properties, such as viscosity, specific heat, refractive index, density and boiling point, of the fruit product. The plot of the log of viscosity against 1/T (K^{-1}) at various shear rates shown in Figure 5 and the constants of Eq. (9) were determined. It was observed that viscosity of puree concentrates diminish with increment in temperature over the range of shear rates. Figure 6 shows that viscosity decreases with a shear rate, which may be because of increased versatility of macromolecules, because of temperature rise, causing less resistance to flow. The change of the magnitude of viscosity was more at shear (10 s^{-1}) contrasted with the little distinction in a shear rate of 50 and 100 s^{-1}. Interaction impact of temperature and shear rate was additionally assessed and was significant for puree at all Brix value (p <0.05).

Activation energy is minimum energy required for particles to get away from the impact of its neighboring particles during the characterizing the viscous flow. The extent of the vitality of initiation for flow increased with increment in the TSS of the puree, it demonstrates that more energy was required to defeat potential vitality hindrance at higher TSS. Both the power law (Eq. (10)) and exponential model (Eq. (11)) were tried to determine the effect of flow activation energy on TSS of Puree. A nonlinear trend of increment in activation energy ($p \leq 0.05$) of puree with the increase in solids content was observed. The exponential model (r >0.75) was more effective in explaining the impact of the TSS on the flow activation energy of the puree than the power law model (r <0.75) as shown in Table (10). A similar type of results was recorded in the case of pomegranate **(Kaya and Sözer, 2005)**. The activation energy ranged between 3.544 – 8.610 $kJ.mol^{-1}$ for puree having different TSS range (3 – 5 °Brix) (Table 11). Higher values of activation energy may be associated with the higher temperature and TSS of the puree **(Ahmed et al., 2007)**. In pseudoplastic vegetable product, the activation energy was proportional to the n i.e., the more pseudoplastic the product, the less the impact of temperature on its apparent viscosity **(Sharoba et al., 2005)**. In this study, it was found that the activation energy was discovered lower for the puree having lower TSS value at a fixed shear rate (Table 11). Similar types of trends were observed by **Hernandez et al. (1995)** and **Vitali and Rao (1984)**. However, it was also observed that for all TSS Value, the activation energy of puree concentrates increase with an increase in shear rate. Higher activation energy values show a more impact of temperature on its viscosity, i.e. faster change in viscosity with temperature **(Sanchez et al., 2009)**. The outcomes demonstrated that temperature and TSS had significantly affected the thickness of puree concentrates. The viscosity value increases altogether with increment insoluble solid substance. Similar results were obtained by **Alpaslan and Hayta (2002); Akbulut et al. (2012); Arslan et al. (2005)**.

Combined Effect of Temperature and TSS on apparent viscosity

From the food process designing perspective, it is necessary to get a single equation, which represents the effect of both temperature and TSS dependency on the viscosity of puree. Few authors have utilized different types of equation to depict the joined impact of temperature and TSS on viscosity of the liquids **(Juszczak et al., 2010; Kaya and Sozer, 2005; Ibarz et al., 2009; Nindo et al., 2005; Nindo et al., 2007; Altan and Maskan, 2005; Juszczak and Fortuna, 2004)**.

Table 12 demonstrates the parameters for the various models for depicting the combined impact of temperature and TSS on the viscosity of puree. The correlation coefficients values of 0.992, 0.998 and 0.999 and % RMSE values of 10.259, 10.324 and 6.887 were obtained for the first order, exponential first order, and exponential second-order models, respectively. The second order exponential condition was ideal, because of high correlation coefficient and low percent RMSE values. The Estimated parameter values of Eqs. (12), (13) and (14) are given in Table 12 and model equation may be represented as (Eqs 17 – 19).

$$\ln \eta = -8.13685 + \left(\frac{602.275}{T}\right) + 6.045833 \quad (17)$$

$$\ln \eta = -6.1791975 + \left(\frac{602.275}{T}\right) + 1.56987034C) \quad (18)$$

$$\ln \eta = -4.900 + \left(\frac{602.275}{T}\right) + 0.89C + 0.085C^2) \quad (19)$$

The viscosity of puree was significantly (p <0.05) influenced by temperature and TSS of puree. The surface plot for the joined impact of temperature and TSS on the consistency of puree at various shear rates is shown in Figure 7 (a – c).

Table 5 Parameters of the Casson model fitted to the data of Roselle leaves puree.

TSS	T (K)	τ_0	K	R^2	RMSE
3	278	35.121	1.077	1.00	1.234
	288	31.509	1.056	0.97	2.012
	303	29.604	0.898	0.98	1.058
	318	25.431	0.660	0.98	4.211
4.35	278	274.146	7.641	0.97	4.121
	288	256.604	6.811	0.98	3.124
	303	238.888	5.988	0.98	2.458
	318	221.455	5.159	0.97	4.354
5	278	967.060	11.756	0.97	4.001
	288	848.185	7.950	0.98	2.055
	303	693.148	5.589	0.98	4.517
	318	746.176	5.744	0.97	3.845

Table 6 Parameters of the Heinz-Casson model fitted to the data of Roselle leaves puree.

TSS	T (K)	τ_0	*n*	K	R^2
3	278	20.788	0.648	15.190	0.95
	288	23.532	0.755	3.460	0.940
	303	22.098	0.761	2.702	0.96
	318	18.129	0.785	1.446	0.95
4.35	278	909.601	0.598	838.955	0.99
	288	923.254	0.594	788.168	0.94
	303	942.032	0.588	734.583	0.91
	318	1047.212	0.569	898.567	0.99
5	278	21310.460	0.601	2439.761	0.92
	288	19364.434	1.017	2215.253	0.92
	303	22154.221	0.351	1984.214	0.90
	318	19238.830	0.561	3617.393	0.91

Table 7 Parameters of the Vocadlo model fitted to the data of Roselle leaves puree.

TSS	T (K)	τ_0	*n*	K	R^2
3	278	9824.763	-23.653	-6.372	89.000
	288	6335.603	20.053	5.296	92.000
	303	8773.116	24.345	5.470	88.254
	318	10552.290	28.807	4.753	0.910
4.35	278	332.122	1.556	2.973	0.990
	288	249.187	1.393	2.373	0.920
	303	181.312	1.232	1.844	92.000
	318	143.135	1.136	1.466	88.254
5	278	592.742	1.107	3.254	0.910
	288	200.640	-0.687	-1.367	0.925
	303	173.914	-0.708	-0.989	0.971
	318	139.636	-0.611	-0.878	0.891

At lower temperatures the magnitude of viscosity increases quickly with a fixed TSS of Puree and increases insignificantly at higher temperatures, this was because of increment in thermal energy of the particles, which increase its intermolecular spacing. A comparable sort of results was accounted for other liquid food **(Nindo et al., 2005; Ibarz et al., 1989)**. Size, shape, nature of solute and its condition of hydration and the type of solute and solvent influences the viscosity determination, thus might account for deviation in various cases. In the case of Roselle leaves, the solids present in the puree may be mainly fiber, diverse sugars (sucrose, fructose), vitamins, minerals, amino acids, proteins, hormones etc. The condition of hydration was distinctive for various TSS, the magnitude of viscosity relies on upon the sort of solids parts present in the puree **(Nindo et al., 2005; Telis et al., 2007)**.

Effect of pH on apparent viscosity

The influence of pH on the apparent viscosity of puree is shown in Figure 8. As the initial puree prepared from leaves is having pH of 1.3 ±0.26, so the puree is an anionic polysaccharide containing a great number of negative charge-bearing groups such as carboxyl groups, which may undergo different degrees of ionization with changing pH value, leading to changes in viscosity and rheological properties **(Lapasin and Pricl, 1995)**. At shear rates less than 50 s^{-1}, the highest viscosity was obtained over the pH range of 1.3 – 5.3.

Table 8 Parameters of the Mirazi-Berk model fitted to the data of Roselle leaves puree.

TSS	T (K)	τ_0	*n*	K	R^2
3	278	31.905	0.480	7.471	0.960
	288	43.776	0.625	3.300	0.950
	303	41.774	0.612	3.008	0.990
	318	31.615	0.527	3.518	92.000
4.35	278	401.213	0.582	28.740	88.254
	288	378.931	0.574	26.671	0.910
	303	358.100	0.582	22.778	0.990
	318	328.848	0.552	22.787	92.000
5	278	2537.131	-157.274	-146.966	88.254
	288	1719.754	1.001	3.736	0.960
	303	1668.560	-3163.220	2.962	0.950
	318	1782.440	-101.650	8.893	0.910

Table 9 The modified Turian parameters for Roselle puree at different concentrations.

TSS (°Brix)	$\log k = \log k_0 - A_1 T$		$n = n_0 + A_2 T$	
	$\log k_0$	A_1	n_0	A_2
3	2.0013	-0.0039	0.9202	-0.0019
4.35	2.1839	-0.0013	0.5829	-0.0009
5	3.8133	-0.0046	1.0489	-0.0030

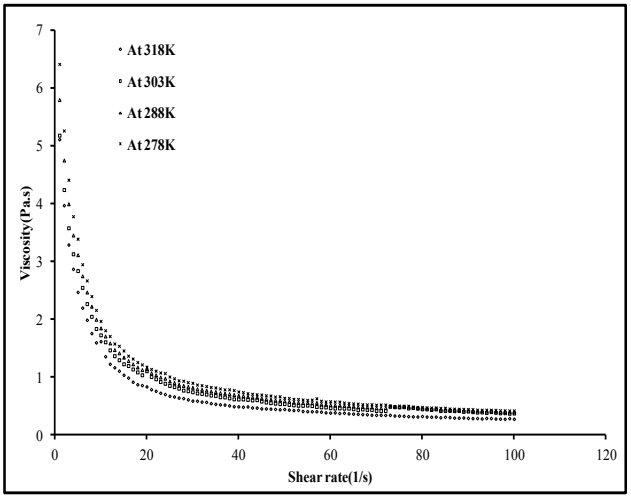


Figure 5 Rheogram of viscosity vs. temperature for the 4.35 °Brix of Roselle leaves puree at various shear rates.

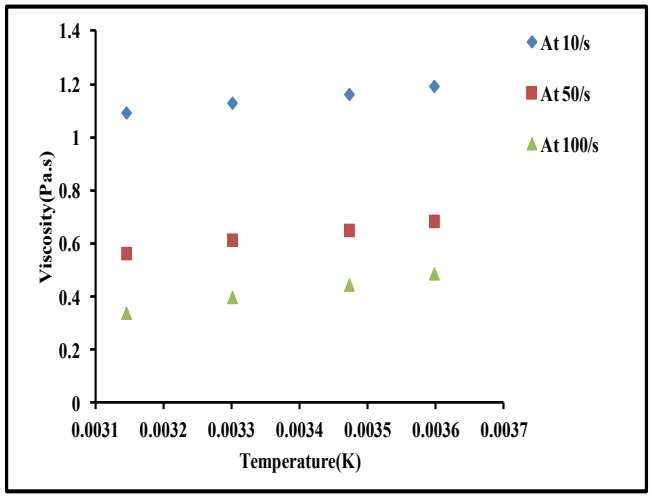


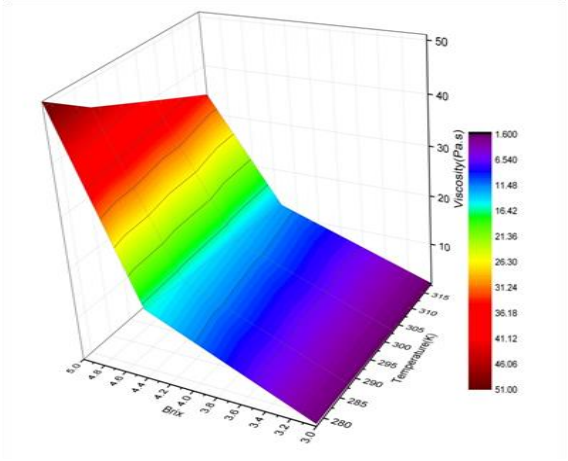
Figure 6 Shear rate vs. viscosity for the 3 ^{0}Brix of Roselle leaves puree at various temperatures (T = 278 K – 318 K).

Table 10 Effect on TSS (°Brix) on activation energy (E_a) values of Roselle leave puree.

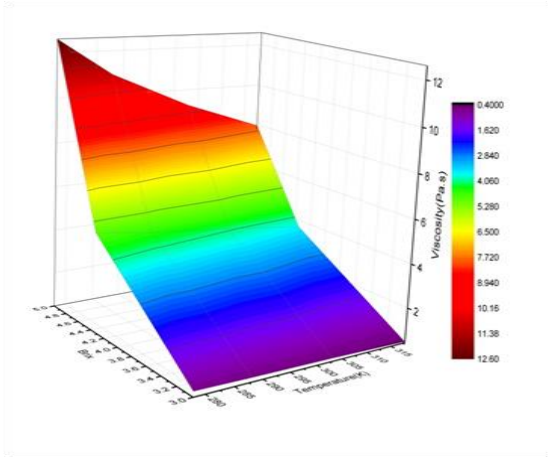
Model	*a* (KJ.mol^{-1})	*b* (%)	r
$E_a = a(C)^b$	0.8741	1.2166	0.7041
$E_a = a \exp(bC)$	1.248	0.3261	0.755

Table 11 Activation energy values at different concentrations and shear rates.

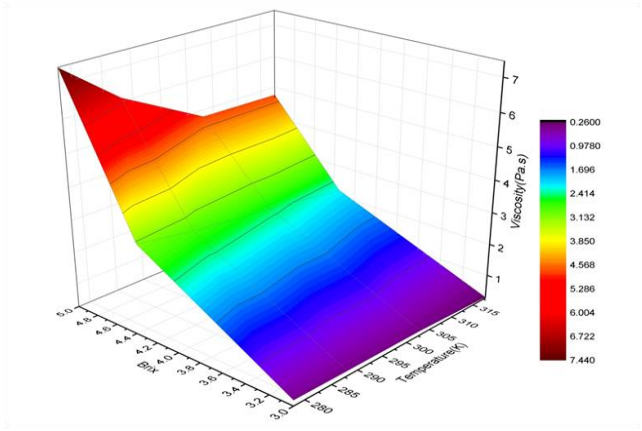
TSS (°Brix)	Shear rate (s^{-1})	η_0	Activation Energy(E_a) (KJ.mol^{-1})	r
3	10	-0.8594	3.5444	0.9977
	50	-3.1631	6.3210	0.9590
	100	-3.9252	7.1869	0.8364
4.35	10	0.9528	4.1428	0.9977
	50	-0.5848	5.0047	0.9934
	100	-1.5313	6.1411	0.9956
5	10	0.7569	7.3348	0.9980
	50	-1.0983	8.3772	0.9986
	100	-1.7639	8.6100	0.8988



(a) At a shear rate of 10 s^{-1}



(b) At a shear rate of 50 s^{-1}



(c) At a shear rate of 50 s^{-1}

Figure 7 Surface plots representing the combined effect of temperature and concentration on the viscosity of Roselle leaf puree at different shear rates: (a) 10 s^{-1}; (b) 50 s^{-1}; (c) 100 s^{-1}.

Table 12 Parameters of different models relating the combined effect of temperature and total soluble solid content of Roselle leaves puree.

Model	a (mPa.s^{-1})	b=Ea/R (K)	c (°$Brix^1$)	c (°$Brix^2$)	r	RMSE
Power Law	0.000293	602.2754	6.045833	---	0.992	10.25937
First order exponential	0.002072	602.27667	1.56987034	---	0.998	10.324
Second order exponential	0.007444	602.27667	0.89111167	0.08590097	0.999	6.887765

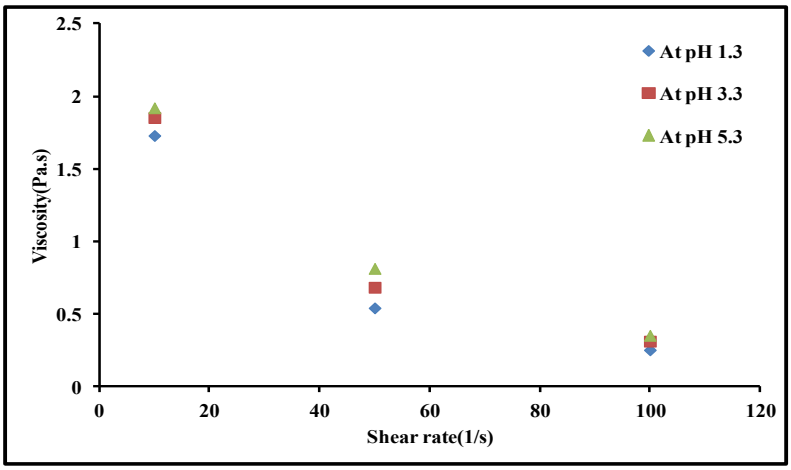


Figure 8 Rheogram of viscosity vs. shear rate for the 3 °Brix of Roselle leaves puree with varying pH (1.3 – 5.3).

There is an increment in viscosity is due to the ionization of carboxyl groups. As per **Feng et al. (2007)**, the viscosity will be at a most extreme when its atomic chains present in puree are entangling with each other. Past research work by the researcher on the impact of pH on viscosity of sweet potato puree and quince puree demonstrated that the consistency of puree increase with pH value **(Ice et al., 1980)**. On the other hand, in the more basic region, the solution consistency dropped at a fixed TSS. As the pH is raised, the functional group present induces electrostatic repulsion that tends to keep the atoms in a developed shape, in this manner producing an exceedingly thick solution **(Onweluzo et al., 1994; Launay et al., 1986)**. Results likewise demonstrated that at higher shear rates (more than 50 s^{-1}); changes in pH had no extensive impact on the viscosity.

In addition, the pH-dependence of viscosity may be due to change in puree confirmation. At low pH, polysaccharide chains tend to appear in coil state with acid groups in free acid form. With increasing pH, acid groups of coils are gradually ionized and the coils are expanded due to increase in electrostatic repulsion between functional groups, leading to more intermolecular interactions among the coils and consequent higher viscosity of the puree **(Feng et al., 2007)**.

The maximum viscosity was obtained around the pH 5.3 for Puree, where the shape of chains may be close to rod conformational state **(Achi and Okolo, 2004)**. This condition usually appears at where acid groups are ionized and electrostatic repulsion reaches a maximum and consequently, tends to keep the molecules in an extended form, leading to a high viscous solution and higher K values **(Medina-Torres, 2000; Coupland, 2013)**. The decrease of viscosity from pH 1.3 to 5.3 may be explained by the neutralization effect of added alkali on the negative charges of the puree, which reduces the hydrodynamic volume of the puree and consequent viscosity **(Chen et al., 2001; Porto et al., 2015)** and puree depolymerization under alkali condition, proposed by **Achi and Okolo, (2004)**.

CONCLUSION

Physicochemical properties of the Roselle leaf puree was found to increase with an increase in TSS value. FTIR spectra confirmed the presence of free alcohol, alkanes, alkene, intermolecular bonded alcohol, aromatic compounds, ketone, amine, phenol, imine, and oxime stretching in the puree. The observation of bands in the IR region made it evident resulting in the chlorophyll molecule masking the other molecules. The rheological analysis also plays a vital role to know the effect of the presence of different compounds on its flow behavior. In the present study, there was a 4 to 5% variation in chlorophyll contents with an increase in TSS contents of puree.

Rheological models were tried to fit with the experimental data of Puree and *best model was selected*. The power-law model display well-fitted data and was considered as a favored model for depicting the rheological properties of puree as impacts of temperatures, TSS and pHs. Puree samples exhibited pseudoplastic behavior (n <1) with the flow behavior index (n) between 0.28 and 0.82. The values of K were found to increase with TSS at a fixed temperature. The Arrhenius model describes satisfactorily, the temperature dependence of viscosity of puree. The activation energy of puree concentrates increase with an increase in TSS and shear rate. The model equation was developed and evaluated by the combined effect of TSS and temperature on the viscosity of puree.

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THE FIELD AND LABORATORY STUDY OF THE COLLECTION SAMPLES OF ONION BREED *ALLIUM CEPA* L.

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ABSTRACT

Thirteen collection samples of *Allium cepa* L. of different ecology-geographical origin grown in the Crimea conditions are given. Morphometric characteristics of the bulbs – the form index, the diameter, the height, the weight, the thickness and the quantity of rich skins are analyzed. The greatest output of standart production (90 – 95.6%) was observed for all breeds. The biochemical values of the *Allium* cepa L. samples under study were examined by the traditional methods. It is marked that the dry substances, the sugars sum, the mono- and disaccharides in the majority of the samples exceed the standart (Yaltinsky rubin) on 14 – 39%, 11 – 48% and 36 – 150% correspondently. The samples with the high concentration of essential oil are singled out: Yaltinsky rubin (Crimea), Tavrichesky (Crimea), Blood red flat (Netherlands) and Brown Beauty (USA) – 4.5, 6.2, 5.6 and 4.4% correspondently. The microsculpture of *Allium cepa* L. leaf was studied by the method of raster electron mi-croscopy and the essential breed differences of the stomata quantity and their arrangement towards the leaf level were distinguished. The results of electron-microscopic research indicate the different level of *Allium cepa* L. samples adaptability. *Allium cepa* L. samples under study of the southern subspecies are recommended for the development of the new breeds with the advanced nutritional qualities.

Keywords: sample; *Allium cepa* L.; bulb onion; morphological and biometric characteristics; essential oils; the parameters of the stomata of the leaves

INTRODUCTION

The peculiarities of the bulb onion breed *Allium cepa* L. growth in Russia are the increase of the cultivated areas in the Southern regions, the yield and the gross collection rise (**Litvinov et al., 2015**). The bulb onion consumption increases not only for the fresh usage, but also for the industrial processing (**Pivovarov, et al., 2001**). For 2006 – 2009 the world gross collection of the bulb onion grew almost on 12% from 66 mln. t to 74 mln. t **(BUSINESSTAT, 2011).** On the world market of *Allium cepa* L. production China is on the first place – 30% of onion in the total production structure; 19% are produced in India; 4.9% in the USA and 2.2% in the Russian Federation. The main production of *Allium cepa* L. in the Russian Federation is placed in three districts: Privolzhsky, Southern and Central. The new breeds and hybrids of *Allium cepa* L. should possess early ripening, yield, stability to diseases and blasts, good storability, small variability of morphological characteristics, the concentration of dry substances in the bulbs 7 – 10% for the salad breeds and 12 – 18% for pungent ones (**Nemtinov et al., 2006**).

The adaptability of the ontogenesis stages, cytological peculiarities of the leaf's epidermis surface, the stomata quantity on 1 mm^2 of intercellular structure of the leaf surface allow to judge about the economical valuable characteristics of the breed **(Yudaeva et al., 2017).** The adaptability of the plants to new conditions depends on the genetic variability in the selection process (**Grinberg et al., 2007; Kalbarczyk, 2008; Bystrická et al., 2014**).

Allium cepa L. is a useful vegetable plant and the source of some biologically active substances. The vitamins and phenol compositions, especially quercetin, possess antioxidant and antibacterial activity **(Ulyanova, 1998; Platonova, 2000; Parr et al., 2000; Romanova et al., 2008; Kavalcová et al., 2015; Silva, et al., 2007; Tawaha, et al., 2007**). The aminoacids – arginine, lysine, leucine, isoleucine, threonine, phenylalanine, alanine, glycine, histidine, serine as well as glutamic and asparlic acids are found in the bulbs. The prolonged consumption of food rich in plant polyphenols guarantees the organism protection from cancer, heart diseases, diabetes, osteoporosis and neurodegenerative diseases (**Pandey et al., 2009**). *Allium cepa* L. antioxidant activity of the bulbs

of different colour is not the same. **Prakash et al. (2007)** and **Cheng et al. (2013)**, experimentally determined the value of the antioxidant activity: 50.6% in the red bulb onion and 13.6% in the white one. **Nuutila et al. (2003)** discovered that the antioxidant activity of yellow and red-coloured breeds is in the range from 32.9% till 44.5%.

Scientific hypothesis

Comparative morphological and biochemical assessment of *Allium cepa* L. varieties of different ecological and geographical origin has not been studied sufficiently. We tested the influence of the *Allium cepa* L. genotype on the economically valuable and morphological features when grown in conditions of Crimea.

MATERIAL AND METHODOLOGY

Objects of research

Investigated of the samples of the bulb onion from 9 countries of Federal Reach Center "All-Russian Institute of Plants Genetic Resources named after N. I. Vavilov" collection was used. The samples differed by origin: 4 samples from Russia (the Crimea: Yaltinsky rubin, Yaltinsky model №3, Tavrichesky and Krasnodarsky Kray: Mestniy); 2 ones from the USA (Southport red, Brown Beauty); as well as from Australia (B12132B0, Azerbaijan (Mestniy), Algeria (Rouge pale), Bulgaria (Trimontzium), Bolivia (Red Wethers field), Netherlands (Blood red flat) and Portugal (Valensiya). Breed Yaltinsky rubin was accepted as the standart.

The researches place and methods

The field researches were held in 2016 – 2017. The onion plants were grown in the collection nursery of Federal State Budget Scientific Institute "Scientific-Research Institute of Agriculture of the Crimea" (FSBSI SRIA of the Crimea). The grounds are presented by the southern calcareous black soil. The agrochemical characteristics of 20 sm ground layer: the humus concentration by Tyurin – 4.5 – 5.4%; pH reaction – 7.85; mineral nitrogen N – NO_3 – 6.3 mg.100g^{-1} of the soil; labile phosphorus concentration P_2O_5 (by Machigin) – 18.4 mg.100g^{-1} of the soil; exchangeable potassium concentration K_2O (by Machigin) – 73.0 mg.100g^{-1} of the soil. The plants were grown in 4 replications on the registration plots with the squares from 0.5 m^2 till 1 m^2.

Environmental assessment of output

The ecological evaluation of the output yield was fulfilled, the photometric peculi-arities of the leaves and bulbs were described – the form index, diameter, height, weight, thickness of rich skins, quantity (rich skins rudiments) **(Vavilova, 2005)**.

Chemicals

All chemical substances chosen forthe analysis were of analytical sortand were bought from Sigma Aldrich (USA) and Merck KgaA (Germany).

Sample preparation

300 g of the average sample was homogenized with the help of high-speed homogenizer (10 000 rpm, 1 min, UltraTurrax T25 Basic, IKA). The extractionaswell as the measurements were held in three-time repetition.

Basic chemical analyses

The following biochemical characteristics of the bulbs quality were determined: dry substance – using the thermographic method **(GOST 2173-2013, 2014)**, ascorbic acid – using the titrimetric method by Murri **(R 4.1.1672-03, 2003)**, the sugars and the reductive sugars sum in accordance with **GOST 8756.13-87 (2010)**, the essential oil concentration – using the distillation chromatographic method **(Timasheva et al., 2018)**.

Electron microscopic examination

The morphologic characteristics of *Allium cepa* L. samples leaves were studied in the Laboratory of Physiology and Biochemistry of Federal State Budgetary Scientific Institution "All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery" by the method of raster electronic microscopy (REM). For the researches the leaves cuttings from the central part of the rosette (in the second decade of June) with the dimensions 5 × 5 mm were placed on a special gluing tape set on the object table of REM JEOL JSM 6010-LA. The epidermis was studied from the leaf abaxial side in accordance with REM operating rules.

Statisic analysis

Results were statistically evaluated by the Analysis of Variance. All the assays were carried out in triplicates and results are expressed as mean ±SD. All calculations were made with the help of Microsoft Office 2013 software package (Microsoft, USA).

RESULTS AND DISCUSSION

Allium cepa L. prolonged vegetation period in the southern regions of Russia and the duration of the bulbs storage period determined the traditionally vested rules: the bulbs onions should be mid-ripening, half-pungent and sweet (salad). The bulb form was an important grading factor and influenced on the consumer's demand for the salad onion of the southern subspecies. The form index 0.5 – 0.7 corresponded to six samples – two from the Crimea (Yaltinsky rubin – standart and Yaltinsky model №3), Mestniy (Krasnodar, Russia), Rouge pale (Algeria), Mestniy (Azerbaijan), Red Wethers field (Bolivia). The rest samples had the index 0.8 – 0.9. The sample B12132B (Australia) had index 1.1. The greatest bulbs diameter (7.4 – 9.0) is characteristic of the samples Mestniy (Azerbaijan), Valensiya (Portugal), Brown Beauty (USA), Yaltinsky rubin (Crimea) – standart and Yaltinsky model №3 (Crimea). The maximal bulbs height is 7.8 sm is typical for the sample B12132B (Australia). Seven samples annually exceeded the standart by the bulbs height. Sample 5 (Tav-richesky) had the bulb diameter of small size 3 – 5 sm, middle size was at 9 samples (1, 2, 3, 4, 6, 7, 8, 10 and 11) and large 8 – 9 sm – at standart and sample 12. Within 2 years the value of the bulbs diameter was less than the standart except the sample Yaltinsky. During the evaluation of the bulbs morphological factors are the thickness, the rich skins and rudiments quantity. The greatest thick-ness of the bulb rich skins (from 5.2 till

7.6 mm) is marked for the samples Brown Beauty (USA), Rouge pale (Algeria), Yaltinsky rubin (Crimea) – standart and Yaltinsky model №3 (Crimea) (Table 1).

The rudiments quantity in the bulb influences the bulb onion reproduction coefficient. The samples Mestniy (Krasnodar, Russia), B12132B (Australia), Tavrichesky (Crimea), Blood red flat (Netherlands), Brown Beauty (USA) and Yaltinsky model №3 (Crimea) showed the rudimentariness less than 2pcs., the rest ones corresponded to the standart level (2 – 2.5 pcs.).The bulbs weight relates to the economical characteristics of the breed. The biggest bulbs weight is marked at the samples Brown Beauty (USA), Southport red (USA), Yaltinsky rubin (Crimea) – standart and Yaltinsky model №3 (Crimea) – 152, 166, 170 and 211 g correspondently. The samples yield at average for 2 years varied in the range 2.2 – 4.5 kg.m^{-2}, the highest one is at the standart 45 kg.m^{-2}. The sample Mestniy (Azerbaijan) was less than the standart on 2.0%. The largest output of the standart products 93.2 – 95.6% was marked at the samples Mestniy (Azerbaijan), Yaltinsky model №3 (Crimea), Brown Beauty (USA), Valensiya (Portugal) and Valensiya (Portugal) (Table 2). It is known the the bulbs colour indicates the presence of the antioxidants – polyphenols: flavonoids (quercetin, rutin) and anthocyans in the products **(Nuutila et al., 2003; Kong et al., 2003; Lu et al., 2011; Cheng et al., 2013)**. As a result it can be supposed that yellow, white and light-pink bulbs contain flavoids (quercetin and rutin), and brown-purple, dark-purple and purple bulbs Yaltinsky rubin (Crimea) – standart, Yaltinsky model №3 (Crimea) and Trimontzium (Bulgaria) – anthocyans. Such breeds are very popular on the onion market (Figure 1).

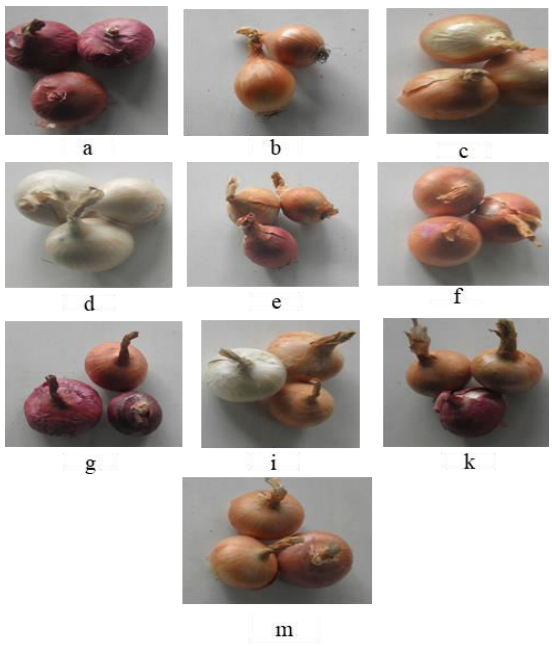


Figure 1 *Allium cepa* L. samples of different bulb colour a: Yaltinsky rubin (Crimea) – purple, b: Mestniy (Krasnodar, Russia) – yellow, c: Southport red (USA) – yellow, d: Trimontzium (Bulgaria) – white, e: Tavrichesky (Crimea) – light pink, f: Rouge pale (Algeria) – yellow, g: Red Wethers field (Bolivia) – dark-purple, i: Blood red flat (Netherlands) – yellow, k: Valensiya -(Portugal) – yellow, m: Brown Beauty (USA) – yellow.

Table 1 *Allium cepa* L. morphometric factors (2016 – 2017).

The sample No., name and origin	The form index	Diameter, (sm ±SD)	Height, (sm ±SD)	Weight (g ±SD)	Thickness of rich skins (mm ±SD)	Quantity (pcs ±SD)	
						rich skins	rudiments
Yaltinsky rubin (Crimea) – standart	0.5	8.20 ±0.20	4.1 ±0.09	169.5 ±8.9	6.4 ±0.5	5.4 ±0.29	2.5 ±0.38
1. Mestniy (Krasnodar, Russia)	0.7	5.8 ±0.59	4.1 ±0.35	124.1 ±13.9	4.5 ±0.65	6.8 ±0.25	1.25 ±0.25
2. B12132B (Australia)	1.1	6.3 ±0.42	7.8 ±0.46	127.6 ±8.2	3.8 ±0.39	6.8 ±0.2	1.33 ±0.17
3. Southport red (USA)	0.9	6.8 ±0.40	6.4 ±0.33	165.5 ±12.0	3.7 ±0.33	7.3 ±0.4	2.44 ±0.34
4. Trimontzium (Bulgaria)	0.9	6.6 ±0.26	6.1 ±0.29	101.7 ±6.8	4.4 ±0.34	6.5 ±0.38	2.1 ±0.26
5. Tavrichesky (Crimea)	0,9	5.2 ±0.15	4.8 ±0.24	99.6 ±8.2	4.2 ±0.36	5.4 ±0.5	1.67 ±0.23
6. Rouge pale (Algeria)	0.7	6.3 ±0.26	4.6 ±0.10	120.6 ±7.6	5.5 ±0.24	5.2 ±0.22	2.33 ±0.17
7. Mestniy (Azerbaijan)	0.7	7.4 ±0.21	5.5 ±0.24	143.8 ±8.6	4.7 ±0.37	5.9 ±0.51	2.11 ±0.20
8 Red Wethers field (Bolivia)	0.7	6.2 ±0.25	4.3 ±0.11	97.8 ±4.3	4.8 ±0.45	5.9 ±0.39	2.11 ±0.26
9. Blood red flat (Netherlands)	0.9	6.8 ±0.34	6.0 ±0.23	127.1 ±11.9	4.1 ±0.39	7.4 ±0.29	1.33 ±0.17
10. Valensiya (Portugal)	0.8	7.6 ±0.25	6.4 ±0.36	134.5 ±14.1	4.8 ±0.28	6.6 ±0.56	2.22 ±0.32
11. Brown Beauty (USA)	0.8	7.8 ±0.23	6.1 ±0.25	151.9 ±6.86	5 ±0.41	7.6 ±0.24	1.56 ±0.34
12. Yaltinsky model №3 (Crimea)	0.5	9.0 ±0.36	4.4 ±0.19	211.0 ±6.46	7.6 ±0.93	6.8 ±0.73	1.5 ±0.29
$HD_{0,05}$	-	1.1	1.0	30.5	1.6	1.6	1.1

Table 2 *Allium cepa L.* yield, growing in the collection nursery (2016 – 2017).

The sample No., name and origin	Yield ($kg.m^{-2}$ ±SD)				The standart products output, %
	2016	2017	average	± to standart, %	
Yaltinsky rubin (Crimea) –standart	4.10 ±0.07	4.87 ±0.31	4.5	-	90.6
1. Mestniy (Krasnodar, Russia)	2.72 ±0.10	3.30 ±0.10	3.0	-33	91.9
2. B12132B (Australia)	0.70 ±0.03	3,77 ±0.07	2.2	-51	90.0
3. Valensiya (Portugal)	2.75 ±0.06	3.50 ±0.09	3.2	-29	95.6
4. Trimontzium (Bulgaria)	2.37 ±0.06	3.35 ±0.25	2.9	-36	90.8
5. Tavrichesky (Crimea)	2.90 ±0.07	2.85 ±0.55	2.8	-38	84.9
6. Rouge pale (Algeria)	1.87 ±0.05	3.12 ±0.14	2.5	-44	84.6
7. Mestniy (Azerbaijan)	3.45 ±0.06	5.22 ±0.29	4.4	-2	93.2
8 Red Wethers field (Bolivia))	1.97 ±0.09	2.40 ±0.12	2.2	-51	78.4
9. Blood red flat (Netherlands)	1.85 ±0.05	3.60 ±0.11	2.8	-38	89.6
10. Valensiya (Portugal)	3.45 ±0.12	3.67 ±0.22	3.6	-20	95.4
11. Brown Beauty (USA)	2.82 ±0.11	3.72 ±0.24	3.2	-29	95.2
12. Yaltinsky model №3 (Crimea)	-	3.70 ±0.21	3.7	-24	94.1
$HD_{0,05}$	0.23	0.33			

Table 3 *Allium cepa* L. biochemical content, growing in the selection nursery (2016 – 2017).

The sample No., name and origin	Dry substance (% ±SD)	General sugars (% ±SD)			Ascorbic acid ($mg.100g^{-1}$ ±SD)	Essential oil (% ±SD)
		total	including			
			monosaccharides	Disaccharides		
Yaltinsky rubin (Crimea) – standart	8.8 ±0.3	11.7 ±0.5	6.7 ±0.3	5.0 ±0.3	18.6 ±0.6	4.5 ±0.4
1. Mestniy (Krasnodar, Russia)	10.5 ±0.3	13.0 ±0.6	2.6 ±0.1	10.4 ±0.5	11.8 ±0.4	2.8 ±0.3
2. B12132B (Australia)	7.7 ±0.2	11.4 ±0.4	5.4 ±0.3	6.0 ±0.4	16.6 ±0.5	2.8 ±0.3
3. Southport red (USA)	10.0 ±0.3	14.4 ±0.5	4.4 ±0.3	10.0 ±0.5	12.9 ±0.4	2.8 ±0.3
4. Trimontzium (Bulgaria)	11.4 ±0.4	14.7 ±0.5	4.3 ±0.3	10.4 ±0.5	16.1 ±0.5	1.6 ±0.2
5. Tavrichesky (Crimea)	11.5 ±0.4	13.2 ±0.4	3.4 ±0.2	9.8 ±0.5	16.2 ±0.5	6.2 ±0.5
6. Rouge pale (Algeria)	12.2 ±0.4	13.7 ±0.5	3.4 ±0.2	10.3 ±0.4	16.3 ±0.5	4.1 ±0.4
7. Mestniy (Azerbaijan)	10.8 ±0.3	13.6 ±0.4	3.8 ±0.2	9.8 ±0.4	18.6 ±0.6	2.7 ±0.3
8 Red Wethers field (Bolivia)	10.8 ±0.3	17.3 ±0.6	6.1 ±0.3	11.2 ±0.5	21.1 ±0.6	4.0 ±0.4
9. Blood red flat (Netherlands)	10.6 ±0.3	12.5 ±0.3	5.7 ±0.3	6.8 ±0.3	19.6 ±0.5	5.6 ±0.5
10. Valensiya (Portugal)	11.4 ±0.4	16.7 ±0.5	5.2 ±0.3	11.5 ±0.5	16.7 ±0.4	4.0 ±0.4
11. Brown Beauty (USA)	11.7 ±0.4	18.1 ±0.6	5.8 ±0.3	12.5 ±0.6	14.8 ±0.3	4.4 ±0.4
12. Yaltinsky model №3 (Crimea)	7.1 ±0.2	11.3 ±0.3	7.4 ±0.3	3.9 ±0.1	27.3 ±0.6	0.9 ±0.1
$HD_{0,05}$	0.6	0.7	0.4	0.8	0.7	0.8

The biochemical composition analysis of the collection samples at the dry substance concen-tration, the sugars sum including mono - and disaccharides showed that the majority of them enlarge the standart on 13 – 46%, 11 – 48% and on 36 – 150%, except the samples B12132B (Australia) and Yaltinsky model №3 (Crimea), in which the concentration of the dry substance on 12 – 19% and the sugars sum on 2% lower than the standart sample, and the fraction of disaccharides is on 22% lower than sample 12 (Table 3). The highest concentration of the sugars sum and disaccharides is marked at samples Red Wethers field (Bolivia), Valensiya (Portugal) and Brown Beauty (USA). In the bulbs of sample Yaltinsky model №3 (Crimea) the increase of monosaccharides and ascorbic acid relatively on 10 and 47% higher than the standart was marked.

The maximal concentration of ascorbic acid in the bulbs contains at samples Red Wethers field (Bolivia) – 21.1 $mg.100g^{-1}$ and Yaltinsky model №3 (Crimea) – 27.3 $mg.100g^{-1}$, this is on 5 – 14% higher in comparison with the standart (18.6 $mg.100g^{-1}$). At *Allium cepa* L. samples under study for the years of the researches the weight fraction of the essential oil in the range from 0.9 till

6.2% that is typical for the salad breeds. The samples singled out according to the studied factors are used in the selection process. The results of electronic-microscopic researches of the tudied *Allium cepa* L. samples leaves allowed to distinguish the peculiarities of cuticle surface micromorphology (the presence of girders and folds, the wax layer), the peculiarities of the stomata position, as well as their length and the quantity on 1 mm^2 of *Allium cepa* L. leaf surface (Table 4).

The highest stomata quantity on the adaxial leaf side of *Allium cepa* L. leaf is observed on samples B12132B (Australia), Valensiya (Portugal), Tavrichesky (Crimea) and Trimontzium (Bulgaria) – 49.9 x 10^2, 47.4 x 10^2, 38.9 x 10^2 and 37.6 x 10^2 $pcs.mm^{-2}$ correspondently. At the majority of the samples the stomata length varies slightly from 8.71 µm (B12132B (Australia)) till 22.49 (Blood red flat (Netherlands)). The largest stomata – 34.32 µm is at sample Yaltinskiy rubin (Crimea), however, the stomata quantity on 1 mm^2 of the leaf surface is not essential – 37.6 x 10^2 pcs.

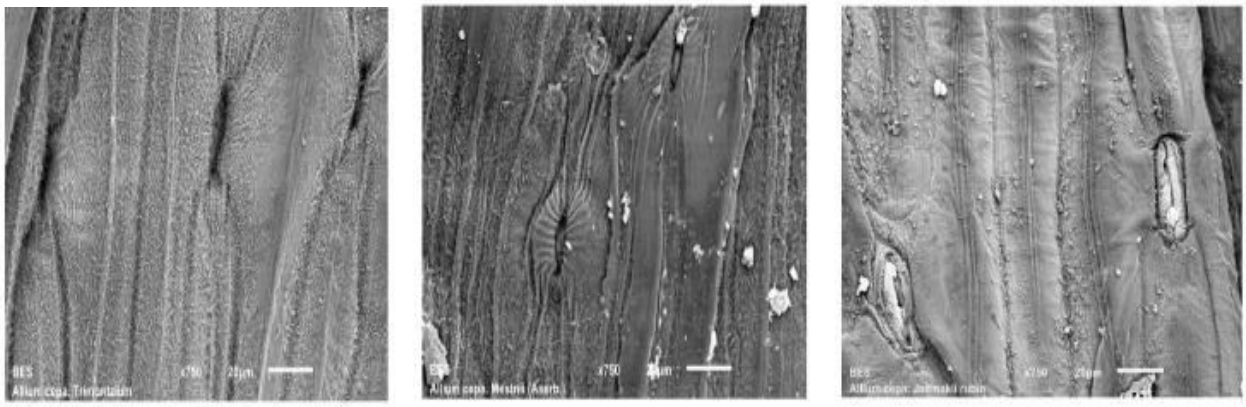
The stomata sculptural peculiarities of three main contrasting collection samples: Trimontzium (Bulgaria); Mestniy (Azerbaijan) and Yaltinsky rubin (Crimea) are presented on Figure 2.

It is determined that the leaf surface microstructure of each *Allium cepa* L. studied samples is specific. The cuticle wax which is mostly developed at Trimontzium (Bulgaria) is clearly seen. The analysis of leaves epidermis low surface ultrastructure showed that the stomata at *Allium cepa* L. studied samples are basically prolonged (the length is bigger than the width) with clearly seen auxiliary epidermal cells covered with wax layer. In relation to the cuticle surface level the stomata are placed differently, for example: at sample Trimontzium (Bulgaria) the stomata are strongly deepened in the cuticle, placed lower than the surface. At the sample Mestniy (Azerbaijan) the stomata are raised above the cuticle surface and have well developed girders that possibly provide the better stomatal pore opening and closing. At the sample Yaltinsky rubin (Crimea) the stomata are placed at the cuticle level and have well-developed stomatal rollers.

It is determined that the stomata length was larger and was 21.15 – 34.32 µm at more productive samples with thick rich skins and their quantities at the level of 5.4 – 5.8 pcs.

Table 4 Morphological factors of *Allium cepa* L. breeds vegetable part**.**

Breed	Stomata length, µm	Fluctuation limits		Stomata quantity $pcs.mm^{-2}$
		min	max	
Yaltinsky rubin (Crimea) – standart	34.32	28.28	38.87	32.1 x 10^2 ±287.4
1. Mestniy (Krasnodar, Russia)	26.98	20.10	34.51	19.7 x 10^2 ±287.3
2. B12132B (Australia)	18.71	16.97	19.31	49.9 x 10^2 ±399.8
3. Southport red (USA)	20.51	16.54	24.95	31.4 x 10^2 ±474.7
4. Trimontzium (Bulgaria)	20.34	16.23	22.92	37.6 x 10^2 ±349.8
5. Tavrichesky (Crimea)	19.67	16.23	25.83	38.9 x 10^2 ±398.4
6. Rouge pale (Algeria)	27.12	23.84	29.47	25.7 x 10^2 ±437.3
7. Mestniy (Azerbaijan)	21.15	14.96	31.23	25.5 x 10^2 ±599.7
8 Red Wethers field (Bolivia)	29.15	27.46	36.54	29.6 x 10^2 ±399.8
9. Blood red flat (Netherlands)	22.49	16.05	25.83	20.6 x 10^2 ±274.8
10. Valensiya (Portugal)	27.13	24.05	32.08	47.4 x 10^2 ±324.8
11. Brown Beauty (USA)	24.92	21.56	29.92	34.9 x 10^2 ±262,4
Average	26.19	20.19	29.29	32.78 x 10^2



1 2 3

Figure 2 The stomata position peculiarities of Alium cepa L. breeds 1 − Trimontzium (Bulgaria), 2 − Mestniy (Azerbaijan), 3 − Yaltinsky rubin (Crimea).

CONCLUSION

The complex evaluation of morphometric factors and biochemical composition of 13 *Allium cepa* L. collection samples of different ecology-geographic origin is given.

It is determined that *Allium cepa* L. samples are characterized by different bulbs colour that indicates about *Allium cepa* L. samples leaves add their morphologic characteristic that indicate the variental differences of the leaf epidermal structures and the various level of adaptability.

The studied collection of the southern subspecies *Allium cepa* L. samples is the base for the acquisition of the best samples according to the factors: the concentration of dry substance, sugars, ascorbic acid and essential oil and is used in the selection work for the improvement of flavouring and curing characteristics of the existing salad onion breeds.

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BIOINFORMATICS ANALYSIS OF AFLATOXINS PRODUCED BY *ASPREGILLUS* SP. IN BASIC CONSUMER GRAIN (CORN AND RICE) IN SAUDI ARABIA

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ABSTRACT
The food contaminants by aflatoxins are inevitable even when all precautions and good agricultural practices are applied. Samples of white rice and corn (yellow, red) grains were collected from different local markets and houses. Three *Aspergillus flavus* strain isolated were identified using molecular characterization of *AFLR* (*aflR*) toxin gene. DNA genome of the three *A. flavus* isolates (namely *A. flavus* _ YC; *A. flavus* _ RC; *A. flavus* _ Rice) which corresponds to isolates from, yellow corn, red corn and white rice respectively were used as a template for PCR to amplify *Aspergillus flavus AFLR* (*aflR*) toxin gene. Partially sequenced was amplified using a specific primer set to confirm its identity, phylogenetic relationships between the three isolates as well as determination of the corresponding antigenic determinants. The epitope prediction analysis demonstrated that there were 1, 2, 3 and 4 epitopes whose score were equal 1 in *A. flavus* _ YC; *A. flavus* _ RC; *A. flavus* _ Rice, respectively. Interestingly, there were great dissimilarity in the epitope sequences among the three isolates except in RLQEGGDDAAGIPA, SPPPPVETQGLGGD, RPSESLPSARSEQG and PAHNTYSTPHAHTQ were found to be similar between all isolates. This work articulates that the molecular identification and characterization of three *A. flavus* using *Aspergillus flavus AFLR (aflR)* toxin gene and the unique antigenic determinants that could be used for design of a broad-spectrum antibody for rapid detection of *A. flavus* in foods and support quality system of food safety.

Keywords: PCR; sequences; phylogenetic tree; protein toxic gene; antigenic determinants

INTRODUCTION
Fungi caused major crops diseases during harvest and storage under higher temperature and humidity conditions **(Bhat et al., 2010; Pasquali et al., 2016)**. While more than 25 different fungi species known to invade stored grains and legumes **(Duan et al., 2007)** some species such as *Aspergillus, Fusarium, Penicillium* are responsible for most spoilage and germ damage during storage **(Boutigny et al., 2012; Aamot et al., 2015; Kachapulula et al., 2017)**. Crop transfers through international trade have made aflatoxins contaminated food a worldwide problem **(Passone et al., 2010)**. Mycotoxins are secondary metabolites produced by fungi, which cause health hazards to animals and human beings; the majority of mycotoxins of greatest concern to human and animal health are produced by the genera *Aspergillus, Penicillium,* and *Fusarium,* the so-called field fungi that frequently infect various food commodities **(Reddy et al., 2010)**. Toxicity of a mycotoxin will be manifested by its effect on the human and animal health and productivity of crops **(Abdel-Wahhab et al., 2006)**. The main routes of mycotoxins exposure are ingestion, inhalation or through skin contact. The toxicity of a mycotoxin is determined by metabolism involving the, transformation, administration, distribution, absorption, excretion and molecular interactions of the toxin and its metabolites. Nowadays the main mycotoxins of interest are aflatoxins (AFs), ochratoxins, **(Frisvad et al., 2019)**, trichothecenes, zearalenone, fumonisins, ergot alkaloids and deoxynivalenol **(Reddy et al., 2010; Cendoya et al., 2014; Covarelli et al., 2015; Singh and Cotty, 2019)**. Mycotoxin producing fungi which are associated with groundnuts, peanuts, cereals such as maize, rice, sorghum, wheat, barley and oats and spices such as black pepper, ginger, nutmeg, chilly, etc. are considered to be of greater significance for all over world **(Kumar et al., 2008; Brožková et al., 2015)**. Several studies have revealed mycotoxin contamination in rice worldwide: for example, aflatoxins in the United Arab Emirates **(Osman et al., 1999)** fumonisins in Iran, Argentina **(Alizadeh et al., 2012; Cendoya et al., 2014)** OTA in Morocco **(Juan et al., 2008)**, ZEA in Nigeria **(Makun et al., 2007)**, DON in Italy **(Lorè et al., 2011)** nivalenol in Korea **(Lee et al., 2011)** and citrinin in Egypt **(Abd Allah and Ezzat, 2005)**. As most of the corn and rice is grown during the wet season; it is

susceptible to mycotoxin contamination. Rice is shown to be a good substrate for toxigenic fungi like *A. flavus, A. ochraceus, Penicillium citrinum,* and *F. proliferatum* **(Aamot et al., 2015; Arino et al., 2007; Sánchez-Hervás et al., 2008)**. Humidity, temperature, storage conditions, and transportation period are the factors that influence mycotoxin production in rice **(Ariño et al., 2007)**. Regarding legumes in Saudi Arabia, very little information exists with respect to its natural contamination with toxigenic fungi and mycotoxins. Aflatoxins were detected in some *Aspergillus* isolates while fumonisin was detected in some *Fusarium* isolates **(Ibrahim et al., 1998; Abdel-Fatah et al., 2017)**. Among food contaminants, mycotoxins may cause substantial economic loss due to lower availability of commodities with acceptable levels of mycotoxins present and possibly greater cost of mycotoxin-safe and acceptable foods **(Mwanza et al., 2013; Samina, 2015)**. Mycotoxins continue to pose various health risks to consumers depending on specific mycotoxin consumed and level of exposure, and health status of individuals in the population **(Voss et al., 2014; Pasquali et al., 2016)**. Many human diseases, especially carcinogenic, teratogenic, hepatic, and gastrointestinal ones, have been found linked with the ingestion of mycotoxin-contaminated products **(Fung and Clark, 2004; Shephard, 2008; Samina, 2015)**. Outbreaks of mycotoxicoses in humans and animals, caused by ingestion of products containing mycotoxins, **(Peraica and Rašić, 2012)**. Risk assessments relating to food safety are frequently hampered by the lack of quantitative data **(Schmidt-Heydt et al., 2007)**. The sequencing fungal genomes and the studies of the molecular basis of fungal pathogenicity provide the study of risk factors associated with continuous exposer of mycotoxins **(Bilodeau, 2011; Taha et al., 2012)**. This paper was shown concentrated on aflatoxins produced by *Aspregillus* sp. and possibility for used the similarity between amino acid toxin gene of *Aspregillus* isolates strain for produced antibodies. Where the database of *Aspregillus* genomes provides a comprehensive resource of genomics data information's an important plant and human pathogenic fungal genus *Aspregillus*. It's given useful for discovery of genes encoding industrial enzymes, and antibiotics which may control in aflatoxin food contaminations **(Spröte et al., 2009; Taha et al., 2012)**. AFs are a group of polyketide-derived furanocoumarins, with at least 16 structurally related toxins that have been characterized. These toxins are produced by a number of different *Aspergillus* species are primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus* **(Geiser et al., 2007; Ito et al., 2001)**. There are four major AFs (AFB1, AFB2, AFG1, AFG2) all of which occur naturally **(Abbas et al., 2010)**. AFB1 is the most commonly occurring of the mold producing compounds **(Abdulkadar et al., 2004)**. AFB1 has been included in category 1A of active carcinogenic compound **(Abou-zeid et al., 1997; Abdel-Wahhab et al., 2006)**. Factors influencing the presence of mycotoxins in foods or feeds include environmental conditions related to storage that can be controlled **(Park et al., 2005)**. So, in this study, we have hypothesized that mycotoxins effects in human populations year to year because other factors as the fungal strain specificity, strain variation, and instability of toxigenic properties are more difficult to control **(El-Manzalawy et al., 2008a; El-Manzalawy et al., 2008b)**. However, by the articulate the molecular identification and characterization of mycotoxins, can be control in mycotoxins effects. In this study our aim advocate to using molecular detection and bioinformatics characterization for study the properties of protein toxin gene of aflatoxins in main consumer grains as rice and corn. Many human diseases occurring in Japan and other Asian countries were attributed to mycotoxins after consumption of mold-damaged rice **(Taligoola et al., 2011)**. Unfortunately, enactment of stringent rules for mycotoxin control in food is not always the best solution **(Samina, 2015)**. The impact of mycotoxin standards is more drastic for the population of developing countries **(Pasquali et al., 2016; Yassin et al., 2010)**.

Scientific hypothesis

Therefore, the aim of this study was to determine the *Aspergillus* species by the molecular identification of toxigenic mycotoxin profiles of those species that are naturally occurring in contaminating corn and rice seeds (as the main crops imported in Saudi Arabia) and protein structural analysis depicted from the gene(s) responsible for toxin biosynthesis. Which can be used as screening test for cost-effective control of mycotoxin and their products. We have hypothesized that by study the molecular characterizations and bioinformatics properties of Aflatoxins could in future be able to produce vaccine for species of *Aspergillus* genera which have higher prevalence rate in development countries **(Bhatnagar et al. 2003; Pasquali et al. 2016)**.

MATERIAL AND METHODOLOGY

Grains samples and isolation of mycotoxigenic *Aspergillus* species

One hundred fifty grains corn (yellow and red grains) and rice were collected from different area of Saudi Arabia (Riyadh, Hail, Qasim, Asir,Tabuk, Jizan, Jouf, Jeddah and Dammam), where collected from storage markets and houses. The collected grains were randomly and its weight between 0.5 – 1 kg of each grain in cleans and dries packaging. Agar plate and blotter tests were used to isolate *Aspergillus* sp. as described by **Neergaard (1977)**. Grains were divided into two groups, the first group was disinfected with sodium hypochlorite 1% for 2 min and the second group was non-disinfected. All grains were washed several times by sterilized water, and then dried between sterilized filter papers. The half of each group was plated on potato dextrose agar (PDA) (Sigma-Aldrich, USA). All dishes were incubated for 5 – 7 days at 25 °C. All isolation process under sterilized conditions to prevent any contamination of grain.

Purification and identification of *Aspergillus* species

Aspregillus species were initially identified to species based on the morphological characteristics **(Leslie, Summerell and Bullock, 2006)** of the macroconidia, microconidia and general mycelium presentation from a single spore isolate grown for 7 – 10 days on SNA with an Olympus BH-2 (Olympus America, New York) light microscope. Potato Dextrose Agar (PDA) was used to identify colony pigment characteristics of aerial mycelium on the agar **(Leslie, Summerell and Bullock, 2006)**.

Carnation Leaf Agar (CLA) (bio-WORLD, USA) was used to identify macroconidia, chlamydospores and the presentation of aerial mycelium single colony was transferred and purified by hypha tip technique onto PDA medium in the presence of streptomycin (50 $mg.ml^{-1}$). The developing fungi were prepared for molecular identification using primers specific for the *Aspergillus flavus AFLR (aflR)* toxin gene. All conditions of isolation and purification of mycotoxins were performed under sterilization for prevent any external agent of seeds pollutions.

Molecular identification of *Aspergillus flavus AFLR (aflR)* toxin gene

Isolation of DNA genome

The mycelium mass of *Aspergillus* species isolates grown on PDA broth medium was harvested by centrifugation at 6000 rpm for 10 min. The pellets were washed twice by PBS buffer and stored at 200 °C. Total DNA of the three isolates was isolated using lysozyme – dodecyl sulfate lysis method as described by **Leach et. al. (1990)**.

Amplification and purification of Aspergillus flavus AFLR (aflR) gene

Specific PCR reactions were conducted to assess the presence of *AFLR (aflR)* gene. The primers used as described by **Cary et al. (2000)** were: Omtl-F (*aflR*) (5′-GCCTTGCAAACACACTTTCA-3′); Omtl-R 5′-AGTTGTTGAACGCCCCAGT3′) and optimal annealing (Tm = 55 °C). The PCR amplification conditions included initial denaturation at 94 °C for 5 min then 35 cycles at 94 °C for 30 s, 55 °C for 60 s followed by extension step at 72 °C for 90 s. and a final extension at 72 °C for 7 min. The amplification reaction was performed by thermal cycler (Applied Biosystems, USA). Purification of PCR product was detected by electrophoresis (PNU, Faculty of science, Research center) using agarose 1.5% in 1x TAE buffer and staining with ethidium bromide (Qiagen, Berlin, Germany) **(Sambrook et al., 1989)**. The resultant fragment of *Aspergillus flavus AFLR (aflR)* toxin gene was excised from the gel and purified using a QIA quick gel extraction kit (Qiagen, Berlin, Germany).

DNA sequencing

The purified PCR products were prepared for Sanger sequencing technology using DNA sequencer technique (Sigma, central lab, PNU, KSA). DNA sequences of *Aspergillus flavus* isolates were aligned using Bio Edit software version 7 (www. Mbio-NCUs. Edu/bio. Edit) and were compared of the often accessions of *Aspergillys* sp. available in the NCBI data base using BIASI- algorithm to identify closely related sequences (http/WWW.NCbI.Nih.Gov). Dendrograms were constructed by using unweighted pair Group method with Arithmetic (UPGMA) on Gen bank.

Epitope prediction and antigenicity

The primary amino acids sequence of the *Aspergillus flavus AFLR (aflR)* toxin gene protein was evaluated from the corresponding nucleotide sequence using MEGA 6.0 software. The linear B-cell epitopes in the primary amino acid sequence of the coat protein was performed using BCPREDS server with default parameters (http://ailab.cs.iastate.edu/bcpreds/) which implements a support vector machine (SVM) and the subsequence kernel method **(El-Manzalawy et al., 2008a)**. Flexible length linear B-cell epitopes were predicted using FBCP red **(El-Manzalawy et al., 2008b)** method with a specificity cut-off; 75%. The antigenicity of each amino acid residue in the primary protein sequence was determined using a semi-empirical method **(Kolaskar and Tongaonkar, 1990)** which makes use of physicochemical properties of each amino acid and their frequencies of occurrence in experimentally known segmental epitopes.

RESULTS AND DISCUSSION

Three *Aspergillus* isolates from tested grains by PDA method was purified by single spore and hypha tip on PDA slant medium. The *Aspergillus* isolates were selected for molecular identification using *Aspergillus flavus AFLR (aflR)* toxin gene sequencing. Three *Aspergillus* isolates represented grains from yellow corn, red corn and white rice and designated as A. *flavus* _ YC; *A. flavus* _ RC; *A. flavus* _ Rice respectively.

Molecular characters of toxin gene

Total DNA was extracted from *A. flavus* _ YC; *A. flavus* _ RC; *A. flavus* _ Rice isolates infected grains. *Aspergillus flavus AFLR (aflR)* toxin gene was amplified from isolated DNA of mycelium using PCR reaction mixture and specific primer sets. PCR amplicons were allowed for sequencing reaction through cycle sequencing method. The DNA amplicons returned as electropherogram files. Electropherogram showed distinct peaks for each base cell as well as high *Q* values for each cell. Sequences obtained for each primer for each isolate had sufficient overlap between them and used to form one continuous sequence (Coting). The nucleotide partial sequence of *Aspergillus flavus AFLR (aflR)* toxin gene in the three isolates was compared with published isolates on Gen Bank. The sequence homology revealed that the gene of interest *Aspergillus flavus AFLR (aflR)* toxin gene and the test fungal isolates was *Aspergillus flavus* isolates. A multiple sequence alignment was constructed using ClustalW software (GNU Lesser GPL) between the three studied isolates. The alignment showed many conserved regions in all sequences as well as distinguished the heterogeneity positions among the aligned sequences (Figure 1a, 1b, 1c). Phylogenetic analysis was performed by construction of phylogenetic tree using a neighbor joining method to unravel the relationships among all *Aspergillus flavus* isolates (Figure 2). The phylogenetic tree resulted in two clades in which *A. flavus* _ Rice (white rice isolate) and *A. flavus* _ RC (red corn isolate) were in the same cluster whilst *A. flavus* _ YC (yellow corn isolate) was separate in a different cluster (Figure 2). Thus, the molecular identification based on sequence homology of the *Aspergillus flavus AFLR (aflR)* toxin gene confirmed the identity and phylogeny of the studied three *Aspergillus flavus* isolates.

The epitope prediction analysis demonstrated that there were 1, 2, 3 and 4 epitopes whose score were equal 1 in *A. flavus* _ YC (yellow corn), B: *A. flavus* _ RC (red corn) and C: *A. flavus* _ Rice (white rice). Also, there were great

variations in the epitope sequences among the three isolates except in RLQEGGDDAAGIPA, SPPPPVETQGLGGD, RPSESLPSARSEQG and PAHNTYSTPHAHTQ were found to be common between all isolates. These residues with high frequencies of occurrences in antigenic determinants were highlighted (yellow) in the antigenicity profile (Figure 3). Figure (3) also show the variability in the positions and types of amino acid residues with high antigenic frequency.

Mycotoxin detection is a major problem in developing countries where contaminated food commodities may readily reach food stores and homes **(Bilodeau, 2011; Boutigny et al., 2012; Taha et al., 2017)**. The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops **(Abdulkadar et. al., 2004; Mwanza et al., 2013)**. Humans are exposed to mycotoxins throughout their life time due to consumption of fungus-contaminated food products and many human diseases, especially carcinogenic, teratogenic, hepatic, and gastrointestinal ones, have been found linked with the ingestion of mycotoxin-contaminated products **(Fung and Clark, 2004; Shephard, 2008; Mwanza et al., 2013)**. Sufficient quantities of mycotoxins in food and feedstuff can adversely affect human and animal health **(Qiu and Shi, 2014)**. Environmental factors and host species have a strong impact on the occurrence of a specific chemotype and the incidence of *Aspergillus* species **(Bhatnagar et al., 2003)**. The distribution of *Aspergillus* species in maize is influenced by optimal climatic conditions, pathogenicity and competition between other fungi **(Bhatnagar et al., 2006)**. The type of environmental factor identified in the incidence of *Aspergillus* species as demonstrated in recent EU maize surveys **(Creepy, 2002)**. In those studies, the prevalence of species varied year-to-year and was believed to be associated with the differences in climatic conditions between years **(Caldas et al., 2002; Scauflaire et al., 2011)**. As was reported by **Covarelli et al. (2015)** the emergence of toxigenic fungi on small grains has a negative impact on the safety and quality of feed and food. in this study We isolate mycotoxins strains of *Aspergillus* sp from contaminated corn and rice grains by a genomic library through amplication mycotoxins structure genes *(aflR)* by polymerase chain reaction (PCR) and sequencing of amplicans this result was agree with **(Chen et al., 2002)**. Data clearly reveal that the PCR technique is efficient in distinguishing mycotoxins **(Bilodeau, 2011; de Souza et al., 2005; Taha et al., 2012; Allam et al., 2015)** from commonly inhabiting stored grains. We isolate *Aspergillus* sp. from both zellow and red corn which used in food and feed in human and animal; also isolated from long and short rice **(Niessen, 2007; Fox and Howlett, 2008)**. *Aspergillus* species being able to grow at moderate to high temperature **(Ehrlich et al., 2003)** and its responsible for spoilage of food commodities during transport and storage. In addition, reduction in nutritive value, insipidness and discoloration are other problems resulted from contamination of grains by *Aspergillus* **(Dean et al., 2012)**. Rapid and accurate identification of *Aspergillus* and/or their metabolites are mandatory for the implementation of preventive measures in the whole food production system as was reported by **Bok and Keller, (2004); Bhatnagar et al. (2003)** and **Dean et al. (2012)**. The molecular characterization of three *Aspergillus* sp. isolated from small grains (yellow corn, white rice and red corn) using the mycotoxins gene, aflatoxin *AFLR (aflR)* allowed for coupled identification and mycotoxins screening in the three *Aspergillus* isolates **(Bhatnagar et al., 2006)**. Mycotoxins-producing fungi were isolated from sorghum grains from Saudia Arabia before **(Mahmoud et al., 2013; Yassin et al., 2010)**. Following the molecular identification of *Aspergillus* sp. B-cell epitopes in the aflatoxin *AFLR (aflR)* gene were predicted. The characterization of B-cell epitopes using computational tools is highly advantageous for the synthesis of specific antibodies for rapid detection of microbial pathogens in their environments. The epitopes prediction saves labor and time for validation experiments. The identification of epitopes plays a crucial role in the vaccine design, immunodiagnostic testing and antibody production **(Sette and Fikes, 2003)**. In this study, BCPREDS server was used to predict epitopes found in the primary amino acids sequence of aflatoxin *AFLR (aflR)* protein. BCPREDS proved high efficiency to predict linear B-cell epitopes in SARS-CoV S protein **(El-Manzalawy et al., 2008a; El-Manzalawy et al., 2008b)**. There was variability in the sequence and numbers of epitopes among the three toxin proteins analyzed. Here, a fixed length of epitopes (14 residues) was observed. The epitope prediction analysis demonstrated that there were 1, 2, 3 and 4 epitopes whose score were equal 1 in *A. flavus* _ YC; *A. flavus* _ RC; *A. flavus* _ Rice respectively. In this study, there were great dissimilarity in the epitope sequences among the three isolates. But we found that there some epitope sequences were common between all isolates in RLQEGGDDAAGIPA, SPPPPVETQGLGGD, RPSESLPSARSEQG and PAHNTYSTPHAHTQ. This result suggesting its exploitation for design of a specific antibody to be used for rapid detection of different *Aspergillus* species in small grains **(Ehrlich et al., 2003; Degola et al., 2007)**. The highly frequent residues with high antigenicity profiles such as valine, leucine, isoleucine, aspartic acid, glutamine and glutamic acid are mostly hydrophobic **(Duan et al., 2007)**. The occurrence of hydrophobic residues in epitopes is frequent and do have a hierarchy signature **(Zhang, 2002; Aftabuddin and Kundu, 2007; Suga and Galel, 2007; Scauflaire et al., 2011)**. Epitope prediction has many implications in pathogen detection and differentiation applications. The consideration of occurrence of *Aspergillus* sp. on small grains is important in risk assessment of mycotoxins and setting up preventive measures proactively. The main problems concentrated in high risk outbreaks of aflatoxicosis have been reported in different countries all over the world; that because the widespread of aflatoxin contamination especially in developing countries **(Pitt 2000a; Pitt 2000b; Kovacs, 2004)**. No doubt, that the safety control in food and feed processing should be by prevention of mycotoxin contamination in agriculture by used as simple and rapid screening tests for cost-effective control of food diseases by production of vaccines **(Sette and Fikes, 2003; Probst et al., 2014; Mwanza et al., 2013; Allam et al., 2015)**.

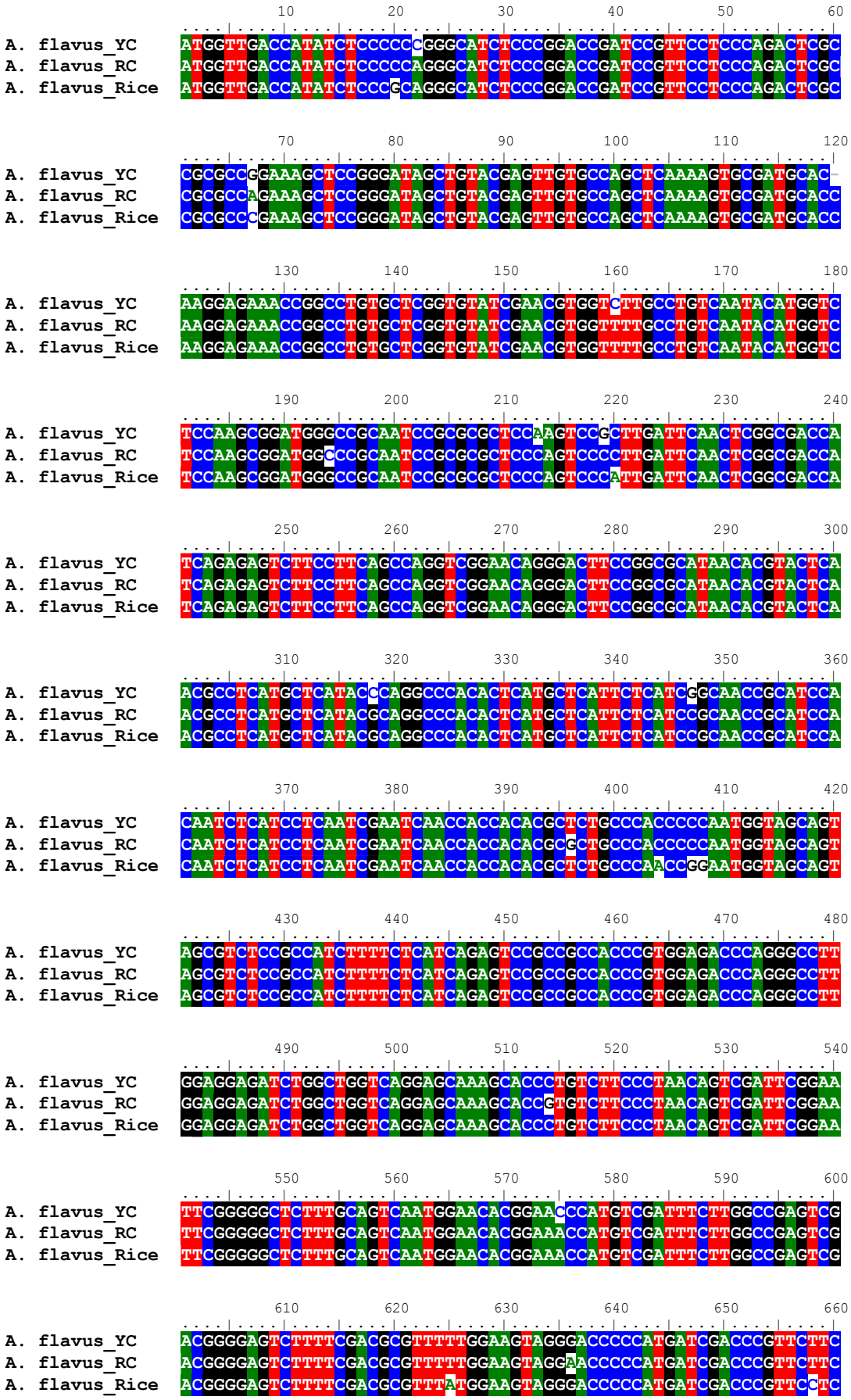

Figure 1a Multiple sequence alignment of the *aflr* gene partial sequence among three *Aspergillus flavus* isolated from yellow corn (YC), red Corn (RC) and rice.

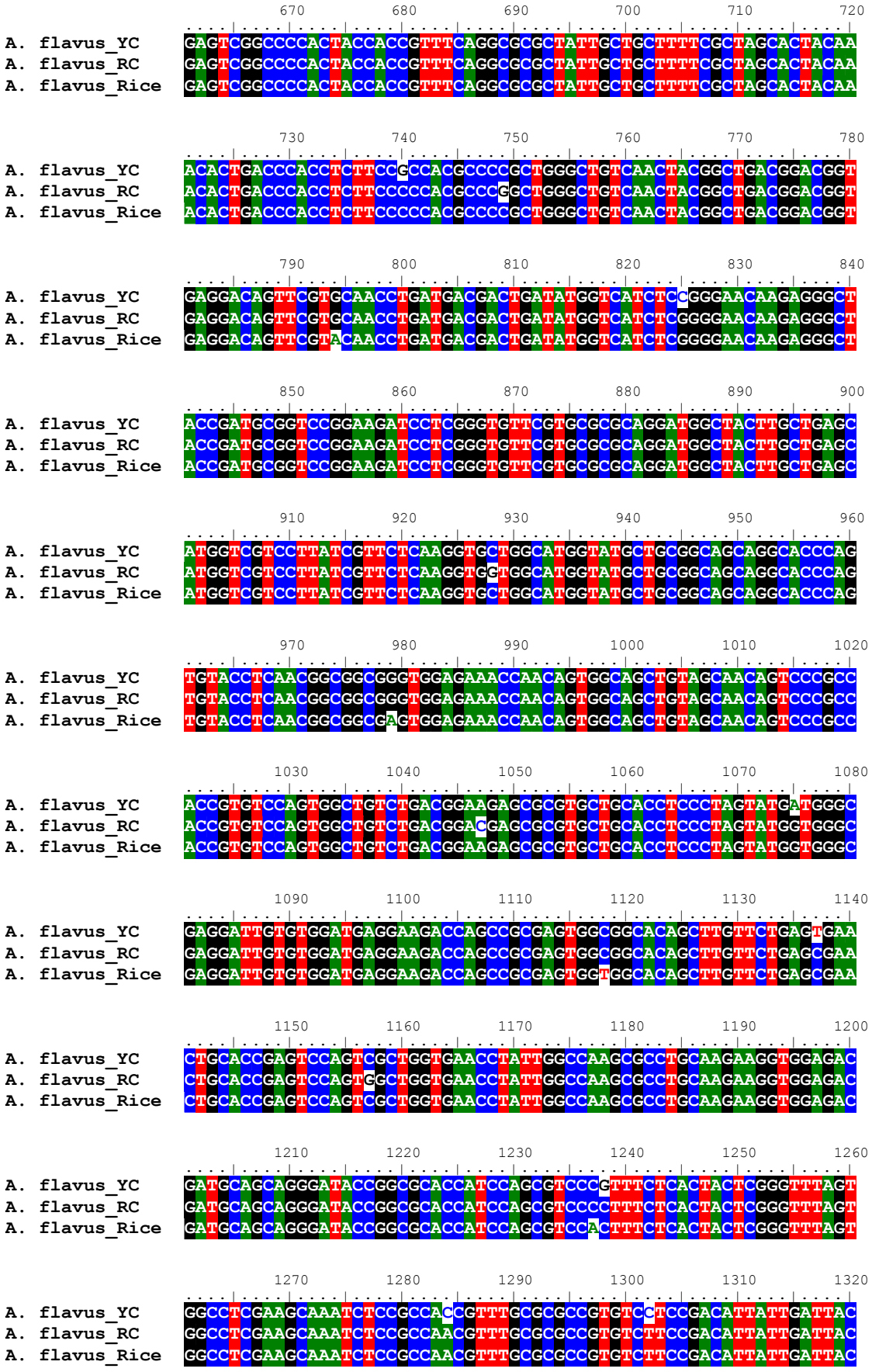

Figure 1b Multiple sequence alignment of the *aflr* gene partial sequence among three *Aspergillus flavus* isolated from yellow corn (YC), red corn (RC) and rice.

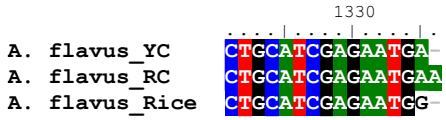

Figure 1c Multiple sequence alignment of the *aflr* gene partial sequence among three *Aspergillus flavus* isolated from yellow corn (YC), red corn (RC) and rice.

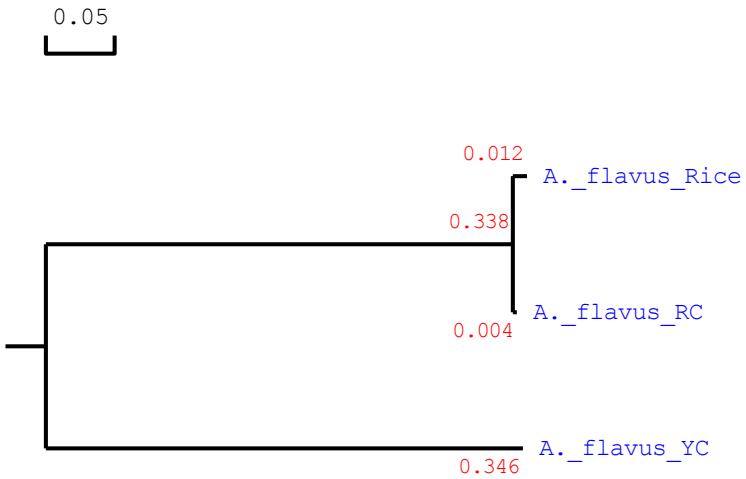

Figure 2 Phylogenetic tree using neighbor joining method among the three *Aspergillus* isolates.

Table 1 Flexible length predictions of epitopes in the amino acids sequence of *AFLR (aflR)* gene sequence protein of the three *Aspergillus flavus* isolates.

No.	Epitope/ A. flavus _ YC (yellow corn)	Score/	Epitope/ A. flavus _ RC (red corn)	Score/	Epitope/ A. flavus _ Rice (white rice)	Score/
1	RLQEGGDDAAGIPA	1	RLQEGGDDAAGIPA	1	RLQEGGDDAAGIPA	1
2	SPPPPVETQGLGGD	1	SPPPPVETQGLGGD	1	SPPPPVETQGLGGD	1
3	DHISPRASPGPIRS	1	DHISPRASPGPIRS	1	GETNSGSCSNSPAT	1
4	PPHALPTPNGSSSV	1	PPHALPTPNGSSSV	1	RRASPGPIRSSQTR	1
5	GETNSGSCSNSPAT	1	GETNSGSCSNSPAT	1	PHALPNRNGSSSVS	1
6	IDPFFESAPLPPFQ	0.997	IDPFFESAPLPPFQ	0.997	IDPFLESAPLPPFQ	0.996
7	MGRNPRAPSPLDST	0.994	MARNPRAPSPLDST	0.995	MGRNPRAPSPIDST	0.992
8	RPSESLPSARSEQG	0.99	RPSESLPSARSEQG	0.99	RPSESLPSARSEQG	0.99
9	PAHNTYSTPHAHTQ	0.936	PAHNTYSTPHAHTQ	0.936	PAHNTYSTPHAHTQ	0.936
10	MEHGTHVDFLAEST	0.847	VRCTKEKPACARCI	0.855	VRCTKEKPACARCI	0.855
11	SSGCLTEERVLHLP	0.842	TDGEDSSCNLMTTD	0.79	SSGCLTEERVLHLP	0.842
12	TDGEDSSCNLMTTD	0.79	VVLIVLKVVAWYAA	0.778	THLFPHAPLGCQLR	0.733
13	KVRCKEKPACARCI	0.773	VGEDCVDEEDQPRV	0.713	VGEDCVDEEDQPRV	0.713

CONCLUSION

A best strategy to control mycotoxins is only by prevention, because most mycotoxins are chemically stable, so they remain unaffected during storage and processing. For this reason, we believed that by using a highly sensitivity and selectivity methods as molecular identification and bioinformatics characterizations of protein toxic gene for mycotoxins gives chance for production of antibody against its toxicity. This paper was show that molecular identification in *Aspergillus flavus* in three isolates in small grains (rice and corn) with the epitope prediction analysis demonstrated that there were great differentiations in the epitope sequences among the three isolates except in four position (as described above) were found to be common between all isolates. This work articulates that the molecular identification and characterization of three *A. flavus* using *Aspergillus flavus AFLR (aflR)* toxin gene and the unique antigenic determinants that could be used for design of a broad-spectrum antibody for rapid detection of *A. flavus* in foods and feeds that conducive to control in aflatoxin levels and cover prevention of mycotoxins.

A

```
1         11        21        31        41        51        60
|         |         |         |         |         |         |
MVDHISPRASPGPIRSSQTRRAGKLRDSCTSCASSKVRCKEKPACARCIERGLACQYMVS 60
..EEEEEEEEEEEEE...................EEEEEEEEEEEEEE...........
KRMGRNPRAPSPLDSTRRPSESLPSARSEQGLPAHNTYSTPHAHTQAHTHAHSHRQPHPQ 120
..EEEEEEEEEEEEE.EEEEEEEEEEEEEE.EEEEEEEEEEEEEE.............
SHPQSNQPPHALPTPNGSSSVSAIFSHQSPPPPVETQGLGGDLAGQEQSTLSSLTVDSEF 180
.......EEEEEEEEEEEEEE.......EEEEEEEEEEEEEE................
GGSLQSMEHGTHVDFLAESTGSLFDAFLEVGTPMIDPFFESAPLPPFQARYCCFSLALQT 240
......EEEEEEEEEEEEEE.............EEEEEEEEEEEEEE...........
LTHLFRHAPLGCQLRLTDGEDSSCNLMTTDMVISGNKRATDAVRKILGCSCAQDGYLLSM 300
...............EEEEEEEEEEEEEE..............................
VVLIVLKVLAWYAAAAGTQCTSTAAGGETNSGSCSNSPATVSSGCLTEERVLHLPSMMGE 360
.........................EEEEEEEEEEEEE.EEEEEEEEEEEEEE.....
DCVDEEDQPRVAAQLVLSELHRVQSLVNLLAKRLQEGGDDAAGIPAHHPASRFSLLGFSG 420
................................EEEEEEEEEEEEEE.............
LEANLRHRLRAVSSDIIDYLHRE 443
.......................
```

B

```
1         11        21        31        41        51        60
|         |         |         |         |         |         |
MVDHISPRASPGPIRSSQTRRARKLRDSCTSCASSKVRCTKEKPACARCIERGFACQYMV 60
..EEEEEEEEEEEEE.....................EEEEEEEEEEEEEE...........
SKRMARNPRAPSPLDSTRRPSESLPSARSEQGLPAHNTYSTPHAHTQAHTHAHSHPQPHP 120
...EEEEEEEEEEEEE.EEEEEEEEEEEEEE.EEEEEEEEEEEEEE.............
QSHPQSNQPPHALPTPNGSSSVSAIFSHQSPPPPVETQGLGGDLAGQEQSTVSSLTVDSE 180
........EEEEEEEEEEEEEE.......EEEEEEEEEEEEEE................
FGGSLQSMEHGNHVDFLAESTGSLFDAFLEVGTPMIDPFFESAPLPPFQARYCCFSLALQ 240
.................................EEEEEEEEEEEEEE...........
TLTHLFPHARLGCQLRLTDGEDSSCNLMTTDMVISGNKRATDAVRKILGCSCAQDGYLLS 300
................EEEEEEEEEEEEEE..............................
MVVLIVLKVVAWYAAAAGTQCTSTAAGGETNSGSCSNSPATVSSGCLTDERVLHLPSMVG 360
.EEEEEEEEEEEEE............EEEEEEEEEEEEEE.................EE
EDCVDEEDQPRVAAQLVLSELHRVQWLVNLLAKRLQEGGDDAAGIPAHHPASPFSLLGFS 420
EEEEEEEEEEEE.....................EEEEEEEEEEEEEE.............
GLEANLRQRLRAVSSDIIDYLHRE 444
.......................
```

C

```
1         11        21        31        41        51        60
|         |         |         |         |         |         |
MVDHISRRASPGPIRSSQTRRARKLRDSCTSCASSKVRCTKEKPACARCIERGFACQYMV 60
......EEEEEEEEEEEEEE.............EEEEEEEEEEEEEE...........
SKRMGRNPRAPSPIDSTRRPSESLPSARSEQGLPAHNTYSTPHAHTQAHTHAHSHPQPHP 120
...EEEEEEEEEEEEE.EEEEEEEEEEEEEE.EEEEEEEEEEEEEE.............
QSHPQSNQPPHALPNRNGSSSVSAIFSHQSPPPPVETQGLGGDLAGQEQSTLSSLTVDSE 180
.........EEEEEEEEEEEEEE......EEEEEEEEEEEEEE................
FGGSLQSMEHGNHVDFLAESTGSLFDAFMEVGTPMIDPFLESAPLPPFQARYCCFSLALQ 240
.................................EEEEEEEEEEEEEE...........
TLTHLFPHAPLGCQLRLTDGEDSSYNLMTTDMVISGNKRATDAVRKILGCSCAQDGYLLS 300
..EEEEEEEEEEEEE.............................................
MVVLIVLKVLAWYAAAAGTQCTSTAASGETNSGSCSNSPATVSSGCLTEERVLHLPSMVG 360
.........................EEEEEEEEEEEEEE.EEEEEEEEEEEEEE..EE
EDCVDEEDQPRVVAQLVLSELHRVQSLVNLLAKRLQEGGDDAAGIPAHHPASTFSLLGFS 420
EEEEEEEEEEEE.....................EEEEEEEEEEEEEE.............
GLEANLRQRLRAVSSDIIDYLHREW 445
.........................
```

Figure 3 Show the amino acids sequence of *AFLR (aflR)* gene sequence protein of the three *Aspergillus flavus* isolates.

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SENSORY PROFILE OF PARENICA CHEESE VARIETIES MADE FROM PASTEURIZED COW'S MILK

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ABSTRACT
Parenica is a steamed, lightly smoked or unsmoked cheese wounded into a roll made from pasteurized cow's milk, with characteristic pronounced fibrous structure of curd. The aim of this work was to set up the sensory profile of smoked and unsmoked parenica cheese varieties made from pasteurized cow's milk and changes in sensory descriptors during 14 days of storage period at the temperature of 4 ±2 °C. Descriptive analysis was carried out by 18 trained assessors, who used a vocabulary of 26 terms to quantitatively describe appearance, aroma, consistency and taste of the experimental samples and also these overall sensory parameters with acceptability. Assessors evaluated the intensity of each descriptor by assigning the score on a 10 points linear scale. Analysis of variance found significant differences between cheese varieties (p <0.05) and the effect of storage period (p <0.05) on sensory quality of experimental parenica cheese varieties. The analysis showed that each sample group in observed representative sensory attributes was significantly different (p <0.05). Multiple factorial analysis showed in parenica cheese samples three selected components that explain more than 69% of the total variation in the dataset at the level of statistical significance p <0.05.

Keywords: cheese; sensory profile; parenica; storage; statistics

INTRODUCTION
The pasta filata cheese is a diverse group of cheeses, which originated mainly in the northern Mediterranean, in countries such as Italy, Greece, the Balkans, Turkey and Eastern Europe **(Zimanová et al., 2016)**.

Parenica is a steamed, lightly smoked or unsmoked cheese wounded into a roll made from pasteurized cow's milk. It is known for the characteristic pronounced fibrous structure of curd. The roll is ussualy bounded with cheese string.

In the last years, there has been a growing attention towards food quality and food safety, as well as an increasing demand for "natural" products, especially those enjoying a 'recognition of quality' status and recognition of 'geographical indications and traditional specialties' (PDO, PGI and TSG) conferred by the European Community to promote and protect the names of quality agricultural products and foodstuffs **(Todaro et al., 2017)**.

Steamed cheese production has a long tradition in Slovakia, although originally made from sheep's milk **(Čuboň et al., 2015)**. However, similar dairy product "Slovenská parenica" cheese obtained the "Protected Geographic Indication" designation (PGI) in 2008, but according the "Methods of Production" described in application for registration of "Slovenské parenica", it could be made from fresh raw, unprocessed sheep's milk or a mixture with fresh raw, unprocessed cow's milk, containing at least 50% sheep's milk **(Council Regulation (EC) No. 510/2006)**.

In comparison to Cheddar and Dutch-type cheeses, pasta-filata cheeses represent a special case, in terms of proteolytic pattern **(Salek et al., 2017)**. The pasta filata cheeses varied in sensory quality, which depends on the quality of milk and processing technology (including the process of pasteurisaztion). These cheeses could be made according the following methods: 1. traditional process of making steamed cheese, 2. a method of making steamed cheese using direct acidification of the milk and continuous processing **(Zimanová et al., 2016)**.

Recent studies aimed to the traditional pasta filata, stretched or steamed cheese making technology have been published **(Di Grigoli et al., 2015; Scatassa et al., 2015; Sulejmani and Hayaloglu, 2016; Cuffia et al., 2017; Paz et al., 2017; Salek et al., 2017; Todaro et al., 2017)**. However, many of them deal with the physicochemical and microbial quality of the pasta filata cheeses.

Cheese is an irreplaceable dairy product in human nutrition and it could be consumed fresh or ripened **(Ozcan et al., 2011)**. Parenica cheese could be lightly smoked during the processing of curd. Smoking is technique of food preservation, but it is also used to enhance sensory properties, taste, color and texture **(Esposito et al., 2015)**. The special taste and textural parameters of traditional

cheese specialties could make them popular **(Farahani, Ezzatpanah and Abbasi, 2014)**.

Generally, smoking of high protein foods enrich the food with aromatic components that provide organoleptic properties as color and flavour to meals and have bacteriostatic and antioxidant effect **(Amran and Abbas, 2011)**. Because of that it is very important to study the qualitative sensory parameters that define the product in order to determine its acceptability to consumers **(Semjon et al., 2018)**. The sensory quality of a product like parenica cheese can be also evaluated using quantitative descriptive analysis.

The aim of this study was to describe sensory profile of smoked and unsmoked parenica cheese manufactured according to the traditional method of production during storage period.

Scientific hypothesis

Hypothesis 1: There exist differences in evaluation of sensory overall attributes (appearance, aroma, consistency, taste and acceptability) in parenica cheese varieties before and after storage of 14 days at 4 ±2 °C.

Hypothesis 2: Sensory profile of parenica cheese varieties changes during storage for 14 days at 4 ±2 °C.

Hypothesis 3: Statistically significant correlations exists between individual sensory descriptors and overall sensory parameters in parenica cheese varieties.

Hypothesis 4: A dissimilarity in sensory parameters exist between each sample groups (smoked/unsmoked parenica cheese samples and samples before/after storage).

MATERIAL AND METHODOLOGY

Processing of experimental cheese

Raw milk was standardised to fat content that final parenica cheese has a minimum fat in dry matter content 30%. Subsequently, raw milk was pasteurised before curdling at a temperature of 72 °C for 20 seconds. The milk was heated to the temperature 30 ±2 °C and inoculated with a fermenting agent composed of mesophilic lactic acid bacteria (*Lactococcus lactis* ssp. *lactis, Lactococcus lactis* ssp. *cremoris*). After adding the rennet was milk curdled at a temperature 29 – 32 °C. Curdled cheese mass was mixed using a cheese harp to produce 1 cm pieces. The cheese mass was left to settle down before being moulded into a lump. The lump was then lifted out of the whey with a cheese cloth and left to drain. After 8 hours of draining and solidifing was the lump placed on a rustproof shelf to ferment. Fermentation continued approximately 24 hours at a temperature 20 ±2 °C to achieve a pH level for the cheese about 5.2. Then the lump cheese was cut into smaller pieces and placed in a water bath (temperature of 60 – 70 °C). When the cheese curd become more plastic, it was removed from the bath and excess water was squeezed out. Subsequently it was streched and folded over several times and a cheese strips were formed by hand, so that they had a length of 1 – 2 m, a width of about 3 cm and a thickness 2 – 3 mm. The cheese strips were placed in a prepared cold saturated salt solution to cool down and after that they were squeezed out. Cheese strips were rolled up and bounded with a cheese strings made separately in water bath from the lump cheese. Then they were dried and a half of them were smoked in a smoking chamber. From a dairy plant to laboratory were the samples packed and transported at the temperature of 4 ±2 °C in plastic bags.

Sampling

To study the sensory profile and quality of parenica cheese were evaluated following sample groups (Figure 1):

- PU (unsmoked parenica cheese made from pasteurized cow's milk),
- PS (lightly smoked parenica cheese made from pasteurized cow's milk).

A total of 72 experimental parenica cheese samples were evaluated in this study. On the initial day of manufacture were from each group (PU, PS) randomly selected samples and divided into the following groups:

Figure 1 Experimentally made smoked and unsmoked parenica cheese varieties.

• the first group consisted of initial samples (n = 18) which were analyzed before storage period (BS),
• the second group contained samples (n = 18) that were placed in plastic bags to prevent desiccation but exposed to normal atmosphere conditions and stored for 14 days at the temperature of 4 ±2 °C (samples after storage – AS).

Plastic bags used in AS sample groups were made from polyethylene with thickness of 70 μm (G-PACK, LLC. Slovak Republic).

All experimental parenica cheese samples were produced in compliance with the traditional methods of production in a dairy plant according to the traditional processing procedures for parenica cheese according **Zimanová et al. (2016)** and modified method of production of "Slovenská parenica" PGI cheese **(Council Regulation (EC) No. 510/2006)**.

Sensory analysis

Samples were received from the dairy plant in plastic bucket containers and stored at 4 ±2 °C until use. The cheeses were portioned into 25 × 25 × 25 mm cubes with approximate weight of 25 g using a wire slicer, served in white plastic dishes and coded with three-digit random numbers. Samples were served at temperature of consumption 20 ±2 °C. Mineral water was provided for mouth rinsing.

Descriptive Analysis

The descriptive sensory evaluation was carried out in a standardized sensory laboratory **(ISO 8589, 2007)** built in the Institute of Postgraduate Education of Veterinary Medicine in Košice according to **Lawless and Heymann (2010)**. The sensory evaluation was performed by a panel from the staff of the University of Veterinary Medicine and Pharmacy in Košice. The group contained 18 panelists aged between 28 and 65. All the assessors were trained in the sensory analysis. Sensory profile was conducted according the General guidance for establishing a sensory profile **(ISO 13299, 2016)**.

Steps in establishing a sensory profile included following steps: establishing a sensory facility (design of test rooms), selection of products by trained experts, selection and traing assessors for the study purpose (selected assessors and recognition of odours), selection of descriptors suitable for the application (vocabulary, flavour profiles, identification of descriptors and texture profile), determination of the perception order of the attributes in the profile (evaluation using scales and magniture estmation), training the assessors to use the selected descriptors and scale (selected assessors and expert assessors), conduction the test (general guidance and flavour profiles) and reporting the results.

Subsequently, the panel evaluated experimental parenica samples before and after the 14th day by the same panel. Assessors evaluated the intensity of each descriptor by assigning the score on a 10 points linear scale (0 – absence of sensation and 10 – extremely intense). Each assessor evaluated also the overall sensory parameters (appearance, smell, consistency and taste) of the served samples.

Statisic analysis

Data analysis was carried out with R-statistics software **(R Core Team, 2017)**. The differences between experimental parenica cheese varieties and the effect of storage were set as the main factors. A two-way analysis of variance (ANOVA) and multiple factor analysis with "FactomineR" **(Sebastien, Josse and Husson, 2008)** and "factoextra" package **(Kassambara and Mundt, 2007)** were conducted and a confidence interval was set at 95%.

RESULTS AND DISCUSSION

Description terminology for the sensory attributes of both varieties of parenica cheese (PU and PS) was introduced during the training sessions of panellist. Descriptors for appearance (white, polished, fibrous, smoked), aroma (lactit, buthyric, yoghurt, nutty), consistency (firm, elastic, cohesive, gummy, juicy, sticky, formable, fibrous, supple greasy) and taste (sour, bitter, sweet, salty, lactic, creamy, spicy, smoked), were selected.

The results of overall sensory characteristics in PU experimental cheese samples during storage period are shown in Figure 2. We observed decreased trend in overall sensory characteristics after storage in PU samples at a statistical significance level $p < 0.05$ (Figure 2). Descriptive sensory analysis performed on unsmoked experimental parenica cheese samples demonstrated statistically significant influence of storage on the intensities of sensory descriptors of white appearance; descriptors of aroma: buthyric, yoghurt, nutty; descriptors of consistency: firm, cohesive, juicy, sticky, formable, fibrous, greasy; descriptors of taste: sweet, lactic, spicy and smoked ($p < 0.001$). In comparison to Cheddar and Dutch-type cheeses, pasta-filata cheeses represent a special case (in terms of proteolytic pattern). Particularly, the casein molecules ("fibres" or "strings") are arranged distinctly after the stretching process **(Costabel, Pauletti and Hynes, 2007)**.The cheese stretching temperature can dramatically influence the behavior of aroma compounds, colour and texture attributes **(Sulejmani and Hayaloglu, 2016).** The immersion of the cheese-curd in the hot liquid is a specific process enhancing its plasticization and stretching properties **(Salek et al., 2017)**.

At the initial stage of storage were found statistically significant differences between experimental cheese varieties in each sensory descriptor except fibrous appearance, nutty aroma, firm, elastic, cohesive, gummy, sticky, fibrous consistency, and sour and spicy taste.

Figure 3 shown that overall sensory paramters in PS samples significantly changed after storage period only in consistency, taste and acceptability ($p < 0.05$).

Descriptive sensory analysis performed on smoked experimental parenica cheese samples demonstrated statistically significant influence of storage on the intensities of sensory descriptors of white and smoked appearance; descriptors of aroma: buthyric and nutty; descriptors of consistency: elastic, cohesive, gummy, juicy, sticky, formable and greasy; descriptors of taste: sweet, lactic and smoked ($p < 0.001$). Consistency is an important viscoelastic property of the product **(Amiri et al., 2018)**. **Cais-Sokolińska, Pikul and Lasik (2012),** found that changes in texture parameters of cheeses as a result of smoking depended on their storage time. We agree with the autors that smoked cheese not subjected to storage was harder than unsmoked cheese. It could be caused by increased dry matter of smoked cheese, or by loss of water content, respectively.

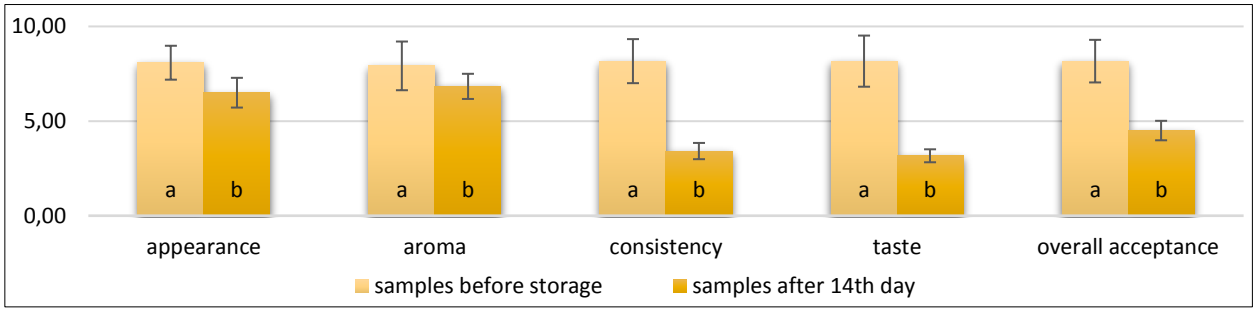

Figure 2 Overall sensory parameters of experimental unsmoked parenica cheese (PU) during storage period.

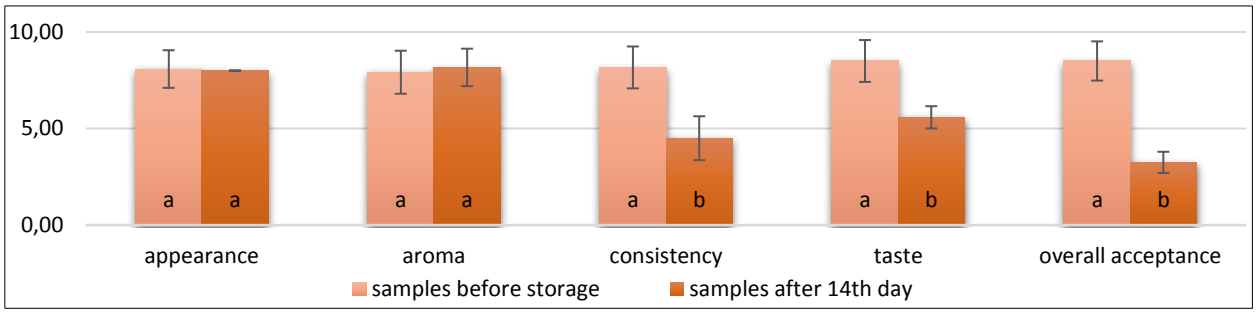

Figure 3 Overall sensory parameters of experimental smoked parenica cheese (PS) during storage period.

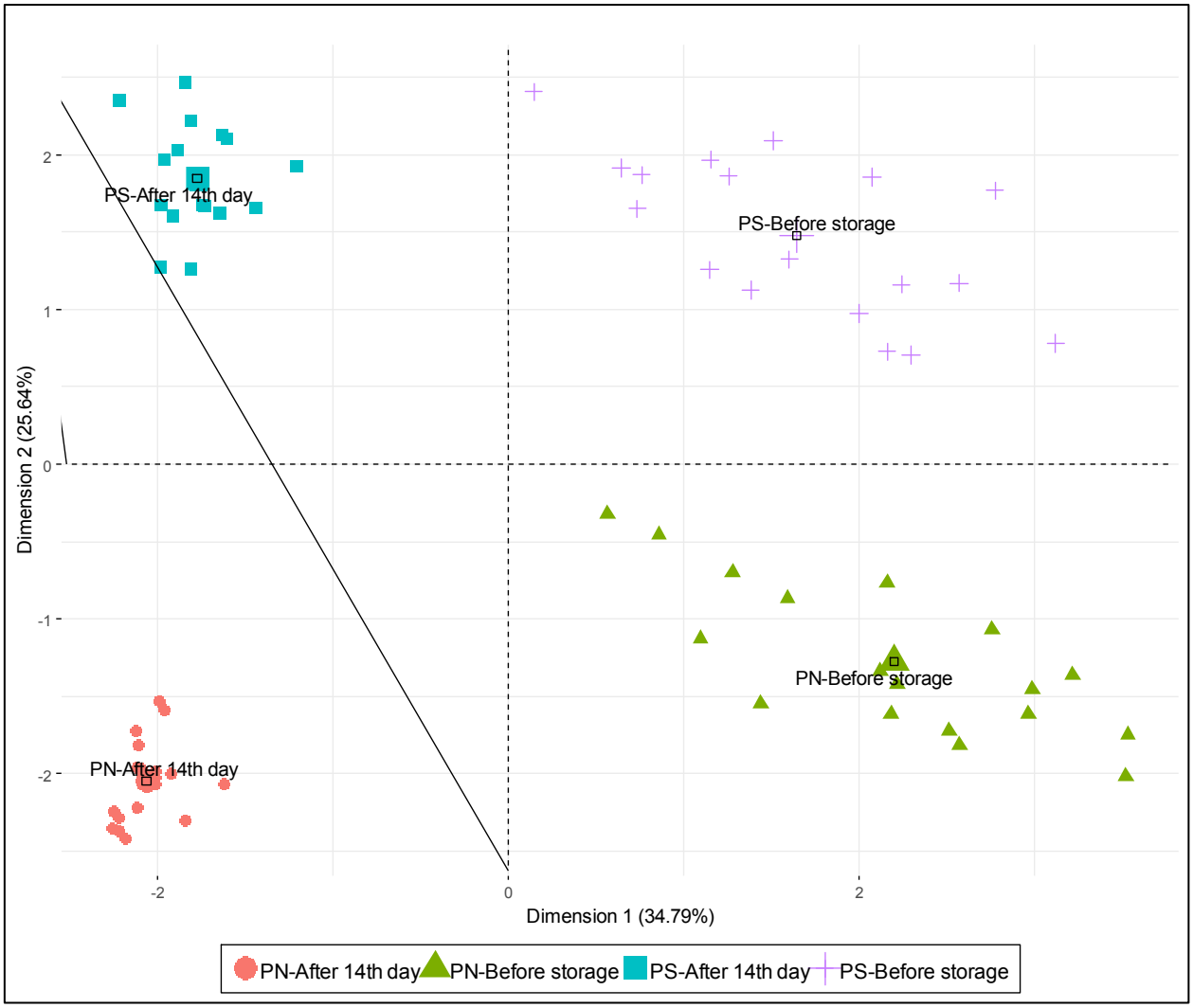

Figure 4 Multiple Factor Analysis plot of parenica cheese varieties individuals during storage.

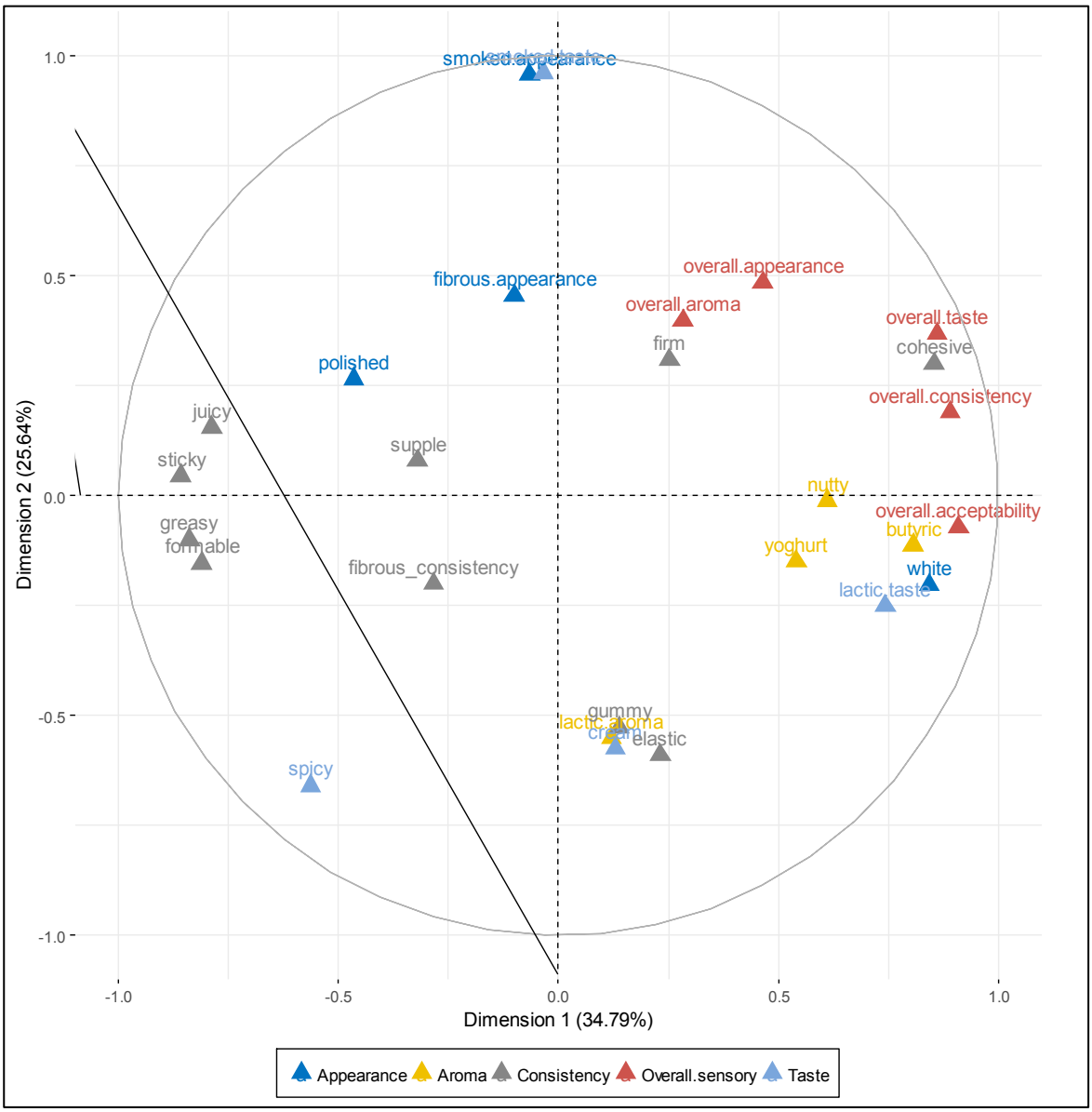

Figure 5 Quantitative variables of Multiple Factor Analysis used for evaluation of parenica cheese varieties during storage.

We observed statistical significant efect of storage on salty taste only in PU samples. Assessors evaluated salty taste in PU samples after storage with higher intensity. On the other hand, intensity of salty taste in PS samples was at the same level before and after storage period of 14 days. We agree with the authors **Todaro et al. (2017)** and **Gaucheron et al. (1999)** who found a slightly increase of salt during 14 days of storage of stretched cheese, and moreover, these authors established a migration of sodium, potassium and chloride ions from the outer layer versus the core of cheese which ended after 5 days.

In our study, multiple factorial analysis (MFA) method was applied to the sensory data, whereas were analysed results of overall sensory characteristics and sensory profile of two varieties of parenica cheese samples during storage period of 14 days. The analysis extracted the most significant variables with a minimum loss of information. Kaiser's criterion (eigenvalue >1) was applied to determine the number of final factors from the initial ones **(Chapman, Lawless and Boor, 2001)**.

The results of MFA showed in parenica cheese samples three selected components that explain more than 69% of the total variation in the dataset. The first dimension (Dim1) explains 34.79% of variation, second dimension (Dim2) 25.64% and third dimension (Dim3) 8.39%.

The highest contribution of the analysed data in Dim1 was related to the effect of storage period (21.93%, r = 0.95) and overall sensory parameters (18.19%, r = 0.89). The highest contribution in Dim 1 included following overall sensory parameters: acceptability (r = 0.91), consistency (r = 0.89), taste (r = 0.86), appearance (r = 0.46) and aroma (r = 0.28), which correlated at statistically significant level (p <0.05).

The first two dimensions explained a total of 60.43% of variance (Figure 4). The highest effect of variety on parenica cheese characteristics was in Dim2 (30.33%, r = 0.84), followed by the descriptors of taste (30.48%, r = 0.52) and descriptors of appearance (21.59%, r = 0.89). In Dim 2 were significantly correlated following descriptors of taste smoked (r = 0.96), lactic (r = -0.25), creamy (r = -0.59) ans spicy (r = -0.66) and following descriptors of appearance: smoked (r = 0.96), fibrous (r = 0.45) and

polished (r = 0.26), at statistically significant level (p <0.05) (Figure 5).

Descriptors of consistency contribute mainly to Dim3 with 25.07% (r = 0.67), followed by the descriptor of aroma 24.64% (r = 0.57). In Dim3 were correlated following descriptors of consistency: supple (r = 0.65), firm (r = 0.54), greasy (r = 0.34), formable (r = 0.32), fibrous (r = 0.28) and juicy (r = 0.24).

MFA analysis showed that each sample group was different in observed sensory characteristics, according to measured attributes data (p <0.05). Figure 4 shown that experimental cheese samples groups were not plotted closely to each other. We agree with the authors **Smit et al. (2005)** that flavor of cheese improved with storage days because during ripening the metabolic processes are responsible for the basic flavor and texture changes. However, further study of individual sensory dexriptors, particularly their intensities, could provide methods such as measurement of intensity in time **(Pavelková, Flimelová and Vietoris, 2012)**.

CONCLUSION

In this experiment we set up the sensory profile of smoked and unsmoked parenica cheese varieties made from pasteurized cow's milk. Panelist evaluate each sensory descriptor of two parenica cheese varieties before and after 14 days of storage period. Sensory profile of parenica cheese varieties changed during storage (p <0.05). We observed decreased trend in overall sensory characteristics after storage in unsmoked parenica cheese samples, but in smoked variety only in consistency taste and overall acceptability (p <0.05).

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HEALTHY EATING INDEX AND DIFFERENT FRUIT DIETARY HABITS IN SLOVAK ADULT FEMALE

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ABSTRACT

The healthy index is a tool for evaluation of nutrition recommendation aimed at prevention of chronic diseases. A lot of studies have been devoted to HEI of different aged groups of people but dates about Slovak population have been still missed. The goal of the study was to evaluate the Healthy Eating Index (HEI) in nutrition of adult female, determine their components in relation of parameters of anthropometry and body composition. Secondly, the research work was also aimed at the comparison of partial score HEI among groups of female with different fruit intake. Daily nutrition was evaluated by 24 hours dietary recalls. In set of female (average age 31.1 ±9.1 years) the average HEI index reached up 53.0 ±8.8 points in accord with medium degree of diet, it means dietary improvement has been highly recommended. In respect of all assayed components of index the best results achieved the variety of diet (8.8 ±1.3 points) and the worst the natrium intake (0.7 ±2.0 points). The average score for the individual components pointed to neccesary of increase in the grain intake and vegetable, on the other hand the intake of cholesterol, saturated fats and especially natrium should be decreased. The differences between groups with various intake of fruit (with recommended intake and insufficient intake) in the rest 9 components have not been proved as significant. The occurrence of risk values of body index, body fats determined by bioelectric impedance and android risk based on circumference of hips can not be considered between groups as significant. Average HEI has been in significance correlation with age ($r = 0.240$; $p < 0.05$), circumference of hips ($r = 0.2312$; $p < 0.05$) and body weight $r = 0.1748$; $p < 0.05$). Future studies have been needed to evaluate diet according to the HEI in different groups of population in Slovakia.

Keywords: diet quality; Healthy Eating Index; fruit intake; anthropometric parameters; Slovak female

INTRODUCTION

Nutritional factors and factors of life style have been significantly contributed to prevalence of chronic diseases. Composition of diet has been influenced by different socially-economic aspects and preferences of consumers **(MH SR, 2001)**.

Healthy Eating Index (HEI) has been implemented as measure of food quality of USDA (United States Department of Agriculture). USDA has been aimed at improvement of health with the diet recommendations for American population **(Guo et al., 2004)**. It has been introduced as a key tool to evaluate the extent to which Americans are following the dietary recommendations for safety and healthy food **(Willett and Skerrett, 2005)**. HEI represents the first and very simple model for generalization and monitoring of changes in dietary habits. Index expresses and measures how well a set of foods aligns with nutrition recommendations, dietary patterns and Food Guide Pyramid **(Variyam et al., 1998; Edelstein, 2011)**. HEI give general image about type and quantity of diet of individuals and their compliance with the specific nutrition recommendations for nutrients and food groups **(Gibson, 2005)**.

The HEI was originally developed in 1995 (HEI-1995) utilized dates from 1989 up to 1990 **(USDA, 1995)**. Dietary intakes of individuals were collected on 2 nonconsecutive days and using the 24-hour dietary recall method including 7500 respondents aged 2 years and older. In this approach it was necessary to separate probands into 3 energetic groups **(Dixon, 2008)**. Subsequently, the index has been periodically evaluated **(Bowman et al., 1998; Basiotis et al., 2002)**. Serving definitions based on Food Guide Pyramid **(USDA, 1997)** for different levels of energetic intake were reflected consistency with the serving definitions among people with different Recommended Energy Allowances as we can see at Table 2. The upper limits for intake of total fats, saturated fats, cholesterol and natrium were determined on the base of consultations with diet and nutrition experts and research of distribution intake of components

(Basiotis et al., 2002). Diet recommendations for American population, Food Guide Pyramid and National Research Council emphasized the importance of diet variety **(National Research Council, 1989a; USDA, 1996; Dietary Guidelines Advisory Committee, 2000)**. Unfortunatelly, nowdays there has not been accepted pattern how to quantify the measure of diet variety. HEI was utilized for evaluation of food quality within NHANES III with dates of reports 1999 – 2001. The results showed that only 10% nutrition of american population could be considered as good, 16% bad and the rest of american nutrition required to be improved. Subgroups of american population also displayed the lower quality of nutrition, especially men 15 – 18 years old, groups with lower incomes, non-hispanic Americans of African origin, persons with lower education. For 1999 – 2000 has been established the average HEI score for american population 63.8 **(Basiotis et al., 2002)**. In the same way **Dubois et al. (2000)** applicated HEI for evaluation of nutrition quality based on Canadian Nutrition Report, Quebec, 1990. HEI has been improved taken into account the Canadian Nutrition Recommendations from 1990 **(Health Canada, 1990)**. It has been also adapted in some countries, considering the local dietary habits **(Fernandes et al., 2015)**. HEI has been evaluated also in relation to plasmatic biomarkers. **Hann et al. (2001)** pointed to the fact that nutrition with high score of HEI has been in positive correlation with plasmatic concentration of carotenoids and ascorbic acid. It has been indicated that choice of food based on food pyramid leaded into healthy nutrition. On the other hand, the increased level of biomarkers has not been uniquely indicated decrease incidence of diseases.

There has been lack of evidence between food intake, biomarkers and diseases. It has been confirmed only a weak correlation between HEI and risk of chronic diseases (cardiovascular and oncological) **(McCullough et al., 2000a; McCullough et al., 2000b)**. Development of new nutrition recommendations for american population in 2005 was motivation for revision HEI (HEI-2005). Standards for food groups have been based on new recommendation of food pyramid („My Pyramid“) **(Britten et al., 2006)**. HEI-2005 has been standardized and can be used in nutrition monitoring, intervention and research as well. It consists of 12 components **(Guenther et al., 2006)**. **Guenther et al. (2008)** found out that score in 9 – 12 components was lower in group of smokers in comparison with non-smokers.

The study of HEI is requisite and necessary for the Slovak population as well **(Fatrcová-Šramková, 2010; Fatrcová-Šramková, 2013)**. The aim of study was to evaluate the Healthy Eating Index (HEI) in nutrition of adult female, to determine their components in relation of parameters of anthropometry and body composition. The research work was also aimed at the comparison of partial score HEI among groups of female with different fruit intake.

Scientific hypothesis

The average HEI of the set of female has been minimally in the medium level according to descriptors (it has not been in accord with the worst level “bad nutrition”).

Group of female with the recommended consumption of fruit achieved the better evaluation in HEI, anthropometric parameters and body composition than female with insufficient intake of fruit.

MATERIAL AND METHODOLOGY

Characteristic of the research sample

The set of analyses was performed using dates from the group of adult female (n = 165). The average age reached up 31.1 ±9.1 (range 21.0 – 49.4 years, median 38.9). The age characteristics and distribution of research sample is presented at Table 1.

Evaluation of the Healthy Eating Index (HEI)

It has been used 24 hour dietary recalls and 3 day – day food intake records. 24 hours dietary recalls were processed by nutrition software Alimenta version 4.3e originated from Food Research Institute, utilised The Slovak Food Composition Data Bank.

Daily consumption of food has been evaluated by 10 components of index HEI (Healthy Eating Index) (Table 1). All components from HEI-1995 have been taken into account. Ten components represented the different aspects of healthy nutrition. Healthy Eating Index from 1995 consists of 10 components (Table 2). The components 1 – 5 (the first partial index) deal with adequency of food composition of individuals reflected recommendations of Food Guide Pyramid from 1992. They measured the compliance of nutrition of individuals with recommendation for 5 basic food groups of Food Guide Pyramid as: cereals (bread, grain, rice and pasta), fruit, vegetable, milk (milk, youghurt and cheese), meat and its substitutions (poultry, fish, legumes, eggs and nuts). The second partial index (components 6 – 10) devotes the part of food that should be intaken minimally: total fats, saturated fats, cholesterol and natrium. The final component examines variety in person´s diet. The each component of index can reach maximum score 10 and minimum 0 **(Kennedy et al., 1995; Basiotis et al., 2002; Guenther et al., 2008; Guenther et al, 2013)**. Food Guide Pyramid expresses the diet recommendations for Americans (Dietary Guidelines for Americaners) **(Dietary Guidelines Advisory Committee, 2000)**.

The recommended servings of food lead to healthy diet. The recommended servings in pyramid have been in accord with energy intake for individuals **(National Research Council, 1989b)**. Table 2 showed recommended servings for different groups of people in relation to age, gender and for energy level 1600 kcal, 2200 kcal and 2800 kcal, i.e. 6700 kJ, 9200 kJ and 11700 kJ **(USDA, 1997)**. According to Food Guide Pyramide is the recommended servings per day (Table 2) for female aged 11 – 24 years and as well 25 – 50 years (energy intake 2200 kcal, i.e. 9200 kJ) for grains 9 servings, vegetable 4 servings, fruit 3 servings, milk 2 servings, meat 2.4 servings **(USDA, 1996)**.

Each component within HEI (Table 1 and Table 2) was scored by minimal point 0 (in case of insufficient nutrition) up to 10 points (in case of sufficient nutrition). High component score indicates intake close to recommended range or amount, low component score indicates less compliance with recommended range or amount. The maximum overall score for the 10 combined

Table 1 Distribution of female in relation to age (%).

Age	Consumption of fruit			
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B	***p*-value**
25 – 30 years	32.2	33.3	32.7	**0.998**
31 – 35 years	6.9	15.4	10.9	**0.384**
36 – 40 years	12.6	12.8	12.7	**0.999**
41 – 45 years	12.6	9.0	10.9	**0.903**
46 – 50 years	35.6	29.5	32.7	**0.871**

Note: group A – recommended consumption of fruit (higher than score 10 point); group B – insufficient consumption of fruit (lower than score 10 point); $p \geq 0.05$.

Table 2 HEI (Healthy Eating Index) **(Basiotis et al., 2002)**.

Parameters	**Score ranges**[a] (points)	Criteria for Maximum Score of 10 points	Criteria for Minimum Score of 0 points
Consumption of different group of food			
1. grain	0 – 10	6 – 11 servings[b]	0 servings
2. vegetable	0 – 10	3 – 5 servings[b]	0 servings
3. fruit	0 – 10	2 – 4 servings[b]	0 servings
4.milk	0 – 10	2 – 3 servings[b]	0 servings
5. meat	0 – 10	2 – 3 servings[b]	0 servings
Nutrition recommendations			
6. total fat intake	0 – 10	30% or less energy from fat	45% or more energy from fat
7. saturated fat intake	0 – 10	Less than 10% energy from	15% or more energy from
8. cholesterol	0 – 10	saturated fat	saturated fat
9. natrium	0 – 10	300 mg or less	450 mg or more
10. food variety	0 – 10	2400 mg or less	4800 mg or more
		8 or more different items in a day	3 or fewer different items in a day

Note: [a] People with consumption or intakes between the maximum and minimum ranges or amounts were assigned scores proportionately. [b] Number of servings depends on Recommended Energy Allowance; see Table 3. All amounts are on a per day basis.

Table 3 The recommended food servings per day according to Food Guide Pyramid **(USDA, 1996; USDA, 1997)**.

Energy (kcal)	**Grain**	**Vegetable**	**Fruit**	**Milk**	**Meat**
1600	6	3	2	2	2
2200	9	4	3	2	2.4
2800	11	5	4	2	2.8

components is 100. Scores were calculated using the population ratio method. The calculation of HEI index was provided in accord with recommendations of **Kennedy et al. (1995); Dietary Guidelines Advisory Committee (2000); Basiotis et al. (2002); Guenther et al. (2008); Guenther et al. (2013)**.

For HEI evaluation the descriptors on the base of USDA recommendation were used **(Gibson, 2005)**. An HEI score over 80 implies a „good" diet, an HEI score between 51 and 80 implies a diet that „needs improvement", and an HEI score less than 51 implies a „poor" diet. Except for HEI score for all components we also aimed at partial score (for selected components). For partial score (evaluation of 5 parameters from 10) the adequate proportion of points (50% evaluation) was used. The following descriptors were utilised for evaluation of the partial score: „good diet" over 40 points, „need improvement" 25.5 – 40 points, „poor diet" less than 25.5 points.

In aspect of for evaluation of partial HEI adult female were separated according to quantitative intake of fruit. It has been established 2 groups of female. Group A („recommended fruit consumption) can be characterised by daily minimally consumption of fruit 3 servings. Group B („insufficient consumption of fruit") mean the low intake of fruit in amount less than 3 servings.

Evaluation of the body composition, anthropometry

It has been characterised and evaluated the anthropometric parameters, parameters of body composition: body weight (kg), height (cm), waist circumference (cm), hip circumference (cm), body fat (absolut in kg, relative in %), body mass index – BMI ($kg.m^{-2}$) as relation weight to height, and waist to hip ratio – WHR (index of centrality). BMI was evaluated according to criteria of **WHO (2018)**, index centrality according to **Kleinwächterová and Brázdová (2001)**. For determination of body composition the bioelectrical impedance analysis (BIA) by Bodystat Quadscan 4000 (Bodystat Ltd, Doubles, Isle of Man, UK) has been used.

Statisic analysis

Statistical evaluation was provided by Statistica.cz (verzia 10, StatSoft, Inc., Česká republika). The differences among groups were tested by one-way t-test and chi-squared test ($\chi 2$–test). Statistical significant

differences were stated as *p*-values ($p = 0.05$). Correlations were expressed by correlation coefficient r.

There has not been confirmed the significant differences in age distribution of female between group A and group B (Table 1).

RESULTS AND DISCUSSION

According to assayed scores the evaluation of components of HEI index has declined tendency in the following order: diet variety > total fat > fruit > milk > meat > grains > vegetable > cholesterol > saturated fats > natrium (Table 4). The highest score reached up the variety of diet (8.8 ±1.3 points). On the other hand, the lowest score had natrium (0.7 ±2.0 points) in exceeded daily intake. The biggest problem in nutrition of female was the daily recommended intake of natrium.

In respect of intaken food, nutrients and quantification of diet variety there has been proved statistically significant differences between real and recommended intake in case of all 10 components of HEI ($p < 0.001$, $p = 0$). Of the first 5 components of the HEI, the most favourable group was represented by fruit and fruit products (7.2 ±3.7 points). On the contrary, the most insufficient was the daily consumption of vegetable. Of the rest nutrition recommendations (components 6 – 9) the highest score achieved diet variety, the mostly insufficient was natrium intake.

Tangney et al. (2001) studied HEI index of elder people and found out that the lowest proportions of perfect scores occurred for grains, vegetables, milk and meat. The highest proportions with perfect scores occurred for total and saturated fat (as a percentage of energy), cholesterol and sodium intakes.

In the base of fruit component the set of female was divided into 2 groups (with recommended intake of fruit – group A and insufficient intake of fruit – group B). For more detailed comparative evaluation the set was dividing according to food group with the best score – fruit. There has been confirmed differences between group A of female meet recommended serving of fruit (minimally 3 servings) *versus* group B of female with insufficient fruit in the rest of 9 components of HEI as well (Table 4 and 5). In group A except for fruit consumption the best evaluation (from maximum 10 points) reached up variety of diet (8.9 ±1.2 points) and total fats (7.9 ±2.8 points) (Table 4). The same order was estimated also in group B: variety of diet 8.8 ±1.4 points and total fats 7.9 ±2.2 points. Similarly, the worst score in group A and B displayed natrium (1.0 ±2.3 points; 0.5 ±1.5 points). The daily natrium intake significantly exceeded the recommendation (group A 9071.1 ±4089.8 mg and group B 8897.2 ± 3999.0 mg). The daily recommended natrium intake represented 2400 mg and less (Table 5).

The insufficient intake of fruit regularly 5 times a day has been evaluated in research work of **Juríková et al. (2016)**. The majority of college students consumed fruit and vegetable only once a day. The vegetable as a source of natural antibiotics has been intaken only once a week as well **(Juríková et al., 2017)**.

It was also interesting observed not only intake of recommended dose of fruit but also vegetable as an important source of bioactive compounds (Table 6).

The results showed that 40% of respondents did not meet criteria for the recommended daily intake of fruit and vegetable, non respondents from group A and majority from group B (0% *versus* 84.6%). Only one of recommendations – fruit was covered by 47.3% of female in group A. There has been proved statistically significant differences between evaluated groups (75.9% *versus* 15.4%, $p = 0.000$). Generally, the recommendations for fruit and vegetable were taken into account in 12.7% female, minimally 1 recommendation (fruit/vegetable) had 60% of probands (all respondents of group A and 15.4% respondents of group B).

In case of fruit the maximum score (10 points) achieved 52.7% female (Table 7). The order of components in accord to maximum score was following: fruit > meat > milk > total fat > variety of diet > cholesterol > vegetable > grains > natrium > saturated fats. Generally, female did not meet the guidelines in parameters of nutrition in consumption of grains (96.4%), natrium intake (98.2%) and saturated fat (all respondents). Our results are in accord with the study of **Ervin (2008)** (56% of older female met the recommendation for diet variety). Similarly, **Shah et al. (2010)** reported that participants according to HEI 2005 consumed more than recommended amounts of sodium, saturated fats, and discretionary calories.

The assayed female groups were significantly differed in component fruit, the rest differences in components has not been proved as significant. The recommended daily intake of milk had more probands of group B (47.4%) *versus* group A (34.5%). On the contrary, the higher consumption of vegetable and total fats was higher in group A (24.1%; 42.5%) *versus* group B (15.4%; 37.2%).

Particularly it was determined the distribution of female in components of HEI at least 50% (score 5 points or more for the individual components) (Table 8). The groups were represented by female has not been covered recommendation of fruit intake (on 50% with score less than 5 points) (B group) and female has been covered at least 50% of recommendations with 5 points and more) (A group). The most significant differences between groups have been determined in consumption of fruit (in group A higher value by 52.6%) and milk (higher value by 14.3% in group B). Differences in consumption of vegetable represented only 5.8%, cholesterol 5.5% and total fats 4.6%. The results pointed to the fact that group B with lower consumption of fruit has achieved weeker results also in another components (in 6 components was determined score under 5 points). Group A displayed also better results in consumption of milk (by 14.3%), total fats (by 4.6%), cereals intake (by 2.8%) and meat (1%). On the other hand, in group B were more female with insufficient diet and score under 5 points in consumption of fruit (by 52.6%), vegetable (by 5.8%), cholesterol (by 5.5%), natrium (by 4.1%), saturated fat (by 0.6%) and diet variety (by 0.6%).

It was monitored the occurence of worst score with 0 points in case of cereals (0.6%), vegetable (16.4%), fruit (13.3%), milk (2.4%) and meat (12.1%) (Table 9). Extremely high proportion of fats (more than 45% of daily energy intake) had only 3.6% probands. The proportion of fats in energy intake must cover 30% (saturated fat acids 10%, monounsaturated fatty acids 10 – 12% and

Table 4 Score for components HEI (mean ±SD).

Components HEI (points)	**Consumption of fruit**		
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B
Grains	6.8 ±2.6	7.0 ±2.8	4.9 ±2.4
Vegetable	5.1 ±3.6	4.4 ±3.7	4.7 ±3.7
Fruit	10.0 ±0.0	4.0 ±3.2	7.2 ±3.7
Milk	6.4 ±3.7	7.7 ±3.4	7.0 ±3.6
Meat	6.7 ±3.8	6.7 ±3.6	6.7 ±3.7
Total fat	7.9 ±2.8	7.9 ±2.2	7.9 ±2.5
Saturated fat	2.2 ±2.0	1.7 ±1.9	2.0 ±1.9
Cholesterol	3.3 ±4.3	2.8 ±4.2	3.0 ±4.3
Natrium	1.0 ±2.3	0.5 ±1.5	0.7 ±2.0
Diet variety	8.9 ±1.2	8.8 ±1.4	8.8 ±1.3

Note: SD – standard deviation.

Table 5 Evaluation of components HEI (mean ±SD).

Components HEI	**Consumption of fruit**		
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B
Grains (servings)	4.3 ±2.1	4.5 ±2.2	4.4 ±2.1
Vegetable (servings)	2.5 ±2.3	1.9 ±1.8	2.2 ±2.1
Fruit (servings)	5.2 ±1.9	1.2 ±1.0	3.3 ±2.5
Milk (servings)	1.6 ±1.2	2.1 ±1.6	1.9 ±1.4
Meat (servings)	2.4 ±2.1	2.4 ±1.9	2.4 ±2.0
Total fat (% of energy)	31.6 ±7.1	32.0 ±5.7	31.8 ±6.5
Saturated fat (% of energy)	8.4 ±3.0	8.6 ±2.4	8.5 ±2.7
Cholesterol (mg)	629.2 ±420.9	583.0 ±338.4	607.3 ±383.7
Natrium (mg)	9071.1 ±4089.8	8897.2 ±3999.0	8988.9 ±4035.8
Diet variety (number of items)	7.12 ±0.96	7.04 ±1.12	7.04 ±1.04

Table 6 Keeping for selected components of HEI (%) – fruit and vegetable.

Number of kept recommendations	**Consumption of fruit**			***p*-value**
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B	
0	0.0	84.6	40.0	-
1 (for fruit and vegetable)	75.9	15.4	47.3	0.000
2 (for fruit and vegetable)	24.1	0.0	12.7	-

Note: p <0.001.

polyunsaturated fatty acids 8 – 10%) **(Kajaba et al., 1999; MH SR, 1997; PHA SR, 2015)**. Limit maximally 30% of daily energy intake has been overlapped by majority of Europeans **(MH SR, 2001)**. The recommended energy value from saturated fats (max. 10%) was highly overlapped by 25% of probands (with values higher than 15% of saturated fat from energy intake).

In USA and Europe saturated fat acids represent 12 – 15% from total energy intake. On every 1% of energy intake in the form of saturated fatty acids has been increased level of LDL cholesterol in comparison with oleic acid by 2 $mg.dL^{-1}$ (0.025 $mmol.L^{-1}$) **(Grundy, 2003)**. The content of n-6 fatty acids should not be 4 – 10 higher in comparison with n-3 fatty acids. Oil originated from sea fish represents a very valuable source of eicosapentaenoic (EPA and docosahexaenoic acid (DHA) decreased the probalibility of trombosis in veins. Nutritionally fats and oils can be considered as healthy if the proportion of fatty acids has been approximate to mentioned relation **(MH SR, 2001)**.

In the set of assayed female was noticed overlapped intake of cholesterol (450 mg and more) among 60% of female with negative impact on incidence of cardiovascular diseases. The better results in cholesterol intake achieved **Ervin (2008)**, 72% of older adults met the guidelines for cholesterol. Human body is able to synthetize essential amount of cholesterol, its excessed intake increase the risk of cardiovascular diseases. It is a reason for recommendation of nutrition experts not consumed more than 300 mg cholesterol **(MH SR, 2001)**. On every 200 mg of intaken cholesterol it has been increased the concentration of serum LDL-cholesterol (LDL – low density lipoproteins) on average by 6 $mg.dL^{-1}$ (0.155 $mmol.L^{-1}$) **(Grundy, 2003)**. Saturated fatty acids increase, polyunsaturated fatty acids decrease the level of cholesterol **(MH SR, 2001)**.

The daily intake of natrium significantly overlapped the recommended dose reached up 4800 mg in case of (83.6%) probands. Female had minimally 0.5 serving from all food groups so diet variety represented the smallest problem in

Table 7 Distribution of female in the evaluated groups based on scores for componenents of HEI.

Components HEI (score)	Consumption of fruit			*p*-value
	Recommended Group A	Insufficient Group B	Together Group A +B	
1. cereals				
<9 porcií	96.6	96.2	96.4	-
≥9 porcií*	3.4	3.8	3.6	
2. vegetable				
<4 porcie	75.9	84.6	80.0	**0.578**
≥4 porcie*	24.1	15.4	20.0	
3. fruit				
<3 porcie	0.0	100.0	47.3	-
≥3 porcie*	100.0	0.0	52.7	
4. milk				
<2 porcie	65.5	52.6	59.4	**0.413**
≥2 porcie*	34.5	47.4	40.6	
5. meat				
<2,4 porcie	58.6	57.7	58.2	**0.999**
≥2,4 porcie*	41.4	42.3	41.8	
6. total fat				
>30 %	57.5	62.8	60.0	**0.921**
≤30 %*	42.5	37.2	40.0	
7. saturated fat				
≥10 %	100.0	100.0	100.0	-
<10 %*	0.0	0.0	0.0	
8. cholesterol				
>300 mg	74.7	78.2	76.4	**0.964**
≤300 mg*	25.3	21.8	23.6	
9. natrium				
>2400 mg	96.6	100.0	98.2	-
≤2400 mg*	3.4	0.0	1.8	
10. diet variety				
<8 položiek	70.1	67.9	69.1	**0.992**
≥8 položiek *	29.9	32.1	30.9	

Note: * criteria for maximum score (10 points); $p \geq 0.05$.

nutrition. On the other hand, the biggest problem of female was overlapped sodium intake.

Average HEI score achieved the mean value 53.0 ±8.8 points (58.2 in the first and 51.4 in the second group, Table 10). This result is in accord with medium level of nutrition according to **Gibson (2005)** with necessary improvement of diet. This value represented also lower value in comparison with **Basiotis et al. (2002)** (HEI 63.8%) monitoring American population in 1999 – 2000. In the same way in the project Chicago Health and Aging was studied adult population 65 year and more (4932 participants) and the average HEI index was calculated 70.7 it means very close to healthy diet **(Tangney et al., 2001)**. According to research study of **Ervin (2008)** only 17% of older adults consumed a "good" quality diet. HEI-2015 for the american population for the years 2013 – 2015 was calculated as following: 59 for 2013 – 2014, 60 for years 2011 – 2012, 59 for 2009 – 2010, 57 for 2007 – 2008, 56 for 2005 – 2006 (from top score 100). HEI-2015 demonstrated that the nutrition of Americans are in medium level and did not align with the nutrition guidelines "2015 – 2020 Dietary Guidelines for Americans" **(USDA, 2015; USDA, 2018)**. Partial score for parameters 1 – 5 was compared with partial score for parameters 6 – 10 (Table 10). The better results were achieved in first partial score for parameters 1 – 5 (30.5 versus 22.5) within set of female and group of A and B with different intake of fruit. Evaluation of partial score 1 – 5 was in accord with medium level of nutrition with needs for improvement of diet, score 6 – 10 is in agreement with bad level of nutrition.

For HEI index, also partial HEI (1 – 5) and HEI (6 – 10) has been confirmed significant differences in comparison real and recommended score ($p < 0.001$; $p = 0$).

In set there have been evaluated anthropometric parameters as well (Table 11). On the basis of BMI classification **(WHO, 2018)** the proportion of female with normal weight took 60%, pre-obesity in 27.3% and overweight in 12.7% (Table 12). Obesity class III or underweight were not be noticed. Cumulative evaluation pointed to high prevalence of pre-obesity and obesity (BMI 25 $kg.m^{-2}$ and more) in 40%. Between groups A and B with different fruit intake there has not been proved statistically significant differences in BMI ($p \geq 0.05$). Normal weight had 54.0% and 66.7% probands in groups A and B, pre-obesity 32.2% a 21.8%, obesity class I – III 13.7% a 11.5%. Only third of respondents in group B displayed risk BMI. Pre-obesity or obesity was identified more often in group A with higher intake of fruit (45.9% *versus* 33.3%). According to index of centrality there has been higher occurence of android body type in group A than in group B (WHR ≥0.85, 16.1% *versus* 0%,

Table 8 Groups of female according to 50% score for HEI components.

Components HEI (score)*	**Consumption of fruit**			*p*-value
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B	
1. grains				
<5 points	20.7	17.9	50.3	**0.977**
≥5 points	79.3	82.1	49.7	
2. vegetable				
<5 points	50.6	56.4	53.3	**0.904**
≥5 points	49.4	43.6	46.7	
3. fruit				
<5 points	0.0	52.6	24.8	-
≥5 points	100.0	47.4	75.2	
4. milk				
<5 points	31.0	16.7	24.2	**0.201**
≥5 points	69.0	83.3	75.8	
5. meat				
<5 points	35.6	34.6	35.2	**0.999**
≥5 points	64.4	65.4	64.8	
6. total fat				
<5 points	14.9	10.3	12.7	**0.846**
≥5 points	85.1	89.7	87.3	
7. saturated fat				
<5 points	94.3	94.9	94.5	-
≥5 points	5.7	5.1	5.5	
8. cholesterol				
<5 points	70.1	75.6	72.1	**0.888**
≥5 points	29.9	24.4	26.7	
9. natrium				
<5 points	90.8	94.9	88.5	-
≥5 points	9.2	5.1	8.5	
10. diet variety				
<5 points	0.0	0.6	0.6	-
≥5 points	100.0	100.0	93.3	

Note: * criteria for evaluation 5 points (50% of the maximum score) according to HEI **(Basiotis et al., 2002)**, $p \geq 0.05$.

Table 9 Groups of female according to minimum score (0 points) for HEI components.

HEI components (criteria)*	**Consumption of fruit**			*p*-value
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B	
Grains (0 serving)	0.0	1.3	0.6	-
Vegetable (0 serving)	14.9	17.9	16.4	**0.965**
Fruit (0 serving)	0.0	28.2	13.3	-
Milk (0 serving)	3.4	1.3	2.4	-
Meat (0 serving)	13.8	10.3	12.1	**0.922**
Total fat (45% of energy intake or more)	5.7	1.3	3.6	-
Saturated fat (15% or more)	24.1	26.9	25.5	**0.982**
Cholesterol (450 mg and more)	58.6	61.5	60.0	**0.985**
Sodium (4800 mg and more)	80.5	87.2	83.6	**0.715**
Diet variety (0 items)	0.0	0.0	0.0	-

Note: * criteria for minimum score (0 points) according to HEI **(Basiotis et al., 2002)**, ($p \geq 0.05$).

$p \geq 0.05$). By measurement of body fat was also observed the higher occurence of health hazard in group A. In the same way **Kant and Graubard (2005)** reported that the HEI score was associated with obesity and biomarkers of cardiovascular diseases and *diabetes mellitus*. The occurence of the risk values of anthropometry and body composition of female with recommended intake of fruit can be caused by total structure of intaken diet and also with presence of nutrition risk factors as well as with

Table 10 Scores for total HEI, partial HEI (1 – 5) and partial HEI (6 – 10) (mean ±SD).

HEI (components)	**Consumption of fruit**		
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B
HEI (1 – 5) [a]	34.9 ±7.2	29.8 ±7.7	30.5 ±7.6
HEI (6 – 10) [a]	23.2 ±7.8	21.7 ±5.9	22.5 ±7.0
HEI (sum 1 – 10) [b]	58.2 ±7.7	51.5 ±8.6	53.0 ±8.8

Note: [a] partial score HEI, [b] total score HEI.

Table 11 Characteristic of female groups with different fruit consumption (mean ±SD).

Parameters	**Consumption of fruit**		
	Recommended Group A	**Insufficient** Group B	**Together** Group A +B
Age (years)	37.6 ±9.7	35.7 ±9.5	36.7 ±9.6
Body height (cm)	165.4 ±5.5	165.3 ±5.7	165.4 ±5.6
Body weight (kg)	68.1 ±12.2	65.7 ±12.7	67.0 ±12.5
Waist circumference (cm)	80.4 ±10.6	77.4 ±10.0	79.0 ±10.4
Hip circumference (cm)	102.6 ±9.8	101.4 ±10.1	102.0 ±9.9
Body fat (%)	21.1 ±8.7	20.0 ±8.8	20.6 ±8.8
Body fat (kg)	30.0 ±7.1	29.4 ±6.9	29.7 ±7.0
BMI (kg.m^{-2})	24.9 ±4.9	24.1 ±4.6	24.5 ±4.6
WHR	0.8 ±0.1	0.8 ±0.1	0.8 ±0.1

Note: BMI – body mass index, WHR – waist to hip ratio.

Table 12 Body mass index (BMI), waist to hip ratio (WHR), body fat (BF) and classification in female groups (%) with different fruit consumption.

Classification	**Criteria**	**Consumption of fruit**			
		Recommended Group A	**Insufficient** Group B	**Together** Group A +B	*p*-**value**
Nutritional status*	**BMI (kg.m^{-2})**				
Underweight	<18,5	0.0	0.0	0.0	**-**
Normal weight	18.5 – 24.9	54.0	66.7	60.0 -	**0.433**
Pre-obesity	25.0 – 29.9	32.2	21.8	27.3 -	**0.524**
Obesity class I	30.0 – 34.9	10.3	7.7	9.1 -	**0.950**
Obesity class II	35.0 – 39.9	3.4	3.8	3.6	**-**
Obesity class III	≥40	0.0	0.0	0.0	**-**
Health risk*	**WHR (kg.m^{-2})**				
Gynoid	<0.85	83.9	100.0	89.1	**-**
Android	≥0.85	16.1	0.0	10.9	**-**
Healthy risk	**BF (%)**				
Without risk	≤25	65.5	80.8	72.7	**0.185**
Risk	>25	34.5	19.2	27.3	**0.185**

Note: $p \geq 0.05$.

lifestyle (the level of moving activity). Overlapped intake of fruit has been in relation with increased intake of sugar over dairy recommendations. Separately it has been evaluated relation between HEI score and anthropometric parameters. HEI index was in significant correlation with age ($r = 0.2402$; $p < 0.05$), body weight ($r = 1748$, $p < 0.05$) and hip circumference ($r = 0.2312$; $p < 0.01$). The mentioned anthropometric parameters were correlated to partial HEI (1 – 5) for group of food and HEI 6 – 10 for diet recommendations. The statistically significant relations have been confirmed between partial HEI (6 – 10) and anthropometric parameters: body weight, body height, waist circumference, hip circumference, BMI ($p < 0.001$) and body fat (% BF, $p < 0.001$; kg BF, $p < 0.05$). There have been proved significant correlation between HEI (1 – 5) and body weight ($p < 0.01$), age, height, waist circumference, body fat (% BF), BMI, WHR ($p < 0.05$).

Higher HEI 2005 scores have been associated with favorable lipid profile **(Shah et al., 2010)** and lower risk of obesity **(Gao et al., 2008)**. We can also conclude that components 6 – 10 significantly contributed to occurence of obesity and chronic diseases (especially increased proportion of total fat, saturated fatty acids, cholesterol or natrium). A version of the HEI calculated from food frequency questionnaires was associated with a lower risk of cardiovascular disease in men in the research study of **McCullough et al. (2000a; 2000b)**. The risk values of anthropometric parameters have been more often occured

Table 13 Correlation between HEI score and anthropometric parameters.

Parameter	HEI (1 – 5)	HEI (6 – 10)	HEI
Age (years)	$r = 0.1740$	$r = 0.1133$	$r = 0.2402$
	$p = 0.025$	$p = 0.147$	$p = 0.002$
Height (cm)	$r = 0.1747$	$r = 0.3715$	$r = 0.1425$
	$p = 0.025$	$p = 0.000$	$p = 0.068$
Weight (kg)	$r = 0.2287$	$r = 0.4715$	$r = 0.1748$
	$p = 0.003$	$p = 0.000$	$p = 0.025$
Waist (cm)	$r = 0.1901$	$r = 0.3288$	$r = 0.0954$
	$p = 0.014$	$p = 0.000$	$p = 0.223$
Hip (cm)	$r = 0.1393$	$r = 0.4450$	$r = 0.2312$
	$p = 0.074$	$p = 0.000$	$p = 0.003$
Body fat (%)	$r = 0.1591$	$r = 0.3405$	$r = 0.1314$
	$p = 0.041$	$p = 0.000$	$p = 0.092$
Body fat (kg)	$r = 0.0747$	$r = 0.1710$	$r = 0.0705$
	$p = 0.340$	$p = 0.028$	$p = 0.369$
BMI ($kg.m^{-2}$)	$r = 0.1806$	$r = 0.3585$	$r = 0.1270$
	$p = 0.020$	$p = 0.000$	$p = 0.104$
WHR	$r = 0.1552$	$r = 0.0028$	$r = 0.1366$
	$p = 0.046$	$p = 0.971$	$p = 0.080$

Note: HEI – healthy eating index, BMI – body mass index, WHR – waist to hip ratio.

in the group of female with recommended intake of fruit and their products. It can pointed to lower quantity level of nutrition despite the incorporation of nutritionally valued food from group of fruit. It has been confirmed also in assessment of the rest HEI components as well.

There has been surprisingly little dietary information and HEI data for Slovak population in literature. Many adults, and in particular, adults with insufficiet fruit intake, may have diets inadequate according to dietary recommendations.

There has not been existed food with ideal nutrition composition so food groups must be selected in the way that daily intake of diet include all sufficient nutrients in ideal proportion **(MH SR, 2001)**. Nutrition education programs and innovative interventions may help meet food and nutrients recommendations and daily nutrition requirements. Keeping of nutrition recommendations are essential for good healthy status of individuals.

CONCLUSION

This is the first study using the complete HEI to assess the quality of foods and nutrients in the diet of Slovak selected adult population. Results of the study indicate the necessity of diet improvements in adults.

The mean score for the individual components showed the needs to increase the proportion of consumed food especially grains and vegetable. On the other hand, it has been seen to be reasonable decrease the intake amount of cholesterol, saturated fats and especially natrium. The assayed results (total HEI 53.0 from 100.0; partial HEI (1 – 5) 30.5 from 50.0; partial HEI (6 – 10) 22.5 from 50.0 points) indicated the needs for distinct improvement of quality of intaken food as well as provide the necessary daily intake of food in accordance with nutrition recommendations. Average HEI has been corresponded with the medium evaluation level. It has been also confirmed correlation between HEI and partial HEI index and body weight. The group of female with insufficient fruit intake reached up the worse evaluation in comparison with group with recommended intake in all 10 HEI components. There has not been established statistically significant differences between groups in occurrence of risk values of the anthropometric parameters.

Future studies have been needed to evaluate diet according to the HEI in different population groups in Slovakia. Studies have been necessary to examine associations between HEI and components with individuals characteristics and additional factors.

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BIOACTIVE COMPOUNDS EVALUATION IN DIFFERENT TYPES OF CZECH AND SLOVAK HONEYS

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ABSTRACT

Honey contains important bioactive compounds (enzymes, phenolic compounds, vitamins, and minerals) with several positive health effects for humans. In the study six types of honey (acacia, rape, floral, multi flower, forest, and honeydew honeys), of Czech and Slovak origin, were evaluated for bioactive compounds by means of color, polyphenols and antioxidant capacity analyses. The brightest color of honeys, the lowest values measured spectometrically, had acacia and rape honeys, followed by floral, and darker multi flower and forest honeys, and honeydew honeys. Polyphenols (PP) amount, determined by spectrophotometric method with Folin-Ciocalteu reagent, was highest for the darkest honeydew honeys, followed by multi flower and forest honey, brighter floral honeys, and rape and acacia honey. Honeys polyphenols were in the range from 54.0 to 254.2 mg GAE.100g^{-1}. The total antioxidant capacity (TAC) was analyzed by spectrometric methods with ABTS and DPPH reagents. Antioxidant capacity values are in agreement with the PP contents order. They were highest also for honeydew honeys (59.2 – 89.6 and 73.1 – 118.7 mg TE.100g^{-1}), followed by multi flower (66.0 and 56.7 mg TE.100g^{-1}) and forest honey (56.0 and 49.1 mg TE.100g^{-1}), then floral honeys (33.0 – 49.2 and 27.8 – 38.7 mg TE.100g^{-1}) and the lowest values for rape (19.0 and 28.1 mg TE.100g^{-1}) and acacia (15.5 and 11.3 mg TE.100g^{-1}) honey. A positive correlation between color, PP amount and TAC was evaluated for analyzed honeys. Darker honey samples showed higher values of phenolic compounds and antioxidant potential, therefore they belong to the honey types with higher amount of bioactive compounds such as antioxidants.

Keywords: honey; color; polyphenol content; antioxidant capacity; DPPH; ABTS

INTRODUCTION

Honey could be generally defined as foodstuff containing mainly sugars, monosaccharides fructose and glucose that form about 70% of sugar content, and about 10% of oligosaccharides (disaccharides and trisaccharides; sucrose, maltose, turanose, isomaltose, maltulose, trehalose, nigerose, kojibiose and trisaccharides maltotriose and melezitose). It is quite energetic food due to sugar content. Further there are enzymes, with important biological activity, such as catalase and glucoseoxidase; amino acids and proteins; organic acids; vitamins (such as ascorbic acid, vitamin E), carotenoid derivatives (β-carotene), minerals, and polyphenols **(Miguel et al., 2017; Bertoncelj et al., 2007; Gheldof et al., 2002)**.

As **Miguel et al. (2017)** mentioned honey is an effective nutraceutical foodstuff and its biological activity is mainly dependent on honey's floral or geographic origin. Composition of honey depends on several parameters such as honey type (blossom nectar or honeydew honey), geographical origin (locality of the collection), flora, soil, weather and season, and also post-harvest conditions and honey storage **(Bogdanov et al., 2008)**.

Honey could be defined as product of honey bees (*Apis mellifera*) as a nectar of flowers, flowering plants, they are floral honeys; unifloral if they are produced from the nectar of one type of flowers; or multifloral. They could be also non-floral, honeydew honeys, which are from honeydew (secretion), a sugar-rich substance secreted by various animals such as secretions of aphids plant sucking insects **(Pita-Calvo and Vázquez, 2017)**. Honeydew honey is chemically different from common blossom nectar honey because nectar is dissimilar from honeydew that is usually darker and has higher mineral content **(Grembecka and Szefer, 2012)**. Its darker color is produced by sugars, minerals and amino acids **(Sanz et al., 2005)**. Even nectar honeys from the same floral origin can vary in their chemical composition.

Honey is quite expensive natural product that was found to be adulterated sometimes **(Bušová and Kouřimská, 2018)**. The situation about the honey quality in the Czech

Republic is specified in the Situational Outlook Report of the Czech Ministry of Agriculture **(MZe, 2017)**. The honey consumption in the Czech Republic is only about 0.7 kg per person per year, in Slovakia is marked higher consummation **(Guziy et al., 2017)**.

Honey is known as a potential therapeutic product with bioactive compounds for the treatment and prevention of various diseases. There were studies of honey and its antioxidant, antibacterial, antiviral, anti-inflammatory, antihypercholesterolemic, vasodilatative, and hypotensive activities **(Viuda-Martos et al., 2008; Hadagali and Chua, 2014)**.

Honey is supposed to be a good natural antioxidant for foods. Honey addition can help to prevent or delay food spoilage due to oxidative reactions, to enhance the oxidative stability of the meat, such as dry honey applied to turkey products **(Antony et al., 2000; Antony et al., 2006)**. Dark honeys have higher content of phenolic compounds and possess better antioxidant activity, so they could be used as a good complement of these products **(Beretta et al., 2005; Bertoncelj et al., 2007)**. Free radical scavenging activity of honeys was found to be related with the water-soluble vitamins **(Chua et al., 2013)**. Also **Gheldof et al. (2002)** found an association between antioxidant activity and water-soluble honey fraction consisted of phenolics, peptides, proteins, organic acids, gluconic acid, ascorbic acid, the enzymes glucose oxidase, catalase and peroxidase.

Due to the study of **Schramm et al. (2003)** consumption of honey (buckwheat honey) increased plasma total phenolic content and also plasma antioxidant and reducing capacities. So phenolic antioxidants from processed honey are bioavailable, and increase antioxidant activity of plasma. It is speculated that phenolic antioxidants may augment defenses against oxidative stress and might be able to protect humans from oxidative stress.

The goal of this study was to evaluate amount of several bioactive compounds of few honey types produced from the Czech Republic and Slovakia by determination of polyphenols and antioxidant capacity.

Scientific hypothesis

The scientific hypothesis of this study was to examine the differences in various types of honeys from Czech and Slovak beekeepers due to their bioactive compounds evaluation measured by polyphenolic content and antioxidant capacity of two methods (DPPH and ABTS tests), and correlations between them.

MATERIAL AND METHODOLOGY

Honey samples

There were evaluated 11 honey samples (nectars and honeydew honeys) of 6 honey types of Czech (produced in Moravian region, CZ) and Slovak origin (SK). There were 1 sample of acacia honey (A1) sample (SK), 1 rape honey (R1; CZ), 3 samples of floral honey (FL1-FL3; all CZ), 1 multi flower honey (MF1) sample (SK), 1 sample of forest honey (FO1; SK), 4 samples of honeydew honeys (HD1-HD4, all CZ), collected from private beekeepers, seasons 2013 and 2014. The samples were stored in glass jars in the dry dark place at room temperature (approximately 20 °C) and after jar opening they were analyzed up to ten days.

Determination of Honey Color

For the determination of honey color a modified spectrometric method of **Beretta et al. (2005)** was used. The honey samples were diluted to 50% (w/v) solution with distilled water. They were sonicated for 5 min and filtered through a paper filter and used for color analyses. Absorbances of samples were measured at two wavelengths, 450 nm and 720 nm against blank on the spectrometer (Libra S6 Biochrom, GB). The difference in absorbances (ΔA) was expressed. Determinations were made in triplicate.

Determination of Polyphenolic Content

The polyphenolic (PP) content in honeys was evaluated by a modified spectrophotometric method with Folin-Ciocalteau reagent **(Socha et al., 2009)**. Samples for PP evaluation were prepared with 10 g honey and 40 mL of distilled water. After sonication (5 min) the solutions were filtered through a paper filter and quantitatively transferred into a 50 mL volumetric flask. Afterwards the samples were made up to 50 mL with distilled water and used for the analyses of PP. To extracts (0.1 mL) with 1 mL of distilled water, Folin-Ciocalteau reagent (1 mL; 10% (w/v); Penta Chemicals, CZ) was added and after agitation it was left for 5 min in the dark at room temperature, then 1 mL of sodium carbonate (10% (w/v); Penta Chemicals, CZ) solution was added and mixed again. After 15 min of standing in the dark at lab temperature absorbance of samples was measured at $\lambda = 750$ nm against blank using a Libra S6 Biochrom spectrometer (GB). Gallic acid was used as a standard and PP values were expressed as gallic acid equivalents (GAE) in $mg.100g^{-1}$ sample. Determinations were made in triplicate.

Determination of Antioxidant Capacity

For the total antioxidant capacity (TAC) determinations modified spectrometric methods using ABTS and DPPH reagents **(Škrovánková et al., 2018; Beretta et al., 2005)** were used.

The procedures for honey samples extraction were same for both determinations. There were mixed 10 g of honey sample and 40 mL of distilled water. After sonication (5 min) the solutions were filtered through a paper filter and quantitatively transferred into a 50 mL volumetric flask. Afterwards the samples were made up to 50 mL with distilled water and used for the analyses.

ABTS method: To 50 μL of honey extract the reactive radical mixture (4 mL), composed of ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; Sigma Aldrich, CZ) (12 mL; 3.5 mM) with $K_2S_2O_8$ (0.06 M; Lukes, CZ) and acetic buffer (pH 4.3), was added. The reaction mixture was shaken vigorously on a Vortex mixer and for 30 min it was left to react without light exposure at room temperature. Honey samples absorbance (A) and absorbance of control samples (AC) were after time limit measured at $\lambda = 734$ nm against blank by spectrometer Libra S6 Biochrom (GB). Inactivation (I) was calculated from the decrease of absorbance (%) according to relation (1). Results of TAC (ABTS) were calculated from

inactivation using calibration curve with trolox as standard. It was expressed as trolox equivalents (TE) in mg.100g^{-1} sample. Average results were obtained from three parallel determinations.

$$I = \frac{AC-A}{AC} . 100 \quad (1)$$

DPPH method: To prepared honey extract (0.2 mL) a DPPH (1,1-diphenyl-2-picrylhydrazyl) solution in ethanol (1.9 mL; 0.02 mM; Sigma Aldrich, CZ) and acetate buffer solution (1 mL; pH 5.5) were added. The mixture was shaken vigorously on a Vortex mixer in capped glass and left without light exposure for 1 h at room temperature. Absorbance of samples (A) and absorbance of control samples (AC) was measured at λ = 517 nm against blank on the spectrometer (Libra S6 Biochrom, GB). Their inactivations (I) were also calculated from the decrease of absorbance according to relation (1) and the values were expressed as trolox equivalents (TE) in mg.100g^{-1} sample. Average results were obtained from three parallel determinations.

Statistic analysis

All analyses were provided in triplicate, and the data were expressed as mean values ± standard deviation (SD). Statistic evaluation of the results was made by Statistica program, StatSoft version 9.0 (Dell, USA) using parametric test comparing mean values of two independent assortments (Student *t*-test). Differences at a 95% confidence level (p <0.05) were considered statistically significant. Correlations between the parameters evaluated were obtained using Pearson's correlation coefficient (r).

RESULTS AND DISCUSSION

Color of honeys

In terms of our measurements and also due to sensorial examination, honey color ranges from white pale, pale yellow to amber and dark brown with the values ΔA from 0.139 to 0.817, the average 0.478 (Table 1). As expected, as the darkest honeys with the highest values were evaluated honeydew honeys (HD samples). Their values (average 0.779) were nearly five times higher than the brightest ones, acacia and rape honeys (average 0.161); and nearly three times to the values of floral honeys (0.268). Multi flower and forest honeys belong to the group of darker honeys. Several compounds, pigments are responsible for honey color. To the most important honey pigments belong water soluble polyphenols, flavonoids, and lipid soluble carotenoids **(Isla et al., 2011)**.

Also mineral composition is important for honey color. As **González-Miret et al. (2005)** evaluated lightness is significantly related with minerals such as S, Ca, Fe, As, Pb, and for the dark honey types (chestnut, and honeydew honeys) also Cd is considerable. Due to these facts, there are therefore expectations of higher content of polyphenols and probably also higher antioxidant values for darker honeys **(Bertoncelj et al., 2007; Kuś et al., 2014)**.

Content of phenolics

In the determination with Folin-Ciocalteau reagent electron-donating antioxidants such as polyphenols, ascorbic acid, and vitamin E are evaluated. The total polyphenols (PP) contents of the honey samples (Table 1) range from 54 to 254.2 mg GAE.100g^{-1} with the average 150 mg GAE.100g^{-1} honey. There were marked differences between honeys (p <0.05, Student *t*-test). Also **Bertoncelj et al. (2007)** mentioned that total phenolic content differs widely among different honey types. As it was expected due to other scientist researches **(Moniruzzaman et al., 2014; Alvarez-Suarez et al., 2010)** brighter, pale honeys had lower polyphenol values. PP results for honeys were in agreement with literature sources; lowest for acacia honey, where also **Beretta et al. (2005)** evaluated the lowest content (5.5 mg GAE.100g^{-1}). It was followed by rape honey and floral ones, forest and multi flower honey, whereas the highest PP content had the darkest honeydew honeys with the nearly five times higher values in comparison to the lowest ones. These findings are in agreement with the values previously reported for other European honeys, as **Kuś et al. (2014)** determined in Polish honeys total phenolic content in the range 12.2 – 117.4 mg GAE.100g^{-1}. The composition of honeys is dependent on the botanical origin, floral source, and also seasonal and environmental factors, as well as processing **(Kıvrak and Kıvrak, 2017; Cavazza et al., 2013; Dimitrova et al., 2007)**.

Honey samples exhibited similar order of samples for color and PP values. To characterize the relationships between color and polyphenolic content the correlation (Figure 1) was evaluated. They are strongly related with a correlation factor r = 0.8294. **Sant'ana et al. (2014)** also discovered that the lowest total phenolic content corresponded to light-colored honey and the highest values to dark honeys. Positive correlation between color and PP determined also other scientists **(Alvarez-Suarez et al., 2010; Kuś et al., 2014).**

Honey color thus seems to be a relatively reliable parameter to indicate high PP content in honey.

Antioxidant capacity

The antioxidant potential that means overall hydrogen/electron-donating activity of present antioxidants, was measured by two methods, with ABTS and DPPH test. The total antioxidant capacity (TAC) results for honey samples are demonstrated in Table 1. The TAC values of ABTS method were in the range from 15.5 to 89.6 mg of trolox equivalents per 100 grams of honey sample with the average 54.5 mg TE.100g^{-1}; and from 11.3 to 118.7 mg TE.100g^{-1} for DPPH method with the average 58.4 mg TE.100g^{-1}, respectively. There were marked differences between honeys (p <0.05, Student *t*-test). Also, **Frankel et al. (1998)** showed in their study that great variations exist in the chemical nature of honey from different floral sources as they found in best honey source 20.3 times higher concentration of antioxidants in comparison with that of the lowest one. The least active honeys in our research were, similarly like in PP evaluation, the brightest honeys **(Beretta et al., 2005; Kuś et al., 2014),** pale acacia **(Bertoncelj et al., 2007)** and rape honeys, for both methods.

Higher potency in scavenging of DPPH free radical and also ABTS test showed floral honeys (average 34.8 and 40.2 mg TE.100g^{-1}, respectively), forest and multi flower honey. Honeys with the best antioxidant potency were evaluated honeydew honeys (average 98.1 mg and 80.6 mg TE.100g^{-1}, respectively). The highest results for TAC (DPPH and ABTS test) were nearly eleven and nearly six times higher, respectively, than the lowest TAC value. Also, **Ferreira et al. (2009)** obtained the highest antioxidant values in the dark honeys.

Table 1 Color evaluation (ΔA), polyphenols content (PP) and total antioxidant capacity (TAC) of honey samples.

Honey sample	ΔA ±SD	PP (mg GAE.100g^{-1} ±SD)	TAC (ABTS) (mg TE.100g^{-1} ±SD)	TAC (DPPH) (mg TE.100g^{-1} ±SD)
A1	0.139 ±0.009^{a}	54.0 ±1.7^{a}	15.5 ±2.4^{a}	11.3 ±1.3^{a}
R1	0.183 ±0.016^{b}	56.8 ±1.2^{a}	19.0 ±0.2^{b}	28.1 ±0.9^{b}
FL1	0.238 ±0.013^{c}	126.5 ±0.6^{b}	33.0 ±1.0^{c}	37.9 ±2.0^{c}
FL2	0.273 ±0.010^{d}	111.3 ±0.5^{c}	49.2 ±6.5^{d}	38.7 ±2.8^{c}
FL3	0.293 ±0.016^{e}	146.2 ±0.9^{d}	38.5 ±1.6^{e}	27.8 ±0.8^{b}
MF1	0.467 ±0.019^{f}	155.3 ±0.4^{e}	66.0 ±3.0^{f}	56.7 ±0.3^{d}
FO1	0.552 ±0.024^{g}	150.1 ±0.5^{d,e}	56.0 ±1.1^{g}	49.1 ±0.9^{e}
HD1	0.745 ±0.027^{h}	164.5 ±0.4^{f}	59.2 ±4.3^{g}	73.1 ±1.3^{f}
HD2	0.753 ±0.025^{h,i}	203.0 ±1.5^{g}	84.5 ±2.0^{h}	99.5 ±1.1^{g}
HD3	0.801 ±0.020^{i,j}	224.6 ±1.3^{h}	89.1 ±9.0^{h}	101.2 ±1.8^{g}
HD4	0.817 ±0.031^{j}	254.2 ±1.4^{i}	89.6 ±2.5^{h}	118.7 ±1.3^{h}

Note: Means within a column with at least one identical superscript are not significantly different by Student's *t*-test (p <0.05).

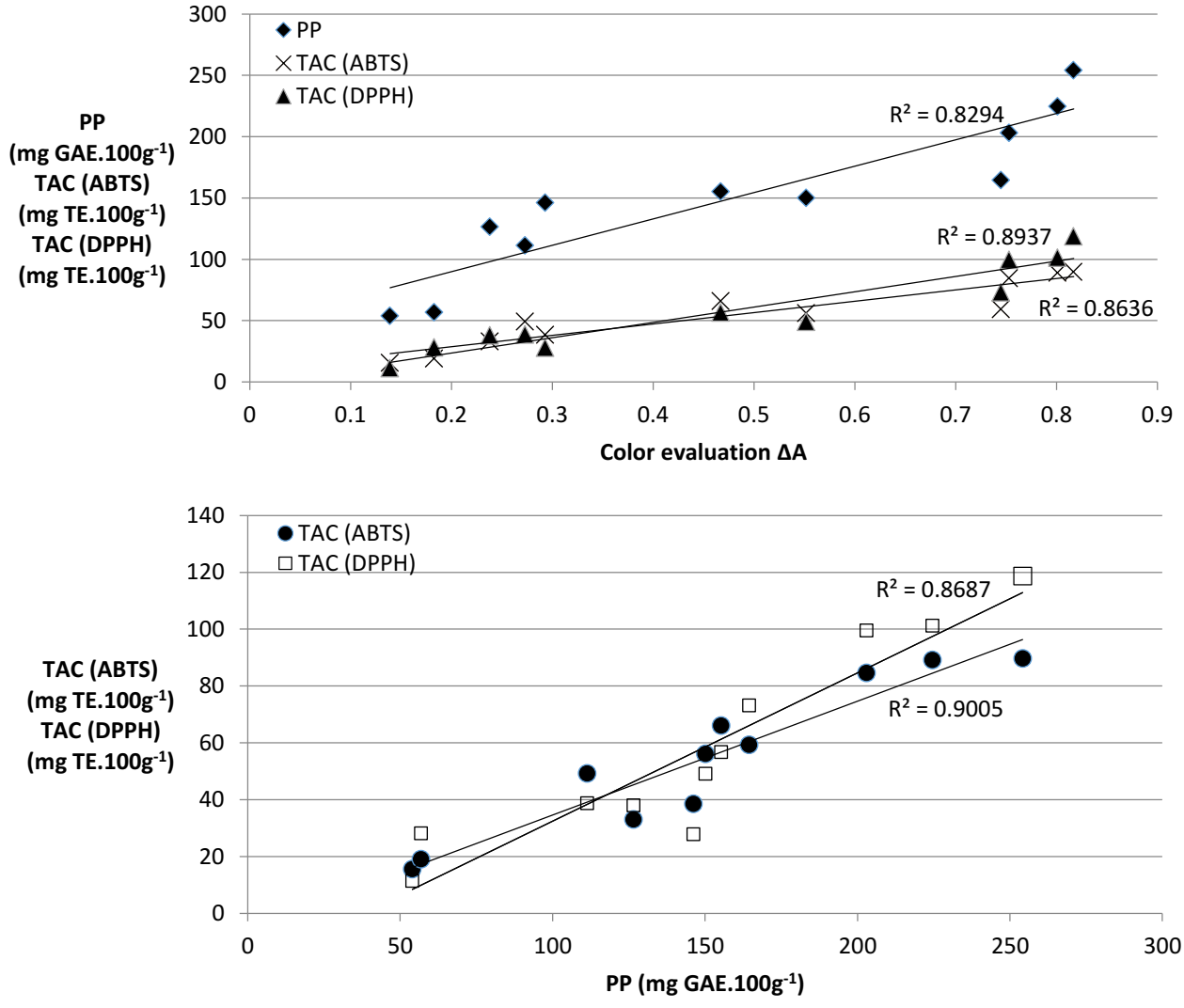

Figure 1 Correlations between color evaluation (ΔA) and PP and TAC values (top), and PP and TAC values (bottom).

Similar descending order of TAC values was observed also for honey color. The correlations to examine the relationships between them (Figure 1) were evaluated. Antioxidant capacity showed a strong relationship comparing both assays and color intensity, for ABTS evaluation $r = 0.8836$, and for DPPH test $r = 0.8937$. High correlation between color and antioxidant capacity was determined also by other researchers **(Sant'ana et al., 2014; Pontis et al., 2014; Beretta et al., 2005; Bertoncelj et al., 2007)**. Honey color thus seems to be a relatively reliable parameter to indicate not only PP content but also antioxidant potential in honey.

Antioxidant capacity determined by both analyses was also strongly positively associated with the polyphenolic content ($r = 0.9005$ for ABTS; $r = 0.8687$ for DPPH), as shown in Figure 1. Therefore high PP contents predicate high TAC values in analyzed honey samples. Also **Wilczyńska (2014), Sant'ana et al. (2014), Pontis et al. (2014), Beretta et al. (2005),** and **Bertoncelj et al. (2007)** found positive correlation between PP and TAC for honey samples. Although **Pontis et al. (2014)** determined high flavonone and dihydroflavonol content in some honeys, no correlations between them and antioxidant potential they observed. Generally, our findings and data in the literature have shown a linear relationship between honey color, phenolic compounds and antioxidant capacity.

CONCLUSION

Honey has several positive health effects for humans. They are related to their bioactive compounds such as enzymes, phenolic compounds, vitamins and some minerals. Honeys with the best polyphenols content and antioxidant capacity values evaluated by both methods are honeydew honeys, the darkest ones. The color of honeys was determined in the progression: pale acacia, then rape honey, floral, multi flower and forest honeys, and dark honeydew honeys. The polyphenol content in honeys was in the range from 54.0 to 254.2 mg GAE.100g^{-1}, in the descending order: honeydew honeys, multi flower and forest honey, followed by floral honeys, then rape and acacia honey. Antioxidant capacity values by two evaluation methods (ABTS, DPPH), are in agreement with polyphenols content order. They were highest also for honeydew honeys (59.2 – 89.6 and 73.1 – 118.7 mg TE.100g^{-1}), followed by multi flower (66.0 and 56.7 mg TE.100g^{-1}) and forest honey (56.0 and 49.1 mg TE.100g^{-1}), then floral honeys (33.0 – 49.2 and 27.8 – 38.7 mg TE.100g^{-1}) and the lowest values for rape (19.0 and 28.1 mg TE.100g^{-1}) and acacia (15.5 and 11.3 mg TE.100g^{-1}) honey. There was established a positive correlation between the color, polyphenolic amount and antioxidant capacity of the evaluated honeys. Darker honey samples showed higher content of phenolic compounds and increased antioxidant capacity.

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MOLECULAR CHARACTERIZATION AND GENETIC DIVERSITY IN SOME EGYPTIAN WHEAT (*Triticum aestivum* L.) USING MICROSATELLITE MARKERS

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ABSTRACT

Wheat (*Triticum aestivum* L.) is the most important and strategic cereal crop in Egypt and has many bread wheat varieties. Seventeen Egyptian bread wheat varieties used in this study with a set of sixteen wheat microsatellite markers to examine their utility in detecting DNA polymorphism, estimating genetic diversity and identifying genotypes. A total of 190 alleles were detected at 16 loci using 16 microsatellite primer pairs. The number of allele per locus ranged from 8 to 20, with an average of 11.875. The polymorphic information content (*PIC*) and marker index (*MI*) average values were 0.8669, 0.8530 respectively. The (GA) n microsatellites were the highest polymorphic (20 alleles). The Jaccard's Coefficient for genetic similarity was ranged from 0.524 to 0.109 with average of 0.375. A dendrogram was prepared based on similarity matrix using UPGMA algorithm, divided the cultivars into two major clusters. The results proved the microsatellite markers utility in detecting polymorphism due to the discrimination of cultivars and estimating genetic diversity.

Keywords: DNA polymorphism; genetic diversity; heterozygosity; PIC; SSR; wheat

INTRODUCTION

Wheat (*Triticum aestivum* L.) is an important and strategic grain crop in the majority of the world. In Egypt, wheat is considered the most important and strategic cereal crop. It represents about 10 percent of the total agricultural production value and about 20 percent of all agricultural imports **(FAOSTAT, 2008)**. Wheat is a self-pollinating polypoid crop that has been bred for a wide array of specific end-use quality traits and various adaptive characteristics, resulting in the development of distinct cultivars tailored to specialized end uses and specific production environments.

Botanists have long used morphological characterization to classify and distinguish genotypes within plant species. The emergence of the Plant Variety Protection (PVP) Act in 1970 had an impact on the protection of plant varieties. Hence, it was necessary to develop the critical tools for classifying and distinguishing genotypes within plant varieties **(Rongwen et al., 1995)**. The polymorphisms of DNA provide a powerful tool for determining and discriminating the levels of genetic variation in plant germplasm. Molecular markers have thus become accurate and reliable tools for identifying and characterizing plant varieties and it has become effective tool for efficient selection of desired agronomic traits since it depends on genotype rather than phenotype. The advances in molecular genetics methodology have led to widespread use of co-dominant molecular markers, especially Simple Sequence Repeats (SSRs), Single Nucleotide Polymorphisms (SNPs) and the amplified fragment length polymorphisms (AFLPs) **(Röder et al., 1998; Bohn et al., 1999; Prasad et al., 1999; Prasad et al., 2000; Roy et al., 1999; Varshney et al., 2000;** for reviews see **Gupta et al., 1999; Gupta and Varshney, 2000; Bered et al., 2005)**.

The first one define microsatellite term was **(Litt and Lutty, 1989)**, as multilocus probes creating complex banding patterns and usually non- species specific occurring ubiquitously. They essentially belong to the repetitive DNA family. Microsatellite are repeated as of only a few bases, like two or three or five, and the whole repetitive region spans less than 150 bp. Therefore, it needs cloning and sequencing for designing the primers **(Weissenbach et al., 1992; Morgante and Olivieri, 1993; Powell et al., 1996)**. Furthermore, these markers have provided high reproducibility and genetic informativeness **(Coombs et al., 2004; Garcia et al., 2007)**.

Microsatellites produced from whole genome sequences, subgenomic sequences, ESTs and gene sequences have also been applied to DNA fingerprinting and the estimation of genetic diversity within a gene pool. Genetic diversity is defining the heritable variation within and between population's organisms **(Ramanatha and Hodgkin, 2002)**. Knowledge of the genetic diversity and population structure within germplasm collections is an

important foundation for crop improvement **(Thomason et al., 2007)**. Progress in plant breeding requires a broad genetic base with a rich and diverse germplasm collection being the backbone of every successful crop improvement program. In this study, we evaluate the potential of 16 microsatellite primer pairs in general and specific SSRs in particular for polymorphism determination, cultivars identification and to evaluate the level of microsatellite based genetic diversity between 17 Egyptian bread wheat cultivars that were potentially useful in wheat breeding programs.

Scientific hypothesis

It is expected that there will be a significant similarity between the wheat varieties used in this experiment due to their genetic resources.

MATERIAL AND METHODOLOGY

Seed Material

In this experiment, some elite Egyptian bread wheat cultivars (17) were selected in different regions in Egypt as shown in Table (1). These cultivars have been obtained from Gene Bank, Egyptian Ministry of Agriculture.

DNA isolation and microsatellite primers

Twenty five seeds of each wheat varieties have been planted in pots in greenhouse. After two weeks of planting, the plants were enough to get fresh leaves and were collected from each sample. The samples were immediately transferred to liquid nitrogen tank to prevent deterioration. Weighed about 1.5 g from plant leaves samples and ground by liquid nitrogen to obtain fine powder. Total genomic DNA was isolated from leaves of each of the seventeen varieties according to the protocol described by **Anderson et al. (1992)**, with a few modifications intended to improve the quality of DNA: two consecutive extractions with phenol: chloroform (1:1) were carried out by an additional wash of 97% (left at -20 °C for one hour) an 70% pre-cooled ethanol, respectively. The yield and quality of DNA were assessed by spectrophotometer and gel electrophoreses.

SSR markers and protocols

Sixteen microsatellite primer pairs (SSR) were selected from **(Röder et al., 1998)**, Table 2. Amplification reactions were carried out in a total volume of 25 µL, which contained 250 nM of each primer (Metabion GmbH, Germany), 0.2 mM of each deoxynucleotide, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase (Bioline, GmbH, Germany) and 50 – 100 ng of template DNA. All reaction volumes were 25 µL overlaid with a drop of mineral oil. The thermocycling program (MJ Research PTC-100 thermal cycler) used was: one cycle at 94 °C for 3 min, 45 cycles at 94 °C for 1 min at either 50, 55 or 60 °C (depending on the individual microsatellite), 2 min at 72 °C, and the final extension step of 10 min at 72 °C. Electrophoresis was done to visualize the PCR amplified product. It was carried out on 2.0% agarose gel and amplified fragments were visualized by staining wit ethidium bromide. Fragment sizes were determined with PyElph 1.4 software, is the commercial program Quantity One from Bio-Rad **(Pavel and Vasile, 2012)**. The fragment size in "Chinese Spring" was taken as standard **Röder et al., (1998)**.

Statistic analysis

The fragment(s) sizes in 'Chinese Spring' were taken as standard, and the size differences of the fragments in other genotypes were considered to be the result of alterations in the repeat number of the simple sequences at the corresponding site(s). All distinct DNA fragments scored as present (1) or absent (0) were used to compute pair-wise similarity coefficients **(Jaccard, 1908)** for each of the markers for the purpose of assessing genetic diversity leading to cluster analysis. PowerMarker software (v3.0, 2004) is statistical software for genetic marker data analysis **(Liu and Muse, 2005)** was used for estimating of allele number, allele frequencies, genotype, heterozygosity and polymorphic information content (PIC). However, Marker index (MI) was calculated according to **Powell et al. (1996)**, MI = Average polymorphic information content (PIC) x Proportion of polymorphic bands x Average number of loci per assay unit.

Cluster analysis

For phylogenetic analysis, data only from the polymorphic SSR loci were subjected to MVSP software statistical software (Kovach Computing Services, Pentraeth, Wales, U.K). All the 17 wheat varieties were clustered based on the estimated genetic distance. The phylogenetic analysis was carried out with the clustering method of the unweighted Pair Group Method Using Arithmetic Average (UPGMA).

Principal component analysis (PCA)

The original 1 – 0 data matrix was used for calculating a correlation matrix between pairs of markers. The correlation matrix was employed for the calculation of eigenvalues, which were then used for determining the coordinates for each genotype that were used for PCA.

RESULTS AND DISCUSSION

DNA polymorphism and genotype identification

The results of PCR amplification of a number of microsatellite loci in seventeen Egyptian bread wheat cultivars using sixteen microsatellite primer pairs are summarized in (Table 2). Altogether, 190 alleles at 16 loci were obtained with average 11.875 alleles per locus. The maximum number of alleles detected at *Xgwm32-3A* belonging to (GA) n was 20 alleles with size ranged from 163-179 bp. However, the minimum number of alleles detected at *Xgwm156-5A* belonging to (GT) n and *Xgwm157-2D* (CT) n was 8 alleles with size from 270-299 bp and 107-113 bp respectively, Table 2. Allele frequency per locus varied from eight (*Xgwm156-5A* and *Xgwm157-2D*) to twenty *Xgwm32-3A*. It is known that microsatellite primer pairs are locus specific and that is meant to be a single locus marker comparing with other molecular markers as RFLP probes, RAPD, ISSR and SCoT primers which multilocus **(Vivodík et al., 2016)**. In the present study, the 16 loci that were assigned to specific chromosomes were able to distinguish between 17 Egyptian bread wheat and thus useful for detecting polymorphism.

Table 1 List of the elite Egyptian wheat cultivars and region of cultivation.

No.	Wheat cultivars	Cultivation region	No.	Wheat cultivars	Cultivation region
1	Misr 1	North Delta - Central & South Delta - New Land - North & East Coast	11	Sids 13	Central Egypt
2	Misr 2	New Land - North and East Coast	12	Sakha 93	North Delta - Central and South Delta - Upper Egypt
3	Gimmasa 7	Nubaria Area - New Land	13	Sakha 94	North Delta - Central and South Delta
4	Gimmasa 9	Nubaria, North Delta - Central and South Delta	14	Sakha 69	North Delta - North Coast Areas
5	Gimmasa 10	Nubaria, North Delta - Central and South Delta	15	Shandaweel 1	Central Egypt - Upper Egypt
6	Gimmasa 11	Nubaria, North Delta - Central and South Delta	16	Bani Sweef 1	Central Egypt - Upper Egypt
7	Gimmasa 12	Nubaria and most of the governorates of the Delta	17	Bani Sweef 4	Central Egypt
8	Giza 168	North Delta - Central and South Delta - Middle Egypt - Upper Egypt - New Land - Nubaria area			
9	Sids 8	Central and Upper Egypt - South Valley			
10	Sids 12	North Delta - Central Egypt - Upper Egypt - New Land - North Coast - Nubaria area			

Table 2 Primers of SSR, locus, repeat motif and annealing temperature (Ann. Temp.) were used.

No.	SSR primers				
	Locus	Forward primer	Revers primer	Repeat motif	Ann. Temp. °C
1	*Xgwm2-3A*	CTG CAA GCC TGT GAT CAA CT	CAT TCT CAA ATG ATC GAA CA	(CA)18	50
2	*Xgwm32-3A*	TAT GCC GAA TTT GTG GAC AA	TGC TTG GTC TTG AGC ATC AC	(GA)19	55
3	*Xgwm33-1A*	GGA GTC ACA CTT GTT TGT GCA	CAC TGC ACA CCT AAC TAC CTG C	(GA)19	60
4	*Xgwm47.1-2A*	TTG CTA CCA TGC ATG ACC AT	TTC ACC TCG ATT GAG GTC CT	(CT)7TT(CT)16	60
5	*Xgwm71.2-2A*	GGC AGA GCA GCG AGA CTC	CAA GTG GAG CAT TAG GTA CAC G	(GT)20	60
6	*Xgwm95-2A*	GAT CAA ACA CAC ACC CCT CC	AAT GCA AAG TGA AAA ACC CG	(AC)16	60
7	*Xgwm113-4B*	ATT CGA GGT TAG GAG GAA GAG G	GAG GGT CGG CCT ATA AGA CC	(GT)12	55
8	*Xgwm114-3B*	ACA AAC AGA AAA TCA AAA CCC G	ATC CAT CGC CAT TGG AGT G	(GA)53	60
9	*Xgwm130-7A*	AGC TCT GCT TCA CGA GGA AG	CTC CTC TTT ATA TCG CGT CCC	(GT)22	60
10	*Xgwm131-1B*	AAT CCC CAC CGA TTC TTC TC	AGT TCG TGG GTC TCT GAT GG	(CT)22	60
11	*Xgwm155-3A*	CAA TCA TTT CCC CCT CCC	AAT CAT TGG AAA TCC ATA TGC C	(CT)19	60
12	*Xgwm156-5A*	CCA ACC GTG CTA TTA GTC ATT C	CAA TGC AGG CCC TCC TAA C	(GT)14	60
13	*Xgwm157-2D*	GTC GTC GCG GTA AGC TTG	GAG TGA ACA CAC GAG GCT TG	(CT)14	60
14	*Xgwm159-5B*	GGG CCA ACA CTG GAA CAC	GCA GAA GCT TGT TGG TAG GC	(GT) 15	60
15	*Xgwm160-4A*	TTC AAT TCA GTC TTG GCT TGG	CTG CAG GAA AAA AAG TAC ACC C	(GA)21	60
16	*Xgwm161-3D*	GAT CGA GTG ATG GCA GAT GG	TGT GAA TTA CTT GGA CGT GG	(CT)15	60

Such a discriminatory set should also ensure the uniform distribution of the microsatellite primers of this set across the three genomes of bread wheat since in several studies, including the present study, microsatellites have been shown to be more frequent in the A and B genomes, than in the D genome **(Röder et al., 1998; Stephenson et al., 1998)**.

Table 3 Locus, expected product size (bp), products size range (bp), allele No., allele frequency, genotype No., gene diversity, heterozygosity, polymorphic information content (PIC) and marker index (MI) of 16 SSR markers assayed in 17 Egyptian bread wheat.

No.	Locus	Expected product size(bp)	Product sizes range (bp)	Allele No.	Allele fre-quency	Genotype No.	Gene diversity	Hetero-zygosity	PIC	MI
1	*Xgwm2-3A*	128	131-145	15	0.2059	15.0000	0.9048	1.0000	0.8979	0.8883
2	*Xgwm32-3A*	169	163-179	20	0.1176	17.0000	0.9377	1.0000	0.9342	0.9231
3	*Xgwm33-1A*	116	105-124	17	0.1471	16.0000	0.9187	1.0000	0.9131	0.8881
4	*Xgwm47.1-2A*	-	159-193	18	0.1176	17.0000	0.9291	1.0000	0.9246	0.9029
5	*Xgwm71.2-2A*	120	105-126	16	0.1765	17.0000	0.9048	0.9412	0.8974	0.8533
6	*Xgwm95-2A*	128	112-133	12	0.1471	14.0000	0.9014	1.0000	0.8929	0.8486
7	*Xgwm113-4B*	148	144-164	10	0.2353	13.0000	0.8685	0.9412	0.8553	0.8324
8	*Xgwm114-3B*	168	138-173	10	0.2059	13.0000	0.8720	1.0000	0.8589	0.8517
9	*Xgwm130-7A*	126	118-133	10	0.1765	14.0000	0.8772	1.0000	0.8645	0.8470
10	*Xgwm131-1B*	165	142-173	9	0.2059	12.0000	0.8547	1.0000	0.8377	0.8299
11	*Xgwm155-3A*	143	124-149	9	0.2059	12.0000	0.8547	1.0000	0.8377	0.8464
12	*Xgwm156-5A*	300	270-299	8	0.2059	10.0000	0.8443	1.0000	0.8251	0.8338
13	*Xgwm157-2D*	106	107-113	8	0.2647	10.0000	0.8339	1.0000	0.8137	0.8198
14	*Xgwm159-5B*	189	177-198	9	0.2059	13.0000	0.8581	1.0000	0.8423	0.8408
15	*Xgwm160-4A*	184	182-200	9	0.2059	12.0000	0.8685	1.0000	0.8543	0.8400
16	*Xgwm161-3D*	154	141-159	10	0.3235	13.0000	0.8356	0.8235	0.8202	0.8019
Mean				11.875	0.1967	13.6250	0.8790	0.9816	0.8669	0.8530

The SSRs relative with them, more alleles detected at (CA) n and (AC) n one loci each, however, (GA) n loci 4 loci, (CT) n and (GT) n loci5 loci each. **Röder et al. (1995)** reported that (GT) n repeats to be more polymorphic than other simple repeats such as (GA) n in wheat. However, in barley more alleles were detected for (GA) n repeats than for (GT) n repeats **(Struss and Plieske 1998)**. The results were obtained agreement with **(Dreisigacker et al., 2004; Dvojkovic et al., 2010; Spanic et al., 2012)** and opposed with **Kumar et al. (2016)**. The loci of microsatellite are multi-allelic and the alleles co-dominant, proved that the microsatellite marker strict in determined DNA polymorphism and highly informative genetic markers **(Devos et al., 1992; Devos et al., 1993; Xie et al., 1993; Mason, 2015)** than other markers as single nucleotide polymorphisms (SNPs) which are biallelic and dominant. The earlier studies proved that four microsatellite markers set each had the ability to distinguish between 24 genotypes in barley and 16 genotypes in tomato **(Rusell et al., 1997; Bredemeijer et al., 1998)**. Furthermore, the microsatellite set could be used to distinguish each genotype in a set of more than 100 wheat genotypes **(Manifesto et al., 1999)**.

Genetic diversity

Polymorphism Information Content (PIC) was estimated for 16 loci (Table 2). The PIC values ranged from 0.8137 of locus Xgwm157-2D to 0.9342 of locus Xgwm32-3A with an average of 0.8669. As well as, the heterozygosity values were estimated as well, ranging from 0.8235 to 1.00 with average 0.9816. The marker index (MI) value over all 16 microsatellite markers was 0.8530. The heterozygosity was detected in three loci (Xgwm71.2-2A, Xgwm113-4B and Xgwm161-3D) with values 0.9412, 0.9412 and 0.8235 respectively, (Table 3). The genotype number per locus was estimated based on allelic frequency data, where the highest number detected 17 of locus Xgwm32-3A and lowest number founded 10 of locus Xgwm156-5A and Xgwm157-2D with an average 13.6250 (Table 2). The data of microsatellite loci and the corresponding alleles were used to calculate the polymorphic information content (PIC) and heterozygosity (H) to evaluate a marker system for its ability to detect high levels of DNA polymorphism in an analysis of genetic diversity. In earlier studies on bread wheat, the PIC values were ranged from 0.23 to 0.79 **(Röder et al., 1995)** and from 0.29 to 0.79 **(Plaschke et al., 1995)** and 0.21 to 0.90 **(Prasad et al., 2000)**. On the other hand, the PIC values obtained by **(Bohn et al., 1999)** were very low (0.30). However, **(Saeidi et al., 2006; Khalighi et al., 2008; Tahernezhad et al., 2010; Mir et al., 2012; Arora et al., 2014)** studies were the opposite of the previous results and consistent with our results where PIC value > (0.7). The marker index is also used to measure the efficiency of polymorphism **Khodadadi et al., (2011)**.

Table 4a The matrix of Jaccard's similarity to 17 Egyptian bread wheat is indicated in Table 1.

	Misr1	Misr2	Gim7	Gim9	Gim10	Gim11	Gim12	Giza168	Sids8
Misr 1	1.000								
Misr 2	0.409	1.000							
Gimmasa 7	0.391	0.348	1.000						
Gimmasa 9	0.391	0.292	0.524	1.000					
Gimmasa 10	0.240	0.429	0.348	0.476	1.000				
Gimmasa 11	0.333	0.348	0.333	0.524	0.476	1.000			
Gimmasa 12	0.391	0.476	0.391	0.333	0.240	0.391	1.000		
Giza 168	0.138	0.185	0.138	0.222	0.231	0.320	0.320	1.000	
Sids 8	0.154	0.261	0.200	0.250	0.261	0.304	0.364	0.192	1.000
Sids 12	0.222	0.231	0.320	0.269	0.231	0.500	0.500	0.308	0.348
Sids 13	0.103	0.148	0.185	0.231	0.240	0.333	0.280	0.320	0.500
Sakha 93	0.143	0.148	0.103	0.231	0.148	0.333	0.280	0.222	0.429
Sakha 94	0.269	0.185	0.222	0.179	0.103	0.222	0.222	0.133	0.292
Sakha 69	0.103	0.033	0.185	0.231	0.069	0.143	0.067	0.138	0.154
Shandawel1	0.103	0.069	0.143	0.103	0.107	0.103	0.067	0.138	0.111
Bani Sweef3	0.065	0.000	0.138	0.138	0.032	0.065	0.065	0.214	0.107
Bani sweef4	0.032	0.033	0.067	0.067	0.069	0.032	0.032	0.031	0.071

Table 4b The matrix of Jaccard's similarity to 17 Egyptian bread wheat is indicated in Table 1.

	Sids12	Sids13	Sakha93	Sakha94	Sakha69	Shandawel1	Sweef3	Sweef4
Sids 12	1.000							
Sids 13	0.435	1.000						
Sakha 93	0.435	0.391	1.000					
Sakha 94	0.308	0.375	0.320	1.000				
Sakha 69	0.269	0.280	0.280	0.375	1.000			
Shandawel1	0.100	0.103	0.103	0.179	0.143	1.000		
Bani Sweef3	0.172	0.222	0.138	0.259	0.320	0.269	1.000	
Bani sweef4	0.065	0.103	0.143	0.138	0.185	0.333	0.375	1.000

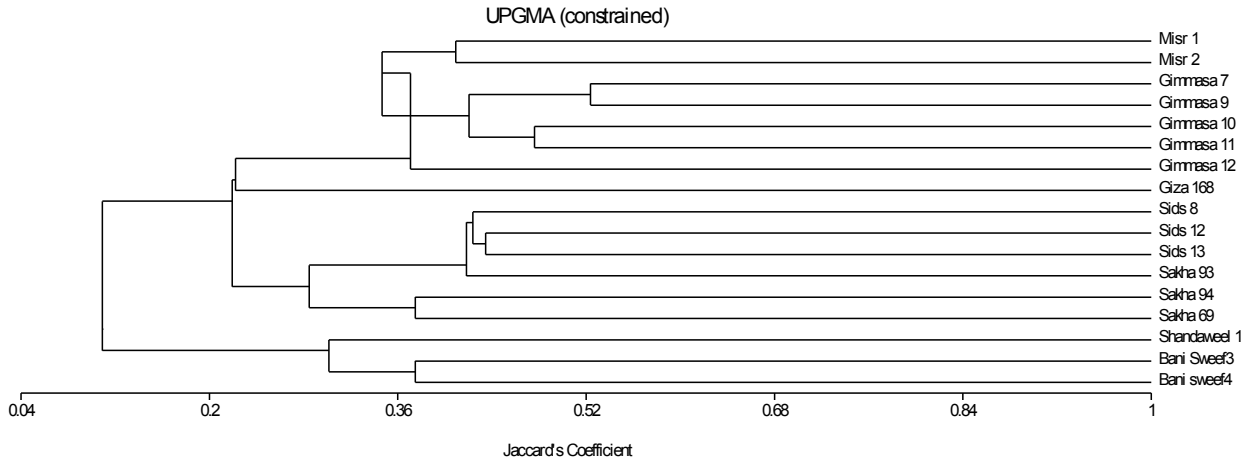

Figure 1 Dendrogram of 17 Egyptian bread wheat based on data on allelic profiles generated using 16 microsatellite primer pairs.

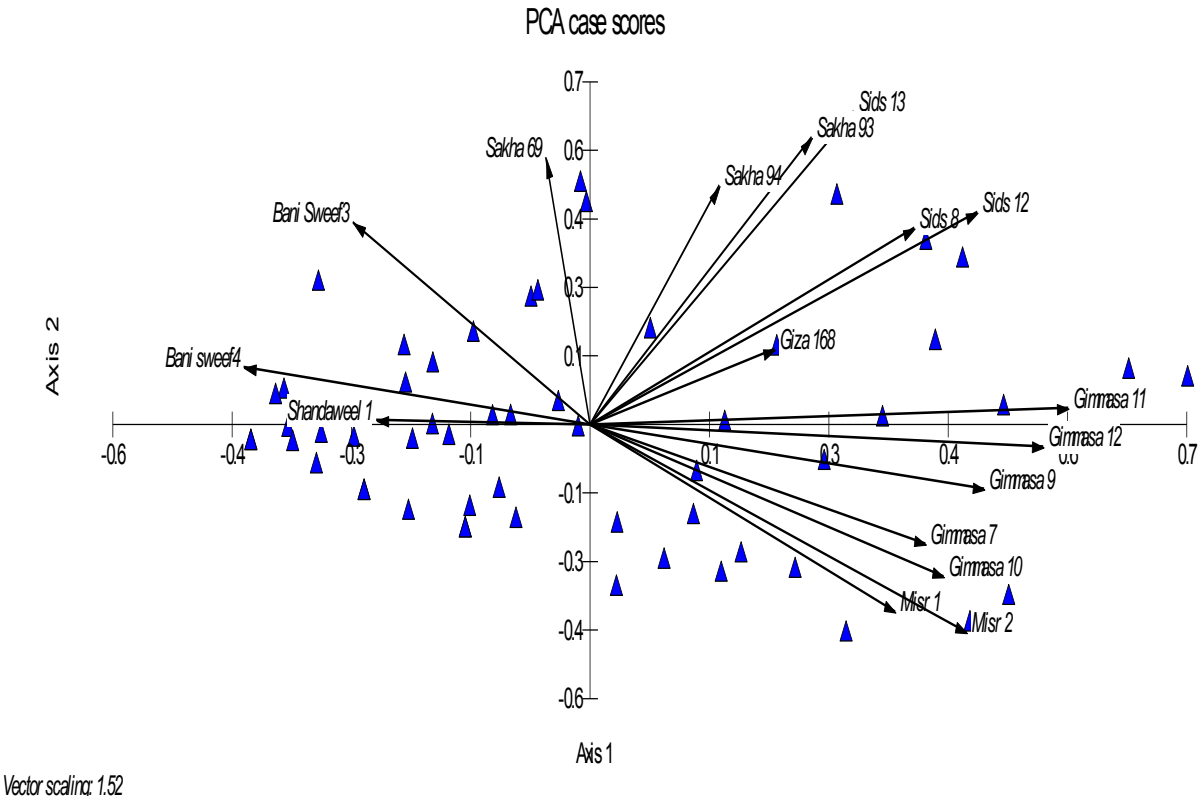

Figure 2 Matrix plot among 17 Egyptian bread wheat cultivars revealed by principle component analysis based on SSR data.

The marker index (MI) value was calculated also for used markers and was close to (0.8530) the MI value (0.70) reported by **(Prasad et al., 2000)**, however those results were higher than the MI value (0.21) obtained by **(Bohn et al., 1999)** on SSRs wheat. When comparing the MI value with other markers found that, SAMPL (9.61) and AFLP (3.41) in bread wheat **(Bohn et al., 1999)**. However, MI value (6.14) that is intermediate between the above two contrasts was also available for AFLPs in soybean **(Powell et al., 1996)**.

Genetic similarity

In order to investigate, genetic relationships between 17 Egyptian wheat genotypes cluster analysis based on Jaccard's similarity coefficients and UPGMA algorithm were calculated for the 43 durum wheat germplasm. A Jaccard's genetic similarity matrix is presented in Table 4a and 4b. The average similarity among 17 Egyptian bread wheat was 0.357. The nearest neighbor cluster analysis obtained from Jaccard's similarity coefficient (Figure 1) illustrated the variability between 17 Egyptian bread wheat. The detected of DNA polymorphism by 16 microsatellite markers allowed of estimates genetic distance and clustering of 17 Egyptian bread wheat cultivars in two major groups. The first group (Group I) included Misr 1, Misr 2, Gimmasa 7, Gimmasa 7, Gimmasa 9, Gimmasa 10, Gimmasa 11, Gimmasa 12 and Giza 168. The second group (Group II) included the other cultivars Sids 8, Sids 12, Sids 13, Sakha 93, Sakha 94, Sakha 69, Shandaweel 1, Bani Sweef 3 and Bani Sweef 4. The similarities between 17 Egyptian bread wheat based on 16 microsatellite markers were ranged from 0.109 in Shandweel 1, Bani Sweef 3 and Bani Sweef 4 cultivars to 0.524 in Gimmaza 7 and Gimmaza 9 with average of 0.357. A Jaccard's genetic similarity matrix was estimated between pairs of Egyptian wheat cultivars using 16 microsatellite markers through cluster analysis. This study used UPGMA cluster analysis based on genetic similarity values for SSR alleles from all the wheat cultivars to construct a dendrogram (Figure 1). The similarity value between 17 Egyptian wheat cultivars was ranged from 0.109 to 0.524 with average value 0.357. This average of genetic similarity value (0.357) can be compared with other studies, whereas SSR-based genetic similarity coefficient values of 0.31 **(Plaschke et al., 1995)** and 0.57 **(Bohn et al., 1999)**. However, STS-based genetic similarity coefficient value of 0.81 **(Chen et al., 1994)** were reported. In these different studies on genetic diversity in bread wheat, undertaken using a variety of molecular markers, the variation in genetic similarity coefficient values may be attributed either to the differences in number of genotypes and the probes/primers used (e.g. 119 RFLP probes were used by **Paull et al. 1998**) or to the relative superiority of microsatellites to detect DNA polymorphism. An unusually low value of RFLP-based genetic similarity (0.18) reported by **Paull et al. (1998)** is certainly due to the larger sample of 124 diverse genotypes and bigger set of 119 RFLP probes used in this study. From the previous study it illustrated the importance and usefulness of the microsatellites (SSR) technique in wheat **(Prasad et al., 1999; Roy et al., 1999)**. Based on the results obtained are considered the

microsatellites technique was sensitive and critical in differentiating between the different varieties of Egyptian wheat under study, as well as in determining the polymorphism, genotype identification, genetic similarity and estimation of genetic diversity.

The principle component analysis was used to visualize the genetic relationships among genotypes shown in Figure 2. Of the total polymorphism, only 40.44% was accounted for by the first two components, implying that the used markers possessed a suitable dispersion of markers in the genome. The 17 Egyptian wheat cultivars were clustered into two groups. The principle component analysis thus is largely compatible to those from cluster analysis obtained from UPGMA.

CONCLUSION

Microsatellites or SSR markers are one of the most common genetic markers and used in many genetic applications. Microsatellites are codominant, highly polymorphic, and Mendelian inherited, all these qualities made it very suitable for such study and the ability to accurately identify differences between wheat varieties in this study. The species specific markers identified would be utilized in future introgression breeding programs.

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WHEAT BRAN STABILIZATION AND ITS EFFECT ON COOKIES QUALITY

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ABSTRACT

Wheat bran are widely used as a source of dietary fiber with enhanced health benefits. However, its hight application level in bakery products caused lower quality of these products (eg. reduced volume and increased hardness of product). Different mechanical, physical or chemical methods are used for modification of wheat bran physicochemical properties. Wheat bran obtained from two wheat variety was used to evaluate the effect of bran stabilization process on qualitative properties of baked goods. Stabilization was performed using hot air and microwave heating. Threated and unthreated wheat bran were blended with wheat flour at 5, 10 and 15% substitution level. Influence of bran addition on rheological properties was evaluated using Mixolab 2. Stabilized wheat bran increased water absorption up to 11.7% (hot air stabilization) and 11.9% (microwave stabilization), respectively. Hot air stabilization of wheat bran improved heat resistance of dough proteins during mixing and the stability of hot gel during heating. Stabilization process of wheat bran using both methods also increased the volume and spread ratio values of enriched cookies. Cookies incorporated with hot air treated bran were significantly softer than cookies contained raw bran. Sensory evaluation showed that addition of stabilized bran improved the shape and taste of cookies. Additionally, it was concluded that the most acceptable cookies were obtained at 5% addition level of bran stabilized using microwave heating.

Keywords: wheat bran; stabilization; dough rheology; cookie

INTRODUCTION

Wheat (*Triticum aestivum*) is a leading cereal crop which is mainly utilised for human consumption and livestock feed. A wheat kernel comprises three principal fractions – bran, germ and endosperm. The outer layers are all parts of the bran. The bran fraction is a by-product of milling and has food and nonfood applications **(Onipe et al., 2015)**. Bran can be processed from red or white, hard or soft, and durum wheat. Besides the obvious color difference, the bran from white wheat has a milder flavor than the bran from red wheat. The bran to be used in a specific application depends on the flavor, color, and appearance desired. The breakfast cereal industry uses both red and white wheat bran, but the bakery industry primarily uses red wheat bran. White bran has excellent potential for use in flour tortillas and pizza dough **(Lauková et al., 2016)**.

Wheat bran has a rich nutritional profile and shows beneficial physiological effects, making consumption of bran-rich food products more interesting from a health perspective than products based on refined flour. Because consumers become more aware of its benefits, wheat bran is increasingly added to mostly cereal-based food products (bread, cookies, breakfast cereals, pasta, snacks, cakes, and more) **(Hemdane et al., 2016)**.

In refined flours, the main components responsible for the water absorption are the gluten network proteins. Wheat bran particles can interfere with the gluten network, decrease dough resilience and impair the framework of gas cells and, thus, gas retention. These effects can lead to low bread specific volume and inferior baking quality **(Prückler et al., 2014)**. During the storage, rancidification can reduce the nutritional value of food, and cause some quality changes, involving flavor, texture, and appearance. Due to the possibility of rapid rancidity there is a necessity for stabilization **(Ertaş, 2016)**. Pretreating the bran before it is added back into the flour is a key to eliminating its adverse effects on the processing and quality of the flour system. Ultrafine milling, extrusion, heating, and biological methods are useful for bran pretreatment **(Zhang et al., 2018)**. Thermal steps like roasting, steaming, cooking under forced air or microwave radiation usually have little impact on the chemical composition, but some effects on the processing properties could be observed, like for example increased loaf volume of bread when containing autoclaved bran **(Prückler et al., 2014)**.

When heating in a forced air oven at 150 °C, the cellulose content decreases with the processing time in wheat. Thermal processing increased the soluble dietary fraction. In general, the changes in the dietary fiber composition

during thermal processing may be partly attributed to the redistribution of the insoluble and soluble components of dietary fiber, and partly to the formation of resistant starch. An increased temperature breaks week bonds between polysaccharide chains and split glycosidic linkages in the polysaccharides. As consequence, the architecture of the fiber matrix may be modified, and insoluble fiber solubilized **(Căpriţă et al., 2012)**.

Scientific hypothesis

Previous work focused to study the effect of wheat bran products has confirmed the negative influence of wheat bran on the quality parameters of bakery products. In this study, different stabilization methods were tested to remove the deterious effect of wheat bran on wheat dough and qualitative parameters of cookies.

MATERIAL AND METHODOLOGY

Wheat flour (moisture 11.36%, wet gluten 35.47%) and other ingredients (sugar, shortening, sodium bicarbonate, and sodium chloride) were purchased in local market. Wheat bran from wheat variety PS Bertold (BB) and PS 215 (WB) were observed from Research and Breeding Station, Vígľaš Pstruša, Slovakia and Research Institute of Plant Production Piešťany, Slovakia.

Wheat bran stabilization

Wheat bran from different wheat variety were stabilized using hot air and microwave heating acording to method described by **Ertaş (2016)**.

Hot air stabilization

Wheat bran was heated in hot air oven (Mora, Czech Republic) at 150 °C for 20 min and cooled for 30 min. Cooled bran was packed to polypropylene bags.

Microwave stabilization

Wheat bran was heated using microwave (Whirpool MWO602, USA) at 800 W for 2 min and cooled for 30 min. Cooled bran was packed to polypropylene bags.

Thermo-mechanical properties of dough

Effect of wheat bran addition (5, 10 and 15%) on thermo-mechanical properties of wheat dough was evaluated using Mixolab 2 (Chopin Villeneuve-la-Garenne, France) according to method described by **Lauková et al. (2018)**. The settings used in the test were 8 min at 30°C with a temperature increase of 4 °C.min^{-1} until the mixture reached 90 °C. There was a 7 min holding period at 90 °C, followed by a temperature decrease of 4 °C.min^{-1} until the mixture reached 55 °C, and then 6 min of holding at 55 °C. The mixing speed during the entire assay was 80 rpm. Determined parameters were: water absorption (WA), dough development time (DDT), stability (ST), initial maximum consistency (C1), protein weakening (C2), starch gelatinization (C3), stability of the starch gel formed (C4), starch retrogradation (C5).

Cookie preparation

The cookies were prepared using a recipe described by **Lauková et al. (2016)**, which included: 100 g wheat flour, 53 g sugar, 26.5 g shortening, 1.1 g sodium bicarbonate, 0.89 g sodium chloride and 12 cm^3 water. Prepared dough was rolled out (3 mm height) and cut into round shape (50 mm diameter). The cookies were baked for 8 – 9 min in hot air oven (Mora, Czech Republic) at 180 °C. After cooling, the cookies were packed in polyethylene bags. In the bran enriched cookies, the wheat flour was replaced by wheat bran at level 5, 10 and 15%.

Qualitative parameters of cookies

Qualitative parameters of cookies were evaluated 2 h after baking. The volume of cookies was measured by rapeseeds displacement method **(AACC Method 10-05.01)**. Volume index of cookies was measured using method acording to **Lauková et al. (2016)**. Baking loss (%) is determined according to dough weight before baking and product weight, which was detected one hour after baking according to **Minarovičová et al. (2018)**. Spread ratio was evaluated according to method described to **Kuchtová et al. (2016)** as the ratio of the diameter to the thickness of cookies. To determine the diameter, six cookies were placed edge to edge and the total diameter was measured. The cookies were rotated at 90 degree for duplicate reading. The average diameter was calculated. To determine the thickness, six cookies were placed on top of one another. The total thickness was measured. This process was repeated twice, and average thickness was calculated.

Texture analysis

Texture properties of baked cookies was determined using a texture analyser Ta.XT plus (Stable Micro Systems, GB) with 3-Point Bending Rig (HDP/3PB) according to modified method described by **Filipčev et al. (2012)**. A pre-test speed 1.0 mm.s^{-1}, test-speed 1.0 mm.s^{-1} and post-test speed 10 mm.s^{-1} were used. The gap distance was 20 mm. Texture determination was repeated 10 times.

Hardness and fracturability of cookies were recalculated using Exponent software (Stable Micro Systems, GB) version 6.1.4.0.

Sensory evaluation

The sensory evaluation of baked cookies was performed according to **Kohajdová et al. (2011)** by panel of 11 judges (staff and students of the Faculty of Chemical and Food Technology, Slovak University of Technology, Slovakia) using five-point hedonic scale (5 – most liked, 1 – most disliked). Descriptors determined were: shape, colour, taste, flavour, hardness. The overall acceptance of cookies was determined using 100 mm graphical non-structured abscissas with the description of extreme points (0% – minimal intensity, 100% – maximal intensity).

Statisic analysis

All determinations were carried out in triplicate except texture analysis and the average values were calculated. Student`s test at p <0.05 was used to establish the significant differences between mean values. As the statistical analysis software was used Microsoft Excel version 2010.

RESULTS AND DISCUSSION

Thermo-mechanical properties of dough

Knowledge of the influence of fibers on rheological properties is essential for the development of good quality products. The most serious technological drawbacks related to the rheological properties of dough include problems with adhesion to work surfaces and changes in shape, color, density and texture of the baked product. These modifications prove inconvenient in the production process and in sensory aspects of the product, both visual and flavor **(Blanco Canalis et al., 2017)**. One of the latest instruments used to determine the rheological quality of dough is Mixolab. Mixolab allows the characterization of the physicochemical behavior of dough when submitted to dual mixing and temperature constraints. Therefore, it is possible to record the mechanical changes due to mixing and heating simulate the mechanical work as well as the heat conditions that might be expected during the baking process **(Xhabiri et al., 2016)**. Effect of wheat bran addition on thermo-mechanical properties of wheat dough is sumarised in the Table 1.

Water absorption (WA) represents the amount of water that flour can absorb to obtain a pre-set dough consistency to produce a torque (peak C1) of 1.1 Nm ±0.05 at 30 °C **(Blandino et al., 2015)**. It was noticed, that WA significantly increased with addition of raw wheat bran from 55.83% (wheat flour) to 60.25% (15% BB) and 57.75% (15% WB), respectively. The higher WA of wheat bran is explained by the greater number of hydroxyl groups in the fiber structure, which allows more water interaction through hydrogen bonding. These effects can be also related to the presence of arabinoxylans, which tightly bind water in the dough system thereby reducing the availability of water for developing the gluten network **(Prückler et al., 2014)**. Stabilization of wheat bran using different methods significantly increased the WA. The highest values (62.40 and 62.50%) were obtained after addition of 15% WB stabilized by hot air and microwave heating.

Dough stability (DS) is indicator for the kneading properties of doughs and thus for the flour strength, whereas stronger doughs have higher values. The addition of wheat bran in general causes shorter DS values, a fact which is due to the interruption of the gluten network **(Prückler et al., 2014)**. From the results, it was noted that DS significantly decreased with increasing addition level of raw wheat bran. Incorporation of wheat bran stabilized using microwave caused shorter DS compared to addition of raw bran.

The C2 indicates a dough consistency loss during exposure to physical-mechanical and thermal stress **(Wang et al., 2017)**. Higher the C2 torque, lower is the protein weakening (Moza and Gujral, 2018). Control C2 value for wheat flour sample was 0.58 Nm. Generally, addition of wheat bran caused decreasing of C2 values to 0.50 Nm (15% BB) and 0.41 Nm (15% WB). The stabilization of wheat bran using a hot air resulted in a lesser weakening of the protein structure compared to raw bran and stabilization using a microwave.

The torque at C3 indicates the starch gelatinization behavior and viscosity of the dough upon heating and it strongly depends upon type and quality of starch **(Moza and Gujral, 2018)**. Wheat flour contributed to a better starch performance of the samples (higher starch gelatinization, C3) than composite flour with bran. This could be ascribed to the competence for water established between the starch and the bran **(Lauková et al., 2018)**. From the results concluded, that C3 values with addition of raw wheat bran ranged from 1.94 Nm (5% WB) to 1.80 Nm (15% WB) which was significantly lower than C3 value for wheat flour (2.11 Nm). Addition of wheat bran using microwave resulted in lower gelatinization of starch (1.92 – 1.65 Nm) compared to adition of raw bran. On the other hand, with the incorporation of hot air heated bran, C3 values were significantly higher (2.01 – 1.84 Nm).

The further reduction in viscosity (C4 value) is the result of the physical breakdown of the granules due to the mechanical shear stress and the temperature constraint **(Dapčević Hadnađev et al., 2011)**. As can be seen from the Table 1, C4 value (hot gel stability) for wheat flour was 2.11 Nm. Increasing addition level of bran caused significant decreasing of hot gel stability. Xhabiri et al. (2016) reported that wheat bran mostly represents the cover of the grain which contains a high amount of α-amylase, which is the cause of decrease of C4 values. From the results concluded, that adition of bran stabilized by hot air increased the stability of hot gel (2.01 – 1.84 Nm) compared to raw bran (1.94 – 1.80 Nm). Incorporation of bran stabilized by microwave resulted in lower stability of hot gel. The C5 values were related to starch gelatinization, pasting, and retrogradation properties **(Koksel et al., 2009)**. From the results concluded that incorporation of wheat bran to wheat dough significantly decreased the C5 values (wheat flour 2.92 Nm). Moreover, addition of stabilized wheat bran using hot air heating caused higher C5 values compared to raw wheat bran. A higher C5 values means more starch retrogradation, thus the decreased value of C5 portrays longer shelf stability and better texture of the end products **(Ding et al., 2018)**.

Cookies quality and texture

Effects of wheat bran addition on qualitative properties of cookies are sumarised in Table 2.

Generally, addition of raw wheat bran resulted in reduced volume from 8.90 cm3 (control sample) up to 7.14 cm3 (15% WB). Reduced volume may also be caused by gluten dilution and physicochemical reactions among fibre components, water and gluten **(Kurek et al., 2016)**. This effect is in agreement with previous study after incorporation of wheat bran from different wheat variety in cookies **(Lauková et al., 2016)**. Compared to cookies contained raw bran, stabilization of wheat bran led to increase the volume about 10% (hot air treatment) and 15% (microwave treatement).

Dough making and handling, cookie baking and quality of the final product are thus largely influenced by cookie dough components. Cookie spread, i.e. the extent to which the dough piece spreads during baking represents one of the major quality parameter **(Dapčević Hadnađev et al., 2011)**. Cookies with higher spread ratios are considered the most desirable **(Ostermann-Porcel et al., 2017)**. Spread ratio for control sample was 9.68. With increasing adition level of raw bran the spread ratio significantly decreased up to 5.69. Stabilization of wheat bran using

both methods improved this parameter of enriched cookies. This is in agreement with results observed by **Nandeesh et al. (2011)** after incorporation of roasted, steamed and microwave treated bran.

Table 1 Thermo-mechanical properties of wheat dough with addition of different treated wheat bran.

Sample		Water absorption (%)	Stability (min)	C2 (Nm)	C3 (Nm)	C4 (Nm)	C5 (Nm)
WF		55.83 ±0.11	10.61 ±0.09	0.58 ±0.01	2.11 ±0.01	1.85 ±0.01	2.92 ±0.02
				Raw bran			
BB	**5%**	59.10 ±0.08*	9.72 ±0.01*	0.51 ±0.01*	1.92 ±0.01*	1.83 ±0.02	3.00 ±0.02
	10%	60.10 ±0.06*	9.78 ±0.02*	0.53 ±0.01*	1.92 ±0.01*	1.78 ±0.02*	2.76 ±0.01*
	15%	60.95 ±0.12*	9.53 ±0.01*	0.50 ±0.00*	1.88 ±0.01*	1.71 ±0.01*	2.71 ±0.01*
WB	**5%**	56.20 ±0.09	10.53 ±0.02*	0.45 ±0.00*	1.94 ±0.01*	1.70 ±0.02*	2.93 ±0.02
	10%	57.52 ±0.11*	8.55 ±0.01*	0.44 ±0.01*	1.93 ±0.01*	1.59 ±0.02*	2.82 ±0.01*
	15%	57.75 ±0.06*	8.15 ±0.03*	0.41 ±0.00*	1.80 ±0.01*	1.51 ±0.01*	2.66 ±0.00*
				Hot air stabilization			
BB	**5%**	60.40 ±0.10*	9.82 ±0.04*	0.53 ±0.01*	1.94 ±0.01*	1.83 ±0.01	3.03 ±0.02
	10%	61.30 ±0.05*	10.38 ±0.01*	0.54 ±0.01*	1.94 ±0.01*	1.80 ±0.01*	2.83 ±0.01*
	15%	62.00 ±0.02*	10.45 ±0.05*	0.52 ±0.00*	1.93 ±0.01*	1.79 ±0.01*	2.82 ±0.01*
WB	**5%**	60.75 ±0.06*	8.16 ±0.01*	0.56 ±0.01*	2.01 ±0.01*	1.48 ±0.01*	2.96 ±0.02
	10%	61.45 ±0.06*	8.37 ±0.01*	0.49 ±0.00*	1.97 ±0.01*	1.46 ±0.01*	2.95 ±0.02
	15%	62.40 ±0.23*	9.55 ±0.02*	0.48 ±0.00*	1.84 ±0.01*	1.43 ±0.00*	2.94 ±0.01
				Microwave stabilization			
BB	**5%**	59.75 ±0.19*	9.56 ±0.08*	0.53 ±0.01*	1.92 ±0.02*	1.77 ±0.02*	2.89 ±0.02*
	10%	60.30 ±0.06*	9.63 ±0.06*	0.50 ±0.00*	1.90 ±0.01*	1.70 ±0.01*	2.78 ±0.02*
	15%	60.90 ±0.14*	9.46 ±0.03*	0.49 ±0.00*	1.90 ±0.01*	1.68 ±0.01*	2.66 ±0.01*
WB	**5%**	60.80 ±0.09*	8.98 ±0.09*	0.45 ±0.00*	1.66 ±0.01*	1.36 ±0.02*	2.92 ±0.02
	10%	61.60 ±0.23*	8.87 ±0.06*	0.44 ±0.00*	1.65 ±0.01*	1.31 ±0.02*	2.82 ±0.02*
	15%	62.50 ±0.17*	8.45 ±0.02*	0.44 ±0.00*	1.65 ±0.01*	1.32 ±0.02*	2.64 ±0.01*

Note: WF – wheat flour, * denotes statistically significant difference at $p < 0.05$ level.

Table 2 Qualitative properties of cookies enriched with stabilised and unstabilized wheat bran.

Sample		Volume (cm^3)	Spread ratio	Volume index (cm)	Baking loss (%)	Hardness (g)	Fracturability (mm)
Control		8.90 ±0.02	9.68 ±0.22	1.73 ±0.00	15.09 ±0.12	1157.58 ±21.36	19.35 ±0.78
				Raw bran			
BB	**5%**	8.43 ±0.04*	7.01 ±0.03*	1.87 ±0.00*	15.64 ±0.40*	1268.95 ±26.14	18.88 ±0.48*
	10%	7.90 ±0.02*	6.59 ±0.02*	2.14 ±0.01*	15.57 ±0.02*	1360.32 ±35.69*	18.69 ±0.75*
	15%	7.22 ±0.03*	5.69 ±0.01*	2.39 ±0.01*	15.27 ±0.16*	1648.77 ±14.82*	18.49 ±0.36*
WB	**5%**	8.20 ±0.03*	8.44 ±0.04*	1.77 ±0.00	15.98 ±0.15*	1213.42 ±36.52	18.80 ±0.64*
	10%	7.64 ±0.03*	7.90 ±0.03*	1.87 ±0.00*	15.35 ±0.23*	1488.20 ±55.26*	18.53 ±0.54*
	15%	7.14 ±0.03*	6.92 ±0.02*	2.24 ±0.01*	15.12 ±0.75*	1753.50 ±25.22*	18.50 ±0.54*
				Hot air stabilization			
BB	**5%**	8.98 ±0.02	7.24 ±0.04*	2.04 ±0.01*	14.80 ±0.16*	1137.69 ±19.22	18.59 ±0.69
	10%	8.20 ±0.03*	6.84 ±0.03	1.99 ±0.00*	14.75 ±0.44*	1291.44 ±25.63*	18.65 ±0.41*
	15%	8.00 ±0.04*	7.07 ±0.02	2.13 ±0.01*	14.20 ±0.31*	1381.30 ±26.22*	19.20 ±0.25*
WB	**5%**	8.88 ±0.03	6.72 ±0.02	2.02 ±0.01*	13.92 ±0.28*	1093.60 ±22.54	18.67 ±0.15*
	10%	8.08 ±0.02*	7.97 ±0.03*	1.93 ±0.00*	13.70 ±0.14*	1268.15 ±26.15*	18.81 ±0.22*
	15%	7.82 ±0.02*	7.71 ±0.04*	2.08 ±0.01*	12.57 ±0.64*	1484.36 ±33.22*	19.18 ±0.51
				Microwave stabilization			
BB	**5%**	8.72 ±0.03*	7.80 ±0.02*	1.76 ±0.00	15.86 ±0.06	1181.40 ±18.91	18.73 ±0.51*
	10%	8.60 ±0.03*	6.88 ±0.03	2.12 ±0.01*	15.03 ±0.21	1227.82 ±11.34	19.08 ±0.56
	15%	8.14 ±0.02*	7.04 ±0.02	2.14 ±0.01*	13.85 ±0.15*	1484.36 ±24.28*	19.11 ±0.44
WB	**5%**	8.92 ±0.03	6.94 ±0.2	2.11 ±0.01*	15.61 ±0.25	1180.30 ±22.33	18.40 ±0.16*
	10%	8.56 ±0.03*	7.64 ±0.04*	2.06 ±0.01*	15.22 ±0.45	1394.67 ±15.96*	18.73 ±0.20*
	15%	8.22 ±0.03*	7.14 ±0.03*	2.19 ±0.01*	14.03 ±0.26*	1586.26 ±31.85*	19.10 ±0.33

Note: * denotes statistically significant difference at $p < 0.05$ level.

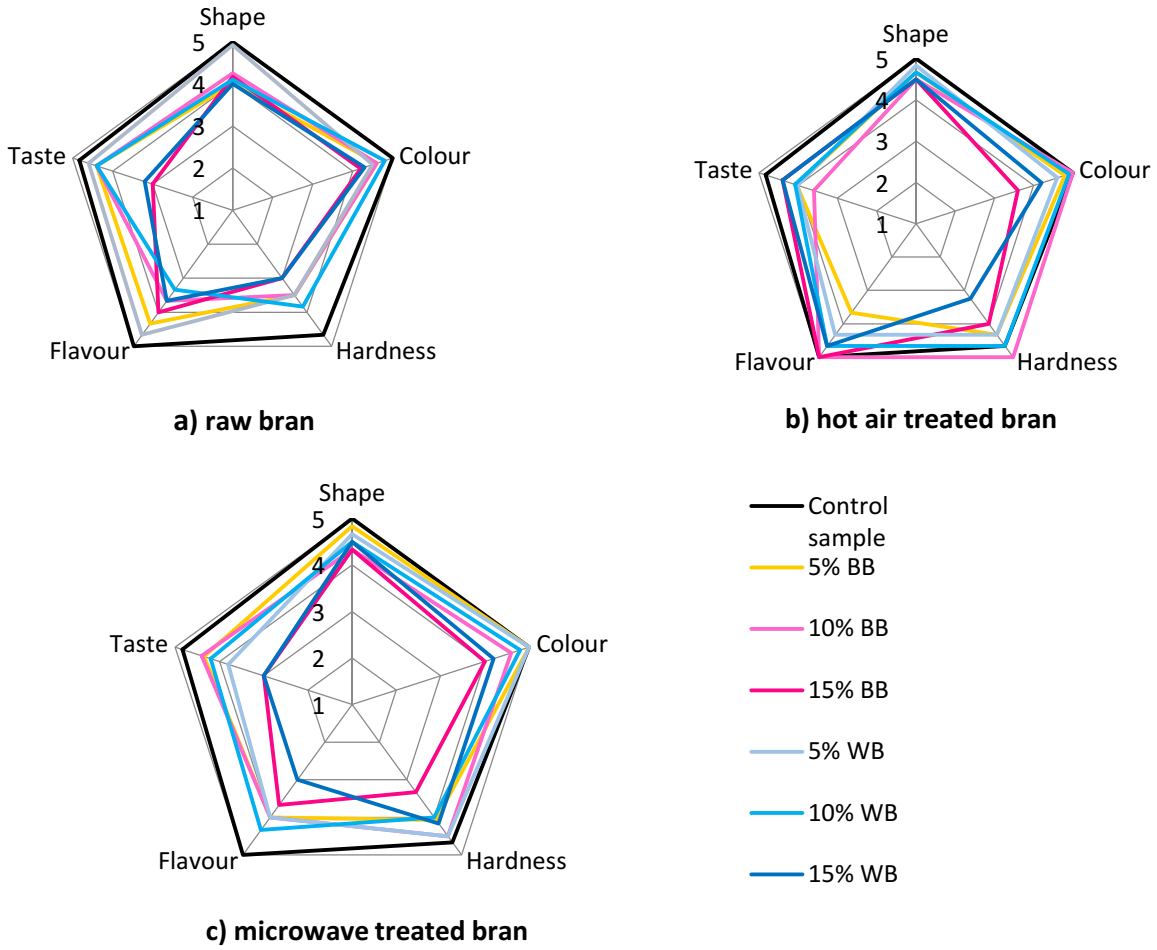

Figure 1 Sensory evaluation of cookies with addition raw wheat bran (a), hot air threated bran (b) and microwave treated bran.

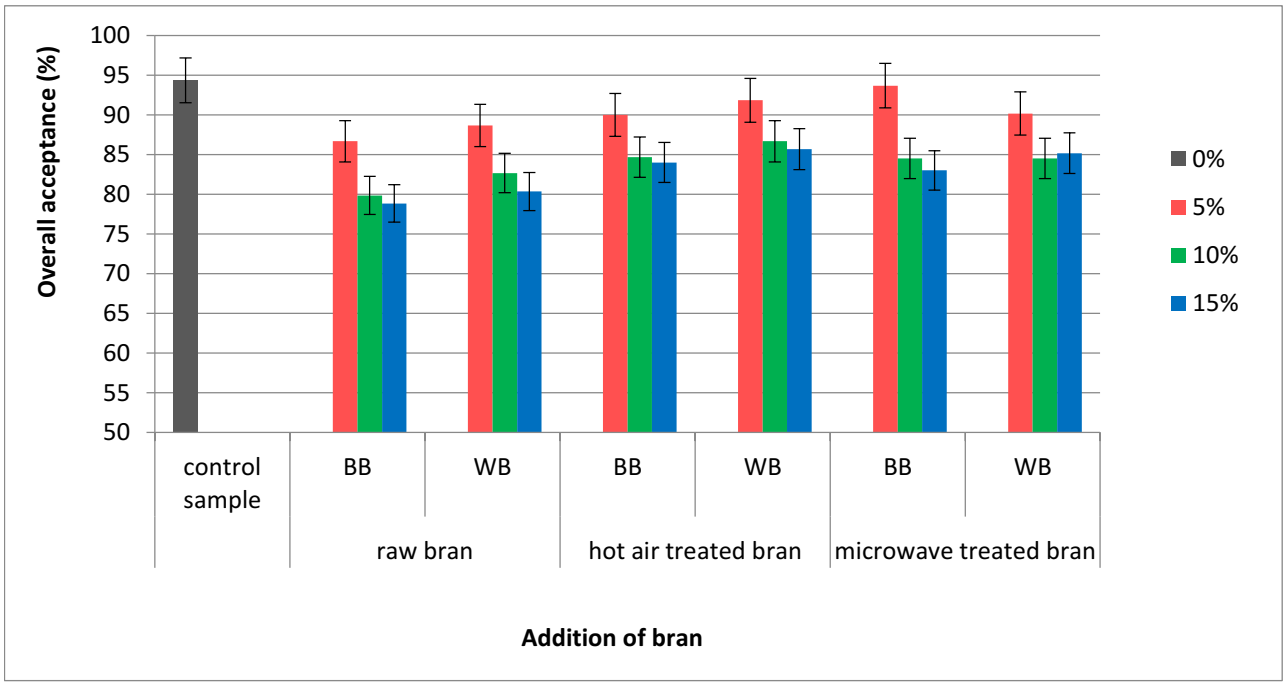

Figure 2 Overal acceptability of wheat bran incorporated cookies. Note: WB – wheat bran from wheat variety PS 215, BB – wheat bran from wheat variety PS Bertold.

Determining the actual baking losses is very important as the finished product after baking must have a defined weight. The loss by baking is influenced mainly by the weight of the product, by shape and moisture content **(Minarovičová et al., 2018)**. Evaluation of baking loss showed that addition of raw wheat bran increased this parameter from 15.09% (control sample) up to 15.98% (5% WB). Higher water evaporation may be due to initially higher water content in the bran-containing biscuit dough as compared to the control because addition of bran to biscuit formulation increases the level of water required **(Filipčev et al., 2017)**. The results also showed that stabilization of bran caused significantly lower values of baking loss (12.57 – 14.80% and 13.85 – 15.86% for cookies enriched with hot air and microwave treated wheat bran, respectively) compared to control sample.

This study shoved that volume index values of cookies enriched with wheat bran were higher compared to control sample of cookies. The highest volume index (2.39 cm) was observed after 15% incorporation of BB.

Texture evaluation of cookies with addition of wheat bran is summarized in Table 2. Maximum peak force recorded from force/distance curve (the maximum force required to break a cookie or maximum resistance of cookie when break) has been reported as hardness **(Dapčević Hadnađev et al., 2013)**. The results showed that supplementation of wheat flour with wheat bran resulted in hight hardness of cookies. This fact is in agreement with study of cookies enriched with roasted, steamed and microwave treated bran by **Nandeesh et al. (2011).** On the other side, cookies incorporated with 15% of bran stabilized using hot air was about 16% softer compared to cookies with addition of raw bran. Stabilization of bran using microwave concluded in about 11.5% decrease of cookies hardness.

Sensory evaluation

Sensory evaluation of cookies incorporated with treated and raw wheat bran is showed in Figure 1 a-c. The surface color of a baked product is, together with texture and taste, a very important element for the initial acceptability of baked goods by consumers **(Ostermann-Porcel et al., 2017)**. From the results concluded that sensorial scores for cookies shape and colour decreased with increasing level of raw wheat bran. Stabilization of wheat bran improved the sensory score of cookies shape. It was also noticed that taste score decreased with increasing addition level of bran. Moreover, the evaluation of taste showed that addition of wheat bran increased the bitter taste of cookies, which is due to content of tannins and phenolic acids **(Heiniö et al., 2016)**. The assessors also describe that incorporation of bran led to dryer cookies. The drying effect of wheat bran might be explained by its water-absorptive properties. Both wheat bran and flour absorb water, but during baking wheat bran may lose water more readily than flour. Biscuits containing wheat bran had less flour; consequently, less water may have remained after baking and the resultant biscuits therefore were perceived as dry by panelists **(Khalil et al., 2015)**. Incorporation of wheat bran in cookies caused lower sensory score of cookies hardness. From the results concluded that stabilization of bran using different methods led to higher hardness acceptance of cookies.

The overall acceptability of cookies with addition of raw and stabilized wheat bran is shown in Figure 2. In general, higher addition levels of bran (10 and 15%) significantly decreased the overall acceptability of cookies. It was noticed that stabilization of bran improved the acceptability of enriched cookies.

CONCLUSION

This study was focused on influence of wheat bran stabilization on qualitative parameters of enriched cookies. Incorporation of wheat bran significantly modified the thermo-mechanical properties of wheat dough. Stabilized bran caused higher protein resistance and stability of hot gel in dough during heating. This study also shoved that pretreatment of wheat bran (using hot air and microwave heating) may improve the qualitative properties of cookies such as volume and hardness. Moreover, the cookies enriched with stabilized bran were more acceptable for assessors.

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CHARACTERISTICS OF TEXTURAL AND SENSORY PROPERTIES OF OŠTIEPOK CHEESE

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ABSTRACT

Oštiepok is a traditional half-fat semi-hard cheese made in Slovakia. The basic raw material used to produce oštiepok cheese is ewe's milk, a mixture of ewe's and cow's milk or cow's milk. Oštiepok cheese is produced either directly at a small-scale mountainside sheep farm, using the traditional on-farm method of production, or at dairies, using the industrial method. Oštiepok cheese was produced as far back as the beginning of the 18th century. An industrial production of Oštiepok cheese using cow's milk were laid by the Galbavý family in Detva (Slovakia) in 1921. The cheese is originally made by cutting off fresh sweet cheese, which is pressed into a wooden, hand-cut and decorated round shape where it is left to stand. Subsequently, it is removed and immersed in warm salty water, left to stand there until the salt penetrates completely in. Then it is necessary that it pass slightly. In its salty water, the ostrich produces its traditional durability, its surface is slightly peeled, mostly yellowish. This cheese may or may not be steamed and may be smoked or unsmoked. Slovenský oštiepok is a protected trade name under the EU's protected geographical indication. A similar cheese is made in the Polish Tatra Mountains under the name Oscypek. The cheeses differ in ingredients' ratios, cheesemaking process and the characteristics of the final products. In this study we have characterized textural and sensory properties of the Oštiepok cheese produced in Slovakia made from ewe's milk, a mixture of ewe's and cow's milk and cow's milk.

Keywords: Oštiepok cheese; Slovak oštiepok cheese; Slovenský oštiepok; cheese; traditional Slovak cheese

INTRODUCTION

Oštiepok cheese (Slovenský oštiepok – Slovak oštiepok) is characterised by its special shape, that of a large egg, pine cone or ellipsoid with decoration. The colour is golden-yellow to golden-brown on the outside after smoking, white to buttery-yellow on the inside. Consistency is solid, firm, slightly fragile, with minor cracks and cavities appearing when cut. Aroma and flavor is savoury, pleasant distinctive cheese flavour, mildly piquant to sour, moderately salty, with a typical smoky aroma resulting from the smoking process; must not be overly acidic, yeasty, tallowy, soapy, rancid, putrid, sharp, spicy or bitter or have other strange flavours. Composition depends on the raw material used and the method of production, a minimum of 48 % dry matter by weight, a minimum of 38.0 % fat in dry matter by weight.
(Council of the European Union, 2007).

Slovenský oštiepok is presented in the Figure 1. The protection of geographical indications and designations of origin for Slovenský oštiepok was estabilished by the Commission Regulation **No. 943/2008**.

Slovenský oštiepok is produced using ewe's milk obtained from grazing ewes, particularly the *Wallachian*, *Improved Wallachian* and *Tsigai* breeds. These breeds are reared and graze in mountainous areas on mountain slopes within the delimited area. Small producers obtain cow's milk by hand or mechanically and process it immediately after milking. On-farm production of Slovenský oštiepok at a *salaš* (small farm), the stages involved are as follows: curdling, shaping, salting, drying, smoking. In the case of the industrial production, the stages involved are as follows: pasteurisation, addition of cultures, curdling, curd stretching, pressing, acidification, steaming, shaping, salting, drying and smoking **(Council of the European Union, 2007)**.

The cooled cheeses are placed in hygienic food packaging. They are packaged whole to ensure that their characteristic shape and decorations are retained (which would not be the case if they were cut and then packed) and that the quality is preserved, the product is not adulterated and consumers are not deceived. All stages of production take place within the delimited geographical area **(Council of the European Union, 2007)**.

The cheese should be produced only from milk meeting the criteria of the Slovak technical standard **STN 57 05 10** Ewe's milk and **STN 57 05 29** Raw cow milk for dairy treatment and processing. Also, the Commission

Figure 1 Slovenský oštiepok (Slovak oštiepok cheese).

Figure 2 Smoking the Slovenský oštiepok (Slovak oštiepok cheese) by the traditional way (Getting, 2016).

Figure 3 Salaš Zbojská. Note: (Salaš is Slovak name for small farm in mountain region).

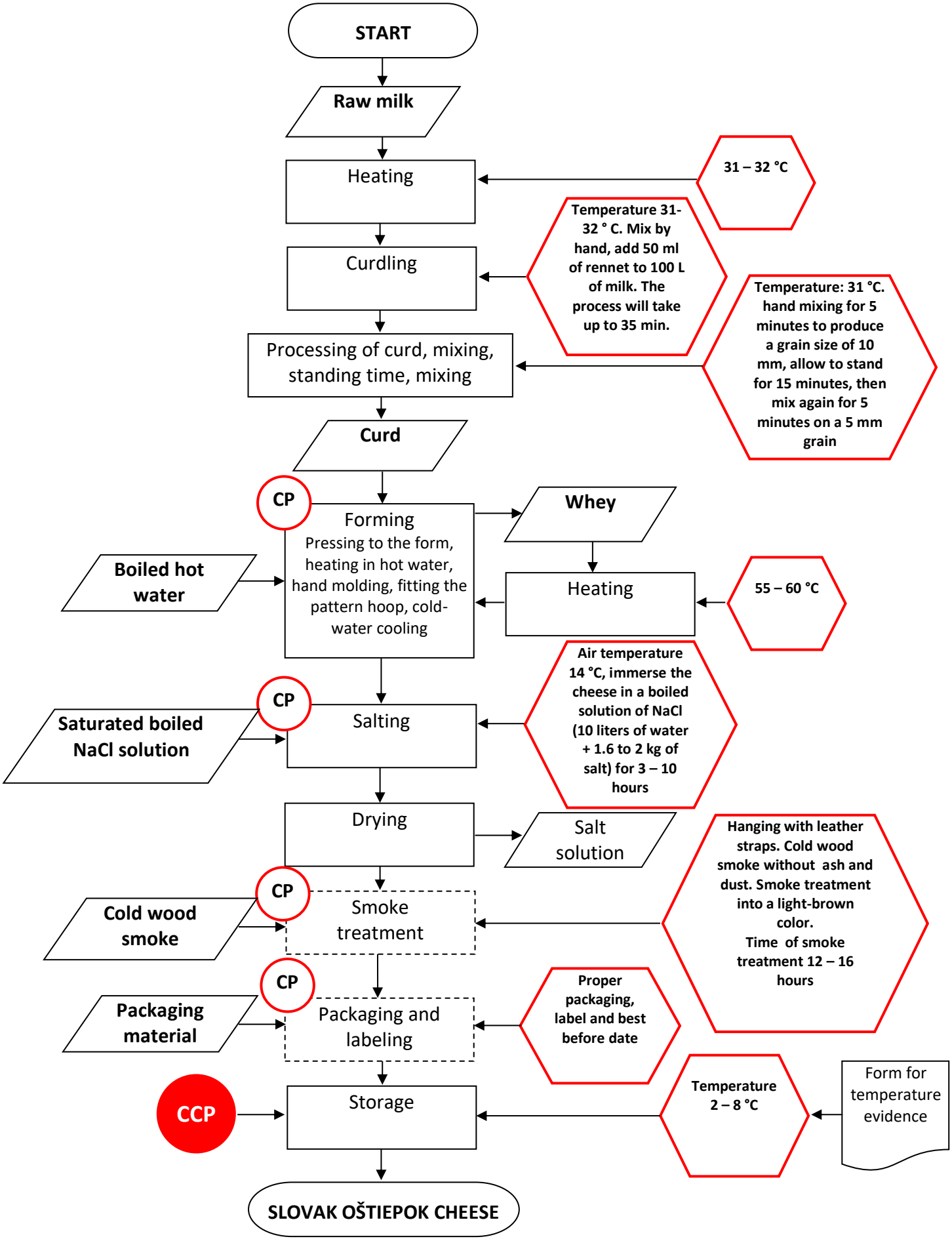


Figure 4 Technological steps of the Slovenský oštiepok (Slovak Oštiepok cheese) production.

Regulation (EC) **No. 1662/2006** and **No. 2073/2005** has to be fulfilled regarding to the hygienic and microbiological criteria.

In this study, we have described in detail the characteristic textural and sensoric properties of Slovenský oštiepok cheese made from ewe's milk, a mixture of ewe's and cow's milk and cow's milk. The hypothesis we have tested was that textural and sensoric characteristics of the Slovenský oštiepok cheese may vary depending on the type of milk used for the production.

Scientific hypothesis

There is a statistically significant difference between Oštiepok cheeses produced in different regions of Slovakia.

MATERIAL AND METHODOLOGY

Cheese samples

Product 1: Ewe's Oštiepok cheese 100% – smoked (Producer: Papúchová D. (Ružomberok, Slovakia), made in: PD Liptovské Revúce), characteristic: dry matter – 48 wt%, fat in dry matter – 50 %, NaCl – max. 2 %, weight – 0.356 kg.

Product 2: Orava ewe's Oštiepok cheese – smoked (Producer: Panči M. (Ružomberok), made in: Šurinák M. (KOLIBA Revišné, Veličná, Slovakia)), characteristic: dry matter min. 45 wt%, fat in dry matter: min. 48 %, NaCl: 4.5 g.100^{-1}g, proteins: 23.1 g.100^{-1}g, carbohydrates: 1.8 g.100^{-1}g, fat: 30 g.100^{-1}g, weight: 0.344 kg.

Product 3: Original ewe's Oštiepok cheese – smoked (Producer: Syrex – Zázrivá, Slovakia), characteristic: fat in dry matter: min. 45 %, salt: 4.5 g.100^{-1}g, proteins: 23.1 g.100^{-1}g, carbohydrates: 1.8 g.100^{-1}g, fat: 30 g.100^{-1}g, weight: 0.218 kg.

Product 4: Ewe's Oštiepok cheese – smoked (Producer: Plachtinská farm – Baránek, Slovakia), characteristic: dry matter: 56 wt%, fat in dry matter: 51 %, salt 1 g.100^{-1}g, proteins: 22.4 g.100^{-1}g, carbohydrates: 1.91 g.100^{-1}g, fat: 29.2 g.100^{-1}g, weight: 0,390 kg.

Product 5: Oštiepok, 100% ewe's cheese – smoked (Producer: Bryndziareň and syráreň – Zvolenská Slatina, Slovakia), characteristic: fat in dry matter: 45 – 60 %, salt: 2.37 g.100^{-1}g, proteins: 22.55 g.100^{-1}g, carbohydrates: 2.61 g.100^{-1}g, fat: 28.65 g.100^{-1}g, weight: 0.542 kg.

Product 6: Ewe's cheese Kolibka Spiš – smoked (Producer: Endel M. – VALACH (Sp. Nová Ves, Slovakia), made in: Kluknavská dairy – OOD Jaklovce, Slovakia), characteristic: fat in dry matter: min. 45 %, salt: max. 2.5 g.100^{-1}g, proteins: 24.5 g.100^{-1}g, carbohydrates: <1 g.100^{-1}g, fat: 26 g.100^{-1}g, weight: 0.328 kg. (Note: Not declared as Oštiepok cheese).

Product 7: Ewe's Oštiepok cheese – smoked (Producer: Badánik – Salaš Krajinka, Slovakia).

Product 8: Oštiepok Ewe's cheese - smoked (Producer: Konečný I. – KONNY (Trenčín, Slovakia), made in: Dairy Krivá, Slovakia), characteristic: dry matter: min. 50 wt%, fat in dry matter: min 40 %, salt: max. 2.5 g.100^{-1}g, fat: 35.26 g.100^{-1}g, weight: 0.242 kg.

Product 9: Ewe's Oštiepok cheese – smoked (Producer: Zúbek M. – OBERT (Ladce, Slovakia)).

Product 10: Ewe's Oštiepok cheese – smoked (Producer: Zvara J. (Detva, Slovakia)).

Product 11: Ewe's Oštiepok cheese – smoked (Producer: Agrosev made in: Plachtinská farm Baránek, Slovakia), characteristic: dry matter: 56 wt%, fat in dry matter: 51 %, salt: 1 g.100^{-1}g, proteins: 22.4 g.100^{-1}g, carbohydrates: 1.91 g.100^{-1}g, fat: 29.2 g.100^{-1}g, weight: 0.284 kg.

Product 12: Oštiepok – Ewe's cheese – smoked (Producer: AgroIna (Banská Bystrica, Slovakia), made in: AgroMagura s.r.o., Slovakia), characteristic: dry matter: min. 40 wt%, fat in dry matter: min. 50 %, salt: 2.5 g.100^{-1}g, weight: 0.458 kg.

Product 13: Original Oštiepok ewe's cheese – smoked (Producer: Chlustová P. (Liptovský Mikuláš, Slovakia), made in: Syrex (Zázrivá, Slovakia)), characteristic: fat in dry matter: min. 45 %, salt: 4.5 g.100^{-1}g, proteins: 23.1 g.100^{-1}g, carbohydrates: 1.8 g.100^{-1}g, fat: 30 g.100^{-1}g, weight: 0.360 kg.

Product 14: Orava ewe's loaf cheese – smoked (Producer: Válek M. (Revišné – Veličná, Slovakia), made in: Šurinák M. – Koliba Revišné (Veličná, Slovakia)), characteristic: dry matter: min. 45 wt%, fat in dry matter: min. 48 %, salt: 4.5 g.100^{-1}g, proteins: 23.1 g.100^{-1}g, carbohydrates: 1.8 g.100^{-1}g, fat: 30 g.100^{-1}g, weight: 0.350 kg. (Note: Not declared as Oštiepok cheese).

Product 15: Ewe's cheese Kolibka Spiš – steamed (Producer: Endel M. Jr. – VALACH (Sp. Nová Ves, Slovakia), made in: Kluknavská dairy – ODD Jaklovce, Slovakia), characteristic: fat in dry matter: min. 45 %, salt: max. 2.5 g.100^{-1}g, proteins: 24.5 g.100^{-1}g, carbohydrates: <1 g.100^{-1}g, fat: 26 g.100^{-1}g, weight: 0.324 kg. (Note: Not declared as Oštiepok cheese).

Product 16: Original ewe's Oštiepok cheese – fresh (Producer: Chlustová P. (Liptovský Mikuláš, Slovakia), made in: Syrex (Zázrivá, Slovakia)), characteristic: fat in dry matter: min. 45 %, salt: 4 g.100^{-1}g, proteins: 19.7 g.100^{-1}g, carbohydrates: 0.8 g.100^{-1}g, fat: 25 g.100^{-1}g, weight: 0.286 kg.

Product 17: Oštiepok Ewe's cheese – fresh (Producer: Dairy Krivá (Krivá, Slovakia)), characteristic: dry matter: min. 50 wt%, fat in dry matter: min. 40 %, salt: max. 2.5 %, weight: 0.270 kg.

Product 18: Orava ewe's Oštiepok cheese – fresh (Producer: Panči M. (Ružomberok, Slovakia), made in: Šurinák M. – Koliba Revišné (Veličná, Slovakia)), characteristic: dry matter: min. 45 wt%, fat in dry matter: min. 48 %, salt: 1.85 g.100^{-1}g, proteins: 26.61 g.100^{-1}g, carbohydrates: 2.15 g.100^{-1}g, fat: 15.18 g.100^{-1}g, weight: 0.374 kg.

Product 19: Original ewe's Oštiepok cheese – fresh (Producer: Syrex (Zázrivá)), characteristic: fat in dry matter: min. 45 %, salt: 4 g.100^{-1}g, proteins: 19.7 g.100^{-1}g, carbohydrates: 0.8 g.100^{-1}g, fat: 25 g.100^{-1}g, weight: 0.296 kg.

Oštiepok cheeses: 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18 and 19.

Ewe's cheese not labelled as the Ošiepok cheese, but it meets the requirements for Oštiepok cheese: 14.

Ewe's cheese, but not the Ošiepok cheese: 6 and 15.

INSTRUMENTAL TEXTURAL ANALYSIS

Sample prepraration

We have pilled of the crust of the cheese and the cheese was cut into 30 equal parts of the size 1 x 1cm. Fifteen parts represented the middle of cheese and 15 parts was from edge parts of cheese **(Figure 6)**. Cutted chees blocks were tempered in fridge to 10 °C.

Materials and instruments

We used:

- texturometer TA.XT Plus (Stable Micro Systems),
- computer with a program Exponent (Stable Micro Systems),
- spherical probe (P/1S),
- fridge,
- cheese samples,
- knife.

The analysis of the textural properties of cheese samples was tested using texturometer TA.XT Plus (Stable Micro Systems) at 10 °C. The instrument was setu up according to the manufacturer's recommendations. We focused on following indicators **(Stable Micro Systems, 2016)**:

- firmness of the cheese (g),
- consistency of the cheese (g.s^{-1}).

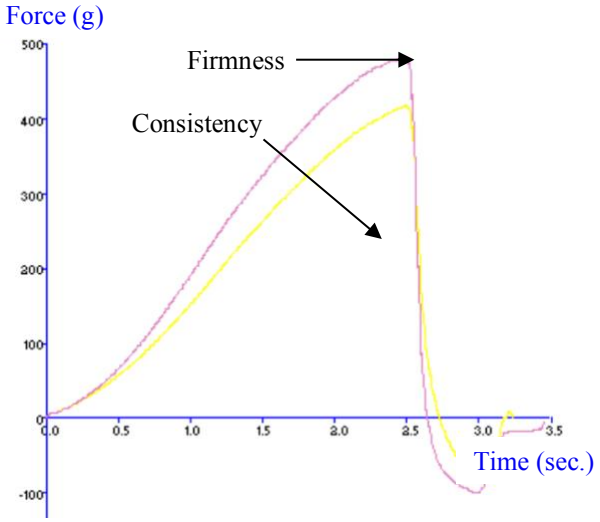


Figure 5 A curve showing the textural properties of cheese in the program Exponent (Stable Micro Systems).

The **Figure 5** shows the textural properties of the measured Oštiešpok cheese. At the highest point of the curve is the firmness of the cheese. The harder the cheese is, the higher is the curve peak (higher the value). The consistency of the cheeses shows the inner area between the curve and the x-axe in the positive area of the figure. The stronger the consistency of the cheese is, the higher is the consistency value this mean the larger is the area in the figure.

SENSORY ANALYSIS

Sample prepraration

We have stripped each Oštiepok cheese and cut into 30 equal parts of the size 1 x 1cm. The sensory analysis was tested using skilled evaluators. The cheese was tempered to 10°C and the temperature in laboratory was 20 °C.

The organoleptic properties of the individual samples were evaluated using a modified profile test. Values for descriptors were: 10 – extremely strong, 9 – very strong, 8 – strong, 7 – moderately strong, 6 – above average, 5 – medium, 4 – weak, 3 – very weak, 2 – treshold, 0 – none.

a) Odor: evaulators focused on two descriptors for odor: cheesy and smoke,

b) Taste: evaulators focused on four descriptors for taste: salty, after smoke, total flavour and other/foreign,

Table 1 Texturometer TA.XT Plus setup

Parameter	Setup
Mode	Measure Force in Compression
Option	Return to Start
Pre-Test Speed:	1.5 mm.s^{-1}
Test Speed:	2.0 mm.s^{-1}
Post-Test Speed:	10.0 mm.s^{-1}
Distance:	5 mm
Trigger Type:	Auto – 2.5 g
Tare Mode:	Auto
Data Acquisition Rate:	400 pps
1“ Spherical Probe (P/1S)	Max. load: 5 kg
	Max. temperature: 200 °C

Note: texturometer setup for measuring firmness and consistency of Oštiepkok cheeses.

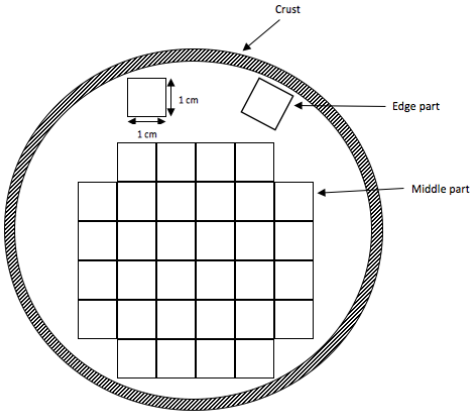


Figure 6 Otiepok cheese **s**ample cutting.

c) Texture: evaulators focused on two descriptors for texture: overall appearance, colour, consistency and scroop.

CHEESE COMPOSITION ANALYSIS

The cheese composition was analysed with standard laboratory methods.

Fat content: ISO 1735:2004 (IDF 5:2004) Cheese and processed cheese products -- Determination of fat content -- Gravimetric method (Reference method).

Protein content: ISO 8968-1:2014 (IDF 20-1:2014) Milk and milk products -- Determination of nitrogen content -- Part 1: Kjeldahl principle and crude protein calculation.

Lactose content: ISO 22662:2007 (IDF 198:2007) Milk and milk products -- Determination of lactose content by high-performance liquid chromatography (Reference method).

Dry matter content: ISO 5534:2004 (IDF 4:2004) Cheese and processed cheese -- Determination of the total solids content (Reference method).

Ash content: Ash content was detected after burning a sample in a muffle furnace at 550 °C for 5 h.

Salt content: ISO 5943:2006 (IDF 88:2006) Cheese and processed cheese products -- Determination of chloride content -- Potentiometric titration method.

STATISTICAL ANALYSIS

Statistical analysis of composition and texture determined by instrument

The results were statistically evaluated using XLSTAT, v. 2018.1 (Addinsoft, USA) statistical program. Firstly, we used the Shapiro-Wilk's test for verifying the normality of data. The significance level (α) was determined at level 0.05 for this test. Consequently, the Kruskal-Wallis one-way ANOVA and Dunn's test were used to test whether there is significant difference between Oštiepok chceeses in composition and texture parameters. We consider the results significantly different at $p < 0.001$. Consequently, we used the principal component analysis (PCA) to show the manin factors of variance between cheeses.

Statistical analysis of data determined by sensory analysis

The results were evaluated using statistical software (XLSTAT, v. 2018.1, Addinsoft, USA). We used a method for a comprehensive assessment of similarity and organoleptic acceptability: Principal component analysis (PCA).

RESULTS AND DISCUSSION

Statistical analysis of composition and texture determined by instrumental analysis

The mean values of composition of Oštiepok cheeses are presented in **Table 2** and were as follows: dry matter 58 wt%, fat ind dry matter 49%, fat 28.42 g.100g^{-1}, carbohydrates 1.81 g.100g^{-1}, protein 22.62 g.100g^{-1}, NaCl 2.85 g.100g^{-1}, other minerals 1.83 g.100g^{-1}.

The results of the normality test showed that the data had a non-normal distribution; the Shapiro-Wilk's test value was <0.05. Subsequently, we performed a Kruskal-Wallis one-way ANOVA and we found that individual products were statistically different ($p < 0.001$) in fat in solid content, solid content, fat, carbohydrates, proteins, NaCl, other minerals and also in textural parameters firmness and consistency. Also, the Dunn's post hoc tests identified, that there are significant diferences ($p < 0.001$) between cheeses in texture parameters determined by texturemeter (consistency and firmness). These results are presented in **Table 3**. The highest values of firmness and consistency parameters were determined for products No. 3 and No. 13 **(Table 3)**. It is because these products has one of the highest values of % of dry metter in comparison of other cheeses analysed in our experiment. The lowest values of firmness and consistency were detected in cheese No. 7 and No. 16 **(Table 3)**.

The mean value of firmness for middle parts of cheeses was 1.332 kg and 1.383 kg for edge parts. The mean value of consistency for middle parts of cheeses was 1.049 kg.s^{-1} and 1.038 kg.s^{-1} for edge parts. There was no significant difference ($p = 0.301$) between middle and edge parts of cheeses in firmness parameter. Also, there was no significant difference ($p = 0.819$) between middle and edge parts of cheese in consistency parameter. The overall mean of firmness of all cheeses was 1.349 ±0.705 kg and Cv 52%. The overall mean of consistency of all cheeses was 1.045 ±0.578 kg.s^{-1} and Cv 55%.

Table 2 The mean values of Oštiepok cheeses composition.

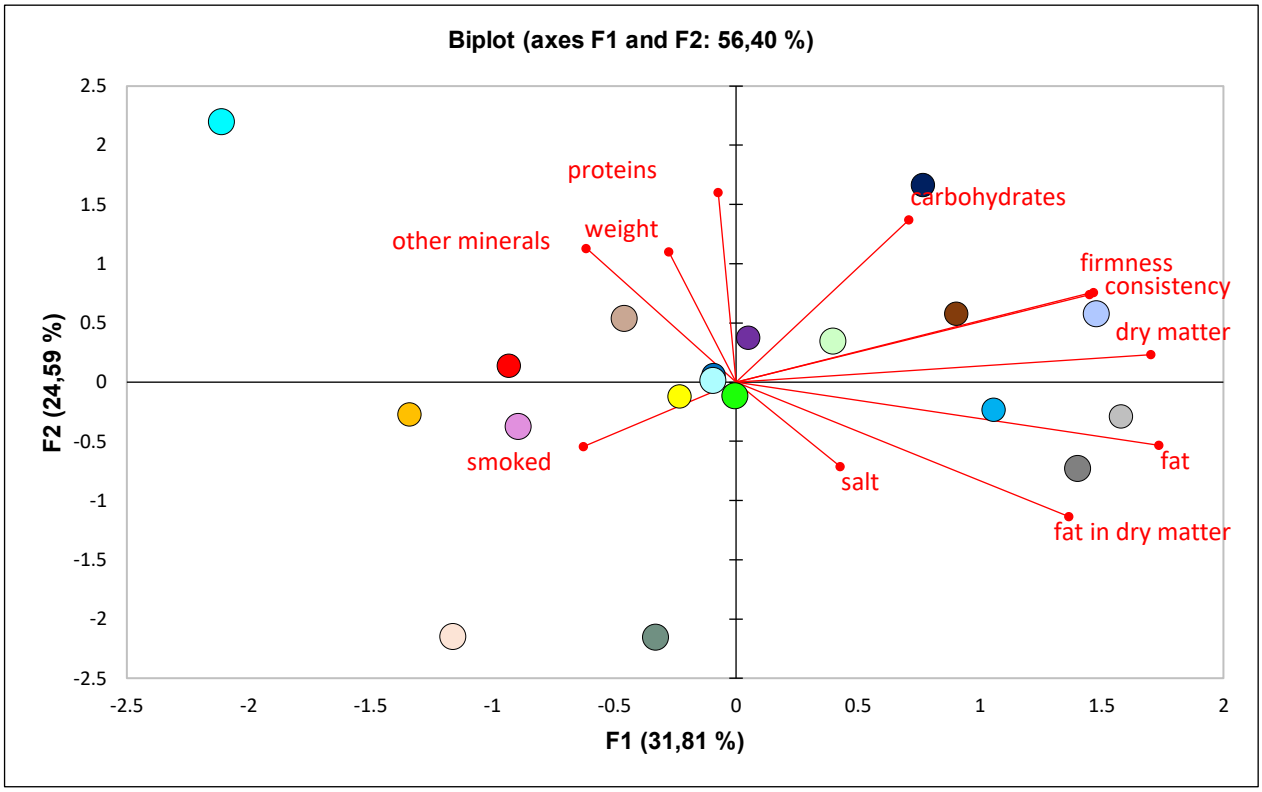
Product no.	Weight (g)	Dry Matter (wt%)	Fat in Dry Matter (%)	Fat (g.100g^{-1})	Carbohy-drates (g.100g^{-1})	Protein (g.100g^{-1})	NaCl (g.100g^{-1})	Other Minerals (g.100g^{-1})	Smoked
1	356	48	47	22.45	1.62	19.53	2.32	1.93	yes
2	344	59	48	28.01	1.83	23.31	3.81	1.82	yes
3	218	62	49	30.21	1.85	23.53	4.44	1.74	yes
4	390	58	50	28.88	1.90	23.13	2.13	1.76	yes
5	542	59	49	28.65	2.62	23.25	2.28	1.83	yes
6*	328	55	48	26.37	1.02	22.96	2.32	1.92	yes
7	356	55	48	26.47	1.47	23.37	1.82	1.89	yes
8	242	65	54	35.35	2.10	23.43	2.48	1.78	yes
9	350	60	51	30.42	2.45	22.17	3.15	1.90	yes
10	368	53	45	24.07	2.02	21.92	3.61	1.59	yes
11	284	58	51	29.54	1.93	23.08	1.52	1.92	yes
12	458	60	51	30.13	2.00	23.22	2.46	1.71	yes
13	360	62	50	30.80	1.86	23.09	4.32	1.82	yes
14*	350	60	49	29.78	1.77	22.73	4.32	1.90	yes
15*	324	57	46	26.34	2.08	24.62	2.41	1.85	no
16	286	51	50	25.59	0.80	19.57	3.66	1.61	no
17	270	63	58	36.10	2.08	20.61	1.85	1.86	no
18	374	53	39	20.46	2.11	26.66	1.69	2.19	no
19	296	56	54	30.34	0.80	19.63	3.49	1.77	no
Mean	342	58	49	28.42	1.81	22.62	2.85	1.83	
SD	74	4	4	3.86	0.49	1.78	0.96	0.13	
Cv (%)	22	7	8	13.58	27.03	7.87	33.73	7.12	

Note: Each product n = 3. Mean, SD and Cv n = 57. *Ewe's cheese, but not the Ošiepok cheese.

Table 3 The mean values of Oštiepok cheeses textural parameters determined by instrumental method.

	Middle part of the cheese						Edge part of the cheese					
Product no.	**Firm-ness (kg)**	**SD (kg)**	**Cv (%)**	**Consi-stency ($kg.s^{-1}$)**	**SD ($kg.s^{-1}$)**	**Cv (%)**	**Firm-ness (kg)**	**SD ($kg.s^{-1}$)**	**Cv (%)**	**Consi-stency ($kg.s^{-1}$)**	**SD ($kg.s^{-1}$)**	**Cv (%)**
1	0.935	0.310	33	0.699	0.265	38	1.285	0.401	31	0.850	0.325	38
2	0.954	0.145	15	0.716	0.102	14	0.779	0.351	45	0.557	0.263	47
3	2.078	0.281	14	1.620	0.259	16	1.767	0.273	15	1.319	0.253	19
4	0.972	0.090	9	0.774	0.074	10	0.876	0.132	15	0.673	0.115	17
5	2.258	0.256	11	1.759	0.248	14	2.189	0.277	13	1.724	0.231	13
6*	2.374	0.459	19	1.894	0.391	21	2.439	0.766	31	1.645	0.584	36
7	0.700	0.094	13	0.507	0.071	14	0.841	0.109	13	0.594	0.070	12
8	1.917	0.297	15	1.528	0.268	18	1.380	0.366	27	1.031	0.269	26
9	1.349	0.533	39	1.161	0.430	37	2.408	1.169	49	1.978	0.989	50
10	0.669	0.144	22	0.486	0.099	20	0.802	0.243	30	0.572	0.171	30
11	0.959	0.273	28	0.744	0.230	31	1.130	0.211	19	0.916	0.199	22
12	1.285	0.256	20	0.983	0.184	19	1.442	0.307	21	1.083	0.271	25
13	2.808	0.406	14	2.259	0.318	14	2.660	0.386	14	2.038	0.394	19
14*	0.751	0.124	17	0.602	0.079	13	0.919	0.237	26	0.676	0.202	30
15*	1.320	0.157	12	1.055	0.137	13	1.255	0.156	12	1.005	0.145	14
16	0.692	0.113	16	0.510	0.090	18	0.673	0.166	25	0.483	0.125	26
17	1.756	0.219	12	1.475	0.169	11	1.625	0.248	15	1.269	0.198	16
18	0.796	0.168	21	0.634	0.142	22	0.946	0.196	21	0.706	0.146	21
19	0.740	0.168	23	0.529	0.145	27	0.861	0.246	29	0.612	0.199	33
Mean	1.332			1.049			1.383			1.038		
SD	0.666			0.547			0.632			0.495		
Cv (%)	50			52			46			48		

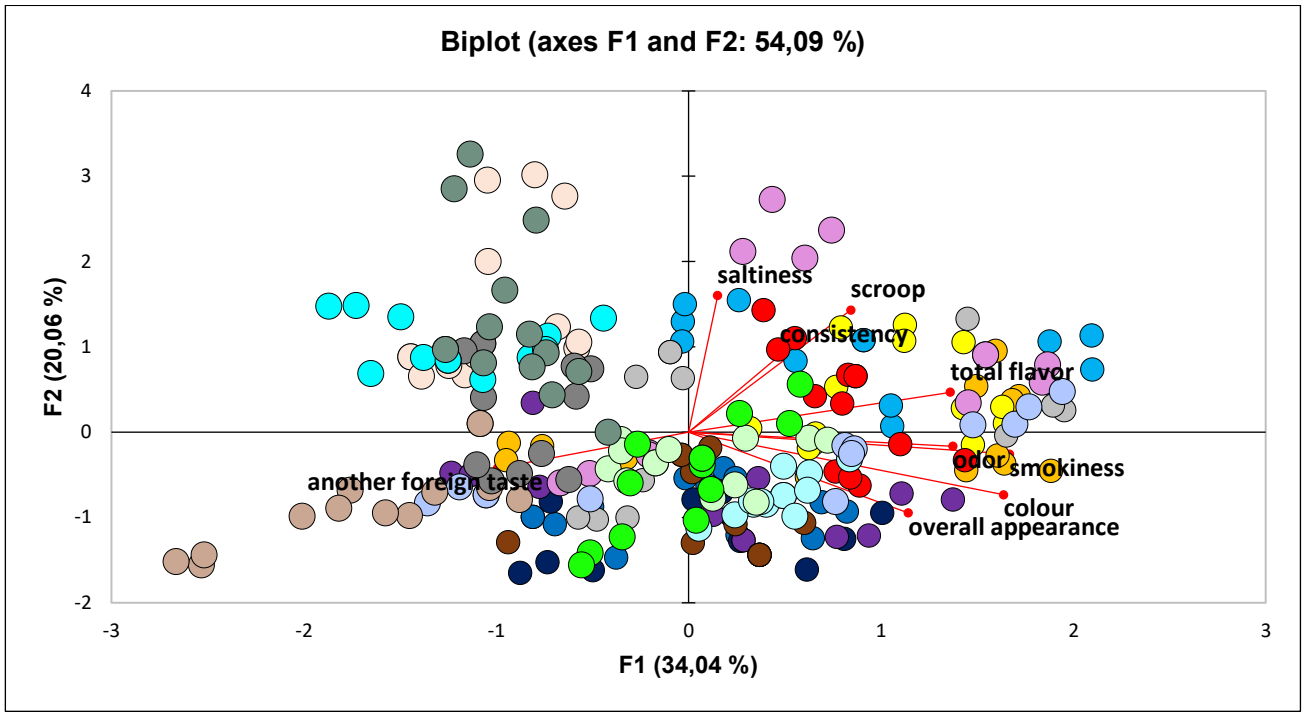
Note: SD, Cv n = 15, General Mean, SD and Cv n = 30. *Ewe's cheese, but not the Ošiepok cheese.



Note: Product No.:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 7 PCA Analysis of Oštiepok cheeses (composition and textural parameters).



Note: Product No.:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 8 PCA Analysis of Oštiepok cheeses (sensory parameters: saltines, scroop, consistency, total flavour, odor, smokiness, colour, overall appearance,another foreign taste).

According to **Olešová (2015)**, the average firmness of her own innovated oštiepok chesee ranged from 5.761 kg to 1.843 kg. The highest firmness had oštiepok after 5 weeks of maturing. Oštiepok cheeses after 2 weeks and 3 weeks of maturing did not differ in firmness. The average consistency of her own innovated oštiepok cheeses varied from 3.438 kg. s^{-1} to 1.175 kg. s^{-1}. The highest consistency had the Oštiepok after 5 weeks of maturing. In our study, we have found the highest consistency in product No. 13 (2.259 kg. s^{-1}).

For consumers, texture is a very important factor of quality. It is mainly influenced by the degree of proteolysis, salt, fat and pH **(Pachlová et al., 2012)**.

Cheese texture, especially firmness and sensory properties, changes during the maturing process **(Forde a Fitzgerald, 2000)**. We agree with this, because evin in our work we have confirmed the change in the textural and organoleptic properties of cheeses during maturation.

Cheese texture, consistency and structure depend on the degrre of primary proteolysis. Insufficient proteolysis can cause inadequate cheese consistency, which is described as a gum **(Rodriguez et al., 2011)**.

The firmness of the cheese can also be influenced by the penetration of NaCl **(Pachlová et al., 2012)**.

We have found statistically significant (p <0.001) difference between the product No. 3 with the highest NaCl content (4.44 g.$100g^{-1}$) and the product No. 11 with the lowest NaCl content (1.52 g.$100g^{-1}$) in firmness parameter 2.078 kg vs 0.959 kg. But in our experiment, this could be influenced also by the difference in Dry matter content 62 wt% vs 58 wt% and Fat in dry matter content 49% vs 51%.

The results of sensory analysis

The results of the normality test showed that the data had a non-normal distribution; the Shapiro-Wilk's test value was <0.05. Subsequently, we performed a Kruskal-Wallis one-way ANOVA and we found that individual products were statistically different (p <0.001) in all sensory parameters tested. Also, the Dunn's post hoc test identified, that there are significant (p <0.001) diferences between cheeses, specially between smoked and not smoked cheeses.

The sensory properties of Oštiepok cheeses were different because the technological process can slightly differ between the producers. Some of the most important factors, which can affect this process are: milk pasteurisation temperature, rennet, rennet dose and power, temperature of milk during curdling, different curd processing and mixing time, pressing to the form, salting, drying, smoking, temperature during storrage and maturation. Differences in these technological steps lead to the differences in textural and sensory parameter, which we were identified in this work. The quality of the traditional product can varies from farm to farm and is also different among farmers.

Similar results and differences in quality of traditional products have also been achieved by **Čuboň et al. (2015)**

The temperature during cheese maturation influences the content of microorganisms **(Kunová, et al., 2015; Cwiková, 2015)**.

In the case of microbial cross contamination, or inadequate pasteurisation, the risk of the presence of microorganisms will increase.

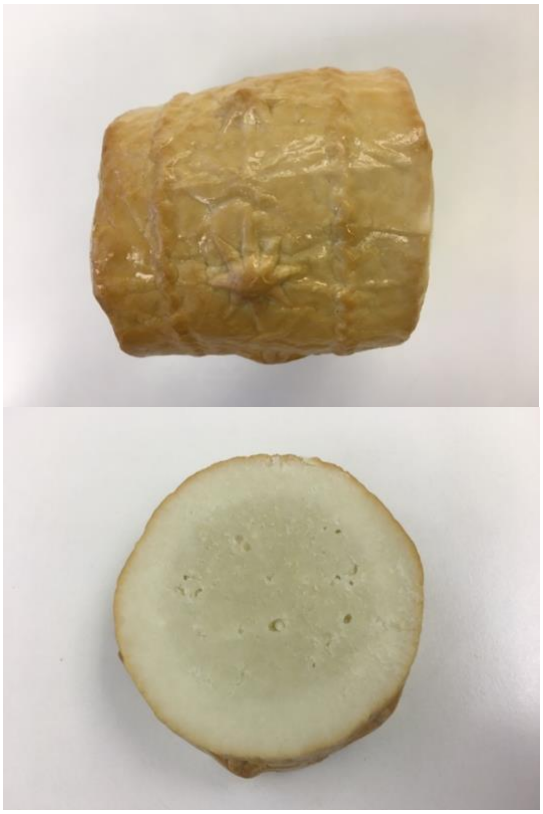


Figure 9 Product 1: Ewe's Oštiepok cheese 100% – smoked (Producer: Papúchová D. (Ružomberok, Slovakia), made in: PD Liptovské Revúce, Slovakia).

Figure 10 Product 2: Orava ewe's Oštiepok cheese – smoked (Producer: Panči M. (Ružomberok, Slovakia), made in: Šurinák M. (KOLIBA Revišné, Veličná, Slovakia).

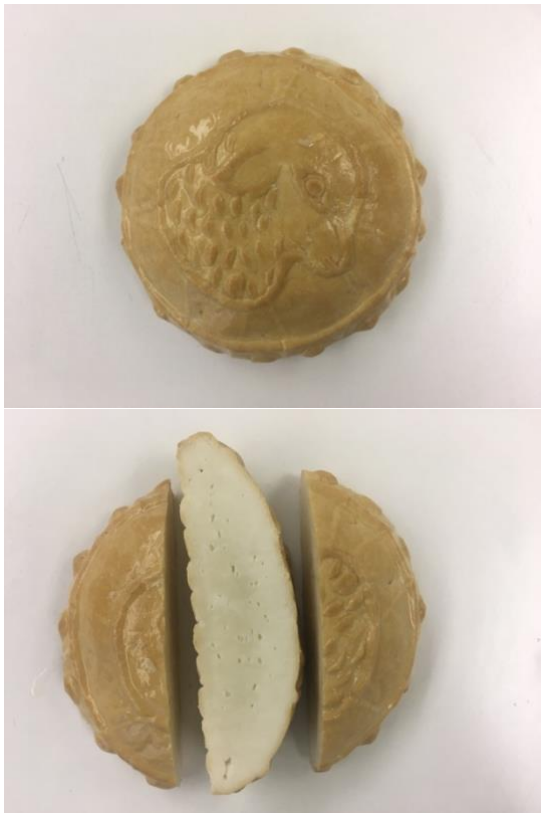


Figure 11 Product 3. Original ewe's Oštiepok cheese – smoked (Producer: Syrex – Zázrivá, Slovakia).

Figure 12 Product 4: Ewe's Oštiepok cheese – smoked (Producer: Plachtinská farm – Baránek, Slovakia).

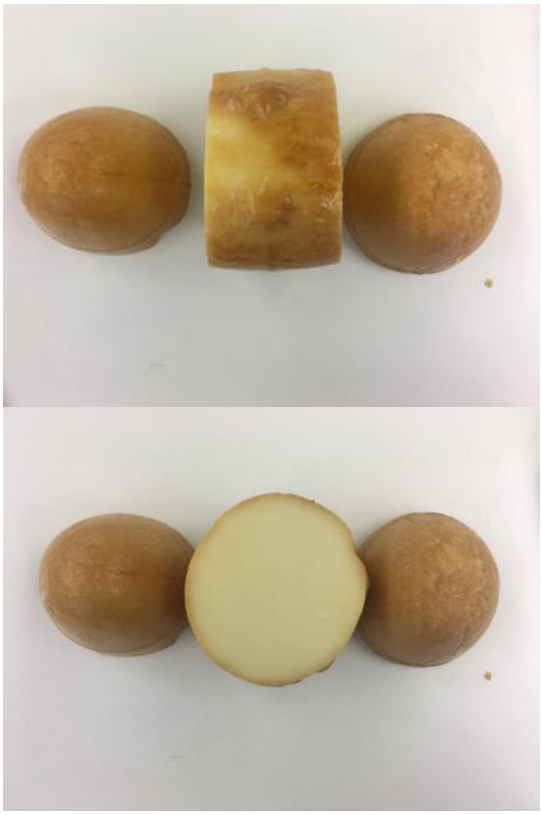


Figure 13 Product 5: Oštiepok, 100% ewe's cheese – smoked (Producer: Bryndziareň and syráreň – Zvolenská Slatina, Slovakia).

Figure 14 Product 6: Ewe's cheese Kolibka Spiš – smoked (Producer: Endel M. – VALACH (Sp. Nová Ves, Slovakia), made in: Kluknavská dairy – OOD Jaklovce, Slovakia), (Note: this is Ewe's cheese, not the Ošiepok cheese).

Figure 15 Product 7: Ewe's Oštiepok cheese – smoked (Producer: Badánik – Salaš Krajinka, Slovakia).

Figure 16 Product 8: Oštiepok Ewe's cheese – smoked (Producer: Konečný I. – KONNY (Trenčín, SLovakia), made in: Dairy Krivá, Slovakia).

Figure 17 Product 9: Ewe's Oštiepok cheese – smoked (Producer: Zúbek M. – OBERT(Ladce, Slovakia)).

Figure 18 Product 10: Ewe's Oštiepok cheese – smoked (Producer: Zvara J. (Detva, Slovakiaka)).

Figure 19 Product 11: Ewe's Oštiepok cheese – smoked (Producer: Agrosev made in: Plachtinská farm Baránek, Slovakia).

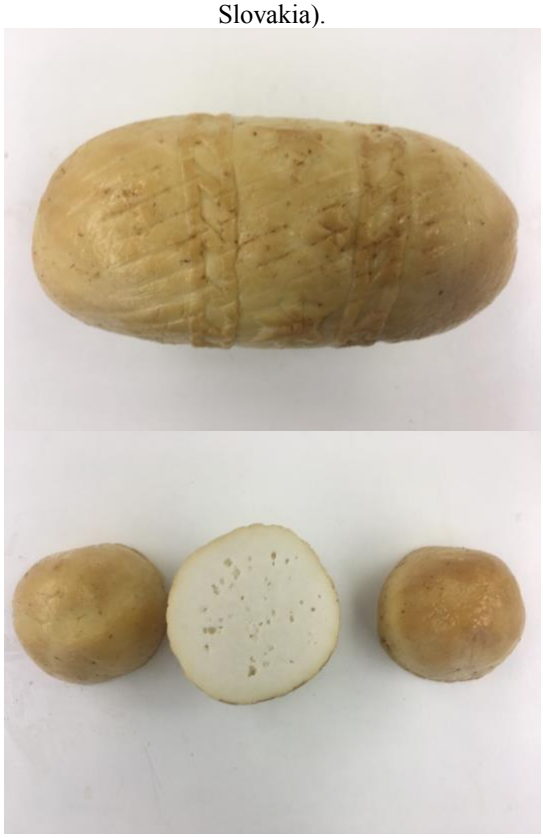


Figure 20 Product 12: Oštiepok – Ewe's cheese – smoked (Producer: AgroIna (Banská Bystrica, Slovakia).

Figure 21 Product 13: Original Oštiepok ewe's cheese - smoked (Producer: Chlustová P. (Liptovský Mikuláš, Slovakia), made in: Syrex (Zázrivá, Slovakia)).

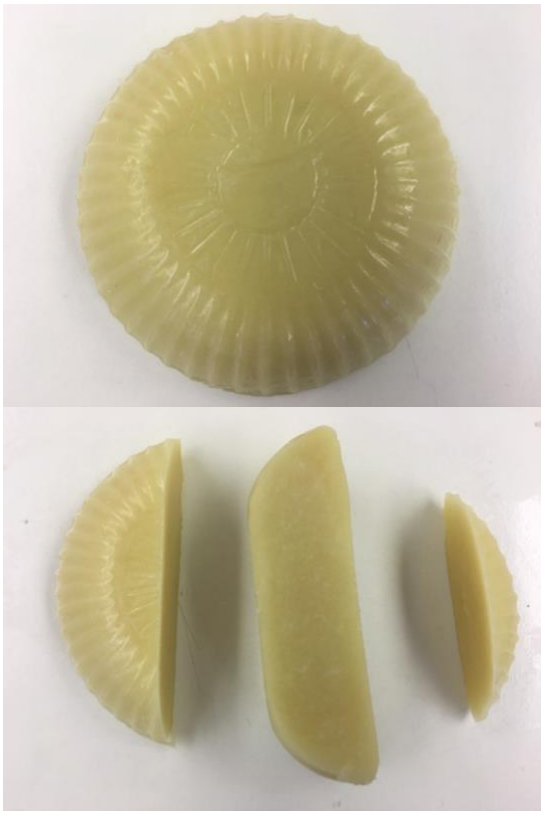


Figure 23 Product 15: Ewe's cheese Kolibka Spiš - steamed (Producer: Endel M. Jr. – VALACH (Sp. Nová Ves, Slovakia), made in: Kluknavská dairy – ODD Jaklovce, Slovakia) (Note: this is Ewe's cheese, not the Ošiepok cheese).

Figure 22 Product 14: Orava ewe's cheese – smoked (not(Producer: Válek M. (Revišné – Veličná, Slovakia), made in: Šurinák M. – Koliba Revišné (Veličná, Slovakia)) (Note: this cheese was not labelled as the Oštiepok cheese, but it meets its requrements).

Figure 24 Product 16: Original ewe's Oštiepok cheese – fresh (Producer: Chlustová P. (Liptovský Mikuláš, Slovakia), made in: Syrex (Zázrivá, Slovakia)).

Figure 25 Product 17: Oštiepok Ewe's cheese – fresh (Producer: Dairy Krivá (Krivá, Slovakia)).

Figure 27 Product 19: Original ewe's Oštiepok cheese – fresh (Producer: Syrex (Zázrivá, Slovakia)).

Figure 26 Product 18: Orava ewe's Oštiepok cheese – fresh (Producer: Panči M. (Ružomberok), made in: Šurinák M. – Koliba Revišné (Veličná, Slovakia)).

There is a lot of microorganisms, which can affect the quality and safety of the product.

The process of cheese maturation affects the growth of probiotic cultures in cheese **(Poľáková, Dudriková and Gallo, 2010; Lovayová et al. 2010)**.

The occurence of *S. aureus, E.* coli and others microorganisms in ewe's milk can lead to the serious health problems **(Poľáková, Dudriková and Gallo, 2011; Medveďová et al. 2010)**.

It is necessary to follow the rules of good hygiene practice, good manufacturing practice, sanitation, to use raw milk of good quality in the production of cheese and to follow the HACCP system guide **(Medveďová et al. 2010; Zajác, Čapla and Golian, 2017a; Zajác, Čapla and Golian, 2017b),** which was preprared and by the Union of sheep and goats breeders in Slovakia and implemented in all small salaš farms in our country.

In our experiment we have identified one product, No. 9 **(Figure 17)** with evident microbial defect. In the cut of this cheese you can see an activity of sporoform microorganisms (a lot of air cavities in cheese). This defect could occur not only for technological reasons but also as a result of transport or storage at the wrong temperature.

Sensory properties can be influenced by the addition of flavorings **(Pavelková, Flimelová and Vietoris, 2012),** but in our experiment we didn't found any cheese with additional flavorings. All of the producers were used only the raw materials authorized by legislation (milk, rennet, NaCl and some of them also $CaCl_2$).

Majcher et al. (2011) states that in shaping curd into a characteristic shape is the cheese pressed into a two-part wooden mold with a cut out ornament, obtaining a Oštiepok

cheese that will be decorated on the surface with an ornament.

We agree, cheeses analysed in this work had characteristic special shape, taste and odor and ovrall appearance. We found that most of the Oštiepok cheese had these properities **(Figures 9 – 27)**, but two of them, product No. 6 and No. 15 does not comply this traditional shape.

The natural preservation method, such as smoking at the salaš (small farm in the mountain region) delivers to Oštiepok cheese a typical golden brown to brown color and typical taste. **(Majcher a Jelen, 2011)** and these aromatic compounds play a very important role in the quality of food **(Attaie, 2009, Benešová, Golian and Zajác, 2018)**.

It is, because cheese taste is the result of the balance between volatile and non-volatile chemical compounds **(Hayaloglu et al., 2013)** and the smoking of cheese add extra portion of these volatile compounds.

From the consumer point of view, it is important to ensure the quality and safety of the products. Because ewe's milk is more expensive than cow's milk, adulteration of Oštiepok cheese may occur. Oštiepok cheese produces with the addition of cow's milk are issued as products made only from ewe's milk. Various laboratory methods can be used to detect adulteration **(Zeleňáková et al., 2016)**.

As part of our experiment, we have tested three ewe's cheeses, which were not the Oštiepok cheese (Products No. 6, No. 14 and No. 15) but sometimes the consumers may buy them instead of the Oštiepok cheese. These products had a different look, they were shaped like a bowl or the loaf. The composition of these cheese were similar to Oštiepok cheeses. The producer of the product No. 14. didn't use the possibility to mark this product as the Oštiepok cheese despite the fact that this product meets the requirements of the Oštiepok cheese. In the case of the Products No. 6 and 15 the difference was in one extra technological step – steaming the cheese in hot water and in the case of the product No. 15 also the the addition of dairy culture. The labelling of these products was correct. The producers correctly did not mark them as the oštiepok cheese.

The consumers can distinguish traditional products, but some may have a problem. It is therefore appropriate to designate such products with a specific protected geographical indication PGI mark of the European Union. At the time of our experiment, no producer of Oštiepok cheese in Slovakia used the protected geographical indication (PGI) mark "Slovenský oštiepok" in the product labelling or in its immediate vicinity of the product during its sale despite the fact that the Slovak Republic has such possibility given by the European Union and Slovak legislation. It means, the competent government authority Ministry of Agriculture and Rural Development of the Slovak Republic and interested parties like the Dairy association od the Slovak Republic or the Union of sheep and goat breeders of the Slovak Republic should do more to inform the producers of the possibility to mark this Slovak national specialty with PGI mark and help them with a bureaucratic process of this matter. It will then be necessary to carry out an information campaign for consumers to recognize such traditional products from ordinary products in the market.

CONCLUSION

Oštiepok cheese is a traditional half-fat semi-hard cheese made in Slovakia. Oštiepok cheese can be made from raw ewe's milk, a mixture of ewe's and cow's milk or cow's milk. Oštiepok cheese is characterised by its remarkable shape, that of a large egg, pine cone or ellipsoid, and is decorated according to the practices and with the typical designs of the individual area in which it is produced. The surface of this cheese is firm, smooth and shiny. Subsequent smoking gives the product its typical colour, smoked aroma and taste. This special process is originated in and is carried out throughout the entire delimited area of Slovakia mainly in small montains farms called salaš or in dairy companies. We have found significant (p <0.001) differences between Oštiepok cheeses from different regions of the Slovakia. The mean values of composition of Oštiepok cheese was: dry matter 58 wt%, fat in dry matter 49%, fat 28.42 $g.100g^{-1}$, carbohydrates 1.81 $g.100g^{-1}$, protein 22.62 $g.100g^{-1}$, NaCl 2.85 $g.100g^{-1}$, other minerals 1.83 $g.100g^{-1}$. The overall mean of firmness of all cheeses was 1.349 ±0.705 kg and Cv 52%. The overall mean of consistency of all cheeses was 1.045 ±0.578 $kg.s^{-1}$ and Cv 55%.

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THE EVALUATION OF SELECTED QUALITATIVE PARAMETERS OF SWEET POTATO (*IPOMOEA BATATAS* L.) IN DEPENDENCE ON ITS CULTIVAR

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ABSTRACT

The sweet potato (*Ipomoea batatas* L.) is relatively known vegetable species, but it is grown only on small area in the Middle European region. Its cultivars are characterized by different colour of tuber flesh which can be white, beige, yellow, orange and purple. The aim of this study was to determine and compare selected qualitative parameters of tubers (total carotenoids, vitamin C and total soluble solids) among orange, white and purple sweet potato cultivars. The field experiments were established at Slovak University of Agriculture in Nitra in 2016 and 2017. Sweet potatoes were grown by hillock system with using of black non-woven textile for soil mulching. The tuber harvest was realised on the 6th October 2016 and 13rd September 2017. The highest content of total carotenoids was found in orange sweet potato cultivars (78.47 – 122.89 mg.kg^{-1} fresh weight) and its values were multiple-fold higher in comparison with purple (4.22 mg.kg^{-1} f. w.) and white (10.71 mg.kg^{-1} f. w.) cultivars. Orange cultivars were also richer source of vitamin C (246.31 – 325.99 mg.kg^{-1} f. w.) compared to white (179.66 mg.kg^{-1} f. w.) and purple (187.75 mg.kg^{-1} f. w.) cultivars of sweet potatoes. The total soluble solids, expressing mainly sugar content, was higher in purple (10.13 °BRIX) cultivar of sweet potatoes, followed by cultivars with orange (8.52 – 9.72 °BRIX) and white (5.57 °BRIX) tuber flesh. Obtained results showed the significant effect of cultivar, characterized by different tuber flesh colour, on the composition and contribution of sweet potatoes for human health.

Keywords: sweet potato; quality; carotenoids; vitamin C; total soluble solids

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) belongs to the family *Convolvulaceae* and genus *Ipomoea* **(Firon et al., 2009)**. **Loebenstein (2009)** indicate, that sweet potatoes (syn. batatas) were domesticated before more than 5000 years in tropical America. According to the database **FAOSTAT (2019)**, world sweet potato production within period 2007 – 2016 was ranged from 101,3 to 105,4 mil. tonnes. **Musilová et al. (2017)** state that sweet potatoes are grown in Slovak republic only by small growers.

Sweet potatoes are universal and delicious vegetable species with high nutritional value because of many medicinally valuable compounds with anticarcinogenic, antidiabetic and anti-inflammatory activity **(Mohanraj and Sivasankar, 2014)**. According to the database **USDA (2019)**, sweet potato tubers are characterized by higher energetic value in comparison with classical potatoes (*Solanum tuberosum* L.). Sweet potatoes have higher ratio amylose to the amylopectin compared to the potatoes. The amylose increases the sugar level in blood more slowly compared to the simple saccharides and it is recommended as healthier food component, including for people suffering by diabetes **(Mohanraj and Sivasankar, 2014)**.

The flesh of sweet potato tubers can be white, beige, yellow, orange and purple **(UPOV, 2010)**. **Allen et al. (2012)**, sweet potato cultivars with orange flesh colour contain are rich sources of carotenoids, known as provitamins A. The dominant carotenoid substance in sweet potatoes is β-carotene **(USDA, 2019)**. **Kammona et al. (2015)** found that β-carotene ratio from total carotenoid content in sweet potatoes was varied from 79.0 (white flesh) to 97.9% (purple flesh). The biochemical change of β-carotene in human body is formed vitamin A; however, it is not possible to state that β-carotene should have the same importance as vitamin A **(Hegedűsová et al., 2016)**. The β-carotene is very effective scavenger of free oxygen radicals which could be responsible for skin damage, eye retina degeneration, cataract formation or various types of cancer. From this reason, β-carotene is ranged to the group of important antioxidant substances **(Pisoschi and Pop, 2015)**.

The vitamin C (syn. ascorbic acid) belongs to the most important vitamins and it is characterized by significant antioxidant properties. The most of plants and animals synthesize vitamin C from glucose; however, the human organism is not able to form vitamin C and is dependent on

its intake from food **(Schlueter and Johnston, 2011)**. The vitamin C is the less stable and most sensible vitamin and it is used as the indicator of change level in relation to the plant product processing **(Giannakourou and Taoukis, 2003)**. **Schlueter and Johnston (2011)** state that vitamin C protects human body against infection of respiratory system and reduces the risk formation of cardiovascular diseases and several cancer types. **Mei and Tu (2018)** indicate that intake of sufficient amount of vitamin C acts against *Helicobacter pylori* which is marked as an important risk factor for stomach cancer formation. According to the study of **Harrison (2012)**, vitamin C plays important protective role against Alzheimer's disease, characterized by decrease of cognitive abilities. The vitamin C is also important for sperm protection against oxidative damage and higher sperm quality of smokers **(Colagar and Marzony, 2009)**.

Scientific hypothesis

The sweet potato is relatively known but a few grown vegetable species in the Middle European region. There is expected a variable content of tested qualitative parameters among sweet potato cultivars with different-coloured flesh tuber.

MATERIAL AND METHODOLOGY

The field experiments with sweet potato were realized on the land of the Slovak University of Agriculture in Nitra in 2016 and 2017. The climate of experimental area is characterized by warm and dry summer and slightly warm, dry or very dry winter. According to the climatic normal 1951 – 2000 for Nitra, annual mean temperature is 9.9 °C and mean rainfall total is 548 mm **(Šlosár et al, 2016)**. Within vegetation period (May – September), the average air temperature was 18.7 °C in 2016 and 19.3 °C in 2017. The rainfall total, during vegetation period, was 312 (2016) and 216 mm (2017).

Plant material

Sweet potato seedlings were purchased from Croatian producer. In Europe, situation with cultivar assortment of sweet potato is often unclear and confusing. A lot of seedlings are produced according to the mother's tuber availability on the market. Thus, the origin of sweet potato seedlings on market is often un-known. Within this study, four cultivars with different tuber flesh colour were tested (Figure 1). The sweet potato cultivar 'Beauregard' was only one certified cultivar and it is very known cultivar with orange flesh colour. Other three cultivars were marked according to the market place from which tubers for seedling production was purchased and used. These cultivars were named as 'Dubaian' (orange cultivar from United Arab Emirates), 'Višnjica white' and 'Višnjica purple' (white and purple cultivar from Croatia). The basic characteristics of all cultivars, evaluated according to the descriptor **UPOV (2010)**, is described in the Table 1.

Experiment organization

The sweet potato is warm-requiring crop which needs warm season lasting at least four months with an average temperature more than 20 °C and without freeze **(Antonio et al., 2011)**. From this reason, out planting of sweet potato seedlings was realised on the 25th May 2016 and 1st June 2017 when the risk of later spring freeze is significantly reduced.

Within soil preparation for sweet potato growing, nitrogen was only applied on the soil supply level of 60 kg.ha^{-1} according to results of agrochemical soil analyses (Table 2). Sweet potato plants were grown by hillock system, similar to the carrot growing (height of 0.30 m). The distance between hillock rows was 1.20 m. In each row, 18 sweet potato seedlings were planted in distance of 0.30 m. Rows for all tested cultivars were divided to three replications with 6 sweet potato plants. The black non-woven textile was used for soil mulching before planting of sweet potato seedlings because of better microclimate around plants.

The harvest of sweet potato tubers was realised on the 6th October 2016 and 13rd September 2017. Harvested tubers of sweet potato were classified according to average weight of tubers in two size classes: >150 g – marketable yield of tubers and <150 g – non-marketable yield of tubers. Qualitative parameters of sweet potatoes were evaluated in marketable tubers. The average sample of each sweet potato cultivar for analyses was prepared from 6 tubers. All tubers were quartered, and opposite quarters were used for qualitative analyses.

Determination of qualitative parameters

Total carotenoid content estimation

The estimation of total carotenoid content was realised in the laboratory of Department of Vegetable Production, Slovak University of Agriculture in Nitra. The total carotenoid content was estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 200 at 445 nm wavelengths. As an extraction reagent, acetone was used acetone **(Hegedüsová et al., 2018)**.

Vitamin C content estimation

The estimation of vitamin C content was realised in the laboratory of Department of Chemistry, Janos Selye University in Komárno. HPLC method of vitamin C content estimation **(Hegedüsová et al., 2018)** was used by the help of liquid chromatograph with UV detector, for separation was used RP C18 column, mobile phase was methanol:water (5:95, v/v), UV detection was adjusted to 258 nm (HPLC Waters 2489 UV/VIS Detector).

Total soluble solids estimation

The juice from the homogenized sample of sweet potatoes was squeezed on the dry block of the digital hand-held refractometer (Kern ORD 45BM, Balingen, Germany). The value of soluble solids was directly read. Measurement was performed at room temperature according to **Hegedűsová et al. (2018)**.

Statistic analysis

The statistical analysis of obtained results was performed by using of the Statgraphic Centurion XVII (StatPoint, USA). Results were evaluated by analysis of variance (ANOVA) and average values were tested by LSD test performed at the significance level of 95% (p <0.05).

Table 1 Evaluated morphological characteristics of sweet potato tubers.

Parameter	Beauregard	Dubaian	Višnjica white	Višnjica purple
Shape	ovate	ovate	ovate	ovate/irregular/oblong
Main skin color	light-purple	red-purple	beige	red-purple
Secondary skin color	orange	-	pink	-
Main flesh color	orange	orange	white	purple
Secondary flesh color	beige	-	-	beige

Table 2 Agrochemical soil characteristics before trial experiments in 2016 and 2017.

Year	pH_{KCl}	Humus (%)	Nutrient content (mg.kg^{-1} of soil)					
			$N_{min.}$	P	K	Ca	Mg	S
2016	7.14 N	4.17 H	13.0 M	198.8 VH	487.5 VH	6100 H	816 VH	26.3 M
2017	7.18 N	3.75 G	10.1 M	147.5 H	477.5 VH	5850 H	762.6 VH	91.3 H

Note: N_{min} – N mineral (N inorganic); N – neutral; G – good; M – medium; H – high; VH – very high.

Table 3 Average content of total carotenoids, vitamin C and total soluble solids in sweet potatoes.

Cultivar	Total carotenoids (mg.kg^{-1} ±SD)	Vitamin C (mg.kg^{-1} ±SD)	Total soluble solids (°BRIX ±SD)
Beauregard	78.47 ±7.74^{c}	246.31 ±86.36^{b}	8.52 ±1.61^{b}
Dubaian	122.89 ±9.57^{d}	325.99 ±127.12^{c}	9.72 ±1.63^{c}
Višnjica white	10.71 ±1.51^{b}	179.66 ±41.52^{a}	5.57 ±0.90^{a}
Višnjica purple	4.22 ±0.81^{a}	187.75 ±71.76^{a}	10.13 ±1.63^{d}

Note: SD – standard deviation.

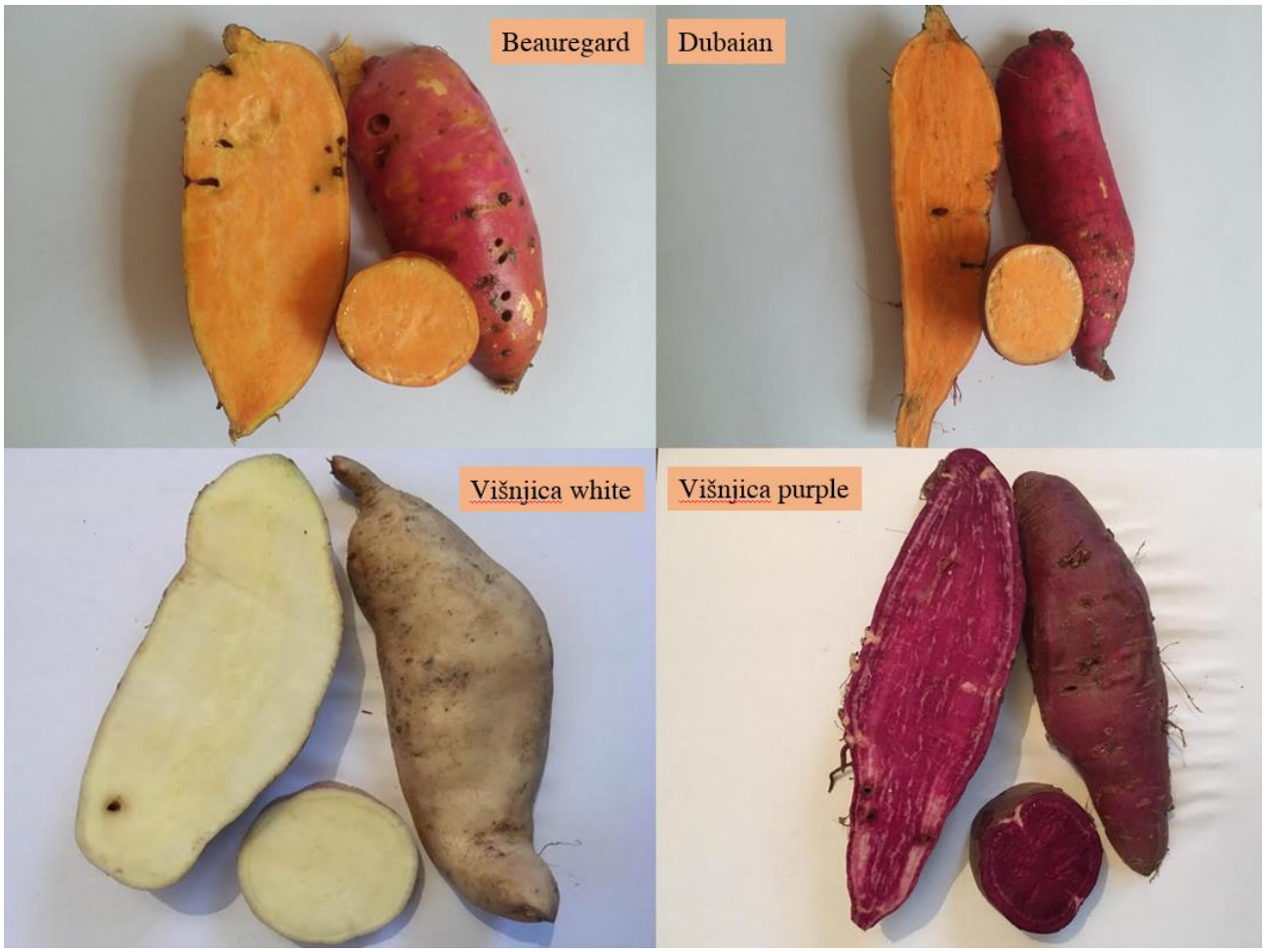

Figure 1 Tested sweet potato cultivars.

Total carotenoid content (TCC)

The statistical analysis of obtained results showed statistically significant (p <0.05) differences of TCC among tested sweet potato cultivars (Table 3). The average values of TCC were ranged from 4.22 ('Višnjica purple') to 122.89 mg.kg^{-1} fresh weight ('Dubaian'). Significant differences of TCC were found among orange and purple/white cultivars; it confirmed similar findings presented by **Allen et al. (2012)** or **Sebuliba, Nsubuga and Muyonga (2001)**. In mentioned studies, authors stated that important source of carotenoids are mainly orange sweet potato cultivars. **Alam, Rana and Islam (2016)** found the variability of TCC in nine orange cultivars of sweet potatoes from 3.8 to 72.4 mg.kg^{-1} f. w. Results found by **Tomlins et al. (2012)** also confirmed that orange cultivars (8.5 – 72.5 mg.kg^{-1} f. w.) are significantly richer sources of carotenoid in comparison with white cultivars of sweet potatoes (0.4 – 1.3 mg.kg^{-1} f. w.). Values of TCC, presented in both studies, were lower than results found in our experiments. The expressive variability of TCC in orange sweet potato cultivars was presented in study of **Ndah and Ojimelukwe (2018)** in which values of TCC was ranged from 18.01 to 180.98 mg.kg^{-1} f. w. Values of TCC in most of cultivars are comparable to orange cultivars tested in our study. **Tang, Cai and Xu (2015)** tested the effect of cultivars with different flesh colour on the TCC in sweet potatoes. The highest TCC was found in orange cultivars (157.9 mg.kg^{-1} f. w.), followed by yellowish-creamy (75.4 mg.kg^{-1}), light-purple (5.19 mg.kg^{-1}), white (4.46 mg.kg^{-1}) and dark-purple cultivars (2.85 mg.kg^{-1}) of sweet potatoes. In comparison to results of our study, TCC was higher in orange cultivars, comparable in purple cultivars and lower in white cultivars of sweet potatoes. **Grace et al. (2014)** found the significantly higher TCC in orange (95.00 mg.kg^{-1} f. w.) sweet potato cultivars compared to the cultivars with yellow (3.40 – 5.16 mg.kg^{-1}) and white (0.55 mg.kg^{-1}) flesh colour. Expressive differences of TCC among sweet potato cultivars, dependent on the colour of tuber flesh, were also found in the study of **Kammona et al. (2015)**. Values of TCC was ranged in following order of tuber flesh colour: orange (389.22 mg.kg^{-1} dry weight) > yellow (138.96 mg.kg^{-1}) > purple (116.28 mg.kg^{-1}) > white (115.18 mg.kg^{-1}). The strong interaction between flesh colour and TCC in sweet potatoes was also presented in studies of **Ellong et al. (2014)**, **Hussein, Billard and Adenet (2014)** and **Leighton. Schönfeldt and Kruger (2010)**.

The most important and dominant carotenoid substance in sweet potatoes is β-carotene **(USDA, 2019)**. According to **Kammona et al. (2015)**, β-carotene ratio from TCC, dependent on the sweet potato flesh colour, was following: purple flesh (97.9%) > orange flesh (93.8%) > yellow flesh (84.1%) > white flesh (79.0%). **Yildirim, Tokuşoğlu and Öztürk (2011)** tested the variability of β-carotene content in ten sweet potato cultivars and it was ranged from 50.1 (yellow) to 70.3 (orange) mg.kg^{-1} f. w. Compared to previous studies, **Suparno, Prabawardani and Pattikawa (2016)** found minimal variability of β-carotene content (62.98 – 64.69 mg.kg^{-1} f. w.) in sweet potatoes. **Wu et al. (2008)** found significantly higher content of β-carotene in orange sweet potato cultivars (55.9 – 231.1 mg.kg^{-1} f. w.) compared to the cultivars with white (23.0 mg.kg^{-1}), yellow (5.2 mg.kg^{-1}) and purple (0.60 mg.kg^{-1}) cultivars. The significant effect of cultivar on the content of β-carotene in sweet potatoes was found in the study of **Vimala et al. (2011)**. Authors tested 42 orange sweet potato cultivars and values of β-carotene was ranged from 10.4 to 143.7 mg.kg^{-1} f. w. Compared to the previous study, **Ukom, Ojimelukwe and Okpara (2009)** found lower β-carotene in orange and white sweet potatoes which was ranged from 5.23 to 11.77 mg.kg^{-1} f. w., dependent on the cultivar. Results presented by **Aywa, Nawiri and Nyambaka (2013)** confirm findings of previous studies. Authors found that sweet potato cultivars are significantly richer source of β-carotene (46.19 – 48.89 mg.kg^{-1} f. w.) compared to cultivars with different flesh colour (20.17 – 26.28 mg.kg^{-1} f. w.).

Vitamin C

The variance analysis of obtained results showed statistically significant (p <0.05) differences of vitamin C content among tested cultivars, except of relation between cultivars 'Višnjica white' and 'Višnjica purple' (Table 3). The average content of vitamin C was increasing in following cultivar order: 'Višnjica white' (179.66 mg.kg^{-1} fresh weight) < 'Višnjica purple' (187.75 mg.kg^{-1}) < 'Beauregard' (246.31 mg.kg^{-1}) < 'Dubaian' (325.99 mg.kg^{-1}).

Rautenbach et al. (2010) tested impact of cultivar on the vitamin C content in sweet potatoes and its values were ranged from 155 to 322 mg.kg^{-1} f. w. These values are comparable to our results. The significant differences of vitamin C content in sweet potatoes (8 different-coloured cultivars) were found in the study of **Ellong et al. (2014)**. The highest vitamin C content was found in yellow cultivars (178 – 291 mg.kg^{-1} f. w.), followed by cultivars with beige (143 mg.kg^{-1}), white (41 – 128 mg.kg^{-1}) and purple (54 mg.kg^{-1}) flesh colour. Compared to our study, vitamin C content was lower in white and purple sweet potato cultivars. The expressive effect of cultivar on the vitamin C content in sweet potatoes was presented by **Yildirim, Tokuşoğlu and Öztürk (2011)** who found its lower values in orange cultivars (252 – 386 mg.kg^{-1} f. w.) in comparison with creamy (252 – 386 mg.kg^{-1}) and yellow (237 – 381 mg.kg^{-1}) flesh colour. In orange cultivars, values of vitamin C content was comparable to our tested cultivars 'Beauregard' and 'Dubaian'. **Aywa, Nawiri and Nyambaka (2013)** found significantly lower values of vitamin C content in comparison with our study. Similarly, authors found higher vitamin C content in orange cultivars compared to white and purple cultivars of sweet potato. The higher content of vitamin C in orange-coloured sweet potatoes in comparison with purple cultivars was also stated by **Grace et al. (2014)**. Compared to our experiment, **Croitoru et al. (2017)** found lower vitamin C content (66 – 117 mg.kg^{-1} f. w.) in tubers of sweet potatoes. The lower values of vitamin C content (129 – 142 mg.kg^{-1} f. w.) was also presented by **Maria and Rodica (2015)**. **Krochmal-Marczak et al. (2013)** determined the vitamin C content in dependency on the flesh colour of sweet potatoes (white, orange, purple). The vitamin C content in orange sweet potato cultivar (242 mg.kg^{-1} f. w.) was lower compared to our tested orange cultivars. On the contrary, authors found higher vitamin C content in white (242 mg.kg^{-1}) and purple (203 mg.kg^{-1}) cultivars of sweet potatoes in comparison with results of our study. The

significantly higher vitamin C content in purple (727 mg.kg^{-1} f. w.) and white (672 mg.kg^{-1}) sweet potatoes was found in the study of **Suparno, Prabawardani and Pattikawa (2016)**.

Within study of **Barrera and Picha (2014)**, the average vitamin C content in cultivar 'Beauregard' (214 mg.kg^{-1} f. w.) was lower than in our experiment. **Mitra (2012)** determined the vitamin C content in 15 orange cultivars of sweet potato. Its values were ranged from 129 to 268 mg.kg^{-1} f. w.; only one of tested sweet potato cultivar was higher compared to orange cultivars in our experiment. **Gichuhi et al. (2014)** found nearly 4-fold lower vitamin C content in cultivar 'Beauregard' (64 mg.kg^{-1} f. w.) compared to this cultivar tested in our experiment.

Total soluble solids

According to **Cejpek (2012)**, the method for estimation of total soluble solids (TSS) are used for testing of sugar content in syrup, fruit and vegetable juices or dairy products and total concentration of monosaccharides and disaccharides in any solutions. **Hegedűsová et al. (2018)** define TSS as additive quantity which expresses the content of dissolved substances, mainly sugars, in vegetable extracts. As the unit of TSS, Brix degrees (°BRIX) are used.

The statistical analysis of gained results revealed statistically significant (p <0.05) differences of TSS content among all tested cultivars of sweet potato (Table 3). The highest average content of TSS was found in purple cultivar 'Višnjica purple' (10.13 °BRIX), followed by orange cultivars 'Dubaian' (9.72 °BRIX) and 'Beauregard' (8.52 °BRIX) and white cultivar 'Višnjica white' (5.57 °BRIX). **Nair et al. (2015)** found values of TSS content in sweet potatoes in the range from 7.9 to 8.8 °BRIX. In cultivar 'Beauregard', authors found lower TSS content (7.9 °BRIX) compared to value found in the same cultivar in our experiment.

The significant differences of sugar content in sweet potatoes were presented in several studies. **Krochmal-Marczak et al. (2013)** found significantly higher content of total sugars in white sweet potato cultivar (3.85 g.100g^{-1} f. w.) compared to cultivars with orange (2.90 g.100g^{-1}) and purple (2.16 g.100g^{-1}) flesh colour. In comparison to our study with TSS, it means opposite order of different coloured flesh of sweet potatoes. On the contrary to previous mentioned study, **Sanoussi et al. (2016)** determined the significantly higher sugar content in orange cultivars (22.45 g.100g^{-1} f. w.) compared to white (17.95 g.100g^{-1}) cultivars of sweet potatoes. **Ellong, Billard and Adenet (2014)** found higher sugar content in purple sweet potato cultivar (33.62 g.100g^{-1} f. w.) in comparison with white cultivars (26.79 – 33.12 g.100g^{-1}). The significant impact of cultivar on the sugar content in sweet potatoes was also found by **Yildirim, Tokuşoğlu and Öztürk (2011)**, **Mitra (2012)** and **Alam, Rana and Islam (2016)**. Compared to previous studies, **Salawu et al. (2015)** found minimal differences of sugar content between sweet potato cultivars with purple (65.17 g.100 g^{-1} f. w.) and white (65.10 g.100 g^{-1}) flesh colour.

CONCLUSION

The sweet potato (*Ipomoea batatas* L.) is relatively known vegetable species, but it is grown only on small area in the Middle European region. It belongs among very important and often used vegetable species in the world, mainly in Asia and Africa. The sweet potato cultivars are characterized by different colour of tuber flesh which can be white, beige, yellow, orange and purple. The most used and typical for sweet potatoes are orange cultivars. The aim of this study was to determine and compare selected qualitative parameters of tubers among orange, white and purple sweet potato cultivars. The highest total carotenoid content was found in orange (78.47 – 122.89 mg.kg^{-1} fresh weight) sweet potato cultivars and its values were multiple-fold higher in comparison with purple (4.22 mg.kg^{-1} f. w.) and white (10.71 mg.kg^{-1} f. w.) cultivars. The vitamin C content in orange cultivars (246.31 – 325.99 mg.kg^{-1} f. w.) was also significantly (p <0.05) higher compared to white (179.66 mg.kg^{-1} f. w.) and purple (187.75 mg.kg^{-1} f. w.) cultivars of sweet potatoes. The total soluble solids, expressing mainly sugar content, was higher in purple (10.13 °BRIX) cultivars of sweet potatoes, followed by cultivars with orange (8.52 – 9.72 °BRIX) and white (5.57 °BRIX) tuber flesh. Obtained results indicate that cultivar of sweet potato, characterized by tuber flesh colour, is very important factor from aspect of its composition, taste and contribution for human health.

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THERMO-DEGRADATIVE CHANGES OF RAPESEED AND SUNFLOWER OILS DURING DEEP-FRYING FRENCH FRIES

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ABSTRACT

The purpose of this study was to investigate changes in TPCs, acid value and peroxide value as well as fatty acids composition in edible oils during french fries production. Lower TPCs content was found in rapeseed oil (3.3%) and the threshold (24%) was achieved on the fourth day. The total time for the deterioration of deep-frying rapeseed oil was $23^{½}$ hours. On the contrary, in fresh sunflower oil at the first day was TPCs content 5.5% and the limit of 24% was reached on the third day. The total time for the deterioration of deep-frying sunflower oil was $17^{½}$ hours. The results indicated significant differences (<0.05) in TPCs content between rapeseed and sunflower oils during deep-frying process. At the beginning of deep-frying French fries in rapeseed oil, the acid number was 0.374 mg KOH.g^{-1} and 1.271 mg KOH.g^{-1} at the fourth day of deep-frying. The measured peroxide value was 4.3 mEq O_2.kg^{-1} at the beginning and at the end of deep-frying 10.5 mEq O_2.kg^{-1}. The initial peroxide and acid values were higher in sunflower oil compared with rapeseed oil, respectively. It should be note, then the acid values and peroxide values, respectively, in the two fresh oils used in this study were below the limit of refined oil according to Slovak legislation (peroxide value – not more than 10 mEq O_2.kg^{-1}, acid value – not more than 0.6 mg KOH.g^{-1}). However, detected values varied during deep-frying process. Monounsaturated fatty acids were predominantly observed in fresh rapeseed oil (61.22%) wherever in sunflower oil they were much lower (29.77%). A slight increase of MUFA was found in both oils. The initial content of saturated fatty acids in rapeseed oil was 6.94%, in fresh sunflower oil was observed slightly higher content of SFA (10.37%). The major groups of fatty acids in fresh sunflower oil were polyunsaturated fatty acids (PUFA) which have in principle a significant effect on oil deterioration. A slight decrease of PUFA was observed in both oils throughout the frying period. The content of PUFA was reduced by about 9.42% in rapeseed oil and by 10.8% in sunflower oil. The initial content was 28.14% and 58.91%, respectively.

Keywords: plant oil; deep-frying; total polar compound; peroxide and acid value; fatty acid methyl ester; oil deterioration

INTRODUCTION

Frying is a unit operation in which food is heated in oil to alter its eating quality, and destroy microorganisms to make safe of food, and for some foods to extend their shelf-life **(Romero et al., 2006; Tabee et al., 2009; Fellows, 2017)**.

Frying is a more efficient process in comparison with other cooking methods and has gained a high popularity both in restaurants and in industry. The benefits of the frying are its speed and operational simplicity. Even though deep-frying is an old and popular process, it is still poorly understood **(Ziaiifar et al., 2008; Zeleňáková et al., 2012; Gertz, 2014)**.

Temperatures used at frying are in the 150 – 200 °C range. In contrast to boiling in hot water the preparing of foods at elevated temperatures provides a desirable appearance (color), texture (crispness) and taste **(Erickson, 2007; Sebastian et al., 2014; Neethu et al., 2016).**

Deep frying is a cooking method in which food is submerged in hot fat, for example oil. Normally, a deep fryer or chip pan is used for this; industrially, a pressure fryer or vacuum fryer may be used **(Aladedunye and Przybylski, 2013; Srivastava and Semwal, 2015)**.

Deep frying food is a process where food is completely submerged in hot oil at temperatures typically between 350 °F (177 °C) and 375 °F (191 °C). Deep frying is commonly used for food preparations such as frozen pre-fried foods, snack foods and fast foods **(Fellows, 2009; Chen et al., 2013)**.

The nature and rate of decomposition products during frying depend mainly on the composition of the oil (fatty acids pattern, unsaponifiable matter content), the mode of frying (intermittent or continuous, shallow-, pan- or deep-frying), frying temperature, length of frying process, and type of food being fried. Many laboratory tests have been proposed for quality assessment of frying oils. There are also used a number of quick tests to screen oils easily at

the fryer **(Szabó et al., 2010; Crosa et al., 2014; Li et al., 2017)**.

The frying-process is a complex system depending on the chemical reactions like oxidation, polymerization of triglycerides (TAGs), and hydrolysis where the physical and chemical properties of the heated fat are altering. It is difficult to estimate the extent of influence of each factor and to keep the frying conditions at an optimum level. Excellent reviews are published in the scientific literature describing the oxidative and hydrolytic changes during frying process. Moisture in foods induces and accelerates oxidation with the hydrolytic compounds **(Gertz 2014; Perkins and Erickson, 2007; Tkáčová et al., 2015; Nieva-Echevarría et al., 2016)**.

Hydrolysis of TAGs occurs due to the presence of moisture from foods, releasing free fatty acids (FFAs), monoglycerides (MAGs) and diglycerides (DAGs). Unsaturated fatty acids in glycerides are prone to be oxidized under high temperature, generating oxidized glyceride compounds **(Dobarganes and Márquez-Ruiz, 2006)**. In addition, dimeric and oligomeric triglycerides are generated in the polymerization reactions **(Firestone, 2007; Choe and Min, 2007; Bansal et al., 2010)**.

TPCs content in deep-fried oils is a reliable indicator of oxidative degradation of frying oils **(Zribi et al., 2014; Aladedunye and Przybylski, 2013; Aniołowska and Kita, 2016)**.

Several authors **(Matthäus, Haase and Vosmann, 2004; Mareček et al., 2010; Sebastian et al., 2014; Crosa et al., 2014; Li et al., 2017)** have determined relative content of fatty acid methyl esters in vegetable oils such as soybean oil, sunflower oil, rapeseed oil, olive oil, coconut oil as well as palm oil (in fresh state and during deep-frying, respectively). Many of them have examined the effects of fatty acids profile on the formation of polar compounds and their retention in French fries, over deep-frying process.

The contents of free fatty acid (FFA) and total polar compounds is usually used for initial oil quality assurance and after-use frying oil quality assessment, respectively **(Lee, 2009; Chen et al., 2013)**.

In context with the above mentioned, the aim of this study was to examine the thermo-degradative changes of rapeseed and sunflower oils during deep-frying French fries.

Scientific hypothesis

The purpose of this study was to investigate thermo-degradative changes of rapeseed and sunflower oils during deep-frying French fries. In this context the purpose of our experiment was to examine the effect of deep-frying process on fatty acids composition, acid value, peroxide value and content of TPCs in used oils.

MATERIAL AND METHODOLOGY

Material

Deep-frozen French fries were purchased at a local supermarket and stored in the freezer at -18 °C until being analyzed. The total quantity of French fries that were used in the experiment was 2.5 kg. For deep-frying French fries were used edible rapeseed and sunflower oils bought from market. Both oils have wide spectrum of using in long-term and short-term thermal preparation of food (cooking, steaming, frying, baking) as well as in cold kitchen (salads, marinades, sauces etc.).

Deep frying French fries

A commercial deep-fat fryer (Siemens TG 15001/01 Kreis Pinneberg, Germany) of capacity 2 L was used for the frying of French fries' samples. Fresh rapeseed oil and sunflower oil was loaded into the fryer separately and heated to 170 °C before frying. The same batch of French fries (100 g) was deep-fried and the same frying conditions (4 min, 170 °C) were applied. Afterwards, the French fries were placed in a plate and extra oil was sucked using tissue paper. The frying procedure was held constantly for 4 continuous days (6 h per day according to reached 24% TPCs content). At the end of each day of frying the deep fryer was shut off and the oil was cooled down. Then, the oil was filtered to remove the solid residues. Time of oil sampling was different according to examined parameters (explained below). All experiments were done at least in duplicates. All analyzes oil samples were carried out at Department of food hygiene and safety, FBFS, SUA in Nitra and at Department of animal nutrition, FAFR, SUA in Nitra as well.

Experimental determinations

Examined parameters: TPCs – total polar compounds, acid value, peroxide value, fatty acids composition.

Measurement of TPCs content by oil tester Testo 270

TPCs estimation was based on dielectric constant changes directly measured on hot oil with deep frying oil tester Testo 270. The following measurements were performed with the Testo 270:

• Temperature of the deep-frying oil: Indicator for correct setting of the deep-fryer.

• TPCs content: Indicator for the deterioration of the deep-frying oil. The sensor works on a capacitive basis and determines the total amount of polar materials in % as the reading.

For quality analysis used oil samples (fresh and deep-fried) were taken every 30 min throughout the deep-frying process. Measurement of TPCs content in oils was carried out at an oil temperature 130 °C. Analyses were terminated when the TPCs content reached ≥24%, which means oil wear.

For quality analysis used oil samples were taken in fresh state as well as deep-fried every 30 min throughout the deep-frying process.

Determination of peroxide value according to ISO 3960:2007 (EN)

The oil samples were dissolved in isooctane and glacial acetic acid, and potassium iodide was added. The iodine liberated by the peroxides was determined iodometrically (visually) with a starch indicator and a sodium thiosulfate standard solution. The endpoint of the titration was determined iodometrically (visually). The peroxide value (PV) expressed in milliequivalents of active oxygen per kilogram (mEq $O2.kg^{-1}$) was calculated by the following equation:

$$PV = \frac{(V - V_0)\ x\ cthio\ x\ cstand\ x\ 1000}{m}$$

where:

V – is the volume of the 0.01 N sodium thiosulfate standard solution used for the determination, in millilitres.

V0 – is the volume of the 0.01 N sodium thiosulfate standard solution used for the blank test, in millilitres.

cstand – is the exact concentration of the 0.01 N sodium thiosulfate standard solution, in moles per litre.

cthio – is the approximate concentration of the 0.01 N sodium thiosulfate standard solution, in moles per litre (= 0.01)

m is the mass of the test sample, in grams.

Determination of acid value according to ISO 660:2009 (EN)

Acid value of fat was determined after dissolution of fat in the extract ethanol-diethyl ether in a 1:1 alkalimetric titration against phenolphthalein. The extracted fat was slightly heated, and fat was dissolved in 25.0 mL of ethanol-ether. The content in extraction flask was titrated with a few drops of the indicator with the potassium hydroxide solution until it turned to slight pink colour. An acid number of fat was expressed in mg KOH per g (mg $KOH.g^{-1}$). A fat acid value is the number of mg of potassium hydroxide required to neutralize free fatty acids per gram of fat extracted from the extracting agent.

For quality analysis used oil samples were taken in fresh state as well as deep-fried every 60 min throughout the deep-frying process.

$$AV = \frac{56.1\ x\ Vx\ c}{m}$$

Where:

AV : acid value

V – is the volume of the potassium hydroxide standard solution, in millilitres.

c – is the exact concentration of the potassium hydroxide standard solution, in moles per litre m is the mass of the test sample, in grams.

For quality analysis used oil samples were taken in fresh state as well as deep-fried every 60 min throughout the deep-frying process.

Determination of fatty acids composition by gas chromatography according to ISO 12966-1:2014

0.1 g oil samples were dissolved in 5 mL hexane; 1 mL 2 M KOH in methanol was added. The tubes were capped and stirred for 30 min to separate into two phases. The upper phase was analysed using gas chromatography. A 6890 GC with a Multi-Mode injector, a 7683B automatic liquid sampler and flame ionization detection (Agilent Technologies, Palo Alto, CA) were used. Separation was performed with a (60 m × 0.25 mm i.d. × 0.15 μm DB-23) column (Agilent 6890 GC). The temperature programme was an initial 50 °C with a 1 min hold, ramp 25 °C per min to 175 °C, then 2 °C per min to 230 °C with a 5 min hold, then 120 °C per min to 245 °C with an 8 min hold. Injector temperature was 250 °C. Carrier gas was H2 with a pressure of 238.96 kPa (2.225 $mL.min^{-1}$). Fatty acid analysis was performed by auto injection of 1 μL of each sample at a split ratio of 1.10^{-1}, constant flow mode, velocity 20.4 $cm.s^{-1}$. The flame ionization detector temperature was 280 °C with H2 35 $mL.min^{-1}$, air 350 $mL.min^{-1}$, and N2 make-up gas flow rates of 30 $mL.min^{-1}$, respectively. The run time for a single sample was 32 min. The fatty acid methyl esters were identified by comparing their retention times and mass spectrum with a mixture standard FAME.

For quality analysis used oil samples were taken in fresh state as well as deep-fried every day after 6-hour of deep-frying process.

Statistic analysis

Mathematical and statistical evaluation of the results was realized by the SAS Enterprise Guide Version 1.5 system program. Measurements of duplicate samples were expressed as means ± standard deviation. The data were subjected to the analysis of variance (ANOVA) in the general linear models (GLM), t-test, Scheffe´s test and Pearson correlation coefficients (rxy). The level of significance associated to the statistical test was 0.05.

RESULTS AND DISCUSSION

Deep frying is cooking method in hot-fat. Typically, deep frying cooks foods quickly: all sides of a food are cooked simultaneously as oil has a high rate of heat conduction **(Koh and Surh, 2015)**. However, only very few researches related to the influences of oil and food types on frying oil quality can be found.

Both goal setting and implementation of the experiment itself were based on requirements of Slovak legislation **(Decree No. 125/2017)**. It requires that deep-fat frying be carried out in accordance with good manufacturing practice and frying fats should not be heated above 180 °C (not longer than 24 hours of continuous frying). The law specifically forbids preparation of fried foods in equipment not provided with temperature control. However, practice shows that, especially, operators of fast food restaurants often violate these requirements and use "frying" oils for several days.

Our research was focused on analysis of thermo-degradative changes of rapeseed and sunflower oils during deep-frying French fries. The following indicators of excessive oil deterioration were investigated: content of total polar compounds, peroxide and acid value, fatty acids composition.

TPCs in oils during deep-frying French fries

The polar compounds were identified as oligomeric triglycerides, dimeric triacylglycerol, oxidized triacylglycerols, diacylglycerols, and free fatty acids **(Aladedunye and Przybylski, 2013; Aniołowska and Kita, 2015)**. Recommended and widely accepted limits are 24% for TPcs and 12% for PTG.

When oxidative alterations strongly predominate over thermal alterations, sensory defects can appear before TPCs and PTG reach recommended values. In that case additional parameters like anisidine value, carbonyl value, or epoxy fatty acids should be considered. There are many available physical and chemical rapid methods. Despite the limited informative value and the possibility of error of rapid tests, they are essential for fryer operators, because

they deliver information about fat quality in real-time **(Weisshaar, 2014)**.

Since the degradation of frying oil is greatly accelerated by foods, the frying condition should be controlled carefully. As is shown in Figure 1, TPCs values measured by Testo 270 grown in both oils continuously since the first deep-frying. Lower TPCs content was found in rapeseed oil (3.3%) and the threshold (24%) was achieved on the fourth day. The total time for the deterioration of deep-frying rapeseed oil was 23½ hours. On the contrary, in fresh sunflower oil at the first day was TPCs content 5.5% and the limit of 24% was reached on the third day. The total time for the deterioration of deep-frying sunflower oil was 17½ hours.

The results in this study indicate significant differences (p <0.05) in TPCs content between rapeseed and sunflower oils during deep-frying process (Table 1). The statistical analysis (Scheffe´s test) showed comparable results in both oils between days and hours of deep-frying French fries. TPCs content was similar or lower at 8 o´clock compared to previous day (at 14 o´clock). However, this finding was not statistically significant (p >0.05). Deeper analysis using Pearson correlation coefficients (rxy) by **Cohen (1988)** showed that between days and hours of deep-frying French fries in rapeseed oil was found high positive linear correlation (p <0.001) between A08 and B08, C14; B08 and C14; B14 and C08. The other linear correlations were other medium negative (p >0.05) or none. However, high positive linear correlation (p <0.001) according TPCs content in sunflower oil during deep-frying time was found at the end of the first frying day and B08, C14, as well as at the beginning of the second frying day and C14 (at the end of the third frying day). In other results were no linear correlations. However, it can be said that deep-frying process directly increases TPCs content (p <0.05).

Similar research was realized by **Zeleňáková et al. (2012)**. Two kinds of rapeseed vegetable oils were used for continuously deep-frying French fries and outside deep-frying process were stored in the room temperature and in the refrigerator, respectively. The both rapeseed oils were from different producers and had different composition related to fatty acid. These factors have significantly affected achieved TPCs amounts. The oil with higher content of oleic acid achieved the 24% TPCs after 22 hours (at room temperature) and 26½ hours (in the refrigerator) of deep-frying. The low erucic acid rapeseed oil was less stable (19 hours and 22½ hours, respectively).

For public health concerns, the content of total polar compounds in frying oil is regulated at not more than 25%, in Taiwan **(Lee, 2009)**. **Chen et al. (2013)** published that the contents of TPCs in soybean oil and palm olein, respectively, were shown to exceed the limit of 25% after 48 h of frying with foods.

Table 1 Changes in TPCs, peroxide value and acid value in sunflower and rapessed oils during deep-frying French fries.

Oil sampling time	TPCs (%)		Peroxide value (mEq O_2.kg^{-1})		Acid value (mg KOH.g^{-1})	
	Rapeseed oil	Sunflower oil	Rapeseed oil	Sunflower oil	Rapeseed oil	Sunflower oil
A08	3.33 ±0.29^{a}	5.50 ±0.01	4.33 ±0.50^{b}	1.00 ±0.80	0.37 ±0.13^{d}	0.60 ±0.13
A14	8 ±0.02^{a}	10.83 ±0.29	5.73 ±0.70^{a}	14.60 ±1.91	0.60 ±0.13^{d}	0.60 ±0.13
B08	7.33 ±0.29^{a}	10.83 ±0.29	4.20 ±0.20^{b}	10.67 ±1.85	0.45 ±0.014^{d}	0.82 ±0.13
B14	12.83 ±0.29^{a}	16.50 ±0.02	6.67 ±0.23^{b}	15.13 ±3.21	0.90 ±0.22^{d}	0.75 ±0.13
C08	12.83 ±0.29^{a}	17.00 ±0.01	5.00 ±0.20^{a}	11.93 ±1.21	0.60 ±0.26^{d}	0.90 ±0.45
C14	17.83 ±0.29^{a}	24.33 ±0.29	7.80 ±0.53^{c}	12.73 ±2.57	1.42 ±0.56^{d}	1.50 ±0.52
D08	18.50 ±0.03	-	8.07 ±0.61	-	1.12 ±0.22	-
D14	24.50 ±0.02	-	10.53 ±0.70	-	1.27 ±0.34	-

Note: Least square means and standard deviations of the variables analysed according to period of deep-frying. Lowercase letters indicate significant differences between means of the rapeseed and sunflower oil according to examined indicators by t-test a(p <0.001), b(p <0.01), c(p <0.05),d(p >0.05). Oil sampling time: A/B/C/D – first/ second/ third/ fourth day of deep-frying 08/14 – at 8 am/at 2 pm.

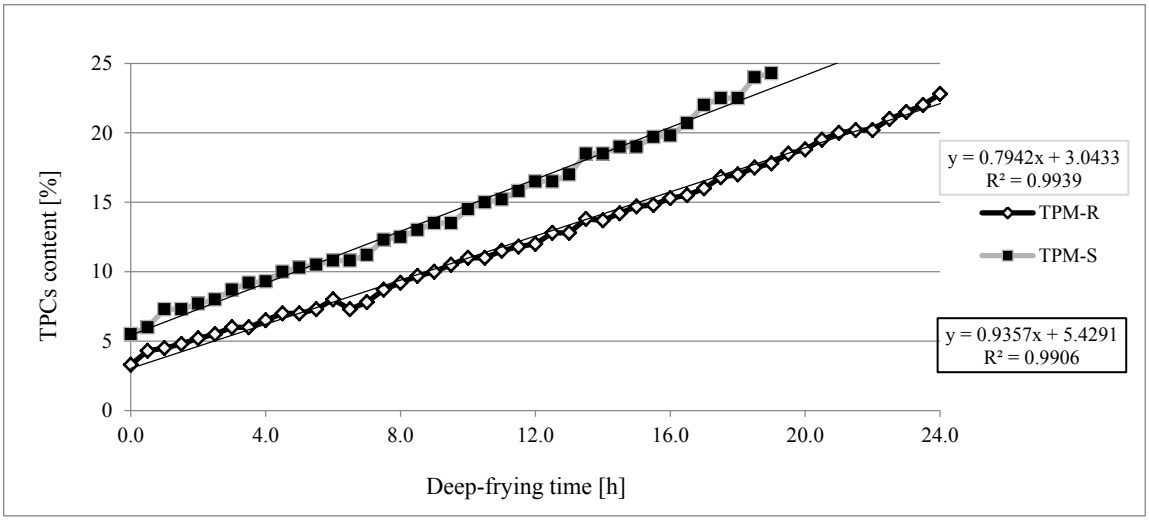

Figure 1 Determination of TPCs content in rapeseed (R) and sunflower (S) oils during deep-frying French fries.

Table 2a Parameters of linear regression for TPCs in sunflower and rapessed oils during deep-frying French fries.

TPCs (%)		
Summary output	**Rapeseed oil**	**Sunflower oil**
Model	y = 3.04 + 0.7942x	y = 5.43 + 0.9357x
Multiple R (*r*)	0.997	0.995
R Square (R^2)	0.994	0.991
p – value	$5.24.10^{-57}$	$3.92.10^{-39}$
Intercept	3.04	5.429
Slope	0.794	0.,936

Table 2b Parameters of linear regression for peroxide value in sunflower and rapessed oils during deep-frying French fries.

Peroxide value (mEq $O_2.kg^{-1}$)		
Summary output	**Rapeseed oil**	**Sunflower oil**
Model	y = 3.48 + 0.2481x	y = 6.49 + 0.4605x
Multiple R (*r*)	0.933	0.689
R Square (R^2)	0.871	0.475
p – value	$4.49.10^{-13}$	0.0006
Intercept	3.48	6.485
Slope	0.25	0.461

Table 2c Parameters of linear regression for acid value in sunflower and rapessed oils during deep-frying French fries.

Acid value (mg $KOH.g^{-1}$)		
Summary output	**Rapeseed oil**	**Sunflower oil**
Model	y = 0.31 + 0.038x	y = 0.29 + 0.063x
Multiple R (*r*)	0.886	0.719
R Square (R^2)	0.785	0.517
p – value	$3.71.10^{-10}$	0.0002
Intercept	3.11	0.289
Slope	0.038	0.063

As it was mentioned, over the frying process, TPCs increased linearly with the frying time in both oils. The progress of each linear function was defined by regression equation and the reliability of all determinations was expressed by determination coefficients (R^2). As shown Figure 1, level of detection reliability (99.4 and 99.1%, respectively) was comparable for both oils. In context with this, between the time of deep-frying and TPCs content was found high positive linear correlation r = 0.997 (rapeseed oil) and r = 0.995 (sunflower oil). The slope of regression line over time for rapeseed oil frying with French fries was 0.794 and that for sunflower oil was 0.936, indicating that the rate of TPCs formation in rapeseed oil frying with foods was lower than that of sunflower oil (Table 2).

Similar results were also recorded by **Chen et al. (2013)** during frying French fries, chicken leg fillets and pork chops. The content or TPCs in both soybean oil and palm olein increased linearly with frying time. The influence of oil type on the content of TPCs in used oils was significant. It is important to mention that TPCs was measured by standard method (column chromatography) and two rapid-measuring devices (Ebro FOM 310 and Testo 270). They found that Testo 270 was suitable for palm oil while Ebro FOM 310 was more suitable for monitoring the quality of soybean oil.

The kinetics of wide spectrum of oxidation products and acrylamide formation depend on the temperature and time of frying determine. This formation starts at temperatures above 120 °C and the maximum rate takes place at temperatures higher than 170 – 180 °C **(Pedreschi and Moyano, 2005; Knol et al., 2009)**. The same batch of French fries (100 g) was deep-fried and the same frying conditions (4 min, 170 °C) were applied in our research.

Polar compounds and TAG oligomers which created during deep-fat frying course with content range of 20 – 27% and 10 – 16%, respectively have been proposed to determinate the rejection of used frying oil **(Zhang et al., 2012)**. However, the measurement of polar compounds doesn't represent the whole content of reaction products formed during the deep-fat frying. Some products were considered as potential detrimental substances to the human body from the nutritional aspect **(Saguy and Dana, 2003; Totani et al., 2008)**.

Organoleptic as well as chemical changes in French fries during frying were investigated by **Andrés et al., 2013; Bingol et al., 2014; Ignat et al., 2015**.

Acid and peroxide value in oils during deep-frying French fries

As was discussed in the **(EFL, 2011)** for fresh oils, the acid value is routinely used as a selective indicator for the degradation status.

Oxidation may be initiated by the formation of lipid peroxides. Initial phases of lipid oxidation can be detected by measuring the peroxide value, which quantifies the levels of peroxides and hydroperoxides formed at that stage **(Bennett et al., 2014)**.

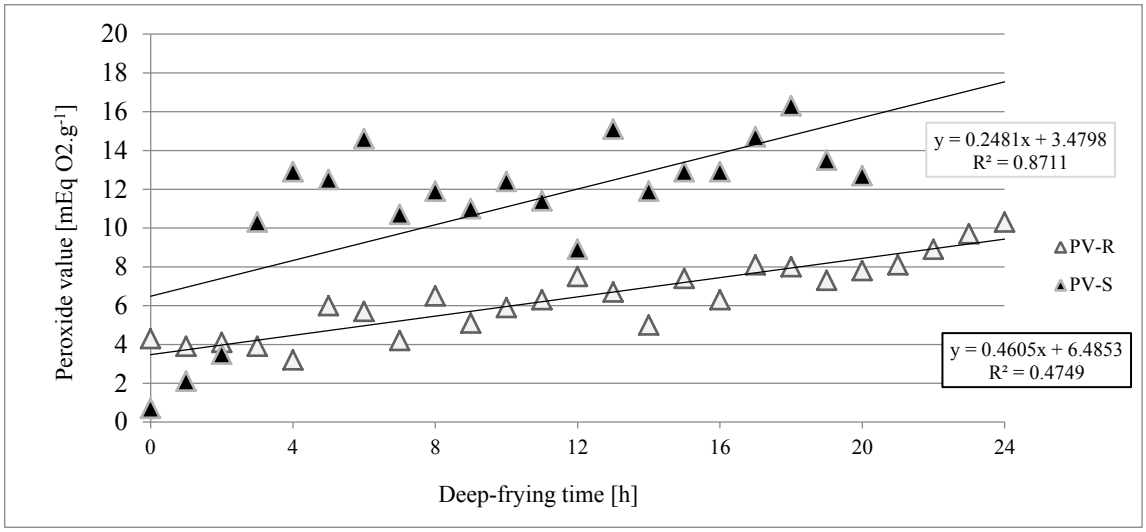

Figure 2 Determination of peroxide value in rapeseed (R) and sunflower (S) oils during deep-frying French fries.

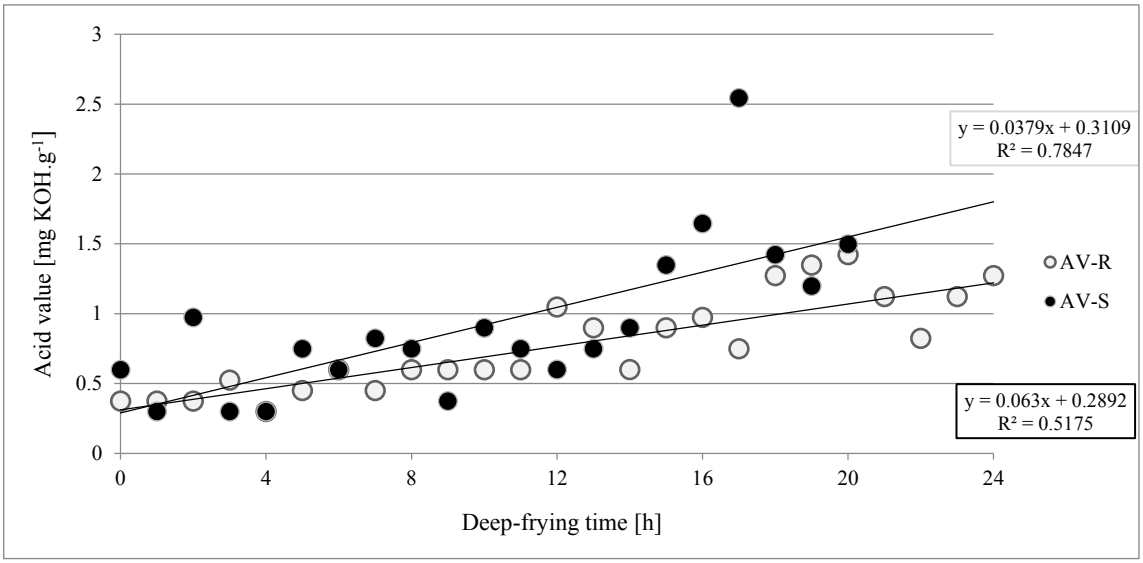

Figure 3 Determination of acid value in rapeseed (R) and sunflower (S) oils during deep frying French fries fries.

Throughout the deep-frying process were in rapeseed oil and sunflower oil measured acid values and peroxide values. At the beginning of deep-frying French fries in rapeseed oil, the acid number was 0.374 mg KOH.g^{-1} and 1.271 mg KOH.g^{-1} at the fourth day of deep-frying. The measured peroxide value was 4.3 mEq O2.kg^{-1} at the beginning and at the end of deep-frying 10.5 mEq O2.kg^{-1}. The initial peroxide and acid values, respectively, were higher in sunflower oil compared with rapeseed oil. The initial acid value in deep-fried sunflower oil was 0.598 mg KOH.g^{-1} and at the end of deep-frying French fries (third day) 1.497 mg KOH.g^{-1}. The measured peroxide value was 0.7 mEq O2.kg^{-1} at the beginning and at the end of deep-frying 12.7 mEq O2.kg^{-1}. It should be note, that the acid values and peroxide values, respectively, in the two fresh oils used in this study were below the limit of refined oil according to Slovak legislation **(Decree No. 424/2012)**. This regulation laying down requirements for edible vegetable fats and edible vegetable oils and their products (peroxide value – not more than 10 mEq O2.kg^{-1}, acid value – not more than 0.6 mg KOH.g)$^{-1}$. However, detected values varied during deep-frying process.

Peroxides are known to be unstable and volatile, as can be seen from Figure 2. It follows that sunflower oil is responsible to thermal degradation and is not suitable for long-term heat treatment.

As it was mentioned, over the frying process, TPCs increased linearly with the frying time in both oils. The linear regression was also used in evaluating the reliability of peroxide and acid value detection (Figures 2 and 3). However, unlike TPCs detection, in these detections was found a lower reliability indicated by R^2. The determination coefficients were 0.8711 (peroxide value) and 0.7847 (acid value) in rapeseed oil and 0.4749 (peroxide value) and 0.5175 (acid value) in sunflower oil. The other parameters of linear regression for both values are shown in the Table 2. This finding is explained by many authors **(Alander and Lidefelt, 2007; Nieva-Echevarría et al., 2016; Li et al., 2017)**.

During deep-frying process (125 °C), rapid oxidation occurs (accumulation of hydroperoxides) and peroxide value increased. However, but upon further heating at high frying temperatures, the peroxide value again decreases, because accumulated hydroperoxides are decomposed

more rapidly and other secondary degradation products are formed. The rise of the peroxide value therefore occurs at a time when the oil cools and is not used.

It can be concluded that measurement of the peroxide value is more suitable for measuring the quality of fresh oil than for measuring the quality of oil during frying and deep-frying, respectively **(Koh and Surh, 2015; Naz et al., 2005; Juárez et al., 2011)**. For public health concerns, the acid value in frying oil is regulated at not more 2.0 mg KOH.g^{-1}, in Taiwan **(Lee, 2009)**.

From the results of **Chen et al. (2013)** soybean oil contained 0.03 mg KOH.g^{-1} of acid value, while palm olein contained slightly higher acid value (0.071 mg KOH.g^{-1}).

Table 3a Fatty acids composition (%) of deep-fried oils during frying process (rapeseed and sunflower oil).

FAMEs (wt %)	Rapeseed oil				
	fresh oil	after 1 day	after 2 days	after 3 days	after 4 days
palmitic acid C16:0	4.52 ±0.00	4.64 ±0.00	4.76 ±0.01	4.91 ±0.00	5.11 ±0.01
stearic acid C18:0	1.49 ±0.00	1.55 ±0.00	1.59 ±0.00	1.67 ±0.00	1.75 ±0.01
oleic acid C18:1cis n9	59.64 ±0.00	59.97 ±0.01	60.29 ±0.01	60.48 ±0.01	60.54 ±0.02
linoleic acid C18:2cis n6	18.96 ±0.00	19.00 ±0.00	18.92 ±0.00	18.93 ±0.01	18.89 ±0.01
arachidic acid C20:0	0.52 ±0.00	0.52 ±0.00	0.53 ±0.00	0.54 ±0.00	0.55 ±0.00
cis-11-eicosenoic acid C20:1 n9	1.23 ±0.00	1.23 ±0.00	1.23 ±0.01	1.22 ±0.00	1.21 ±0.00
behenic acid C22:0	0.31 ±0.00	0.31 ±0.00	0.32 ±0.00	0.34 ±0.00	0.36 ±0.00
lignoceric acid C24:0	0.11 ±0.01	0.12 ±0.00	0.12 ±0.00	0.13 ±0.00	0.15 ±0.00
caprylic acid C8:0	-	-	-	-	0.12 ±0.00
myristic acid C14:0	-	-	-	-	-
palmitoleic acid C16:1	0.22 ±0.00	0.22 ±0.00	0.23 ±0.00	0.23 ±0.00	0.23 ±0.00
α-linolenic acid C18:3 n3	9.19 ±0.00	8.58 ±0.00	8.00 ±0.00	7.28 ±0.01	6.41 ±0.02
erucic acid C22:1 n9	0.13 ±0.00	0.13 ±0.00	0.14 ±0.00	0.14 ±0.00	0.14 ±0.01
cis-13.16-docosadienoic acid C22:2	-	-	-	0.06 ±0.08	0.19 ±0.00
PUFA polyunsaturated fatty acids	28.14	27.58	26.92	26.26	25.49
MUFA monounsaturated fatty acids	61.22	61.56	61.88	62.06	62.11
SFA saturated fatty acids	6.94	7.14	7.33	7.59	8.03
ratio Σn3/Σn6	0.48	0.45	0.42	0.38	0.34
ratio Σn6/Σn3	2.07	2.22	2.36	2.60	2.95

Table 3b Fatty acids composition (%) of deep-fried oils during frying process (rapeseed and sunflower oil).

FAMEs (wt %)	Sunflower oil			
	fresh oil	after 1 day	after 2 days	after 3days
palmitic acid C16:0	6.13 ±0.00	6.91 ±0.01	7.77 ±0.01	8.59 ±0.01
stearic acid C18:0	3.05 ±0.00	3.16 ±0.00	3.29 ±0.00	3.43 ±0.00
oleic acid C18:1cis n9	29.51 ±0.01	30.26 ±0.01	30.99 ±0.01	31.81 ±0.04
linoleic acid C18:2cis n6	58.82 ±0.00	57.31 ±0.00	55.27 ±0.07	52.55 ±0.06
arachidic acid C20:0	0.22 ±0.00	0.23 ±0.00	0.24 ±0.00	0.25 ±0.00
cis-11-eicosenoic acid C20:1 n9	0.16 ±0.00	0.16 ±0.00	0.16 ±0.00	0.16 ±0.00
behenic acid C22:0	0.73 ±0.00	0.74 ±0.01	0.76 ±0.01	0.77 ±0.00
lignoceric acid C24:0	0.25 ±0.01	0.26 ±0.00	0.27 ±0.00	0.28 ±0.01
caprylic acid C8:0	-	-	-	0.17 ±0.00
myristic acid C14:0	-	-	0.11 ±0.00	0.13 ±0.00
palmitoleic acid C16:1	0.11 ±0.00	-	0.05 ±0.08	0.05 ±0.08
α-linolenic acid C18:3 n3	0.09 ±0.00	-	-	-
erucic acid C22:1 n9	-	-	-	-
cis-13.16-docosadienoic acid C22:2	-	-	-	-
PUFA polyunsaturated fatty acids	58.91	57.31	55.27	52.55
MUFA monounsaturated fatty acids	29.77	30.42	31.22	32.03
SFA saturated fatty acids	10.37	11.31	12.44	13.63
ratio Σn3/Σn6	0.00			
ratio Σn6/Σn3	628.64			

Other studies **(Gerde et al., 2007; Tabee et al., 2009)** also showed that the acid value in palm olein were higher than those in soybean oil.

Table 1 lists the acid value and peroxide value in both analysed oils with statistical evaluation. The influence of oil type on the peroxide value in used oil was significant (p <0.05), but the effect of oil type on acid value was not observed (p >0.05).

Our results partially correspond with those from **Chen et al. (2013)**, who used palm and soybean oil for frying French fries. They found significant influence of oil type on acid value in these oils.

The One-way ANOVA test was used to determine the statistical significance between two types of frying oils according to changes of peroxide value and acid value during the frying time. Post-Anova (Scheffe´s test) was used where the F value was significant.

No significant differences in the peroxide value of rapeseed oil were observed in the first two days of frying. The first significant differences were found between B14 and A08, B08. Interestingly, no significant differences in the peroxide value were observed at the beginning of the third frying day and A08, A14, B08 and B14, but there were significant differences at the end of the third frying day and A08, A14, B08, C08. At the beginning of the last frying day, there were observed significant differences compared with A08, A14, B08 and C08. The peroxide value was significant different at the end of the last frying day compared with the first three frying days.

In sunflower oil, there is a significant difference in peroxide value measured at the beginning of frying (A08) and all other measurements. There were observed no significant differences between A14, B08, B14, C08, C14.

In the determination of acid value in both oils, there were no significant differences among days and hours of deep-frying rapeseed oil and sunflower oil, respectively.

Fatty acid methyl esters composition in oils during deep-frying French fries

Fatty acids composition is one of the most crucial factors determining the oxidative stability of oil. Fatty acids profile changed during frying process.

In the present study, fatty acid profiles in rapeseed and sunflower oil were analysed and compared over the deep-frying process of French fries at temperature 170 °C. As is shown in the Table 3, the content of PUFA, MUFA, and SFA in fresh oils was different but other changes in quantity due to the deep-frying in these oils were comparable (increasing and decreasing, respectively).

Monounsaturated fatty acids (MUFA) were predominantly observed in fresh rapeseed oil (61.22%) wherever in sunflower oil they were much lower (29.77%). A slight increase of MUFA (from 61.22 to 62.1 and from 29.77 to 32.03%) was found in both oils. Moreover, the saturated fatty acids (SFA) in fresh oils were determined and the trend of increasing due to deep-frying was quite similar to MUFA. The initial content of saturated fatty acids in rapeseed oil was 6.94%, over the deep-frying process increased to 7.14, 7.33, 7.59 and 8.03%. In fresh sunflower oil was observed slightly higher content of SFA (10.37%) and it increased with the increase of frying time (to 13.63%).

The major groups of fatty acids in fresh sunflower oil were polynusaturated fatty acids (PUFA) which have in principle a significant effect on oil deterioration. A slight decrease of PUFA was observed in both oils throughout the frying period. The content of PUFA was reduced by about 9.42% in rapeseed oil and by 10.8% in sunflower oil. The initial content was 28.14% and 58.91%, respectively.

The impact of the fatty acid composition on lipid oxidation was evaluated by measuring the relative percentage of unsaturated, polyunsaturated and saturated fatty acids. **Roman et al. (2013)** reported a higher reactivity of PUFA than MUFA (-linolenic acid > linoleic acid > oleic acid) in sunflower oil, as had previously been shown by different studies **(Parker et al. 2003; Martin-Polvillo et al., 2004)**.

The major fatty acid in fresh rapeseed oil was oleic acid (59.64 ±0.00%) while in fresh sunflower oil dominated linoleic acid (58.82 ±0.00%). A slight increase of oleic acid (C18:1) was observed in both fried oils examined. At the end of frying period, oleic acid increased by 1.5% in rapeseed oil and by 7.79% in sunflower oil. Initial content of oleic acid in sunflower oil was 29.51 ±0.01%.

In contrary, amount of oleic acid decreased slightly from 38.70% to 32.06% in palm oil over the frying process. However, oleic acid content in the other two kinds of oils (palm kernel oil and coconut oil) remained unchanged (approximately 17% and 8%, respectively) during frying period **(Li et al., 2017)**. The high oleic acid is reported as a better oil compared to regular sunflower, soybean, corn and peanuts oils due to its good thermal and oxidative stability during traditional frying **(Smith et al., 2007; Marmesat et al., 2012)**. Chemometric analysis showed, that there was no correlation between the polar compounds level and saturated fatty acids profile. It can be also assumed that high-oleic oils show a lower polar compounds level after a period of deep-frying compared to the oils with less oleic acid **(Abdulkarim et al., 2007; Zribi et al., 2014)**. Among frying oils, those with high oleic acid content such as palm olein have better health profile and heat stability **(Tabee et al., 2009)**.

In the case of palmitic (C16:0) and stearic acids (C18:0), they increased with the increase of frying time as well in both kinds of oils (from 4.52 to 5.11% and 1.49 to 1.75%, respectively, in rapeseed oil; from 6.13 to 8.59% and 3.05 to 3.43% in sunflower oil). Notably, even though no caprylic acid was observed in fresh rapeseed and sunflower oils, it increased to 0.12% and 0.17%, respectively, at the end of the frying procedure. The similar results were found in content of myristic acid (C14:0).

Caprylic acid and capric acid were observed reduced in refined palm kernel oil and refined coconut oil. The medium-chain fatty acids are volatile under the high temperature **(Amri, 2011)**. According to **Zribi et al. (2014)**, the fatty acids composition influences the generation of polar compounds over the frying process. Higher percentage of unsaturated fatty acids (UFAs) in the oils resulted in a higher level of polar compounds during the frying process. However, this was inconsistent with the present study.

Figure 4 French fries prepared by rapeseed oil (A) and sunflower oil (B).

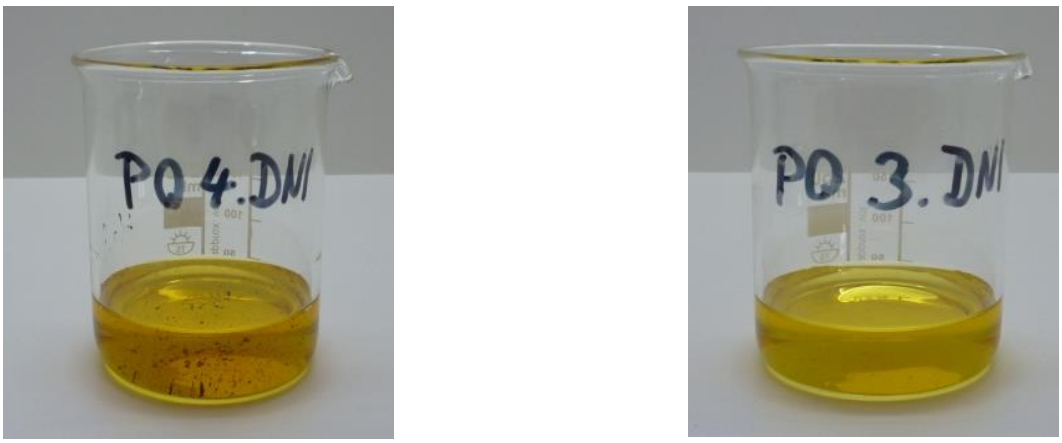

Figure 5 Visual changes in rapeseed oil (A) and sunflower oil (B) after 4 and 3-day deep-frying French fries.

In our study fresh rapeseed oil was found to have 18.96% of linoleic acid (C18:2) and 9.19% of linolenic acid (C18:3). The level of linoleic acid in fresh sunflower oil (58.82%) was higher than this in the rapeseed oil analysed. On the contrary, fresh sunflower oil contained only 0.09% of linolenic acid. The linoleic acid content was almost unchanged during frying process in rapeseed oil. Slight decrease was found in sunflower oil (from 58.82 to 52.55%).

It is necessary to note, that linolenic acid concentration was not detectable from the second day of deep-frying French fries in sunflower oil. The deterioration of (α) linolenic acid was more pronounced, with its contents being decreased by 30.25% in the last day of deep-frying French fries in rapeseed oil. Similar results were found by **Chen et al. (2014)** who detected by 20.86% lower linolenic acid content in palm oil. **Aladedunye and Przybylski (2009)** reported decreases of 13.3% in linoleic acid and 47.1% in linolenic acid when fresh canola oil was heated at 215 °C for 7 days.

Fresh soybean oil was reported to have 21.5% of oleic acid (C18:1), 53.4% of linoleic acid (C18:2) and 5.1% of linolenic acid (C18:3) and saturated fatty acid **(Juárez et al., 2011)**. It is important to note that soybean oil is commonly used for deep fat frying by Korean school meal services.

In our study, rapeseed oil has n-6/n/3 ratio around 2. This result is similar to this from **Dubois et al. (2007)** who analysed soybean oil (around 6). Flaxseed oil stands out for containing high levels of n-3, and sunflower oil stands out for introducing high n-3 values. The mixture of these oils can result in a mix with higher concentrations of polyunsaturated fatty acids and different n-6/n-3 ratios.

Recent studies have been demonstrated that mixtures of vegetable oils might modify the fatty acid profile and improve the stability of oils **(Wang et al., 2006; Meinhart et al., 2017)**.

Industrial food producers should understand the thermo oxidative changes in their frying oils, especially below 15% polar compounds. In fast food restaurants where the products are prepared for the direct consummation the limits of degradation are much higher (i.e. 24% polar materials) and may be monitored by units that measure and related dielectric constant to the degree of degradation **(EFL, 2011)**.

Finally, there is a discussion of the effect of frying on the sensory characteristics of foods, changes to their nutritional value, healthy concerns over fried foods and methods to reduce their fat contents.

CONCLUSION

Good understanding of the frying process helps in optimizing the manufacturing processes with regard to quality of food and use life of fat and of energy consumption. To guarantee a good quality of the fried end product it is necessary to install a management system which includes all critical points of the frying process. Variables involved in the process include frying conditions, replenishment of fresh oil, original oil quality, food materials, and fryer type. Oil for frying should be selected by its performance during the frying process. Nowadays, besides thermal-oxidative stability, handling (good melting profile), availability and price, the

nutritional-physiological features (good fatty acid balance, low trans-fatty acids, no allergens, no GMO) are important points of decision. There is no ideal fat suitable for all frying applications.

Our results, supported by other studies, provided the basis for choosing the suitable vegetable oil as well as rapid-measuring device to control the quality of frying oil in restaurants.

Based on our findings, we suggest:

- for frying and deep-frying to use only oils for that purpose,
- to keep the set temperatures and time with respect to the type of food,
- to keep the established ratio between food and oil,
- to filter the used oil at the end of the frying day to remove food debris,
- to store the oil in a dark and cool place,
- regularly check the oil quality,
- immediately to change oil showing the deterioration (exceeded smoke point and TPCs of 24%).

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GOAT YOGHURT DRINKS WITH ELEVATED α-LINOLENIC ACID CONTENT AND ENRICHED WITH YACON FIBER

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ABSTRACT
Goat milk and goat milk products are very valuable in human nutrition because of their favorable nutrient composition which can be further boosted by the addition of prebiotic fiber and probiotic bacteria. It has also been possible to change the fatty acid profile of goat milk through feed composition. The aim of this study was to increase the nutritional value of goat milk by producing a probiotic yoghurt drink made from milk with elevated omega-3 fatty acids and enriched with natural yacon prebiotics. Goat nutrition is one of the key factors how we can naturally increase omega-3 fatty acid content in goat milk. In our study, twenty four White Shorthair goats were divided into the control and experimental group which was supplemented with 55 mL of linseed oil per day for eight weeks to increase the monounsaturated and polyunsaturated fatty acid content in the milk. The yoghurt milk drinks were formulated from individual goat milk samples with added bifidobacteria and yacon prebiotics. Our results showed that goat feed supplementation with linseed oil indeed positively changed fatty acid profile of goat milk in which α-linolenic acid content increased while, at the same time, lauric, myristic and palmitic acid contents decreased. Also, yoghurt drinks enriched with yacon prebiotics have shown higher bifidobacteria counts compared to the control.

Keywords: fatty acid; goat milk; α-linolenic acid; linseed oil; yacon

INTRODUCTION
In the last few years, the number of goat farms (in particular operating under organic farming practices) has been increasing not only in the Czech Republic but also worldwide leading to greater production of goat milk **(Josrová, 2018)**. Therefore, farmers and dairy producers are actively seeking to develop new products and formulas which would attract consumers' attention. Goat milk represents a welcome alternative to products made from cow's milk. For instance, products made from goat milk are considered to be healthier because goat milk shows better nutritional characteristics like digestibility due to smaller diameter of fat globules (3.5 μm in goat milk compared to 4.5 μm in cow's milk) which causes the fat to be better dispersed in the milk **(Park et al., 2007)**. Goat milk contains also a higher amounts of caproic, caprylic and capric acids (in total 15 – 18% compared to 5 – 9% in cow's milk) that were shown to be beneficial to humans **(Sanz Sampelayo et al., 2007)**. These short-chain fatty acids have been proven to help patients with malabsorption syndrome, intestinal disorders, coronary heart diseases, cystic fibrosis and other conditions **(Haenlein, 2004; Ribeiro and Ribeiro, 2010)**. Furthermore, it is possible to increase the content of omega-3 fatty acids in goat milk by supplementing regular goat feed with moderate amounts of linseed oil which is rich in α-linolenic acid as described in **Borková et al. (2018)**. Differences in milk casein content were also found to impact human health. Goat α_{S1}-casein content is lower (typically 10 – 13% of total casein content) compared to cow's milk and depends on the particular genetic variant for α_{S1}-casein. The presence of null alleles for α_{S1}-casein such as 0_1, 0_2, 0_4 and N cause the absence of α_{S1}-casein in goat milk **(Caboni et al., 2016)** which is an important factor in decreasing the allergic sensitation of α_{S1}-casein **(Ballabio et al., 2011; Hochwallner et al., 2014)**.

Many more possibilities are available to boost nutritional impact of goat milk products as using milk high in omega-3 essential fatty acids, probiotic bacteria and prebiotics. Probiotic bacteria are well known for their antimicrobial, antioxidant and immunomodulatory effects **(Lee et al., 2011; Tomáška et al., 2015)**. Prebiotics are more or less indigestible components of foods comprising fructooligosaccharides (FOS) which positively influence the growth and activity of probiotic bacteria in the gastrointestinal system **(Valcheva and Dieleman, 2016)**. FOS are oligosaccharides consisting of D-fructose chains connected by β-(1→2) glycosidic bonds and capped by glucose. The yacon (*Smallanthus sonchifolius*) plant belongs to the *Asteraceae* family and grows in eastern Andes from Venezuela to northern Argentina **(Caetano et al., 2016)**. Yacon tuber is a great source of FOS **(Topolska**

et al., 2015) which do not undergo hydrolysis by human intestinal enzymes and are instead selectively fermented by bacteria in the colon leading to a more balanced composition of gut microbiota. Dry yacon tubers contain up to 50% (very rarely up to 70%) of FOS **(Velez et al., 2013)**.

The aim of this study was to increase the nutritional value of goat milk by producing a probiotic yoghurt drink made from goat milk with elevated omega-3 fatty acids and enriched with natural yacon prebiotics.

Scientific hypothesis

This research aimed to confirm the fact that milk from goats supplemented with linseed oil can be successfully used to produce dairy products with elevated polyunsaturated fatty acids. A special interest was paid to the use of natural yacon prebiotics to elevate the numbers of bifidobacteria in such products to increase their health benefits.

MATERIAL AND METHODOLOGY

Animals, animal diet, feed analysis

The experiment was carried out on a private organic farm near Liberec (Czech Republic) using the following set-up: Twenty four White Shorthaired dairy goats (on their third lactation) were selected based on age (3 years), date of kidding (between March and April 2017) and litter size (2 kids) and divided into experimental (LO) and control (C) groups, each comprising twelve animals. The goats were housed indoors and their feed consisted of: hay (*ad libitum*), haylage (3 kg per animal per day) and grain mix of 50% corn, 25% barley and 25% oat (1 kg per animal per day). Goats in the LO group were fed basic ration supplemented with 55 mL of linseed oil (LO) per animal per day and the C group was fed the same diet without LO supplement. The LO supplementation begun on May 17 and lasted for eight weeks till July 11, 2017. LO was supplied by 1. zemědělská a.s. (Chorušice, Czech Republic).

The animal feed was sampled on July 11, 2017 and analyzed according to methods published in **Regulation (EC) No 152/2009** for dry matter, ash, crude protein, ether extract and crude fiber. The fatty acid analysis in feed including lipid extraction was carried out as described by **Kubelková et al. (2013)**. Results are given in Table 1.

Milk sampling and analysis

Individual milk yield measurements and milk analyses were carried out at three time points – at the beginning (week 0), in the middle (week 4) and at the end (week 8) of the experiment. Results are shown in Table 2. Milk samples were analyzed for fat, protein and total solids by IR milk analyzer DairySpec FT (Bentley Instruments). The instrument has been calibrated using a set of goat milk samples which were simultaneously analyzed by reference methods in accredited laboratory (MILCOM a.s., Prague).

Two pooled milk samples were created to mimic standard practices on the farm. Pooled samples were created from individual milk samples (taken on July 11, 2017) obtained from the C and LO goat groups. One pooled sample was created from milk in the control group (to produce one PCY drink) and another one from milk in the experimental group fed linseed oil (to produce one PLY drink).

The fat in yoghurt drinks was extracted according to **ČSN EN ISO 1211 (2011)**. Fatty acids (FA) were then re-esterified into the corresponding methyl esters (FAME) and were analyzed by gas chromatography according to method described by **Borková et al. 2018**. FAME were identified using external analytical standard (Supelco, USA). The content of a particular fatty acid was calculated as a ratio of its peak area.sum^{-1} of peak areas of all fatty acids and given in g.100 g^{-1} total fatty acids.

Yoghurt drink formulation

Milk samples for yoghurt drinks were taken the last day of the experiment (July 11, 2017) during morning milking. Twelve yoghurt drinks (denoted as ILY) from the group fed linseed oil and twelve yoghurt drinks (denoted as ICY) originating from the control group were individually produced. All these yoghurt drinks in both groups contained 5 g of yacon tuber powder (see below). Also, twelve individual yoghurt drinks were prepared from the group fed linseed oil but without the addition of yacon tuber powder (denoted as IL) to enable the investigation of yacon powder addition on bacterial growth. Both yoghurt drinks made from pooled samples (PLY or PCY) contained each 5 g of yacon tuber powder.

Yoghurt drinks were prepared as follows: 5 g of lyophilized Peruvian yacon tuber powder (Gloobe corp., Czech Republic) were added into 95 g of goat milk, pasteurized at 84 °C for 10 min and fermented using 0.1% CCDM 528 (*Streptococcus thermophilus* and *Lactobacillus delbruckii* ssp. *Bulgaricus*, Laktoflora®) and 1% *Bifidobacterium animalis* ssp. *lactis* (Bb12, Chr. Hansen) at 30 °C for 16 – 18 h.

Yoghurt bacteria counts (in colony forming units, CFU) were estimated according to **ČSN ISO 7889 (2004)**, bifidobacteria counts (in CFU) according to **ČSN ISO 29981 (2010)** and FOS analysis as described in **Bohačenko and Pinkrová (2014)**.

Statisic analysis

The data were analyzed in Statistica (ver. 12, StatSoft). Milk yield and milk composition were tested by repeated measures ANOVA. The FA content, the numbers of CFU of yogurt bacteria and bifidobacteria were tested by one-way ANOVA. Tukey's *post-hoc* HSD test (p <0.05) was used to evaluate differences between groups. Results are expressed as mean value with the standard error of mean (*SEM*).

RESULTS AND DISCUSSION

Our previous work **(Borková et al., 2018)** revealed that feed supplemented either with linseed oil or linseed extrudate increased the amounts of omega-3 fatty acids in goat milk which can be successfully used to produce yoghurt drinks with added value. We found out that feed supplemented with linseed oil yields better results than linseed extrudate. Therefore, we decided to modify the previous experiment using feed supplementation with linseed oil to get enriched omega-3 goat milk which was used to produce yoghurt drinks with added yacon prebiotics to boost the drink's health benefits. We also

Table 1 Composition of basic diet and linseed oil.

	Hay	Haylage	Grain mix	Linseed oil
DM (g.100 g^{-1} FW)	78.8	51.3	87.5	–
Crude protein (g.100 g^{-1} DM)	6.26	12.9	9.88	–
Ether extract (g.100 g^{-1} DM)	1.40	2.49	2.48	–
Crude fibre (g.100 g^{-1} DM)	42.7	23.2	7.75	–
Ash (g.100 g^{-1} DM)	6.42	9.11	13.7	–
FA (g.100 g^{-1} FA):				
SFA	34.9	20.3	23.0	9.58
MUFA[6]	34.4	14.8	19.5	16.0
PUFA[7]	30.7	64.9	57.5	74.4
n-6 FA	16.9	34.2	38.6	15.5
n-3 FA	13.8	30.0	18.9	58.9
ALA[8]	12.4	28.6	17.5	58.9

Note: DM = dry matter; FW = fresh weight; Crude protein = nitrogen content × 6.25; Ether extract = crude fat; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid, ALA = α-linolenic acid.

Table 2 Milk yield and milk composition.

	C	LO	*SEM*	*p* value
Milk yield, 0 week (kg.day^{-1})	1.25	1.38	0.11	NS
Milk yield, 4 week (kg.day^{-1})	1.03^{b}	1.62^{a}	0.12	$p < 0.05$
Milk yield, 8 week (kg.day^{-1})	1.13^{b}	1.56^{a}	0.10	$p < 0.05$
Milk fat, 0 week (g.100 g^{-1})	3.59	3.23	0.16	NS
Milk fat, 4 week (g.100 g^{-1})	3.07	3.08	0.09	NS
Milk fat, 8 week (g.100 g^{-1})	2.81	2.84	0.11	NS
Milk protein, 0 week (g.100 g^{-1}	3.18	3.05	0.06	NS
Milk protein, 4 week (g.100 g^{-1})	2.98	3.01	0.05	NS
Milk protein, 8 week (g.100 g^{-1})	2.93	2.92	0.04	NS
Total solids, 0 week (g.100 g^{-1})	12.0	11.7	0.21	NS
Total solids, 4 week (g.100 g^{-1})	11.1	11.2	0.13	NS
Total solids, 8 week (g.100 g^{-1})	10.7	10.9	0.16	NS

Note: NS = not found to be significantly different ($p > 0.05$); C = goats in the control group (N = 12); LO = goats fed linseed oil (N = 12); *SEM* = standard error of mean.

increased the length of linseed oil supplementation to goats to eight weeks to prove that it has a long-lasting effects on omega-3 fatty acid elevation (and other quality indicators) in goat milk. Our last experiment published in 2018 found a minor (but statistically insignificant) increase in milk yield, milk fat and total milk solids in milk samples from goats fed linseed oil. Results from the current study revealed that after four weeks of linseed oil supplementation the milk yield has increased ($p < 0.05$) and this effect lasted until the end of experiment. The linseed oil supplementation did not affect other quality parameters like fat and protein content or total solids (Table 2). Thus, the increased milk yield and quality (see below) after linseed oil supplementation may positively impact farm's economy offsetting the costs for feed supplementation.

From Table 3 (listing some major and nutritionally important minor fatty acids) it is evident that linseed oil supplementation (ILY) changes fatty acid profile in the final product. To sum up the results, saturated fatty acid (SFA) levels were significantly decreased ($p < 0.001$) while the levels of monosaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) increased ($p < 0.01$, $p < 0.001$ resp.). Contrary to our 2018 study, linseed oil supplementation was able to reduce short-chain SFAs and C12:0 in yoghurt drinks (in particular C6:0, $p < 0.05$; C8:0, $p < 0.05$; C10:0, $p < 0.01$; C12:0, $p < 0.05$). These are interesting results because the data in scientific papers so far either found an increase of short-chain SFA levels as in **Bernard et al. (2009)** or no change at all **(Martínez Marín et al., 2011)** after linseed oil supplementation. In the case of medium-chain SFAs (C14:0 and C16:0), we saw their levels to decrease ($p < 0.01$) after linseed oil supplementation which is in accordance with the data published in scientific literature **(Bernard et al., 2009; Martínez Marín et al., 2011)** and our last paper **(Borková et al., 2018)**. According to **Martínez Marín et al. (2011)** the observed reduction of short and medium-chain SFAs was likely caused by the increased intake of PUFAs from linseed oil added to the feed which might have affected some crucial enzymes in the *de novo* fatty acid synthesis pathway such as acetyl-CoA carboxylase and fatty acid synthase. The *de novo* synthesis in the mammary gland is the major source of fatty acids with less than sixteen carbon atoms. Unlike our previous short-term supplementation experiment, the long-term supplementation with linseed oil decreased the levels of C14:0, C16:0 and also the levels of C6:0 to C12:0.

The increased amounts of *trans*-C18:1 and *trans*-C18:2 isomers, ALA and conjugated linoleic acid contributed to the elevated levels of MUFAs and PUFAs. These results are in accordance with findings of **Martínez Marín et al. (2011) and Bernard et al. (2009)** who also observed such increases after linseed oil supplementation. The increase of the unsaturated fatty acids mentioned above in yoghurt drinks was caused by the ALA present in linseed oil. Fatty acids longer than eighteen carbons get into milk from lipoproteins present in blood plasma (which themselves originate from food or animal's fat depots). The PUFAs present in feed undergo biohydrogenation in rumen during digestion which leads to the formation of C18:0 and other isomers like *t*11-C18:1 (vaccenic acid) and *t*11,*c*15-C18:2 **(Chilliard et al., 2007)**. These fatty acids are subsequently absorbed in the colon and transported through blood into the mammary gland where they might be desaturated by stearoyl-CoA desaturase activity. Contrary to our previous report in **Borková et al. (2018)**, the linseed supplementation did not decreased *c*9-C18:1 in yoghurt drinks. The PUFAs biohydrogenation in goat rumen is a very complex process influenced by many external and internal factors. The composition of metabolites resulting from biohydrogenation process is dependent on the supplement type and feed itself which both impact the lipogenesis in the mammary gland and the activity of key enzymes. Unlike our previous experiment, we did not see the desaturase activity to drop significantly in the yoghurt drinks (see the desaturase index in Table 3) made from the experimental group (ILY). This fact is the likely cause why we did not observe the *c*9-C18:1 fatty acid to decrease. It is worth to mention that the present study confirmed again that ALA levels in yoghurt drinks remained increased.

From the nutritional perspective it is important to underscore the increased content of *trans*-fatty acids in the ILY drinks. As a matter of fact, *trans*-fatty acids in food can negatively influence consumers' health. However, this notion is not true for all *trans*-fatty acids **(Anadón et al., 2010; Jacome-Sosa et al., 2014; Ganguly and Pierce, 2015)** like vaccenic acid (*t*11-C18:1) which is the main biohydrogenation intermediate of ALA.

All our yoghurt drinks (with or without the addition of yacon tuber powder) made from enriched omega-3 milk (LO goat group) have met the requirements of the **Regulation no. 397/2016 Col. of Czech Republic** specifying that all yoghurt products must contain at least 7 log CFU.g^{-1} (which equals to 10^7 CFU.g^{-1}). Yoghurt drinks containing yacon tuber powder (ILY) showed significant increase in the number of bifidobacteria than yoghurt drinks without the yacon addition (IL) (Table 4). This result is likely due to the presence of fructooligosaccharides in the yoghurt drinks which are a welcomed substrate for the probiotic bacteria leading to their increased counts.

Two pooled milk samples (one from the experimental, the other from control group) were created to mimic standard practices on the farm because farmers usually do not have access to individual milk samples but only to the pooled milk from the herd. Both pooled samples were used to manufacture yoghurt drinks with yacon tuber powder (one drink per each pooled sample denoted as PLY and

Table 3 Fatty acid composition of goat yoghurt drinks.

FA (g.100 g^{-1} total FA).	ICY	ILY	*SEM*	*p* value
C4:0	2.43	2.60	0.051	NS
C6:0	2.52^{a}	2.30^{b}	0.055	p <0.05
C8:0	2.62^{a}	2.25^{b}	0.078	p <0.05
C10:0	8.92^{a}	7.33^{b}	0.303	p <0.01
C12:0	3.84^{a}	3.26^{b}	0.121	p <0.05
C14:0	10.4^{a}	9.20^{b}	0.224	p <0.01
C16:0	27.0^{a}	23.8^{b}	0.573	p <0.01
C18:0	10.6	11.2	0.389	NS
t-C18:1	2.10^{b}	4.91^{a}	0.386	p <0.001
*c*9-C18:1	19.1	18.9	0.408	NS
t-C18:2	0.89^{b}	2.73^{a}	0.231	p <0.001
*c*9,*c*12-C18:2	2.13	2.32	0.070	NS
*c*9,*c*12,*c*15-C18:3 (ALA)	1.00^{b}	1.53^{a}	0.078	p <0.001
CLA	0.56^{b}	1.17^{a}	0.082	p <0.001
*c*9-C18:1/C18:0	1.82	1.77	0.084	NS
SFA	71.5^{a}	64.8^{b}	0.997	p <0.001
MUFA	23.6^{b}	27.1^{a}	0.620	p <0.01
PUFA	4.91^{b}	8.10^{a}	0.425	p <0.001
n-6 FA	2.32	2.55	0.072	NS
n-3 FA	1.13^{b}	1.66^{a}	0.077	p <0.001

Note: Different small caps in the superscript indicate differences between groups (at p <0.05), NS = not significantly different (at p >0.05); ICY = control group (N = 12); ILY = goats fed linseed oil (N = 12); *SEM* = standard error of mean; *t*-C18:1 = *trans* isomers C18:1 including e.g. vaccenic acid (*t*11-C18:1); *t*-C18:2 = *trans* isomers C18:2 including e.g. *t*11,*c*15-C18:2; ALA = α-linolenic acid; CLA = conjugated linoleic acid (mixture of isomers *c*9,*t*11-C18:2 and *t*9,*c*11-C18:2); *c*9-C18:1/C18:0 = desaturase index; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

Table 4 Comparison of the number of colony forming units in yoghurt drinks.

	ILY	IL	*SEM*	*p* value
∑ yoghurt bacteria (log CFU.g^{-1})	8.35	8.50	0.056	NS
bifidobacteria (log CFU.g^{-1})	6.96^{a}	6.80^{b}	0.033	p <0.05

Note: Different small caps in the superscript indicate differences between groups (at p <0.05); NS = not significantly different (at p >0.05); ILY = yoghurt drinks with yacon tuber powder from goats fed linseed oil (n = 12); IL = yoghurt drinks without yacon tuber powder from goats fed linseed oil (n = 12); *SEM* = standard error of mean.

Table 5 Composition of yoghurt drinks made from pooled samples.

	PCY	PLY
Fructooligosaccharides (g.100 g^{-1})	0.73	0.81
∑ yoghurt bacteria (log CFU.g^{-1})	8.56	8.51
bifidobakteria (log CFU.g^{-1})	7.04	6.88
Fatty acids (g.100 g^{-1} total FA)		
C4:0	2.59	2.41
C6:0	2.56	2.30
C8:0	2.61	2.28
C10:0	9.03	7.42
C12:0	3.90	3.24
C14:0	10.6	9.09
C16:0	27.3	23.1
C18:0	10.2	11.7
t-C18:1	2.03	5.20
*c*9-C18:1	18.9	18.7
t-C18:2	0.87	2.85
*c*9,*c*12-C18:2	2.08	2.35
*c*9,*c*12,*c*15-C18:3 (ALA)	0.96	1.59
CLA	0.55	1.17
SFA	71.9	64.4
MUFA	23.3	27.3
PUFA	4.78	8.32
n-6 FA	2.26	2.59
n-3 FA	1.10	1.71

Note: PCY = yoghurt drinks with yacon tuber powder and bifidobacteria from control group (n = 1); PLY = yoghurt drinks with yacon tuber powder and bifidobacteria from experimental group (goats fed linseed oil) (n = 1); *t*-C18:2 = *trans* isomers C18:2 including e.g. *t*11, *c*15-C18:2; ALA = α-linolenic acid; CLA = conjugated linoleic acid (mixture of isomers *c*9, *t*11-C18:2 and *t*9, *c*11-C18:2); SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

PCY) because we were interested if there is any difference in fatty acid profile in yoghurt drinks made from the pooled sample versus yoghurt drinks made from individual milk samples. The results for both groups are shown in Table 5. The yoghurt drinks made from pooled milk samples from goats supplemented with linseed oil (PLY) had increased contents of MUFAs, PUFAs and a lower content of SFAs similar to findings for ILY yoghurt drinks. PLY yoghurt drinks had 66% higher ALA content (compared to the PCY group used as a control). The same comparison for yoghurt drinks from individual milk samples (ILY and ICY) has shown only 53% increase in ALA content. The higher amount of ALA in the pooled samples (66%) was caused by some goats having higher milk yield with the highest ALA content (unpublished results). Fructooligosaccharide content in yoghurt drinks from the pooled samples was 0.73 – 0.81 g.100 g^{-1} of yoghurt drink thus these yoghurt drinks can be a valuable source of prebiotics, probiotics and α-linolenic acid.

CONCLUSION

Goat yoghurt drinks with bifidobacteria and yacon tuber powder are novel products that might be interesting for health conscious consumers providing them with added value. These drinks are a good source of fructooligosaccharides and bifidobacteria compared to regular products without any added prebiotics. In case of the milk produced by goats fed linseed oil it is possible to manufacture a product which has an increased omega-3 fatty acid content and decreased content of saturated fatty acids. These yoghurt drinks eventually contained significantly higher amounts of α-linolenic acid and lower levels of lauric, myristic and palmitic acid.

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UNUSUAL ASPECTS OF THE FAT CONTENT OF MEALWORM LARVAE AS A NOVEL FOOD

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ABSTRACT

The work focuses on some aspects of content and properties of the fat in mealworm as a novel food. The total fat content of this species is a markedly variable nutritional value that is significantly dependent on the breeding conditions. In this work, the fat content of a mealworm from various Czech suppliers ranged from 202 g.kg^{-1} to 282 g.kg^{-1} dry matter, determined using the Soxhlet extraction method. The total average value from all suppliers was 235.8 ±40.8 g.kg^{-1}. This is a range that can be expected by the customer when buying mealworm larvae from a random Czech supplier. Furthermore, the work graphically compares the values of the total fat content with other comparable commodities of animal origin, e.g. chicken or fish. Finally, the aim was to obtain initial information about the comparison of the sensory properties of the mealworm fat with other fats of animal origin using a simple electronic nose. There was a difference between the fat obtained from insect larvae and the conventional unprocessed fats. This work brings a wider view of fat as a taste carrier in a new food - a mealworm.

Keywords: mealworm; fat; breeder; electronic nose

INTRODUCTION

The inclusion of edible insect as novel foods in human nutrition is affected by several factors such as nutritional value, consumer gastronomic requirements, as well as economic and ecological aspects **(Cerritos, 2011; Chae et al., 2012; Fontaneto et al., 2011; Mariod, Abdel-wahab and Ain, 2011; Premalatha et al., 2011)**. The attitude of consumers towards entomophagy varies in different parts of the world. In developing countries, the edible insect is a common basic food with an interesting nutritional value, such as protein and fat content. On the contrary, in the developed world, especially in Europe and North America, it is predominantly an enjoy-ment food **(De Foliart, 1992; Ramos-Elorduy et al., 2011)**. By changes of European legislation, edible insect became a novel food that is being increasingly promoted on European markets **(EFSA 2015; EFSA, 2018)**. The reason is the growing consumer interest in repeated eating of edible insect, not only because of its interesting nutritional value but also for its specific sensory properties **(Adámek et al., 2018)**.

Insect is consumed mostly culinary processed (baked, blended into rice, soups, pasta, or salads) **(van Huis, 2015)**. Fat as a carrier of taste is generally rich in edible insect. However, its quantity varies considerably among species. Its value for mealworm (*Tenebrio mollitor*) is most often in the range of 150 to 300 g.kg^{-1}. Not only the amount of fat but also the fatty acid profile is dependent on various aspects (diet, developmental stage, breeding temperature). In terms of purchasing insect in native state, however, many aspects are unknown for the consumer. Information on total fat content is important to consumers because of the use of edible insect material as part of food products and meals. The fat content can determine the final product properties. In the case of including insect in the food basket of the Czech consumer, it is important to compare it with commonly consumed foods of animal origin. For the consumer, not only the nutritional value but also the organoleptic properties play an important role. The primary assessment of a consumed food is its visual appearance and aroma, on the basis of which the consumer chooses to consume. A firmly defined state of a food with a certain flavor can be recorded using the electronic nose to distinguish this firmly defined state of the food (commodity) from other states. This can be used, for example, to determine the authenticity and safety of foods, the maturity of a particular raw material, and so on.

Scientific hypothesis

A) Average values of the total fat content of the mealworm as a novel food by individual breeders do not differ by more than ±10% from the total average fat content of all selected suppliers. B) The total fat content of the mealworm as a novel food is comparable to other commodities of animal origin from common livestock. C) The results of

comparison of the fat sensory evaluation of mealworm as novel food with other fats of animal origin using a simple electronic nose will be different.

Aim

The aim of this work was to evaluate some aspects gained and analyzed during the monitoring of nutritional values of mealworm, especially for fat. The work observes and compares the total fat content of mealworm from different breeders including the determination of its average value and the standard deviation and its comparison with other fats of animal origin commonly used. Furthermore, the aim was to obtain initial information on the comparison of the sensory properties of the fat of the mealworm with other fats of animal origin using a simple electronic nose.

MATERIAL AND METHODOLOGY

Material

Mealworm (*Terebrio mollitor*) larvae in the last and the penultimate developmental stages (full length of the body just before the pupae) were used. The larvae were taken from the breed, left to starve for 48 hours, killed with boiling water (100 °C) and immediately dried at 105 °C. The samples prepared this way were homogenized and stored in a refrigerator at 4 – 7 °C until analysis.

The larvae were purchased from three companies in the Czech Republic. In addition, the data of the author described in **(Adámková et al., 2016; Adámková et al., 2017)** were used for comparison. Furthermore, the average values and standard deviations from available literature were compared with a focus on breeding and sales in the Czech Republic.

Methods

Determination of fat content using Soxhlet extraction method

Determination of fat content was carried out by Soxhlet extraction method **(Soxhlet, 1879)** using Gerhardt Soxtherm machine (C. Gerhardt, Germany). 5 g of dried and homogenized samples (with an accuracy of 0.0001 g) were placed in the extraction cartridge and extracted with 150 ml petroleum ether (program: 70 °C for 120 minutes). The extracted sample was then dried at 103 °C and repeatedly weighed to a constant weight.

E-nose

Fat samples were further analyzed using the simple electronic nose described in **Adámek et al. (2018)**. Samples were analyzed at 20 °C – 23 °C.

Statisic analysis

The data were analyzed using Excel 2013 (Microsoft, USA). Results were expressed by average ± standard deviation. For the calculation of the general average fat values for mealworm and their comparison, also the values from the available literature complemented by the optimal conditions of breeding focused on the breeding area in the Czech Republic were used together with the measured values.

In case of measurement by E-nose, data was evaluated using Excel 2013 (Microsoft, USA) and Gnuplot 5.0: an interactive plotting program **(Williams et al., 2016)**.

RESULTS AND DISCUSSION

Comparison of the total fat content in samples from each supplier

To determine the overall value of the total fat content of the mealworm larvae and its comparison, the total fat content in samples from three suppliers (2 direct breeders + 1 supplier with unknown breeder) was determined in the first step. The basic results determined by the Soxhlet method are shown in Table 1.

From the results shown in Table 1. average values and standard deviations for individual suppliers were calculated and these values were compared with other literary sources focusing on the area of the Czech Republic, as shown in Table 2.

Table 2 shows that even under optimal breeding conditions, the total dry fat content of mealworm larvae (*Tenebrio molitor*) samples may differ statistically, even if the breeders are from one geographical area. By random purchase, the consumer gained insect with a total fat content ranging from 170 $mg.g^{-1}$ to 360 $mg.g^{-1}$ of fat in dry matter. The mean value calculated from the experimental values and available literature sources for Europe for the optimal breeding conditions stated is 243 ±57 $mg.g^{-1}$.

Table 1 Basic results of total fat content determination in samples from three breeders.

Supplier / Number of sample	Weight (g)	Cartridge (g)	Cartridge with fat (g)	Fat (g)	Fat (%)
1/1	5.0730	125.7388	126.8781	1.1393	22.5
1/2	4.9918	127.5941	128.5756	0.9815	19.7
1/3	4.9943	126.6273	127.7322	1.1049	22.1
1/4	5.0119	127.1224	128.0918	0.9694	19.3
1/5	5.0684	125.5152	126.4834	0.9682	19.1
1/6	5.0132	127.1985	128.1483	0.9498	18.9
2/1	5.1480	140.6099	142.0639	1.4540	28.2
2/2	5.0160	144.0048	145.4104	1.4056	28.0
2/3	5.1116	143.0519	144.5135	1.4616	28.6
2/4	4.7388	143.3339	144.6375	1.3036	27.5
2/5	5.0217	141.0421	142.4845	1.4424	28.7
2/6	4.9480	140.4960	141.8926	1.3966	28.2
3/1	5.0095	140.4526	141.4555	1.0029	20.0
3/2	4.9979	140.0654	141.1921	1.1267	22.5
3/3	5.0967	143.0847	144.1158	1.0311	20.2

Table 2 Comparison of total fat content from analyzed samples from three suppliers with other literary sources focusing on the area of the Czech Republic.

Supplier	Breeding conditions	M [mg/g]	SD [mg/g]	
Supplier no. 1	optimal	202.7	15.9	
Supplier no. 2	optimal	282.2	4.3	
Supplier no. 3	optimal	209.3	14.0	
Mean (three suppliers)		**235.8**		
Supplier (reference)	**Breeding conditions**	**M**	**SD**	**Reference**
Carassius, Prague, Czech Republic	optimal	167.0	1.1	**(Baštová, 2017)**
Radek Frýželka, Brno, Czech Republic	optimal	170.0	1.0	**(Adámková et al., 2016)**
Radek Frýželka, Brno, Czech Republic	optimal	361.0	53.0	**(Bednářová, 2013)**
Fabryka Owadów, Warsaw, Poland	optimal	247.0	15.0	**(Zielińska et al., 2015)**
Alicante, Spain	optimal	301.0	7.0	**(Barroso et al., 2014)**
Krmiva Hostivice, Hostivice, Czech Republic	optimal	245.6	31.0	**(Adámková et al., 2017)**
Krmiva Hostivice, Hostivice, Czech Republic	optimal	251.7	27.3	**(Adámková et al., 2017)**
Krmiva Hostivice, Hostivice, Czech Republic	special	146.7	10.2	**(Adámková et al., 2017)**
Krmiva Hostivice, Hostivice, Czech Republic	special	245.6	31.0	**(Adámková et al., 2017)**
Krmiva Hostivice, Hostivice, Czech Republic	special	233.2	36.2	**(Adámková et al., 2017)**
Kreca, Ermelo,The Netherlands	special	250.0	single analysis	**(van Broekhoven et al., 2015)**
Kreca, Ermelo,The Netherlands	special	263.0	single analysis	**(van Broekhoven et al., 2015)**
Kreca, Ermelo,The Netherlands	special	276.0	single analysis	**(van Broekhoven et al., 2015)**
Kreca, Ermelo,The Netherlands	special	189.0	single analysis	**(van Broekhoven et al., 2015)**
Mean (optimal breeding conditions)	**optimal**	**243.0**		

From a standard deviation of 23.4% of the average total fat content, it can be estimated that the consumer is not able to estimate the amount of fat in a particular insect sample with sufficient accuracy. The real extreme values can occur in breeds where welfare is not respected by the breeder and especially the seller **(Adámková et al., 2017)**.

The analyzed data show the total fat content of mealworm is not affected by the area of breeding. The content of the fat will be influenced by the breeding temperature, seasons, stress and nutrition in managed breeds **Broekhoven et al. (2015); Nowak et al. (2016)**. Here, too, it is confirmed that nutrition is one of the important factors influencing the quantity and variability of fat as it is for other commodities of animal origin (patent). The fat content of mealworm can be compared with the fat content of the parts of the body of common livestock (Figure 1) **(Pipek, 1995; Steinhauser, 1995)**. Due to the size of the insect, from the whole body of the insect, whereas in common livestock it is possible to extract the fat from individual parts thereof. In addition, it is necessary to consider that fat consumption from ordinary livestock can take place after a short heat treatment or even in the raw state, insect fat can be used for food purposes only after chemical extraction. This increases the cost of this raw material. On the other hand, in the longterm storage process there are no significant biochemical and microbiological changes and no specific storage conditions **(Adámek et al., 2018)**.

When comparing the total fat content in the dry matter of the mealworm with the total fat content of the conventional meat, the analyzed values for the mealworm are in a wide range from 16% to 36%. This can be compared with both lean chickens (14%) and, for example, salmon (37.6%). This range includes, for example, beef sirloin, mackerel or turkey meat.

From the health point of view, not only the total fat content but also the fatty acid profile, especially linoleic acid and linolenic acid, is important. EFSA Recommendation on fat and other lipophilic substances ingestion from 2010 **(EFSA, 2010)** no longer state recommendation for the n-6: n-3 ratio but, indicate that linoleic acid intake should not fall below 4% and linolenic acid below 0.5% of total energy intake. In the case of mealworm meal consumption and required energy intake of 10,000 $kJ.day^{-1}$, linolenic acid in the amount of 205 g and 708 g of linolenic are needed. Considering the linolenic acid intake, the quantity contained in the dry matter is insufficient and must be supplemented from other sources. On the contrary, the amount of linoleic acid is sufficient, and the dry matter can serve as the source of this acid **(Adámková, 2017)**. Although mealworm is included among livestock since 2015 **(EFSA, 2015)**, it must be considered a specific species with special biological properties. For this reason, insect has to be bred under the defined breeding conditions to achieve the desired properties.

In the next part, the flavor of raw pork and beef fat was measured with a simple electronic nose and compared with fat obtained by the extraction from mealworm larvae. The results in Figure 2 show the difference between insect fat and pork and beef fat.

The most significant difference was recorded by the MQ-8 type sensor, which responds in particular to Hydrogen (H_2), next to alcohol, LPG and cooking fumes. Therefore, the difference between fats can be caused by the treatment of fat from insect during extraction or different fat composition.

In today's food industry, modern techniques such as electronic nose, eye or tongue are used to capture sensory properties. The disadvantage of these techniques is that they cannot replace the human sensory organ in full. The

advantage is good repeatability even in longterm measurements (human can be tired) and in some cases a better sensitivity, resolution or range wider than a human can possess (e.g. electronic eye). An electronic nose used to record flavors from commodities of animal origin is described, for example, by **Gopal (2015)**, who in his study used the Peres' electronic nose to assess the freshness and durability of the meat. The electronic nose plays another important role in detecting food counterfeiting and assessing its authenticity **(Peris and Escuder-Gilabert, 2016)**. In the case of edible insect as a novel food that is being introduced to the market, however, this technology has been used only minimally. The introduction of this technology is one of the arguments for persuading the Czech (European) consumer that food from edible insect is safe and has properties similar to other commodities of animal origin. It is necessary to bear in mind that the insect fat is extracted.

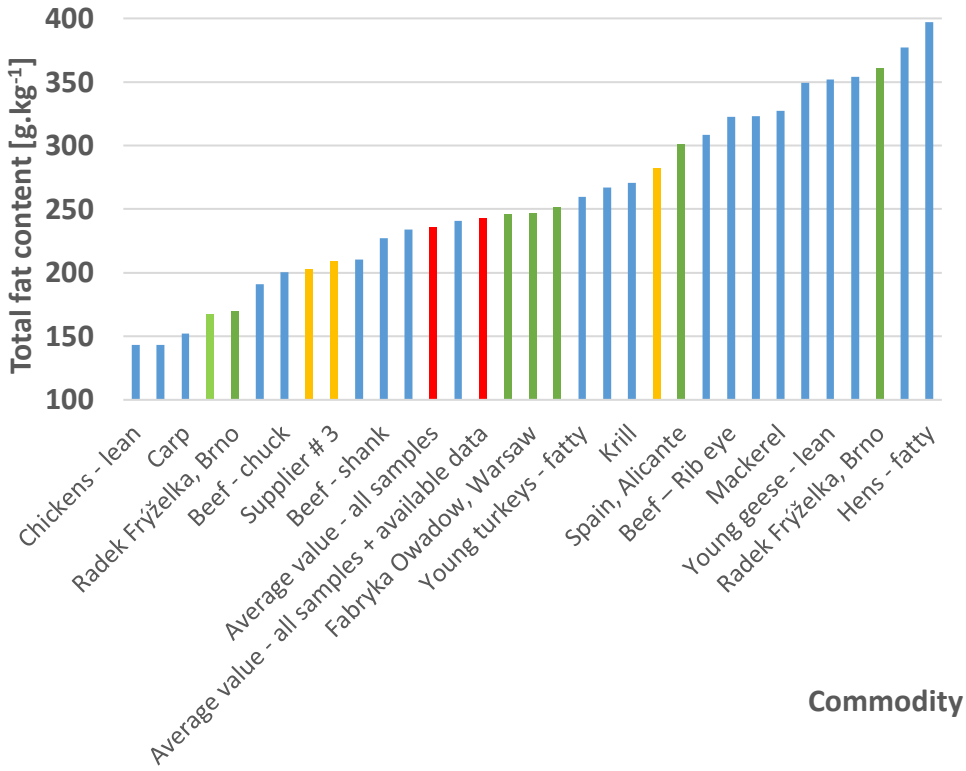

Figure 1 Comparison of total fat content in mealworms and conventional meat **(Pipek, 1995; Steinhauser, 1995)**.

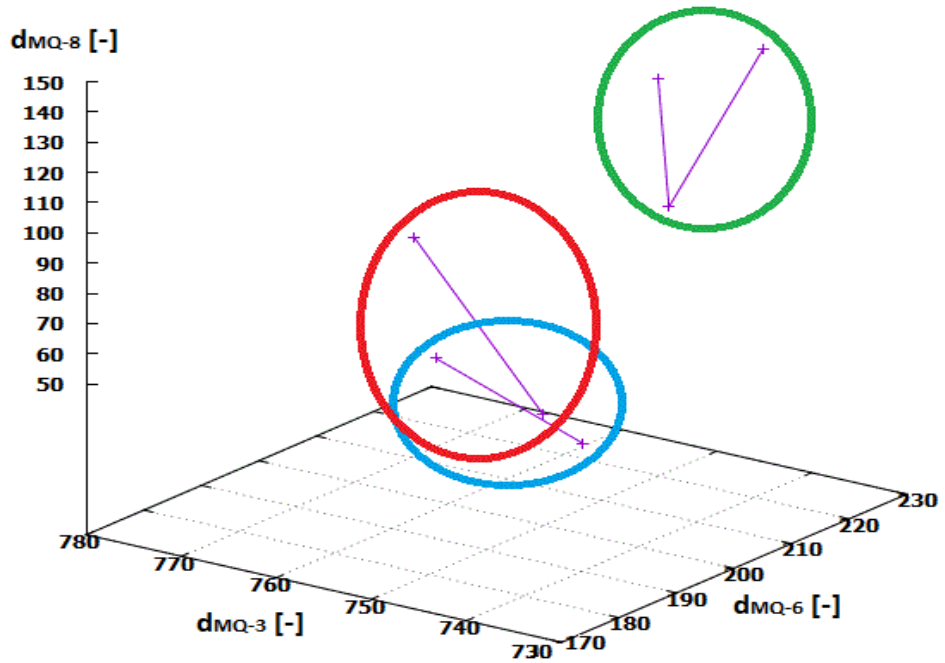

Figure 2 Comparison using a simple electronic nose of pork (blue) and beef (red) crude fat with fat obtained by extraction from mealworm (green).

CONCLUSION

The total fat content of the mealworm is a significantly variable nutritional value, which is highly dependent on the breeding conditions.

In this work, the fat content of the mealworm from various Czech suppliers ranged from 202 $g.kg^{-1}$ to 282$g.kg^{-1}$ dry matter. The overall mean value of all suppliers 235.8 ±40.8 $g.kg^{-1}$ does not confirm the hypothesis that the fat content of the dry matter will not differ by more than 10% of the average fat value. The result demonstrated the wide range of fat content even though the supplier has stated the optimal breeding conditions for the species. If a consumer needs to obtain insect with a specific fat content, the solution to this problem can be to communicate directly with the breeder and ensure adequate breeding conditions to achieve the required nutritional value. Considering the wide range of fat content in mealworm samples it is possible to compare the fat content with, for example, lean chickens (14%) and salmon (37.6%). This range includes, for example, beef sirloin, mackerel or turkey meat. On the other hand, measurement using an electronic nose demonstrated the differences between insect fat and unprocessed (raw) pork and beef fat. The article brings some new information on some aspects of edible insect as a source of fat.

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Characteristics of flour and dough from purple and blue wheat grain

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ABSTRACT
The characteristics of flours and doughs prepared from wheat grains containing purple pericarp (variety PS Karkulka and Jumiko) and wheat grain containing blue aleurone (variety Skorpion) were tested and compared with commercial wheat to evaluate the applicability of colored wheat in bread making. The fine flours prepared from colored wheat grains significantly differed in the activity of the amylase enzyme, expressed as Hagberg falling number. Zeleny sedimentation volume of flour prepared from grains of the PS Karkulka variety (36 mL) was significantly higher than the values of AF Jumiko and Skropion (34 mL) varieties. The results of uniaxial deformation test indicated that doughs prepared from wheat varieties PS Karkulka and Skropion can be elongated; the dough is, however, weak and can be expected to rupture more easily than dough prepared from variety AF Jumiko, as well as commercial flour. Even if some variations in the values of farinographic dough development time and stability were also observed, clear differences in the behavior of doughs prepared from colored and commercial flours were not found. The differences in dough behavior during heating test were also negligible. It can be concluded that none of the tested colored wheat grains exhibited characteristics completely different from the others or the commercial one. The results may indicate the applicability of all tested colored wheat grains in yeast-leavened bread production.

Keywords: fine flour; rheology; quality; bread making

INTRODUCTION
Wheat is grown on more land than any other commercial crop and continues to be the most important food grain source for humans. Global production is about 700 million tons per year. Wheat provides 20% of daily protein and is a significant source of calories for 4.5 billion people **(FAO, 2014)**. The unusual properties of wheat storage proteins (gluten proteins) allow flour to be transformed into dough with suitable properties for bread making **(Goesaert et al., 2005)**.

Wheat grain may also be a good source of phytochemicals, compounds with health-promoting effects. When taken regularly and in adequate amounts, these molecules can have long-term benefits on human health through reducing the likeliness of obesity, diabetes, and cancer and degenerative diseases, such as cardiovascular disease **(Borrelli and Trono, 2016)**. Some of these phytochemicals significantly influence grain color. Anthocyanins are accumulated in the aleurone or pericarp layer resulting in or a combination of blue and purple. Phlobaphenes present mainly in the outer layer are responsible for a reddish color **(Lachman et al., 2018)**.

Colored wheat grains can be used in food production. Since the phytochemicals are situated in the aleurone or pericarp layer, whole grain products are preferred. The applicability of purple wheat grain in biscuit and pasta production has already been studied. Biscuits prepared from whole wheat extracted from purple grains exhibited higher antioxidant activity than biscuits prepared from common wheat grain. Purple biscuits, moreover, showed lower levels of lipid-derived carboxylic acids and higher levels of alcohols and aldehydes than common biscuits, indicating lower oxidative degradation of lipids **(Pasqualone et al., 2015)**.

Ficco et al. (2016) found the sensory properties of pasta from the purple genotype not to be significantly different from commercial whole wheat pasta. In vitro glycemic index was also lower.

Wheat grain with purple pericarp (PS Karkulka and Jumiko) and wheat grain with blue aleurone (Skorpion) **(Lachman et al., 2018; Martinek et al., 2013)** were studied. The aim of this study was to compare the characteristics of flour and dough prepared from colored wheat grain with commercial wheat flour and dough, and to evaluate the applicability of these wheats' grains in the production of yeast-leavened bread.

Scientific hypothesis
The doughs prepared from colored wheat grains exhibit different behavior than dough made from commercial wheat during uniaxial deformation, farinographic and heating tests, which may limit their use in the production of yeast-leavened bread production.

MATERIAL AND METHODOLOGY

Material

Fine flours from wheat grain with purple pericarp (PS Karkulka and Jumiko) and wheat grain with blue aleurone (Skorpion) were used in this study. Fine flours are characterized by being made up of at least 96% of particles that pass through a 257 μm sieve opening and a maximum of 75% of particles passing through a 162 μm sieve opening **(Decree No. 333/1997)**. The Babiččina volba commercial fine flour manufactured by GoodMills Česko s.r.o. was bought in a local supermaket.

Flour characteristics

The flours were characterized by Hagberg falling number and Zeleny sedimentation volume. Hagberg falling number is used to describe the activity of flour amylase enzymes. It was determined according to **ČSN EN ISO 3093 (2011)**. Zeleny sedimentation volume is a key factor of the wheat quality. It is used to predict the baking strength of flour. This parameter was determined according to **ČSN EN ISO 5529 (2011)**.

Dough rheological characteristics

Uniaxial deformation test was performed on dough samples made by mixing flour, water (according optimal farinograph water absorption) and salt (1.5%). The dough was made into thin rolls, put onto the lubricated surface of a Teflon mold and compressed with a lubricated top plate. Test pieces were formed into 5 cm long chunks with a trapezoidal cross-section (3 mm, 5 mm, 4 mm). The doughs were left resting for 40 ±1 min at 30 ±1 °C. The test was performed using textural analyzer TA.XT plus (Stable Micro Systems, UK) equipped with an SMS/Kieffer Dough and Gluten Extensibility Rig. During testing the dough sample was stretched by the hook until it fractured. Test speed of the hook was 3.00 $mm.s^{-1}$, trigger force 5 g. The force required to stretch the dough sample and the displacement of the hook were recorded as a function of time. The values of resistance to elongation *R* (N), extensibility *E* (mm) and extension area *A* (N.mm) were calculated. Force *R* represents the dough resistance to extension, extensibility *E* represents the distance at which this peak force occurs, and extension area *A* is the area under the curve which is related to the energy required to stretch the test piece to its rupture. Each test was performed on dough samples prepared at least in six replicates. The given results are represented as mean values.

Dough behavior during mixing was studied using Mixolab (Chopin Technologies, Paris, France). The dough was prepared by mixing flour with distilled water. Chopin S protocol simulated the operation conditions of the Farinograph. The obtained parameters were: flour water absorption *WA* (%), dough development time *DT* (min), dough stability *ST* (min) and degree of softening *DS* (FU). The standard Chopin+ protocol was used to test dough behavior during heating and cooling, following a 30 – 90 – 50 °C pattern. The extent of dough weakening α ($Nm.min^{-1}$), starch gelatinization β ($Nm.min^{-1}$) and cooking stability γ ($Nm.min^{-1}$) were evaluated. Each test was performed on dough samples prepared at least in six replicates. The given results are represented as mean values.

Statistical analysis

The results were statistically analyzed using analysis of variance (ANOVA). The differences were tested on $p = 0.05$ significance level using Fisher LSD test. The analysis was performed using Statistica 13 software (StatSoft, CR).

RESULTS AND DISCUSSION

Flour characteristics

The flours were characterized by Hagberg falling number and Zeleny sedimentation volume (Table 1). The flour prepared from Skorpion variety was the only one exhibiting optimal value (251 s) for bread making **(Kurt and Evers, 2017)**. The flours from the AF Jumiko and PS Karkulka varieties exhibited significantly (p <0.05) higher values (407 – 460 s), indicating low activity of amylase enzymes **(Ranken, Kill and Baker, 1997)**. Zeleny sedimentation volume of flour prepared from grains of PS Karkulka variety (36 mL) was significantly (p <0.05) higher than the values of AF Jumiko and Skropion (34 mL) varieties. This higher value of sedimentation volume can be explained by better swelling and flocculating properties of the insoluble proteins associated with good bread making characteristics **(Kurt and Evers, 2017)**.

Dough behavior during uniaxial deformation

Dough extensibility *E* did not significantly (p <0.05) differ among the tested doughs (Table 2). Dough resistance to elongation *R* was significantly lower in doughs prepared from colored wheat grain (0.17 – 0.30 N) than in the dough from commercial wheat flour (0.34 N). The recorded weak resistance to extension can also be related to the decrease in the extension area values *A* recorded in dough from colored wheat flours. The decrease was, however, significant (p <0.05) only in the dough prepared from Skorpion variety. The combination of good resistance to elongation and extensibility, expressed as *R/E* ratio, is expected in doughs with good bread making quality **(Burešová and Hřivna, 2011; Goesaert et al., 2005; Tsiami et al., 1997a, b)**. The dough prepared from AF Jumiko variety exhibited an *R/E* ratio similar to dough from commercial flour. The values obtained in dough from PS Karkulka and Skorpion varieties were significantly (p <0.05) lower. Significantly weak resistance to extension recorded in these samples can be the factor decreasing the values of *R/E* in these doughs. The results of uniaxial deformation test indicated that doughs prepared from PS Karkulka and Skropion varieties can be elongated; these doughs are, however, weak, and can be expected to rupture more easily than doughs prepared from AF Jumiko variety, as well as commercial flour.

Farinographic characteristics

Farinographic characteristics are used to describe dough formation and behavior during kneading at constant temperature. The amount of water required to prepare dough with optimal consistency of 500 FU, expressed as water absorption, is also obtained.

Table 1 Hagberg falling number (*FN*) and Zeleny sedimentation volume (*SEDI*) of flours from colored wheat grains.

Flour	FN (s ±SD)	SEDI (mL ±SD)
AF Jumiko	460 ±10^{c}	34 ±2^{a}
PS Karkulka	407 ±9^{b}	36 ±2^{b}
Skorpion	251 ±3^{a}	34 ±2^{a}

Table 2 Dough behavior during uniaxial deformation test.

Flour	R (N ±SD)	E (mm ±SD)	A (N.mm ±SD)	R/E (10^{-3} N.mm^{-1} ±SD)
AF Jumiko	0.30 ±0.04^{c}	44 ±9^{a}	9.9 ±0.7^{ab}	7.0 ±0.9b
PS Karkulka	0.21 ±0.02^{b}	56 ±9^{a}	9.2 ±0.9^{ab}	3.8 ±0.7a
Skorpion	0.17 ±0.04^{a}	47 ±9^{a}	8.2 ±0.7^{a}	3.6 ±0.6a
Commercial	0.34 ±0.03^{d}	52 ±9^{a}	12.4 ±0.9^{b}	6.5 ±0.9b

Note: Dough resistance to elongation (*R*), extensibility (*E*), extension area (*A*), *R/E* ratio.

Table 3 Rheological characteristics of dough.

Flour	WA (% ±SD)	DT (min ±SD)	ST (min ±SD)	DS (FU ±SD)
AF Jumiko	60.0 ±0.4^{b}	4.9 ±0.3^{b}	36 ±2^{b}	46 ±7^{a}
PS Karkulka	61.4 ±0.4^{c}	3.6 ±0.4^{b}	28 ±1^{a}	44 ±3^{a}
Skorpion	62.4 ±0.8^{d}	1.0 ±0.4^{a}	39 ±5^{b}	38 ±9^{a}
Commercial	57.6 ±0.5^{a}	2.9 ±0.1^{ab}	29 ±1^{a}	44 ±5^{a}

Note: Farinographic water absorption (*WA*), dough development time (*DT*), dough stability (*ST*) and degree of softening (*DS*).

Table 4 Rheological characteristics of dough. Mixolab dough weakening (α), gelatinization rate (β) and stability (γ).

Flour	α (10^{-3} Nm.min^{-1} ±SD)	β (Nm.min^{-1} ±SD)	γ (Nm.min^{-1} ±SD)
AF Jumiko	-69 ±9^{a}	0.09 ±0.05^{a}	-0.07 ±0.02^{a}
PS Karkulka	-113 ±9^{a}	0.39 ±0.02^{ab}	-0.06 ±0.03^{a}
Skorpion	-94 ±6^{a}	0.48 ±0.06^{b}	-0.08 ±0.01^{a}
Commercial	-57 ±4^{a}	0.22 ±0.09^{ab}	-0.05 ±0.04^{a}

The water absorption WA of all tested flours prepared from colored wheat grain was significantly (p <0.05) higher than the absorption of commercial flour (Table 3). Dough development time DT was significantly higher in dough prepared from AF Jumiko and PS Karkulka varieties than in dough from Skorpion and commercial flour. Dough stability ST of dough prepared from PS Karkulka was closer to the commercial flour. The stability of dough ST prepared from PS Karkulka and AF Jumiko was, however, significantly (p <0.05) higher. Dough softening DS did not differ among tested flours. The farinographic test revealed that the flours from colored wheat required a higher amount of added water to reach optimal consistency of 500 FU. Even if some variations in dough development time and dough stability were also observed, clear differences in the behavior of doughs prepared from colored and commercial flours were not found.

Dough behavior during heating

Changes of dough viscosity during baking significantly impact dough ability to trap and hold gas in enclosed cells and expand along with it. Thus, optimal dough viscosity is essential for obtaining high quality, spongy bread crumb **(Collar, Bollain and Rosell, 2007; Mondal and Datta, 2008)**. Viscosity changes occurring in dough during baking are generally attributed to protein breakdown, gelatinization rate and cooking stability.

At the beginning of heating (30 – 50 °C), doughs exhibited weakening indicated by negative values of α (Table 4). Dough weakening can be partially related to water released from proteins after reaching the temperature of their denaturation, and partially to water released from dough macromolecules due to mechanical stress applied on the sample. Dough viscosity rose in the temperature range of 60 – 80 °C, which is evident from positive values of β, indicating dough strengthening. While in the previous phase of heating, the dough characteristics were mainly affected by protein denaturation, starch gelatinization occurred in this heating phase. Light dough weakening was observed during the final phase of the heating test, which is evident from negative values of γ. Although the doughs exhibited some differences during the heating test, these differences were not significant during the initial and final phase of the test. The only significant (p <0.05) differences were found during the gelatinization phase in which dough from AF Jumiko variety exhibited reduced gelatinization extent (0.09 Nm.min^{-1}) than dough from Skorpion variety

(0.48 Nm.min^{-1}). The differences in gelatinization rate among the other doughs were not significant.

CONCLUSION

The fine flours and doughs prepared from wheat grains with purple pericarp (PS Karkulka and Jumiko varieties), along with wheat grain with blue aleurone (Skorpion variety) were tested and compared with commercial flour to evaluate the applicability of colored grain wheat in bread making. The flours prepared from colored wheat grains exhibited variations in the values of Hagberg falling number and Zeleny sedimentation volume. The dough behavior during uniaxial deformation, farinographic and heating tests also varied. The differences were, however, generally weak. None of the tested colored wheat grain exhibited characteristics completely different from the others or the commercial one. The results may indicate the applicability of all tested colored wheat grains in bread making. Experimental baking tests, however, are yet to be performed to finally confirm it.

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NATURAL FRUIT BEVERAGES FORTIFIED BY BIOLOGICALLY ACTIVE SUBSTANCES OF GRAPE VINES

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ABSTRACT

Based on the study of general knowledge of biochemical and all subsequent developmental studies of organic matter, especially products of grapevine and selected fruit products, a comprehensive study of processing technologies is prepared. Use of a combination of vine products and fruit products in the form of natural grapes. Beverages are researched and developed to be purely natural on the basis of grape musts, blue and white, either individually and again separately in targeted combinations, both biochemically, organoleptically and colorfully, with fruit sources. The core of grape value of biologically active substances is an integral and essential new part and condition of designing these beverages. Their increased biological values, which create the preconditions for containment and if properly managed on the basis of scientific knowledge, may in some cases almost result in the elimination of synthetic additives. It should be noted that 20 – 25% of the adult population suffers from many unexpected allergies, for example, to the sulphite content, although its content in the final product does not exceed the health-approved normatives. And there are many other, interrelated relationships. Beverages are technologically dealt with both without alcohol fermentation and with this fermentation, but only based on their compositional natural resources. They are therefore suitable for the entire population profile. The whole set contains 7 variants and a combination of natural beverages from different fruits. Including natural beverages with or without alcoholic fermentation from the must of white wine grapes, the juice of apple puree with those of biologically active substances from the products grapevine. Three months of monitoring and determination of basic (oenological) values and biologically active substances were performed on these products. The high-performance liquid chromatography method with a refractometric detector determined amount of sugar and alcohol, whilst titrating determined total and volatile acids and free sulfur dioxide. Yeast assimilable nitrogen, total anthocyanins and polyphenols were determined by spectrophotometry, antioxidant activity by DPPH and ABTS methods.

Keywords: biologically active substances; natural stability; isolation of selected substances; natural beverages with or without alcohol

INTRODUCTION

The values of biologically active substances are monitored by biochemical analysis in connection with the determination of the oenological, microbiological and other characteristics. Critical points of instability of the biologically active substances value are determined, possibilities of restraint until the causes of this instability are eliminated. Values of endogenous sulfur dioxide content and possibilities of reduction until its elimination **(Wells and Osborne, 2011; Ivanova, Petruseva and Mitrev, 2015)**. Correlation of concurrent laboratory and organoleptic values, which create the first sense of customer interest and interest in manufacturing and commercial applications. The equilibrium of the total polyphenol values demonstrates the stability of the biologically active substances values, thus demonstrating the correctness of the design of the technological processes **(Mlček et al., 2016; Tarko et al., 2015)**. The protein content test proves their stability, which is a measure of protection against sunburn.

In this case, however, completely different technological processes, due to the prepared conditions for yeast activity, are also of completely original origin **(Hernández et al., 2018)**. The yeast for the preparation of the fermentation support comes from the same variety and locality, including agrotechnical practices and technology for the cultivation of grapevine **(Lachman et al., 2009)**. The fermentation process is prepared from grapes harvested 5 to 7 days before the harvest for the entire production process. The exact start date of the preparation of the

fermentation support is determined by the temperature relations of the habitat and the duration of the preparatory processes for the technological process of fermentation and its course **(Yu et al., 2018)**. The temperature sessions need to be regulated in the range of 18 – 28°C and YAN conditions (assimilable N). In the case of unfermented must in the range of 120 – 200 mg.L^{-1} (on the experimental parcels of the submitted project, it ranged in three sampling times at 177 – 166 – 134 mg.L^{-1}, the yeasts were not referred to as "life", they did not consume nitrogen). In fermentation, nitrogen value has fallen further since the beginning of the fermentation process and reached 50 – 44 – 40 mg.L^{-1}. However, it is necessary to emphasize that the resources for obtaining the raw material needed for its processing are consistent but the technological procedures for the respective goals are quite varied.

The technological bond of the basic raw material, ie the apple pulp, has been created to obtain apple juice and the sources of tripenten biological substances contained in apple peel. Apples contain a range of substances that the technologist must register and monitor their development **(Baron, Dénes and Durier, 2006; Valdramidis et al., 2009)**. Mutual relationships of organic acids and sugar content, which is represented by fructose, glucose. During fruit maturation, sucrose passes gradually into fructose. Ripening also creates conditions for the transformation of starch (in immature fruit) into sugar. Furthermore, it is necessary to monitor proteins, pectin substances, fiber and others, especially vitamins and aromatics **(Qin, Petersen and Bredie, 2018)**. Biological activity is heavily enriched with biologically active substances from grape vines, which which belongs to high-incidence plants. The combination of both components – i.e. apples and natural additives, i.e. biologically active substances from grapevine products, creates a value that is purposefully controlled on the basis of the relevant analyzes **(Vrancheva et al., 2018)**.

Scientific hypothesis

The aim of the research is to increase the share of biologically active substances and to increase their stabilization throughout the technological processes from the stage of their sources, the analytical procedures in the course of research and development and the complete observance of the development rules throughout the production process. Monitor biological agents and their development during storage and validate values using appropriate matrix-adapted methods.

MATERIAL AND METHODOLOGY

Materials

The whole set contains 4 variants and a combine natural beverages from different fruits. Includes natural beverages with or without alcoholic fermentation from the must of white wine grapes, the juice of apple puree with those of biologically active substances from the products grapevine.

Methods

Determination of free SO_2 by OIV-MA-AS323-04B : R 2009

The modified method of **Snopek et al. (2018)** which is based on the methodology of **OIV (1990)**. 50 mL of wine sample is pipetted into a 500 mL volumetric flask, we add 3 mL of 16% H_2SO_4 (Penta, Prague, Czech Republic), 1 mL EDTA 3 solution having a concentration of 1%, 5 mL of starch solution is titrated against a white background I_2 (Ing. Petr Lukeš, Uherský Brod, Czech Republic) solution having a concentration of 0.02 mol.L^{-1} to blue color.

Spekctrophotometric methods

Spectrophotometric measurements were performed on a Lambda 25 UV-VIS spectrophotometer (PerkinElmer, USA) in 10 mm optical quartz cuvettes.

Determination of total polyphenol content (TPC)

The spectrophotometric method using the Folin-Ciocalteau reagent is used to determine the total phenolic compound (TPC) content. The essence is the reduction of the phosphomolybdate-tungsten complex by phenolic substances in an alkaline environment. The modified method of **Singleton and Rossi (1965)** according to **Snopek et al. (2018)** was used. Determination is carried out after a 30 min incubation at a wavelength of 765 nm. TPC of substances was expressed as gallic acid equivalent (GAE) in mg.L^{-1}. For the preparation of calibration solutions, use distilled water (20 mL), four volumetric flasks (volume 50 mL) and a micropipette. Split the standard solution, then the Folin-Ciocalteau (Penta, Prague, Czech Republic) reagent (1 mL) and mix. After 2 minutes, 5 mL of 20% sodium carbonate solution are added. The volumetric flask thus prepared is filled with distilled water to the mark. Incubation is followed (60 minutes) and we measure dye intensity in a 10 mm diameter cell at 765 nm against a blank. In the same way, determine the absorbance of the samples. According to the regression curve equation we calculate the content of polyphenols, expressed as mg of gallic acid equivalent (GAE).L^{-1}.

Determination of total antioxidant activity (TAA) by DPPH metod

Total antioxidant activity was assessed by modification method of **Rop et al. (2010)** according to **Snopek et al. (2018)**. The stock solution is prepared by dissolving 24 mg of 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) (Penta. Ing. Petr Švec, Prague, Czech Republic) in 100 mL of methanol (Penta, Prague, Czech Republic). To prepare the working solution, use 10 mL of stock solution and 45 mL of methanol. The working solution is measured spectrophotometrically at a wavelength of 515 nm against methanol as blank. A sample of 450 µL of beverage is pipetted into a test tube. 8.55 mL of DPPH working solution are added. Incubation is followed (60 min) in the dark and then the sample is measured at the indicated wavelength. Absorption loss was recalculated using the linear regression equation to equivalent Trolox (TE).L^{-1}.

Determination of total antioxidant ativity by ABTS metod

The analysis was performed using the modified method of **Re et al. (1999)** and **Hosu, Cristea and Cimpoiu, (2014)**. The $ABTS^{+}$ cationic radical was obtained by reacting a 7 mmol.L^{-1} 2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulphonic acid) ABTS (Merck KGaA, Darmstadt, Germany) solution of a diammonium salt with a solution of 2,45 mmo.L^{-1} $K_2S_2O_8$ mixed 1:1 (v/v). The solution was incubated for 16 hours at room temperature in the dark. Subsequently, 0.5 mL of the sample was added to 3 mL of $ABTS^+$ solution diluted to give an absorbance of less than 0.800. Absorbance was measured at 734 nm for 30 minutes. Absorption loss was recalculated using a linear regression equation to the Trolox equivalent (TE).L^{-1}.

Determination of total anthocyanins content (TAC)

A sample extract was prepared for the assay, to which 5 mL of the extraction mixture (70 methanol : 29 distilled water : 1 acetic acid) was added to 1 mL sample. Subsequently, they were placed in a water bath and shaken for 1 hour at 50 °C and placed for ultrasound for 20 minutes. The extract thus prepared is pipetted in a volume of 0.5 mL into two tubes. To one was added 2.5 mL of 0.025 M KCl buffer of pH 1 (0.186 g of KCl was dissolved in 98 mL of distilled water, followed by pH adjustment by concentrated HCl), and the same quantity of 0.4 M acetate buffer pH 4.5 (5.443 g of sodium acetate trihydrate was dissolved in 96 mL of distilled water, the pH value checked by pH meter and optionally adjusted with HCl). Absorbance was measured in all tubes on a spectrophotometer at a wavelength of 510 nm (absorption maximum of major cyanidine-3-glucoside) and at 700 nm against the extraction mixture. This determination was carried out by a modified method according to **Giusti and Wrolstad (2001)** and **Orsavová et al. (2019)**

Determination of sugar and alcohol

Before sampling, samples were diluted 1:10 with distilled water and then filtered through nylon micro filters (Syringe Filter, Nylon 13 mm x 0.45 μm). The determination of ethanol was carried out using a high performance liquid chromatography (RP-HPLC) method on UltiMate 3000 (Dionex, Sunnyvale, CA, USA) using a Phenomenex Rezex RCM-Monosaccharide Ca + 2 (100 x 7.8 mm, 8 μm) Torrance, CA, USA) with a RI detector. Water, isocratic elution, with a flow rate of 0.4 mL.min^{-1}, was used as the mobile phase. Column temperature 80 °C. Detector temperature RI 35 °C.

The qualitative evaluation was performed on the basis of analysis of the individual sugars and ethanol standards. A quantitative evaluation where the resulting value was determined as the average of the six measurements was performed by the calibration curve method and subsequent calculation of the concentration of the substance in the sample. The fructose, glucose and sucrose content was expressed as the equivalent amount of g standard in a 1 liter sample. The alcohol content was expressed as volume %.

Determination of volatile and total acids

To measure total acids, measure 30 mL of water, 1 mL of indicator and 20 mL of sample (CO_2-free) into a conical flask. We will titrate in green blue with a standard 0.1 M NaOH solution (pH = 7.0). All titratable acids are calculated as the tartaric acid content in g.L^{-1}.

The Berh S2 apparatus is used for volatile acids, a 25 mL sample (CO_2-free) is dripped into the distillation vessel. The heated distillate is heated to 60 – 70 °C, add 2 drops of phenolphthalein and titrate with 0.1 M NaOH to pink color. Calculate the volatile acid content as acetic acid in g.L^{-1}.

Determination of yeast assimilable nitrogen (YAN)

According to **Gump et al. (2002)** and **Petrovica et al. (2018)** free amine nitrogen and ammonia as well as YAN components are measured separately by an enzymatic test using K-PANOPA Megazy (Ireland) and ammonia using Enzytec Fluid Ammonia (R-Biopharm). This was done spectrophotometrically on a 20XT instrument (Thermo Fisher Scientific, Walthan). These individual values for free amine nitrogen and ammonia provide the total amount of available YAN and expressed as the nitrogen content in mg .L^{-1}.

Statisic analysis

The data obtained was expressed as mean value ± standard deviation (SD) and the Microsoft Office Excel program (Redmond, WA, USA) was used to calculate them. All analyzes were performed five times in two replicates. Differences between observed results were detected by t-test (Statistica, 2018, StatSoft, USA). A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Samples of natural beverages from grape vines and apple juice were supplemented with biologically active substances from grapevine and free sulfur dioxide, total antioxidant activity (TAA) by methods using DPPH and ABTS, total polyphenol content (TPC), total anthocyanins content (TAC), sugar (fructose, glucose and sucrose), alcohol (ethanol), free and volatile acids and yeast assimilable nitrogen (YAN) were determined at regular monthly intervals during beverage storage under the prescribed conditions at 5 ° C.

Free sulfur dioxide in test samples of natural beverages (Table 1 – 4) ranges from 1.84 to 15.05 mg.L^{-1}. The smallest content was found in the natural beverage from apple juice with values of biologically active substances from vine products without alcoholic fermentation after 3 months of storage, on the contrary, the highest value was in natural beverage from white grape must with alcoholic fermentation after the first determination. If we compare the achieved values for free SO_2 (Table 1 – 4) with a study of sulfur dioxide **(Snopek et al. 2018)** the measured values are compared to those in organic quality.

To determine the total polyphenols content, a spectrophotometric method using Folin-Ciocalteau reagent was used in samples of a natural beverages of grape vines and fortified beverages of apple juice, using gallic acid as a standard. For beverages tested, the higher value of total polyphenols in fortified apple beverages was made by biologically active substances from grapevine and its change during storage was only fractionally. For alcoholic beverages, they exceeded 677.26 mg GAE.L^{-1} after first measurement to 619.54 mg GAE.L^{-1} after 3 months of storage.

Table 1 Results of individual provisions for natural beverage from white grape must without alcoholic fermentation.

Length of storage [month]		1st	2nd	3rd
Free SO_2	**(mg.L^{-1}) ±SD**	2.25 ±0.12^{a}	2.21 ±0.18^{a}	2.02 ±0.24^{a}
TPC	**(mg GAE.L^{-1}) ±SD**	303.55 ±10.14^{a}	339.30 ±8.17^{b}	329.30 ±12.87$^{a, b}$
TAA (DPPH)	**(mg TE.L^{-1}) ±SD**	436.60 ±12.54^{a}	503.42 ±17.24^{b}	431.77 ±11.72^{a}
TAA (ABTS)	**(mg TE.L^{-1}) ±SD**	632.53 ±14.57^{a}	616.58 ±18.74^{a}	660.63 ±20.14^{a,b}
TAC	**(mg C3G.L^{-1}) ±SD**	5.95 ±0.98^{a}	6.26 ±1.01^{a}	6.14 ±0.52^{a}
Sugar (fru,glu,suc)	**(g.L^{-1}) ±SD**	226.64 ±2.41^{a}	228.18 ±3.24^{a}	226.11 ±2.25^{a}
Alcohol	**(% vol.)**	0.21 ±0.01^{a}	0.22 ±0.01^{a}	0.19 ±0.02^{a}
Volatile acids	**(g.L^{-1}) ±SD**	0.04 ±0.01^{a}	0.06 ±0.01^{b}	0.06 ±0.01^{b}
Total acids	**(g.L^{-1}) ±SD**	8.81 ±0.25^{a}	8.85 ±0.31^{a}	8.91 ±0.27^{a}
YAN	**(mg.L^{-1}) ±SD**	177.04 ±1.24^{a}	166.58 ±2.01^{b}	134.45 ±2.18^{c}

Table 2 Results of individual provisions for natural beverage from white grape must with alcoholic fermentation.

Length of storage [month]		1st	2nd	3rd
Free SO_2	**(mg.L^{-1}) ±SD**	15.05 ±0.74^{a}	7.45 ±0.51^{b}	6.74 ±0.59^{b}
TPC	**(mg GAE.L^{-1}) ±SD**	305.03 ±8.25^{a}	334.00 ±8.87^{b}	306.58 ±9.24^{a}
TAA (DPPH)	**(mg TE.L^{-1}) ±SD**	552.38 ±11.29^{a}	621.34 ±14.52^{b}	472.02 ±9.93^{c}
TAA (ABTS)	**(mg TE.L^{-1}) ±SD**	825.32 ±18.47^{a}	810.04 ±17.74^{a,b}	785.70 ±15.98^{b}
TAC	**(mg C3G.L^{-1}) ±SD**	4.35 ±0.42^{a}	3.91 ±0.33^{a}	3.23 ±0.28^{b}
Sugar (fru,glu,suc)	**(g.L^{-1}) ±SD**	10.44 ±0.74^{a}	9.58 ±0.98^{a}	9.37 ±0.74^{a}
Alcohol	**(% vol.)**	11.74 ±1.01^{a}	11.76 ±0.94^{a}	11.72 ±0.89^{a}
Volatile acids	**(g.L^{-1}) ±SD**	0.12 ±0.01^{a}	0.15 ±0.01^{b}	0.15 ±0.01^{b}
Total acids	**(g.L^{-1}) ±SD**	9.24 ±0.71^{a}	9.08 ±0.67^{a}	8.84 ±0.55^{a}
YAN	**(mg.L^{-1}) ±SD**	50.15 ±2.17^{a}	44.84 ±1.87^{b}	40.18 ±0.98^{c}

Table 3 Results of individual provisions for natural beverage from apple juice with values of biologically active substances from vine products without alcoholic fermentation.

Length of storage [month]		1st	2nd	3rd
Free SO_2	**(mg.L^{-1}) ±SD**	2.84 ±0.25^{a}	2.37 ±0.71^{a,b}	1.84 ±0.46^{b}
TPC	**(mg GAE.L^{-1}) ±SD**	677.26 ±12.02^{a}	672.34 ±8.07^{a}	619.54 ±8.96^{b}
TAA (DPPH)	**(mg TE.L^{-1}) ±SD**	644.64 ±10.99^{a}	609.95 ±9.92^{b}	655.58 ±10.85^{a}
TAA (ABTS)	**(mg TE.L^{-1}) ±SD**	975.73 ±15.94^{a}	916.60 ±13.34^{b}	956.89 ±14.20^{a}
TAC	**(mg C3G.L^{-1}) ±SD**	8.29 ±0.57^{a}	9.51 ±0.74^{b}	9.19 ±0.84^{a,b}
Sugar (fru,glu,suc)	**(g.L^{-1}) ±SD**	156.54 ±5.51^{a}	148.24 ±4.23^{b}	147.65 ±4.63^{b}
Alcohol	**(% vol.)**	0.12 ±0.01^{a}	0.13 ±0.01^{a}	0.09 ±0.01^{b}
Volatile acids	**(g.L^{-1}) ±SD**	0.01 ±0.01^{a}	0.03 ±0.01^{b}	0.03 ±0.01^{b}
Total acids	**(g.L^{-1}) ±SD**	10.45 ±0.47^{a}	10.40 ±0.53^{a}	10.47 ±0.61^{a}
YAN	**(mg.L^{-1}) ±SD**	156.42 ±5.17^{a}	133.81 ±4.33^{b}	124.37 ±3.86^{c}

Table 4 Results of individual provisions for natural beverage from apple juice with values of biologically active substances from vine products with alcoholic fermentation.

Length of storage [month]		1st	2nd	3rd
Free SO_2	**(mg.L^{-1}) ±SD**	10.02 ±0.74^{a}	6.87 ±0.67^{b}	6.21 ±0.71^{b}
TPC	**(mg GAE.L^{-1}) ±SD**	312.94 ±6.24^{a}	328.41 ±8.57^{b}	312.18 ±7.09^{a}
TAA (DPPH)	**(mg TE.L^{-1}) ±SD**	459.22 ±7.41^{a}	553.96 ±9.04^{b}	479.44 ±7.61^{c}
TAA (ABTS)	**(mg TE.L^{-1}) ±SD**	825.36 ±11.27^{a}	794.44 ±9.98^{b}	741.36 ±9.54^{c}
TAC	**(mg C3G.L^{-1}) ±SD**	3.31 ±0.23^{a}	2.57 ±0.19^{b}	2.66 ±0.21^{b}
Sugar (fru,glu,suc)	**(g.L^{-1}) ±SD**	25.78 ±0.89^{a}	24.14 ±0.91^{a}	22.97 ±0.95^{b}
Alcohol	**(% vol.)**	9.02 ±0.78^{a}	9.25 ±0.69^{a}	9.37 ±0.91^{a}
Volatile acids	**(g.L^{-1}) ±SD**	0.52 ±0.02^{a}	0.54 ±0.05^{a}	0.52 ±0.04^{a}
Total acids	**(g.L^{-1}) ±SD**	9.92 ±0.68^{a}	9.84 ±0.88^{a}	9.93 ±0.76^{a}
YAN	**(mg.L^{-1}) ±SD**	99.45±3.54^{a}	97.17 ±2.94^{a}	76.34 ±3.58^{b}

Note: Table 1 to 4: SO_2 – sulfur dioxide; TPC – total polyphenol content; TAA – total antioxidant ativity using DPPH and ABTS – radical scavenging activity; TE – trolox equivalent; GAE – gallic acid equivalent; C3G – cyanidine-3-glucoside equivalent; fru – fructose; glu – gluctose, suc – sucralose; YAN – yeast assimilable nitrogen; ±standard deviation. The different superscripts in rows indicate statistically significant differences between data groups (statistically tested on level of significance $\alpha = 0.05$).

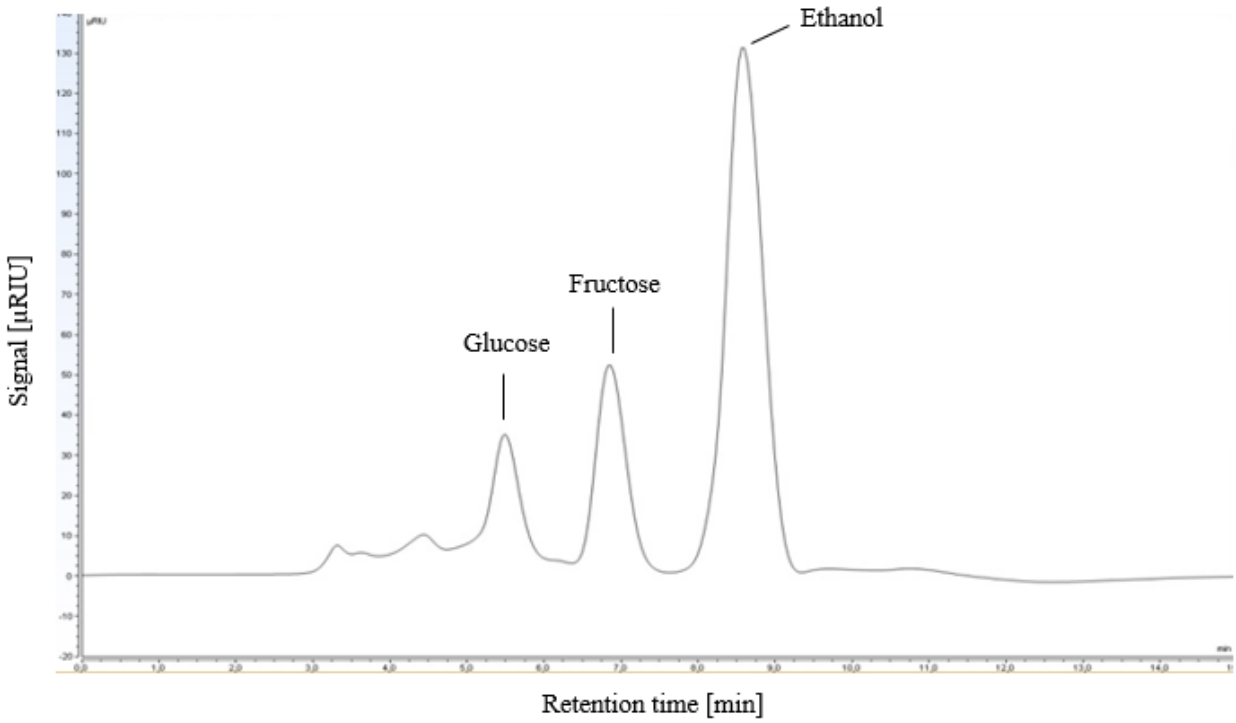

Figure 1 Chromatogram of determination of sugars and alcohol in a sample of white grapes (HPLC – RID).

The least total polyphenols were determined for a natural drink of white grape vines with alcoholic fermentation. It reached from 305.03 mg GAE.L^{-1} after the first measurement to 306.58 mg GAE.L^{-1} after three months of storage.

Paixao et al. (2007) evaluated the content of polyphenols in a fermented beverage of white grape vines. The average measured content was 369 mg GAE.L^{-1}, another study **(Hurtado et al., 1997)** reported the TPC of the beverage of 292 mg of GAE.L^{-1}. These published values are comparable to our values. The results of the work by **Ricci, Parpinello and Versari (2017)** for a TPC were reported at 222 mg GAE.L^{-1} which is in correlation with our results. Fortified beverage achieved above-average results and can be evaluated very positively. **Tarko et al. (2015)** published comparable values for apple juice, indicated as raw materials with high TPC.

To determine the antioxidant activity of natural beverage samples, the DPPH method was used, based on the reaction of the test substance with a stable 1,1-diphenyl-2-picrylhydrazyl radical. In addition, the ABTS method was used. It is characterized by reacting the test substance with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). Both methods used trolox as standard. The highest antioxidant activity in natural beverages (Table 1 – 4) was determined by both methods in non-alcoholic natural beverage from apple juice with fortified bioactive substances from white grape vines. The value ranged from 655.58 – 644.48 mg TE.L^{-1} determined by the DPPH method and 975.73 – 956.89 mg TE.L^{-1} using the ABTS method during storage. The smallest antioxidant activity was determined in a beverage sample of white grapes without alcohol.

The determination of total anthocyanins content was another of analyzes that support the high content of biologically active substances in a natural beverage based on fortified apple juice. Its content ranged from 9.51 mg C3G.L^{-1} to 8.29 mg C3G.L^{-1}. There followed a non-alcoholic natural drink of white grape vines with values ranging from 5.95 mg C3G.L^{-1} to 6.14 mg C3G.L^{-1}. A decrease in total anthocyanin values was observed for alcohol drinks during a three-month storage period with a white grape drink from 4.35 mg C3G.L^{-1} to 3.23 mg C3G.L^{-1} and for fortified apple juice with alcohol then 3.31 mg C3G.L^{-1} to 2.66 mg C3G.L^{-1}. Our measured values of the content of anthocyanin in apples reached the published values by **Wolfe, Wu and Liu (2003)**. Losses were attributed primarily to the technological modifications that took place during the production of the beverage.

Chromatographic techniques were most suitable and accurate for the identification and quantification of mono- and oligosaccharides in food **(Duarte-Delgado et al., 2015)** and according to the guidelines of the Association of Official Analytical Chemists **(AOAC, 1993; Sims, 1995)** for quantification of sugars. Table (1 – 4) lists the sugars (fructose, glucose and sucrose) content in natural beverages samples at the beginning of storage determined by the HPLC method. Figure 1 shows the chromatogram of determination of sugars and alcohol in a sample of white grapes (HPLC – RID). When the highest peak is ethanol, the other peaks represent the individual sugars. Table 1 – 4 shows that alcohol-free beverages contain up to ten times more sugars than alcohol drinks. Most of the sugars were determined for a grape wine without alcohol 226.64 g.L^{-1}, which was retained after 3 months of storage and the alcohol content fluctuated between 0.19% (vol.) and 0.22% (vol.). In fortified apple juice the sugar value dropped from the initial 156.54 g.L^{-1} to 147.65 g.L^{-1}, and its alcohol content also reduced from 0.12% (vol.) to 0.09% (vol.). Conversely, for a white grape alcohol drink,

which reached an average of 11.74% (vol.) alcohol content, it contained only 9.37 g.L^{-1} to 10.44 g.L^{-1} of sugars.

Fortified apple juice contained less alcohol from 9.02 – 9.37% (vol.), the sugar content ranged from 25.78 – 22.97 g.L^{-1}. **Pickering et al. (1998)** determined the effect of ethanol concentration (range 0-14% vol.) on perception of the "fullness" of beverages. It has been found that ethanol concentrations were highly correlated with perceived intensity, physical viscosity and density measurements **(Nurgle and Pickering, 2005).**

Volatile and total acids were also determined. Volatile acids were determined in very small amounts in all analyzed samples. Most of them were determined for fortified apple juice with an alcohol of 0.52 – 0.54 g.L^{-1}, on the other hand, the least amount in the same sample without alcohol, and in the range of 0.01 – 0.03 g.L^{-1} during the three month storage. Total acids were at highest as measured for fortified apple juice without alcohol in the range of 10.45 – 10.47 g.L^{-1}. For all other beverages, the total acid value was approximately the same as 9 g.L^{-1}.

Asymilatable nitrogen were considered to be the initiator of fermentation, affecting its kinetics, aromatic content, acetic acid and others. Table 1 – 4 shows the results of determining the amount of assimilable nitrogen in natural beverages. Highest values of assimilated nitrogen were determined for non-alcoholic beverage variants. The highest was determined for a drink from white grapes wine 177.04 mg.L^{-1} and dropped to 134.45 mg.L^{-1}. The fortified apple juice was 156.42 mg.L^{-1} and dropped to 124.37 mg.L^{-1} during 3 month storage. Conversely, for alcoholic beverages, the values were lower. Table 2 shows a decreasing value from 50.15 mg.L^{-1} to 40.18 mg.L^{-1}. Table 4 shows a high drop from 99.45 mg.L^{-1} to 76.34 mg.L^{-1}.

This confirmed **(Steidl, 2002)** that the more ammonium ions are in the environment, the more yeast consume them.

CONCLUSION

It is essential to select and evaluate sources to prepare raw material base for the production of natural beverages without and with alcoholic fermentation using exclusively natural starting values. During production it is necessary to assess natural values and quality of these basic rescources.

The high content of biologically active substances from grape vines and their incorporation into all musts is an integral part of the entire technological, production process and in combination with the corresponding fruit sources it appears to be a positive step towards enriching food with natural additives.

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MICROBIOTA OF DIFFERENT WINE GRAPE BERRIES

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ABSTRACT

The wine grape berries share a complex microbial ecology including filamentous fungi, yeasts and bacteria. The microbiota reveals different physiological characteristics and depends on the grape ripening stage and the availability of nutrients with different effect on wine production. The microbiota of grape berries (n = 12) was isolated and identified in the present study. The samples were collected in September 2018. Grape berries were obtained from Vrbovo vineyard located in Slovakia. The grape berries investigated belonged to Blue Frankish, Cabernet Sauvignon, Chardonnay, Dornfelder, Feteasca regala, Green Veltliner, Irsai Oliver, Müller Thurgau, Pálava, Pinot Blanc, Rhinriesling and Welschriesling varieties. The microorganisms were cultivated on Malt extract agar (MEA) at 25 °C for five days in aerobically for microscopic filamentous fungi and Tryptone Soya agar (TSA) at 37 °C for 24 – 48 h aerobically for bacteria and yeasts. Total bacterial counts on different wine grape berries ranged from 2.57 ±0.09 in Chardonnay to 4.39 ±0.21 log CFU.g^{-1} in Pálava. Microscopic filamentous fungi count ranged from 1.18 ±0.03 in Blue Frankish to 2.60 ±0.17 log CFU.g^{-1} in Welschriesling. MALDI-TOF MS Biotyper mass spectrometry was used for identification of microorganisms (bacteria and yeasts) and microscopic filamentous fungi with manuals. The most identified microscopic fungal species was *Alternaria* sp., for yeasts *Issatchenkia orientalis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* for bacteria.

Keywords: microbiota; grape berries; identification; MALDI-TOF MS Biotyper

INTRODUCTION

The *V. vinifera* phyllosphere is colonized by bacteria and fungi which modulate health, development and quality of grape and the produced wine characteristics **(Barata, Malfeito-Ferreira andLoureiro, 2012)**. The grape surface inhabiting microorganisms are sensitive to environmental changes during wine fermentation and cannot survive in low pH, ethanolic and anaerobic conditions. At the same their metabolic activity on the grape surface can have consequences for wine quality, e.g. metabolic changes produced by phytopathogenic fungi **(Hong et al., 2011)**.

The grape microbiota more often demonstrates the beneficial effect, and the participation of microbiota in wine fermentations can improve the sensory characteristics of wines **(Ciani et al., 2010)**. Studies of microbiota involved in wine fermentations allowed a discovery of microbial species, which show positive enological properties. The application of those microorganisms with *Saccharomyces* yeasts was commercialized in winemaking **(Ciani et al., 2010)**. With improvement of detection and identification methods in winemaking, a growing number of microorganisms were recognized as active contributors in wine fermentations with significant improvement of sensory qualities of wine **(Ciani et al., 2010)**.

Regional wines characteristics potentially are influenced by microbial biogeography, which is another important factor for winemaking. Traditional winemaking relies mostly on native grape microbiota for fermentations. This practice thought to enhance the regional typicity. The role of microorganisms is well described for grape health, fruit and wine quality **(Barata, Malfeito-Ferreira and Loureiro, 2012)**, but the effect of grape microbiota on regional characteristics of wines is still of limited knowledge. The effect of geographic region, grape variety, and climatic factors influence the bacterial and fungal communities of wine grapes was shown through the growing years **(Bokulich et al., 2014)**. The regional fungal biodiversity of grapes was demonstrated also globally **(Taylor et al., 2014; Gayevskiy and Goddard, 2012; Pinto et al., 2015)**. The local *Saccharomyces cerevisiae* strains, the principal yeast species for wine fermentations purposes, resulting in distinct wine chemical compositions, thus the role of regional microbiota is important for winemaking **(Knight et al., 2015).**

Study of microbiota of grape berries was conducted in Slovakia **(Kačániová et al., 2018).** A total of 33 species of 8 Gram-negative (G$^-$, 20.72%) and 10 Gram-positive (G$^+$, 31.53%) bacteria and 10 yeasts species of 8 genera (47.74%) were identified with MALDI-TOF Mass Spectrometry. These results show that the yeasts were the most common group pf microorganisms isolated from grapes, but the yeasts and bacteria were isolated from each

grape variety. Bacteria counts were higher than yeast. The highest counts of yeast species were identified in Irsai Oliver (10.06%), Pálava, Pinot Blanc and Rheinriesling (9.43%) grape varieties **(Kačániová et al., 2018).**

Scientific hypothesis

Grape berries contain bacteria, yeasts and moulds, which could be identified with MALDI-TOF mass spectrometry. Microbial ecology of grapes could affect the wine grape berries health. Accurate identification of wine grape berries microbiota is essential to understand the grape microbial ecology.

MATERIAL AND METHODOLOGY

Wine grape berry samples

Twelve grape samples from wineyard in Vrbové located in the Small Carpathian wine region were used in this experiment. Ripe grape bunches were collected into sterile polyethylene bags and transported to the laboratory for microbiological analyses. The grape samples of following varieties were investigated: Blue Frankish, Cabernet Sauvignon, Chardonnay, Dornfelder, Feteasca regala, Green Veltliner, Irsai Oliver, Mūller Thurgau, Pálava, Pinot Blanc, Rhinriesling and Welschriesling.

Microbiological analyses of grape berries samples

Five grams of grape berries from each variety were diluted with 45 mL of sterile physiological saline (0.85%). Berries were stirred on a horizontal shaker for 30 min. After that, the dilutions of 10^{-2} and 10^{-3} were prepared for cultivation of sampled with spread plate method. A 0.1 mL of each dilution (10^{-2}, 10^{-3}) was cultivated on Plate count agar (PCA) (Oxoid, UK) and on Malt extract agar base (MEA) (Oxoid, UK) supplemented with bromocresol green (0.020 g.L^{-1}) (Centralchem®, Slovakia). Inoculated PCA agars were cultivated at 37 °C for 24 – 48 h aerobically. Microscopic filamentous fungi were cultivated at 25 °C for five days aerobically. The identification of fungal species was done according to the manuals of **Samson et al. (2002)**, **Samson and Frisvad (2004)**, **Pitt and Hocking (2009)**.

Growing colonies with macroscopic morphological differences were recultivated on TSA (Tryptic Soya agar, Oxoid®) and inoculated plates were cultivated at 30 °C or 25 °C for 24 h for bacteria and yeasts, respectively. After cultivation, the proteins were extraction was done.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial and yeast isolate was transferred into an Eppendorf vial and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13 000 g, 2 min). After removal of supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial and yeast pellets were resuspended in 20 – 50 µl of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13 000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile / 2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification **(Kačániová et al., 2018)**.

Climatic conditions during the wine grape harvest

Climatic conditions during the harvest were characterized by air temperature (Figure 1), soil temperature (Figure 2), and cumulative rainfall (Figure 3).

Statistic analysis

All experiments were carried out in triplicate and standard deviations for replication were calculated. The experimental data were subjected to analysis of variance (Duncan's test) at a 95% confidence level (software XL STAT, 2019).

RESULTS AND DISCUSSION

In our study, the total bacteria counts isolated from different wine grape berries ranged from 2.57 ±0.09 in Chardonnay to 4.39 ±0.21 log cfu.g^{-1} in Pálava (Table 1). Microscopic filamentous fungi count ranged from 1.18 ±0.03 in Blue Frankish to 2.60 ±0.17 log cfu.g^{-1} in Welschriesling. **Kántor et al. (2015)** found the bacteria counts on Acetobacter agar (AA) from 1.76 to 2.80 log cfu.mL^{-1}. The highest counts of acetic acid bacteria on AA agar was found in grape variety Blaufränkisch (2.80 log cfu.mL^{-1}). Lactic acid bacteria (LAB) counts on MRS agar ranged from 0.48 to 2.06 log cfu.mL^{-1}, but the LAB was not isolated from white grape varieties.

Table 1 Microorganisms counts isolated from wine grape berry varieties in log cfu.g^{-1}.

Grape type	TSA	MEA
Blue Frankish	4.42 ±0.16^{a}	1.18 ±0.03^{c}
Cabernet Savignon	4.42 ±0.09^{a}	2.51 ±0.09^{ab}
Chardonnay	2.57 ±0.09^{d}	2.35 ±0.16^{b}
Dornfelder	3.77 ±0.12^{b}	1.25 ±0.06^{c}
Feteasca regala	3.75 ±0.07^{b}	2.44 ±0.11^{ab}
Green Veltliner	3.43 ±0.20^{c}	2.35 ±0.17^{b}
Irsai Oliver	3.85 ±0.09^{b}	2.37 ±0.14^{ab}
Mūller Thurgau	3.55 ±0.07^{bc}	2.49 ±0.06^{ab}
Pálava	4.39 ±0.21^{a}	1.18 ±0.04^{c}
Pinot Blanc	3.64 ±0.13^{bc}	2.44 ±0.15^{ab}
Rhinriesling	3.78 ±0.19^{b}	2.43 ±0.08^{ab}
Welschriesling	3.41 ±0.16^{c}	2.60 ±0.17^{a}

Note: TSA-Tryptic Soya agar , MEA-Malt extract agar; mean ± standard deviation; different letters in column mean that values were significantly different

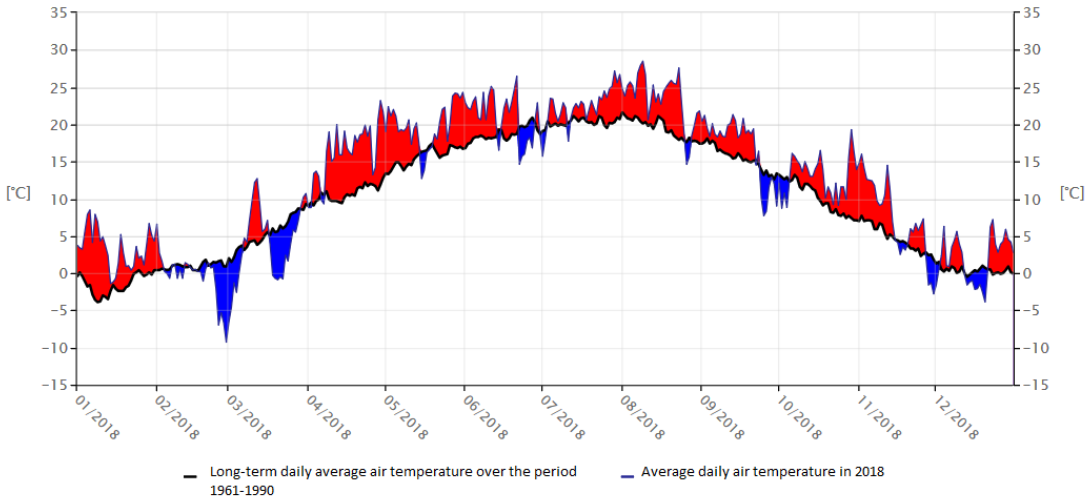

Figure 1 Average daily air temperature in Vrbové in 2018 (www.shmu.sk).

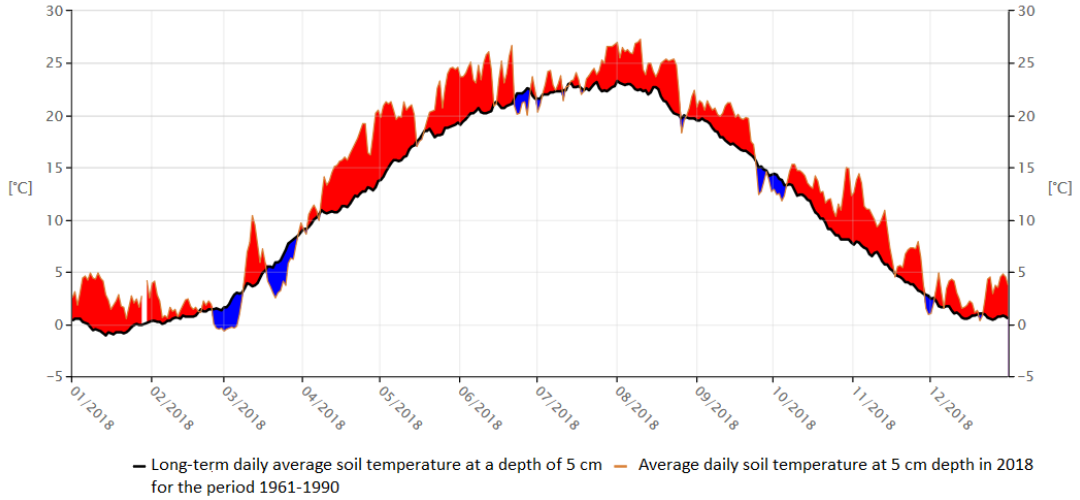

Figure 2 Average daily soil temperature in Vrbové in 2018 (www.shmu.sk).

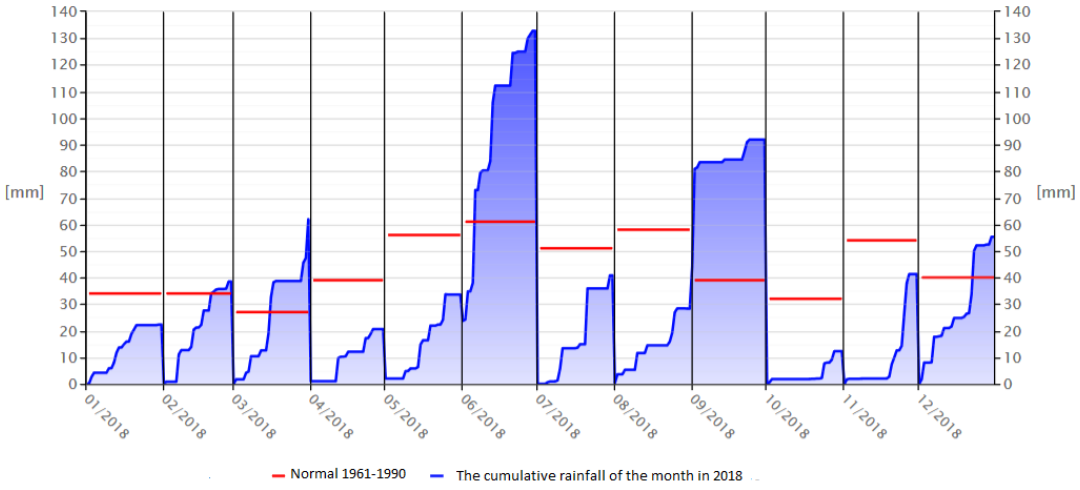

Figure 3 The cumulative rainfall in Vrbové in 2018 (www.shmu.sk).

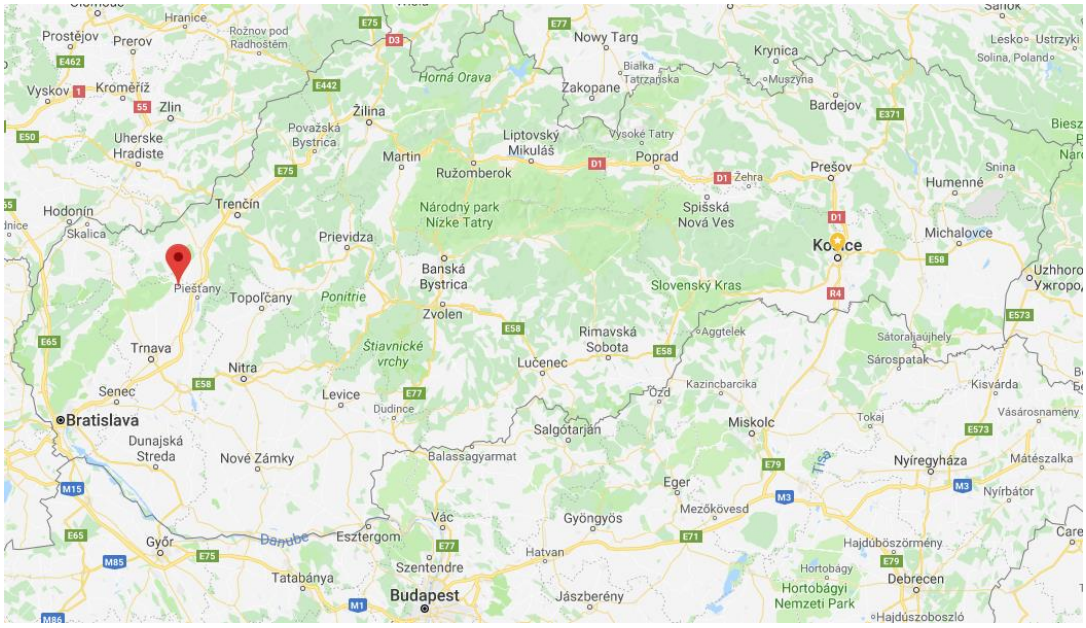

Figure 4 Vrbové, Slovakia – location.

Table 2 Microorganisms isolated from different wine grape berries varieties

Grape variety	Isolated microorganisms
Blue Frankish	*Alternaria* sp., *Arthrobacter koreensis, Bacillus cereus, Candida magnoliae, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus acidophilus, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Pantoea agglomerans, Rhodotorula glutinis, Staphylococcus epidermidis, Stenotrophomonas maltophilia, Yarrowia lipolytica*
Cabernet Savignon	*Alternaria* sp., *Arthrobacter koreensis, Bacillus cereus, Botrytis cinerea*, *Cladosporium* sp., *Enterobacter cloacae, Hanseniaspora uvarum, Ignatzschineria indica, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus acidophilus, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Micrococcus luteus, Staphylococcus epidermidis, Stenotrophomonas maltophilia, Yarrowia lipolytica*
Chardonnay	*Alternaria* sp., *Bacillus endophyticus, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus fermentum, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica*
Dornfelder	*Alternaria* sp., *Arthrobacter koreensis, Bacillus cereus, Hanseniaspora uvarum, Ignatzschineria indica, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Pantoea agglomerans, Rhodotorula glutinis, Yarrowia lipolytica*

Table 2 Microorganisms isolated from different wine grape berries varieties (continue)

Grape variety	Isolated microorganisms
Feteasca regala	*Bacillus endophyticus, Candida magnoliae, Escherichia coli, Hanseniaspora uvarum, Kazachstania exigua, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Micrococcus luteus, Penicillium expansum, Staphylococcus epidermidis*
Green Veltliner	*Alternaria* sp., *Bacillus cereus, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica*
Irsai Oliver	*Bacillus endophyticus, Cladosporium* sp., *Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Penicillium expansum, Rhodotorula glutinis, Staphylococcus epidermidis*
Mūller Thurgau	*Bacillus cereus, Cladosporium* sp., *Hanseniaspora uvarum, Ignatzschineria indica, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Micrococcus luteus, Penicillium expansum, Stenotrophomonas maltophilia*
Pálava	*Aeromonas hydrophila, Alternaria* sp., *Aspergillus niger, Cladosporium* sp., *Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Metschnikowia pulcherrima, Pantoea agglomerans, Staphylococcus epidermidis, Stenotrophomonas maltophilia, Yarrowia lipolytica,*
Pinot Blanc	*Bacillus endophyticus, Botrytis cinerea, Cladosporium* sp., *Hanseniaspora uvarum, Ignatzschineria indica, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Penicillium expansum, Stenotrophomonas maltophilia*
Rhinriesling	*Alternaria* sp., *Bacillus endophyticus, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Metschnikowia pulcherrima, Pantoea agglomerans, Penicillium expansum, Rhodotorula glutinis, Staphylococcus epidermidis, Stenotrophomonas maltophilia*
Welschriesling	*Alternaria* sp., *Bacillus licheniformis, Candida magnoliae, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Staphylococcus epidermidis, Stenotrophomonas maltophilia*

The highest LAB counts were found in grape variety Cabernet sauvignon of 2.06 log cfu.mL^{-1}, and the highest counts of LAB were detected in Blaufränkisch grape variety. The yeasts count on Sabouraud dextrose agar (SDA) ranged from 2.47 log cfu.mL^{-1} to 2.76 log cfu.mL^{-1}, and the highest counts were on Blaufränkisch grape variety surface. In generally, limited yeast diversity and low bacteria counts (10 – 10^3 CFU.mL^{-1}) were detected on immature grape berries, but the yeasts count increased to 10^4 – 10^6 CFU.mL^{-1} as the grapes were ripe enough to harvest. During ripening, the sugars diffuse from the inner tissues of the grape to the surface, that facilitating yeast

growth. Unripe grapes mostly harbour *Rhodotorula, Cryptococcus* and *Candida* species. These species could be isolated from mature, ripe grapes, however, the apiculate yeasts as *Hanseniaspora* (anamorph *Kloeckera*) and *Metschnikowia*, were mostly distributed. *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* has increased incidence on the damaged grapes **(Fleet, 2003)**.

In our samples, different bacterial, yeasts and fungal species were found (Table 2). The most abundant microscopic filemanous fungi were: *Alternaria* sp., *Aspergillus niger, Botrytis cnerea, Cladosporium* sp. and *Penicillium expansum*. *Alternaria* sp. was found in 8 (66.7%) wine grape berries samples. Altogether nine yeast species were isolated: *Candida magnoliae, Hanseniospora uvarum, Ignatzschineria indica, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Metschnikowia pulcherrima, Rhodotorula glutinis* and *Yarrowia lipolityca. Issatchenkia orientalis* was the most abundant yeast, which was found in 10 grapes varieties (83.33%). In total, 14 bacterial species were isolated: *Arthrobacter koreeniss, Bacillus endophyticus, B. cereus, B. licheniformis, Escherichia coli, Enterobacter cloacae, Lactobacillus acidophilus, L. paracasei, L. feremntum, Leuconostoc mesenteroides* subs. *mesenteroides, Micrococcus luteus, Pantotea agglomerans, Staphylococcus epidermidis* and *Stenotrophomonas maltophilia.* The most distributed bacterial species was *Leuconostoc mesenteroides* subs. *mesenteroides*, which was isolated from 10 grape berry varieties (83.33%).

The microorganisms can contaminate from different environmental sources. The origin of microorganisms could be the vineyard, can be residents of the winery flora, or can be transmitted with insects such as fruit flies and, bees **(Fleet et al., 2002)**. Over twenty yeast species have been identified in wines **(Renouf et al., 2007)**. *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida stellata* are thought to be the principal yeasts of grapes. In some studies, *Hanseniaspora* was reported to be the dominant species **(Beltran et al., 2002; Combina et al., 2005; Hierro et al., 2006)**, while *Candida* assumed to be widespread as well **(Clemente-Jimenez et al., 2004)**. Majority of *Candida stellata* isolates from wine are actually *Candida zemplinina* **(Csoma and Sipiczki, 2008)**.

Kačániová et al. (2018) isolated from the surface of grape berries different species of microorganisms. The most abundant G^- bacteria were *Stenotrophomonas maltophilia* and *Ignatzschineria indica.* Same results were found in the present study with wine grape berries. Within 22 different species of G^+ bacteria, *Bacillus endophyticus, Paenibacillus glucanolyticus, Paenibacillus lautus* and *Staphylococcus succinus* were the most isolated among bacteria *Rhodotorula mucilaginosa* was the most abundant among yeasts.

Kunová et al. (2018) found fungal counts ranged from 2.85 log cfu.g^{-1} in Cabernet Sauvignon to 4.83 log cfu.g^{-1} in Feteasca regala. After identification of 627 isolates of microscopic fungi, moulds belonged to genera *Alternaria* and *Penicillium* were the most widespread and were isolated from 100% of samples. *Alternaria* sp. was the most abundant fungal species in our study also. The high prevalence of *Aspergillus* (76.92%) and *Cladosporium* (76.92%) was found (Table 2).

Alternaria, Cladosporium and *Penicillium* were the most abundant moulds after identification of 1377 cultures of microscopic fungi isolates **Felsöciová et al. (2017)**. The identified prevalence found was similar to our results (100%). The higher prevalence was detected for *Fusarium* (100%), *Epicoccum*, *Rhizopus* (87.5%), *Botrytis*, *Aspergillus* (75%) and *Mucor* (62.5%). Different fungal genera with higher prevalence in comparison with our study were identified. **Kántor et al. (2017)** found 11 genera of G^- (11%), 11 of G^+ (27%) bacteria and nine genera of yeasts (62%) among 200 isolates of 19 Slovak grape samples. The most frequently isolated G^- bacteria were *Acinetobacter* (22%), *Pseudomonas* (22%) and *Sphingomonas* (13%). The most common genera of G^+ bacteria were *Bacillus* (20%), *Lactobacillus* (19%), *Leuconostoc* and *Staphylococcus* (11%). The most common yeasts genera were *Hanseniaspora* (37%), *Metschnikowia* (31%), and *Rhodotorula* (10%). Our results on diversity of microbial species in grape samples corresponded to **Kántor et al. (2017)** results.

Similar results were described in **Kántor et al. (2016)** study, who studied similar grape wine varieties as sampled in our study. The most dominant species was *Saccharomyces cerevisiae* isolated from all 15 new wine samples, that was a very good wine quality indicator. Altogether, seven different *S. cerevisiae* strains were identified with mass spectrometry, the second most common species was *Kloeckera apiculata (Hanseniaspora uvarum)* found in seven new wine samples. Also, other non – *Saccharomyces* yeasts such as *Metschnikowia pulcherrima*, *Pichia occidentalis* and *Pichia kluyveri* were identified.

CONCLUSION

Natural microflora of grape berries is very diverse. In our study, the bacteria were the most distributed in comparison with other groups of microorganisms. The highest bacterial counts were found in Palava grape variety, followed by Welschriesling. In our study, 5 different yeast, 9 moulds and 14 bacteria species on grape berries were identified.

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QUALITATIVE PARAMETERS OF PROTEIN GELS FROM ALBUMEN BASE

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ABSTRACT
The aim of this research was to monitor strength of egg albumen gels depending on addition additives - salt, sugar, corn syrup, citric acid, citric acid in combination with sugar, whey protein and apple fiber. The egg albumen gel was prepared under two temperature limits at 70 and 90 °C. The highest strengths of egg albumen gel were achieved at 90 °C in the albumen gel with the addition of 1% citric acid and 3.5% sugar with a strength of 7.38 N, with the lowest strength of 1.61 N being achieved with the albumen gel with 0.1% salt. For an egg albumen gel prepared at 70 °C, the strength ranged from 1.34 N (0.1% salt) to 6.63 N (1% citric acid + 3.5% sugar). On average, the pH of egg albumen gels ranged from 4.67 (1% citric acid + 3.5% sugar) to 9.05 (0.1% salt). For the strength of egg albumen gel and pH with additives of various additives at a given concentration, a statistically significant difference was found.

Keywords: hen eggs; albumen gel; strength; additives; texture analysis; temperature; pH

INTRODUCTION
Egg albumen is one of the most important sources of animal proteins that make up the main component of dry matter, and their content ranges from 10 to 12% in native protein. Egg albumen is rich in ovalbumin (about 54%), ovotransferrin (about 12%), ovomucoid (about 11%), and lysozyme (about 3.4%) **(Mine, 2002)**. It is gelation is a complex process involving protein denaturation, aggregation and formation of gel network **(Mine, 1995)**. Egg proteins are especially valuable for the high content of essential amino acids that are essential for humans because they cannot synthesize themselves in the body. The digestibility of egg proteins is in the range of 98 – 100%.

Eggs, especially egg albumen, have several functional properties that are used in the food industry. These properties include mainly gel forming ability, foaming capability and emulsifying properties. Changes in egg albumen during egg storage affect its functional properties **(Mine and Yang, 2010; Wang et al., 2010)**. Most functional properties of egg white depend on exposure of hydrophobic groups, molecular surface and interactions of these groups **(Li-Chan, Powrie and Nakai, 1995)**. Proteins produce gels by polymerization in a number of molecules, providing a three-dimensional network, and this process takes place by transforming a viscous liquid into a viscous elastic matrix **(Hermansson, 1979)**. The formation of the gel structure takes place in two phases where the first phase involves changes in conformation (mostly induced by heat) or partial denaturation of protein molecules. In the protein dispersion thus formed, the first features of the elastic solid appeared. In the second stage, aggregation of denatured proteins results in increased viscosity and formation of a fixed network. The second phase should be slower to make a gradual build-up of the organized grid network, because if accelerated at this stage, a non-organized structure would form, a coagulum that would not be able to retain water, which would lead to syneresis. The nature and properties of egg whites may be affected by several factors, predominantly protein concentration and pH values of the solution. Egg albumen gel formation can be conditioned by the use of both heating and cooling, depending on the nature of the protein and the process itself **(Alleoni, 2006)**.

The ability to coagulate egg albumen is used in many food products – such as surimi, baker's products, desserts, meat products and currently in very popular foods called "superfoods" with a high protein content, which important especially for athletes or people with alternative diets. Commercially available egg albumen products have a wide range of uses and are produced in various shapes (dice, burgers, rollers). These products can be enriched with other nutritionally valuable substances, such as fiber **(Zayas, 1997)**.

Egg albumen coagulates at pH 7 from 65.0 °C and at pH 9 to 69.5 °C. The ability of egg albumen coagulation is utilized in many food products **(Croguennec, Nau and Brulé, 2002)**. The albumen is brought to a solid state at a temperature of 61 – 70 °C. At 70 – 74 °C, the gel increases elasticity and stabilizes the gel at 89 °C. The strongest albumen gels are in the temperature range of 71 – 83 °C **(Simeonovová et al., 2013)**. Factors influencing gel formation are temperature, warm-up time, pH value and ionic strength. Egg protein properties change due to chemical changes in glycoprotein and ovomucin **(Kirunda and McKee, 2007)**. These changes lead to

a decrease in the height of the solid egg by lowering its viscosity and losing its gel-like structure **(Hammershøj and Quist, 2001; Lomakina and Míková, 2006)**. The egg albumen gels are produced without flavour and with flavours in a salty and sweet form. They can be used in cold kitchens (e.g. grated, salad cuts) or in hot kitchens (they can be baked, choked, cooked). The addition carbohydrates and/or salt in products are common in the food industry and therefore it is important to consider their effect with respect to protein gel formation **(Raikos, Campbell and Euston, 2007)**.

Gelling traditionally requires warming, but can also be caused by high pressure, acidification, enzymatic amplification, salt or urea use. The characteristics of each gel are different and depend on the degree of degradation and protein concentration **(Kaewmanee, 2010)**.

Texture is one of the major quality factors in foods in addition to appearance, flavour and nutrition. In many cases, sensory texture is correlated with rheological properties measured by instruments **(Pollak and Peleg, 1982)**. The quality of the resulting egg albumen gel is evaluated mainly by its strength.

Scientific hypothesis

The main hypothesis of this work is detection of dependence of egg albumen gel strength, pH and height on species and amount of addition.

MATERIAL AND METHODOLOGY

For the determination of the strength of the egg albumen gel were use hen eggs, hybrid Hisex Brown from a commercial breeding from South Moravia, Czech Republic. The hens were fed a complete feed mixture. Hen eggs were imported on a laying day and stored at 4 °C and 75% relative humidity. For fortification was used different salt additions (0.1 and 0.4%), sugar (10%), corn syrup (10%), citric acid (1%), citric acid in combination with sugar (1 + 3.5%), whey protein (1 and 3%), apple fiber (1 and 3%), where the strength of the egg gel was determined.

The egg albumen gel was made from the fresh eggs, where the egg was manually crushed, and the individual egg components were separated. The egg albumen was thoroughly homogenized, and the additive was added thereto at a certainly concentration. The albumen protein preparation thus prepared was poured into the sampler and placed in a water bath at 90 °C for 30 min and for comparison at 70 °C for 30 min. For measuring the strength of the gel, a universal instrument for measuring physical characteristics – TIRAtest (type 27025, TIRA, Germany) was used. A flat-plate pressure test with a crossbar speed of 100 mm.min^{-1} was performed using this instrument. From each gel was cut 8 cylinders with a height and a diameter of 1 cm.

The concentration of hydrogen ions is expressed by the hydrogen exponent (pH), which is negative logarithm of hydrogen ion concentration. Digital pH was used for determination PORTAMESS 911 pH KNICK with injection electrodes and the height of the egg albumen gels was measured with a sliding gauge.

Statistic analysis

Statistical analysis of the differences was based on Statistica12 (StatSoft, Czech Republic), namely single-factor ANOVA – Duncan's test. Microsoft Excel version 2010 (Microsoft, USA) was used to evaluate the results. The statistically inconclusive difference was considered to be a result whose probability value reached $p > 0.05$.

RESULTS AND DISCUSSION

In this study, we focused on proteins precisely albumen gels on egg albumen base. Additives were added to the homogeneity of the egg albumen mixture at a given concentration. As standard in this case, the egg albumen was used without any addition. The strengths of the egg albumen gel at a preparation temperature of 90 °C are shown in Table 1. Strengths was observed in the specimens thus prepared, when the result showed that the highest value was achieved by the gels with the addition of 1% citric acid + 3.5% sugar (7.38 N) and the lowest values were achieved in egg albumen gels with the addition of 0.1% salt (1.61 N).

Compared to the standard pure egg albumen gel, the highest value increased by 4.71 N and the lowest value dropped by 1.05 N. When using a lower sample temperature (70 °C), there were very similar results when the highest strength of the egg albumen gel was achieved in the sample with 1% citric acid + 3.5% sugar and the lowest value in the sample with 0.1% salt. **Raikos, Campbell and Euston (2007)** says that the highest achieved strength of the egg albumen gel with the addition of 3% sugar + 3% salt when the value is 14.21 N, which is higher than our results by 48%. Adding 1% apple fiber to albumen gel strength increased by 1.56 N over egg albumen gel without addition at 90 °C. Higher amounts of fiber resulted in a decrease in the strength of the protein by 0.61 N. Similarly, an albumen gel prepared at 70 °C was obtained when the addition of 1% of apple fiber increased the egg albumen gel strength by 2.03 N against the gel without addition.

When comparing the results of the egg albumen gel strength at 90 and 70 °C, it can be seen that the lower strength of the resulting albumen gel occurred at a lower sample preparation temperature. On the other hand, the lowest value was achieved by in its results in the sample with the addition of 6% salt, where the value was 4.66 N **(Raikos, Campbell and Euston, 2007)**. This value approximated our results for egg albumen gels with the addition of 1% whey protein (4.60 N) at 90 °C and at 70 °C for samples with 1% citric acid (4.86 N) added.

Holt et al. (1984) showed egg albumen gel strength were highest in gels with a treatment combination of 85.2 °C, pH 9.0, and 0.08 M NaCl. Temperature had the greatest effect on all three rheological parameters. Gels heated above 80 °C were of unusual character, exhibiting syneresis and shrinkage. The average pH values of egg albumen gel with additive additions in a given concentration are shown in Table 2. **Croguennec, Nau and Brulé (2002)** states that the highest strength of the egg albumen gel occurs at pH 5, resulting in gross aggregation, and the resulting coagulate has a low viscoelastic property whereas egg albumen gels at pH 7 and 9 are more viscoelastic. The highest strength values for egg albumen gel were obtained at pH 9.05 (0.1% salt) and the lowest ones at pH 4.67 (1% citric acid + 3.5% sugar).

Handa et al. (1998) showed that at pH 7 and 9 had egg albumen gels a fine and uniform network structure that may have contributed to the excellent gel properties. It is clear from our results that the additive additives we have chosen, have a significant effect on the measured pH values, when compared to the standard which was 9.04 for the egg albumen gel without addition, the average pH values ranged from 9.05 to 4.67. **Li et al. (2018)** states that pH values also had affect eggs' other functional properties as a foam and foam stability.

Egg albumen gel height values (Table 3) were also observed, with the highest value achieved by the albumen gel with the addition of 1% citric acid + 3.5% sugar (4.23 cm) and the lowest value of 1.98 cm protein albumen gel with the addition of 10% corn syrup.

Between individual samples a statistically significant difference was observed due to different pH values. Adding 1% whey protein reduced the egg albumen gel height by 0.8 cm. However, after the addition of 3% whey protein, the height increased by 0.31 cm in the sample at 90 °C. Egg albumen gel height results correspond to pH values, where it can generally be said that lower height albumen gel has been reached in the alkaline environment than in the acidic environment.

Table 1 Effect of type and amount of addition additives on egg albumen gel strength at 90 and 70 °C.

Quantity and type of addition	Strength [N] 90 °C	Strength [N] 70 °C
Without addition	$2.66^{b,c}$	1.98^{b}
0.1% salt	1.61^{e}	1.34^{c}
0.4% salt	2.55^{b}	1.76^{a}
10% sugar	$3.13^{a,c}$	$2.56^{a,b}$
1% apple fiber	4.22^{d}	4.01^{d}
3% apple fiber	3.61^{a}	3.15^{c}
1% citric acid	5.56^{f}	4.86^{e}
1% citric acid + 3.5% sugar	7.38^{g}	$6.63^{c,e}$
1% whey protein	4.60^{d}	4.11^{d}
3% whey protein	$3.35^{a,d}$	$2.57^{a,c}$
10% corn syrup	3.38^{a}	3.08^{a}

Note: a, b, c, d, e, f, g – different superscripts in a line indicate a statistically significant difference at $p < 0.05$.

Table 2 Effect of type and amount of addition additives on pH value egg albumen gel.

Quantity and type of addition	pH [-]
Without addition	9.04^{a}
0.1% salt	9.05^{a}
0.4% salt	9.04^{a}
10% sugar	8.94^{a}
1% apple fiber	8.77^{c}
3% apple fiber	8.23^{b}
1% citric acid	5.08^{d}
1% citric acid + 3.5% sugar	4.67^{a}
1% whey protein	6.75^{c}
3% whey protein	7.23^{b}
10% corn syrup	6.79^{b}

Note: a, b, c, d – different superscripts in a line indicate a statistically significant difference at $p < 0.05$.

Table 3 Effect of type and amount of addition additives on height of the egg albumen gel.

Quantity and type of addition	Height of the egg albumen gels 90 °C [cm]	Height of the egg albumen gels 70 °C [cm]
Without addition	2.45a,b	2.38a,b
0.1% salt	2.43a,b	2.42a,b
0.4% salt	2.51a,b	2.48a,b
10% sugar	2.49a,b	2.47a,b
1% apple fiber	2.52a,b	2.50a,b
3% apple fiber	2.75a	2.73a
1% citric acid	3.78c	3.78c
1% citric acid + 3.5% sugar	4.23d	4.22d
1% whey protein	2.37a,b	2.33a,b
3% whey protein	2.68a	2.66a
10% corn syrup	1.98b	2.00b

Note: a, b, c, d – different superscripts in a line indicate a statistically significant difference at $p < 0.05$.

CONCLUSION

There was monitored the effect of influence of the type and amount of additive additions on the rheological properties especially strength, height and pH of the egg albumen protein gels using two temperature stages of sample preparation.

Average egg albumen gel strength values ranged from 1.61 N (0.1% salt) to 7.38 N (1% citric acid + 3.5% sugar) for samples prepared at 90 °C. A native egg albumen gel sample was considered as standard without an additive addition having an average strength of 2.66 N. The closest to this strength was a sample of the albumen gel with the addition of 0.4% salt with an average value of 2.55 N.

For egg albumen gels prepared at 70 °C, average gel strengths were in the range of 1.34 N (0.4% salt) to 6.63 N (1% citric acid + 3.5% sugar). As a standard, native albumen gel was using again without addition of additives with an average value of 1.98 N, which was again most closely approximated by egg albumen gel with the addition of 0.4% salt (1.76 N).

Changes in pH values were also observed for samples after the addition of selected additives, with an average value of 9.04 in the non-admixture of the albumen gel sample. The same values were obtained with a sample of 0.4% by the addition of salt. The highest average pH was achieved by the sample with 0.1% salt addition and the lowest pH sample with 1% citric acid + 3.5% sugar (4.67).

It can be stated that the various additive additives significantly affect the functional properties of hen egg masses and the egg albumen gel, which can be used in innovations and the creation of new recipes for the food industry.

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GLUTEN-FREE RICE MUFFINS ENRICHED WITH TEFF FLOUR

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ABSTRACT

In recent years, demand for gluten-free products has grown. More and more people suffer from allergies, so the market should expand to products for this group of people. It is also important to improve the gluten-free nutritional content diets by incorporating alternative gluten free grains that are naturally rich in nutrients. Teff is a valuable ingredient of gluten-free products because it increases their nutritional quality. Teff is rich in fibre, carbohydrates and has a complete set of essential amino acids, is also high in iron and has more copper, zinc and calcium than other cereal grains. The effect of teff flour addition (25, 50 and 75%) to rice muffins on qualitative and sensory parameters was evaluated. The antioxidant activity of raw materials and products was also determined. Utilization of teff flour up to 50% provided satisfactory results. Incorporation of higher addition levels of teff flour (75%) negatively affected qualitative and textural properties of muffins; the muffins were harder, crumbly and less springy. High antioxidant potential of teff was reflected in increasing antioxidant activity of baked products. Muffins enriched with teff flour had pleasant flavor, sweet and nutty taste. Sensory evaluation revealed that rice muffins incorporated with teff flour at level 25% were the most acceptable for assessors.

Keywords: muffin; gluten-free; teff flour; rice flour, sensory

INTRODUCTION

In recent decades, gluten has attracted great attention due to the increasing number of diagnosed patients with intolerance to this protein fraction, relating to the improved sensitivity of the detection methods and the increasing awareness of the existence of the disease. Three pathologies are associated with gluten intake, which appear to be increasing in importance: i) food allergy, ii) coeliac disease, which is an autoimmune disorder caused by the ingestion of gluten not only from wheat, but also rye, barley and some varieties of oats and iii) gluten sensitivity, a pathology of intolerance to gluten **(Rosell et al., 2014)**.

Celiac disease is a cell-mediated autoimmune disease whereas wheat allergy is an immunoglobulin E (IgE) – mediated reaction. The symptoms of these disorders may vary, depending on individual sensitivity and disease severity. Celiac disease causes villous atrophy of the small intestine, resulting in various gastrointestinal and extraintestinal/systemic complications. Like other food allergies, depending on the severity, the symptoms of wheat allergy may range from mild itching to life-threatening anaphylaxis. Since there is no cure available, avoidance of gluten/wheat in the diet is the best option for patients **(Sharma, Pereira and Williams, 2015)**. The production of high-quality leavened baked goods made from ingredients other than wheat flour represents a major technological challenge, due to the absence of the visco-elastic gluten compound **(Hager and Arendt, 2013)**.

Teff is a cereal native to Ethiopia and Eritrea. It has an excellent adaptability to harsh environmental conditions and plays an important role in food security. In recent years, teff is becoming globally popular due to the attractive nutritional profile such as gluten free and high dietary fiber content **(Zhu, 2018)**.

Teff (*Eragrostis tef*) is a tropical cereal that belongs to the family of *Poaceae*, subfamily *Eragrostoidae*, tribe *Eragrosteae* and genus *Eragrostis*. About 350 species are known in the genus *Eragrostis*, of which teff is the only cultivated species **(Gebremariam, Zarnkow and Becker, 2014)**. There are about 33 improved tef varieties and hundreds of farmers' local varieties in Ethiopia, differing in seed size and color from milky-white to almost dark-brown **(Shumoy and Raes, 2017)**. For marketing purposes, teff is classified on the basis of seed color: netch (white), qey (red/brown) and sergegna (mixed) **(Gebremariam, Zarnkow and Becker, 2014)**.

Teff is the smallest grain in the world, taking 150 grains to weigh as much as one grain of wheat. The extremely small grains are 1 – 1.5 mm long and there are 2500 – 3000 seeds to the gram. Because of its small size, teff is made into whole-grain flour (bran and germ included), resulting in a very high fiber content and high nutrient content in general **(Mohammed, Mustafa and**

Osman, 2009; Gebremariam, Zarnkow and Becker, 2014).

Teff grain is gluten free and has great potential to be formulated into a range of food/beverage products to aid people with celiac disease. As a result of the unique chemical composition and the whole grain form, a range of health benefits have been associated with teff. For example, teff showed in vitro anti-oxidative activities, and can improve the haemoglobin level in human body and help to prevent malaria, and incidence of anaemia and diabetes **(Zhu, 2018)**.

Scientific hypothesis

The purpose of this study was to prepare gluten-free muffins with known additions of teff flour, determine the physical and textural properties of muffins, the antioxidant activity and the color of individual raw materials and products. It was also important to perform a sensory analysis of finished products.

MATERIAL AND METHODOLOGY

Fine rice flour (moisture 8.17%), whole grain teff flour (moisture 9.56%) and other ingredients (vegetable oil, salt, sugar, milk, eggs and baking-powder) were purchased in local market.

Muffins were prepared according to **Tess et al. (2015)**. Rice flour was replaced with 0%, 25%, 50% and 75% teff flour. Milk (174.2 g), oil (53.4 g) and egg (76 g) were mixed together with an electric hand mixer. Flour (200 g), sugar (51 g), baking powder (5.6 g) and salt (4 g) were mixed together in a separate bowl, and then were mixed into with the wet ingredients. Muffin pans were filled with the butter and were baked for 21 minutes at 190°C in a preheated oven (Mora MB05103GX, Czech Republic). Then were muffins removed from the pans and allowed to cool on wire racks for one hour after which analyses were performed. Baked muffins are presented in Figure 1.

Qualitative parameters of muffins

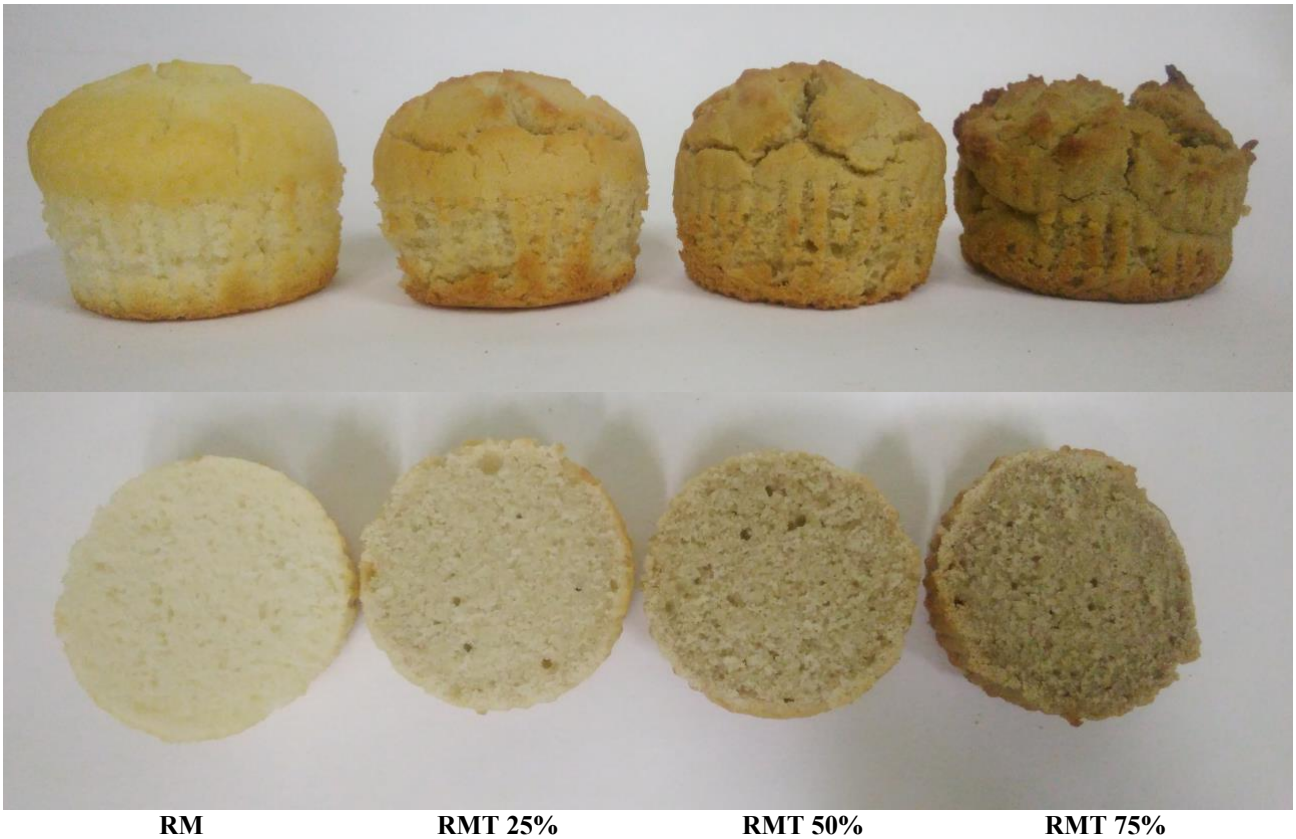
Qualitative parameters of muffins were evaluated 2 h after baking.

The muffin height and width was measured from the highest part of the muffin to the bottom part and at the widest point using a calliper **(Martínez-Cervera, Salvador and Sanz, 2015)**.

Cambering of muffins was calculated as a ratio of muffin height and width **(Lauková, Kohajdová and Karovičová, 2016)**.

Moisture of muffins was determined according to method AACC 44-19.01 **(AACC, 2000)**.

Baking loss (%) is characterized as the muffin weigh reduction after baking. The muffins were weighed before (W3) and after baking and 2 h cooling (W4). The weighting mean mass loss during baking was calculated as follows: weight loss = (W3-W4)*100/W3 **(Martínez-Cervera, Salvador and Sanz, 2015)**.



RM **RMT 25%** **RMT 50%** **RMT 75%**

Figure 1 Photo of muffins.
Note: RM – rice muffins without teff flour. RMT – rice muffins with teff flour (25, 50 and 75%)

Textural analysis

Muffin firmness was determined according to modified method described by **Acosta, Cavender and Kerr (2011)** using a texture analyzer (TA-XT Plus, Stable Micro Systems, Godalming, Surrey UK). Firmness and springiness were measured using Method MUF1/P36R. Firmness was defined as the force (in grams) required compressing the product by a pre set distance. A simple way of looking at the springiness property is to record the force after 30 seconds and divide this by the maximum force and then multiply by 100%. The closer the resulting value is to 100% the more like a „spring" the product is. Cross sections of 2.5 cm thickness were cut from the center of each muffin and subjected to a modified compression test fitted with a 36 mm diameter cylindrical probe. Each sample was compressed to 40% of the sample's initial height at a probe speed of 1.0 $mm.s^{-1}$.

The textural profile analysis (TPA) was conducted on the muffins using a texture analyzer. The quality attributes measured were hardness, springiness, cohesiveness and chewiness **(Gupta, Sharma and Sharma, 2007)**. Hardness is defined as the maximum peak force during the first compression cycle (first bite). Springiness is related to the height that the food recovers during the time that elapses between the end of the first bite and the start of the second bite. Cohesiveness is defined as the ratio of the positive force during the second compression to that during the first compression **(Tess et al., 2015)**. Chewiness is obtained by multiplying harness, cohesiveness and springiness **(Cornejo and Rosell, 2015)**. Gumminess is defined as a product of hardness x cohesiveness **(Bourne, 2002)**. The test was performed on cubes (2.5 cm side) taken from the center of the muffin. The test speed was 1.7 $mm.s^{-1}$; the post test speed was 10 $mm.s^{-1}$ and there was a 5 s interval between the two compression cycles. A trigger force of 5 g was selected. The compression of 40% was performed with a 36 mm cylindrical probe, and the cubes were compressed twice **(Tess et al., 2015)**.

Color measurement

The color was determined using a Cary 300 Spectrophotometer (Agilent Technologies, USA). The color of the rice flour, teff flour and muffins from these flours was measured. A crumb of muffins was dried and grinded with a kitchen robot (Eta 0010, Czech Republic) before measuring. The individual color values were expressed using CIELab* and Metric L*Ch*. The color parameters were L* (L* = 0, black and L* = 100, white), a* (-a* = greenness and +a* = redness), b* (-b* = blueness and +b* = yellowness), C – Chroma and h* – hue angle. The spectrophotometer was calibrated with a white calibration tile **(Kraithong, Lee and Rawdkuen, 2018)**. The total color difference (ΔE) was determined using the equation according to **Ghanem et al. (2012)**.

Determination of antioxidant activity

Antioxidant activity was evaluated by measuring free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity according to **Cai et al. (2014)**. Sample (0.1 g) was extracted with 1 mL of pure methanol at 25 °C for 2 h with continuous shaking under a dark environment and centrifuged at 1,200 × g for 10 min. The extract (0.05 mL) was reacted with 1 mL of 0.1 mM DPPH solution at 25 °C for 30 min, and absorbance was measured at 517 nm. Antioxidant activity was calculated as percent discoloration of DPPH = $[1 – (A1/A0)] \times 100$, where $A1$ is the absorbance of sample extract at the end of the reaction (t = 30 min) and $A0$ is the absorbance of the pure methanol control at the beginning of the reaction (t = 0). Measurements were conducted in duplicate, and the data were reported as percentage of discoloration.

Sensory evaluation of muffins

The sensory evaluation of muffins was made by five point hedonic scale which ranged from 5 = most liked to 1 = most disliked. The panel was made up of staff and students of the Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia. The overall acceptability of muffins was determined using 100 mm graphical non-structured abscissas with the description of extreme points (minimal or maximal intensity, from 0 to 100%) according to **Lauková, Kohajdová and Karovičová (2016)**.

Statistic analysis

All measurements were carried out in triplicate and the average values were calculated. The results were expressed as mean value ± standard deviation. Significant differences between mean values at significance level $p < 0.05$ were compared using Student`s test. Microsoft Excel version 2010 was used as the statistical analysis software.

RESULTS AND DISCUSSION

The qualitative (cambering, moisture and baking loss) and textural (firmness and springiness) parameters of muffins are shown in Table 1.

The cambering value of control sample (RM) was 0.79. From the results concluded that addition of teff flour increased the cambering of muffins up to 0.88 (RMT 75%).

Moisture content of muffins showed no significant differences after addition 25 – 50% of teff flour. Addition of 75% of teff flour increased muffins moisture to 41.20%. A high level of moisture content may be indicating short self life of composite muffins as they encourage microbial growth leads to spoilage **(Man et al., 2014)**.

Determining the actual baking losses is very important as the finished product after baking must have a defined weight. The loss by baking is influenced mainly by the weight of the product; by shape and moisture content **(Minarovičová et al., 2018)**. Increasing level of teff flour caused decreasing of baking loss values.

In baking industry, the products having a specific shape and definite texture determine the acceptance or rejection of the product by the consumers. Texture of product shows its quality **(Younas et al., 2015)**. Texture evaluation demonstrated that muffins including 25 and 50% of teff flour had similar firmness compared to control sample (RM). However, the 75% replacement of rice flour resulting in 39.15% increase of muffin firmness. Similar trend was observed when the hardness was measured using TPA (Table 2). Comparable results were also described by

Table 1 Qualitative and textural parameters of muffins.

	Cambering	Baking loss (%)	Moisture of crumb (%)	Firmness (g)	Springiness (%)	Overall acceptance (%)
RM	0.79 ±0.01	18.18 ±0.36	40.07 ±0.14	3278.92 ±164.00	62.51 ±1.13	91.73 ±5.15
RMT 25%	0.78 ±0.02	20.42 ±0.69	39.91 ±0.59	3452.83 ±170.04	57.58 ±1.30	91.10 ±2.34
RMT 50%	0.81 ±0.02	17.51 ±0.67	40.27 ±0.52	3536.74 ±168.88	54.60 ±0.79*	90.40 ±5.92*
RMT 75%	0.88 ±0.04*	15.92 ±0.70*	41.20 ±0.16*	4562.75 ±169.26*	49.73 ±1.61*	79.60 ±8.33*

Note: RM – rice muffins without teff flour, RMT – rice muffins with teff flour (25, 50 and 75%), * denotes statistically significant difference at p <0.05 level.

Table 2 TPA parameters of muffins.

	Hardness (g)	Gumminess	Chewiness	Springiness	Cohesiveness
RM	5534.36 ±239.64	3752.88 ±177.28	3716.79 ±127.88	0.95 ±0.00	0.67 ±0.00
RMT 25%	5642.03 ±184.51	3747.68 ±209.43	3482.48 ±196.98	0.93 ±0.01	0.64 ±0.01
RMT 50%	5562.78 ±266.82	3071.74 ±231.41*	2713.38 ±193.85*	0.88 ±0.03*	0.55 ±0.02*
RMT 75%	6329.06 ±185.45*	2589.47 ±11.85*	2067.76 ±30.77*	0.79 ±0.01*	0.42 ±0.00*

Note: RM – rice muffins without teff flour, RMT – rice muffins with teff flour (25, 50 and 75%), * denotes statistically significant difference at p <0.05 level.

Table 3 Color parameters of raw materials and muffins.

	L*	a*	b*	C	h*	ΔE
RF	89.06 ±0,05	0.11 ±0.00	5.18 ±0.01	5.18 ±0.01	88.74 ±0.01	-
TF	73.56 ±0,10	2.09 ±0.00	12.97 ±0.05	13.14 ±0.04	80.86 ±0.04	-
RM	77.81 ±0.05	1.38 ±0.03	14.44 ±0.13	14.51 ±0.13	84.52 ±0.06	-
RMT 25%	72.33 ±0.02	2.14 ±0.01*	16.37 ±0.04*	16.51 ±0.04*	82.57 ±0.02*	17.19 ±0.13
RMT 50%	68.00 ±0.11*	2.85 ±0.02*	16.96 ±0.24*	17.20 ±0.24*	80.47 ±0.06*	52.49 ±1.76
RMT 75%	64.58 ±0.05*	3.13 ±0.01*	16.01 ±0.01*	16.31 ±0.01*	78.93 ±0.03*	90.42 ±0.70

Note: RF – rice flour, TF – teff flour, RM – rice muffins without teff flour, RMT – rice muffins with teff flour (25, 50 and 75%), * denotes statistically significant difference at p <0.05 level.

the authors **Tess et al. (2015)** in muffin enriched with teff flour.

Springiness is associated with freshness in a product with a high quality muffin having higher springiness values **(Tess et al., 2015)**. The increase in the muffin firmness is related to the decrease in muffin springiness. With higher addition levels of teff flour the muffins were less springy.

TPA parameters of muffins are summarized in Table 2. Gumminess is defined as the energy required to disintegrate a semisolid food to a state of readiness for swallowing **(Bourne, 2002)**. In this study was observed that addition of teff flour at higher levels (50 and 75%) caused significantly lower gumminess of muffins.

Springiness is a measurement of how much the crumb springs back after being compressed once and it can be defined as the elasticity of the crumb, it is also an important parameter to determine the staling degree of product **(Lauková et al., 2017)**. Substituting of rice flour in muffins with teff flour resulted in lower springiness, similarly to the protocol MUF1/P36R which was used in textural analysis.

Cohesiveness is defined as how well the product withstands a second deformation relative to how it behaved under the first deformation **(Boz and Karaoğlu, 2013)**. It was noticed that muffins with 25% of teff flour had comparable cohesiveness with control sample (RM). Higher substitution levels caused lower cohesiveness. These results are in agreement with study of **Tess et al. (2015)**.

Chewiness is related to the work needed to chew a solid sample to a steady state of swallowing **(Boz and Karaoğlu, 2013)**. Results in Table 2 also showed that increasing level of teff flour led to significantly lower chewiness of muffins.

The color of bakery products is affected by ingredients, process, and ingredient process interactions, such as Maillard or caramelization reactions **(Kırbaş, Kumcuoglu and Tavman, 2019)**. Color also depends on the concentration of a certain ingredients **(Bhadury, 2013)**. Rice flour is white in color and teff flour can range in color from ivory to light brown. This fact was confirmed with result presented in the Table 3. The highest lightness (L*) was observed in rice muffins (RM). Significant decrease of this parameter was detected in samples containing 50 and 75% of teff flour, which is the consequence of darker color of initial teff material. Incorporation of teff flour caused in higher a* and b* color parameters. Chroma (C*), considered the quantitative attribute of colorfulness, is used to determine the degree of difference of a hue in comparison to a grey color with the same lightness. The higher the C* value, the higher is the color intensity of samples perceived by humans **(Granato and Masson, 2010)**. Higher color intensity (C*) of muffins was related to high C* value identified in teff flour. These findings are reflected in color differences (ΔE), which had increasing trend up to 90.42 (RMT 75%).

The antioxidant activity (percentage of discoloration) was measured in raw material and also in baked products

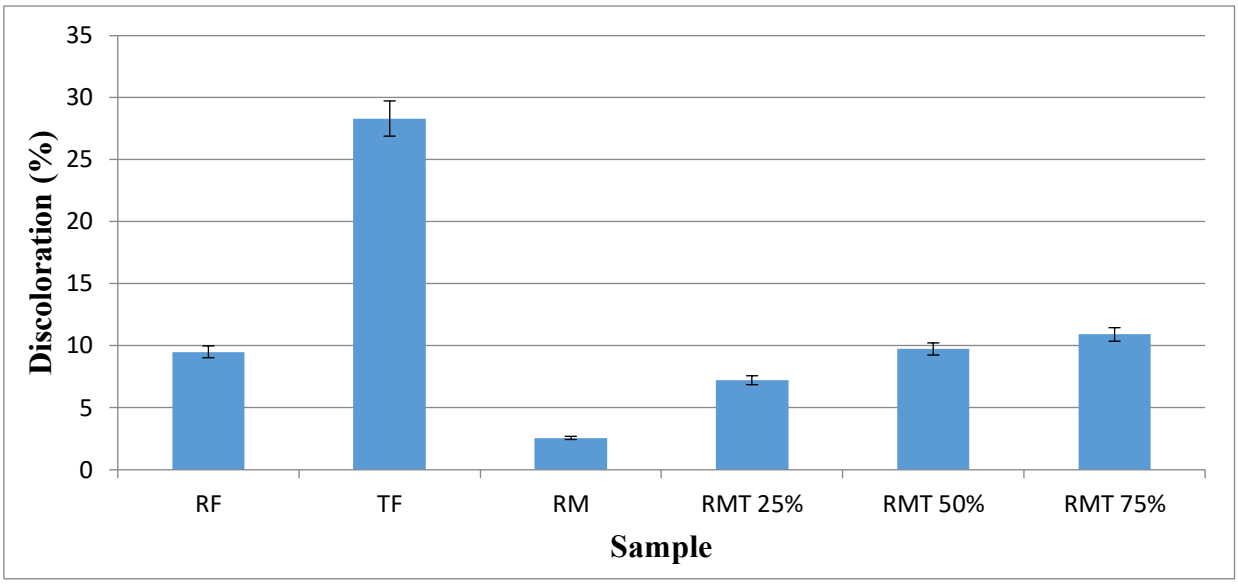


Figure 2 Antioxidant activity of raw materials and muffins.
Note: RF – rice flour, TF – teff flour, RM – rice muffins without teff flour, RMT – rice muffins with teff flour (25, 50 and 75%).

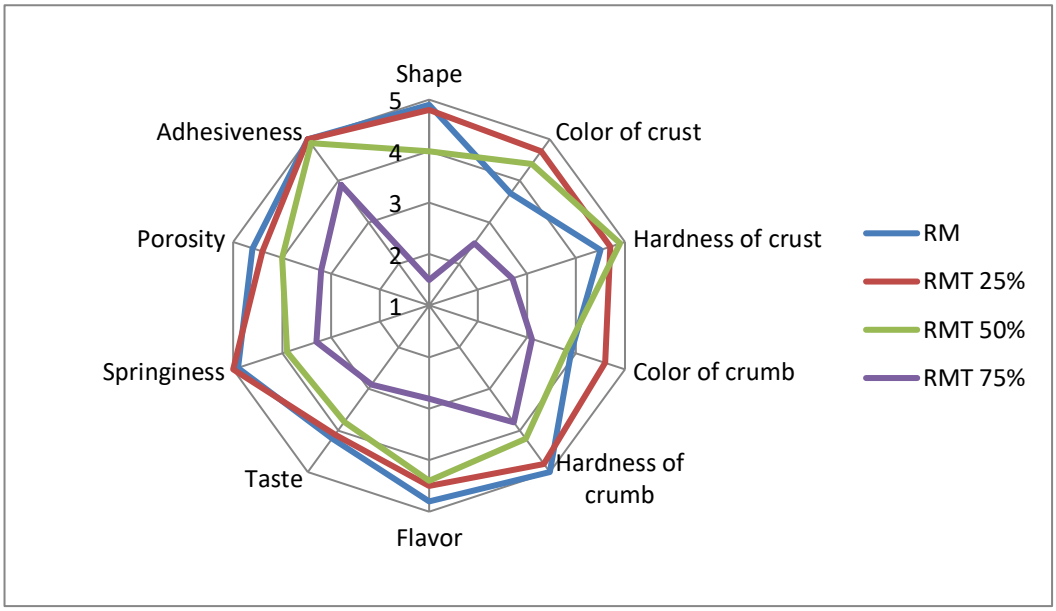


Figure 3 Sensory evaluation of muffins.
Note: RM – rice muffins without teff flour. RMT – rice muffins with teff flour (25, 50 and 75%)

(Figure 2). As can be seen from the results, teff flour had about 3-times higher antioxidant activity (28.32%) than rice flour (9.51%). Thereupon the teff enriched muffins also had higher antioxidant activity (7.22 – 10.91%). The effects of teff flour on sensory parameters of muffins are presented in Figure 3. Generally, teff supplementation of rice flour resulted in decreasing of shape score of muffins. The highest addition level of teff led to cracked and less compact shape of muffins. Color is an important attribute of the baked food products because it affects to the consumer's perception to the acceptability of the product **(Bhadury, 2013)**. The results showed that color of enriched muffins, both for crust and crumb, was more acceptable for assessors up to addition level 50% than control sample (RM). The score for flavor of muffins was not significantly affected by teff addition, except for sample including 75% of teff. The muffins enriched with 25 and 50% of teff flour had similar sensory score of taste with control sample (RM). Moreover, the assessors describe the pleasant sweet and nutty taste of teff incorporated muffins. The assessors also described that muffins contained high levels of teff were harder and less springy compared to control sample (RM). Results also showed that incorporation of teff at higher levels caused that muffins had less porosity.

The overall acceptance results of muffins are summarized in Table 1. It was concluded that the most acceptable enriched muffins (91.10%) were prepared with 25% of teff flour, which was comparable with overall acceptability of control sample RM (91.73%), while higher supplementation level caused the lower acceptance of muffins. Similar decreasing trend was described by **Tess et al. (2015)** for rice muffin enriched with teff flour.

CONCLUSION

In this study it was noticed that lower addition of teff flour in the muffins had similar quality parameters like control rice muffins. Moreover, enriched muffins had better color, flavor and taste. In general, it was concluded that muffins with acceptable qualitative and sensory parameters can be prepared by addition of teff flour at level 25 and 50%.

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MYCOTOXIN-PRODUCING *PENICILLIUM* SPP. AND OTHER FUNGI ISOLATED FROM GRAPES FOR WINE PRODUCTION IN SMALL CARPATHIANS WINE REGION

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ABSTRACT
The diversity of mycobiota associated with grapevine in Vrbove, Slovakia at the harvest time in 2018 was evaluated. Fourteen samples of grapes were analyzed by plating methods and by plating methods with surface disinfection. The identification of fungi was performed using the morphological and microscopical characteristics. From the 1001 strains detected and identified from exogenous mycobiota, the most frequent genera were *Alternaria, Rhizopus* and *Sordaria*. Their relative density was low, except *Alternaria*. The most frequently encountered moulds and with the highest relative density from endogenous mycobiota were *Alternaria, Cladosporium* and *Penicillium*. Most of all genera had relative density less than 1%. *Penicillium* contributed small proportion in both sources. *Penicillium citrinum* was the most dominant species in exogenous and endogenous mycobiota. *Penicillium expansum* and *P. glabrum* were recorded in exogenous source and *P. hordei, P. chrysogenum* and *P. griseofulvum* in endogenous. Potentially toxigenic *Penicillium* species were tested for their toxigenic ability by thin layer chromatography method. Out of 15 tested isolates representing five potentially toxigenic species 11 produced at least one mycotoxin. Positive toxinogenity was detected in all tested strains of *Penicillim citrinum* (9/9).

Keywords: grapes; *Penicillium*; microfungi; mycotoxins; TLC

INTRODUCTION
Fresh grapes are prone to fungal contamination in the fields, during harvesting, transporting, marketing and during storage under domestic conditions. If the spoiling fungi are toxigenic or pathogenic, they could pose a health risk for the consumer. Grape fruit contain high levels of sugars and other nutrients, and they possess and ideal water activity for microbial growth, their low pH makes them particularly susceptible to fungal spoilage **(Tournas and Katsoudas, 2005)**.

The most frequent filamentous fungi found in grapes were species of *Cladosporium, Penicillium, Botrytis, Alternaria* and *Aspergillus* **(Serra et al., 2005; Fredj et al., 2007)**. The grape mycrobiota may change in response to various factors such as: the climate, grape variety and geographical region **(Raspor et al., 2006)**.

Penicillium species are one of the most common fungi occurring in a diverse range of habitats, from soil to vegetation, air, indoor environments and various food products **(Visagie et al., 2014)**. Species of *Penicillium* are economically, ecologically and medically important microorganisms playing vital roles in natural ecosystems, agriculture and biotechnology **(Visagie, 2008)**. Many species of the *Penicillium* are among the common postharvest pathogens on a wide range of fruits and vegetables. *Penicillium expansum*, the main cause of green (blue) mold of apple, pear and fruits of other deciduous trees, is an example of destructive pathogens that causes much of post-harvest economic losses on food during storage and marketing stages **(Barkai-Golan, 2008)**. Two main genera are responsible for mycotoxin production in grapes: *Aspergillus* and *Penicillium*. The mycotoxin production is characteristic of the species and therefore by identifying the species one can predict potential mycotoxin hazards **(Serra et al., 2006)**. Green mould produces mycotoxins for example patuline which is however degraded during fermentation and by sulphurization. Berries affected by green mould have an off-flavour and even a small amount of infected berries add a mouldy taste to the wine **(Kassemeyer and Berkelmann-Löhnertz, 2009)**. Ochratoxin A (OTA) producing fungi are members of the genera *Aspergillus* and *Penicillium*. Natural occurrence of OTA has been reported from temperate to tropical climates mainly on cereals and their products, beverages such as bread, beer, coffee, dried fruits, grape juice and wine among others. Only some OTA producing species are known to be a potential source of OTA contamination of these commodities, including *A. niger* aggregate, *A. carbonarius, A. ochraceus, A, westerdijkiae,*

A. steynii, Penicillium verrucosum and *P. nordicum* **(Cabañes et al., 2010)**.

In our work the goal was to assess the fungi present on the surface and inside of healthy grapes destined for commercial winemaking at harvest time. Emphasis was given to *Penicillium* species due to their relevance for mycotoxin production.

Scientific hypothesis

During maturation of grapes, the spoilage agents, *Aspergillus, Penicillium* and *Rhizopus*, increase their incidence. When the temperature is higher then 37 °C, species in *Aspergillus* section *Nigri* become predominant **(Valero et al., 2007)**. At harvest time, the conditions are optimal for fungal invasion, especially if physical damage had occurred on grape. Certainly the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions.

MATERIAL AND METHODOLOGY

Study area

The grapes (*Vitis vinifera*) used in this study originated from a small family winery founded in 2002. Their goal is to produce quality wines mainly from grapes from their own production. The farm is situated in Vrbovsky subregion, in the village of Vrbove, which is part of the Small Carpathians wine region. In the years 2014 – 2016, 10 ha of their own vineyards were planted in Vrbove. Young vineyards are great advantage because they had the opportunity to choose many varieties according to the climate, soil type, but also their own experience so that they could obtain a high-quality raw material for the production of unique wines. They planted old varieties that were grown in Vrbove in the past, for example Green Veltliner, Müller Thurgau, Sauvignon, but also modern varieties such as Pálava, Cabernet Sauvignon and Alibernet.

The Small Carpathians wine region has a medium climate and abundant moisture. The spring of 2018 was an extremely abnormal temperature in Slovakia. April and May were two recordable warm months in Hurbanovo. The average monthly air temperature reached 16.2 °C in April and 19.7 °C in May. Summer was extremely hot with the absence of longer, cooler days. Intense rainfall occurred at the beginning of September **(Siman, 2019)**.

Grape samples

Wine company provided the grapes grown for commercial winemaking. A total of 14 samples were taken: ten from white varieties (Green Veltliner, Seteasca Regala, Chardonnay, Rheinriesling, Welschriesling, Sauvignon, Pálava, Pinot Blanc, Irsai Oliver, Müller Thurgau and the remaining 4 from red varieties: Dornfelder, Blaufränkisch, Alibernet, Cabernet Sauvignon, during the period from the end of August to the beginning of September 2018, in the maturation stages corresponding to harvest. The samples comprised 10 bunches of grapes collected across two diagonal transects. Grape samples were put directly each into a sterile plastic bag. Samples were brought into the laboratory and kept at 5 °C till fungal analysis.

Isolation and identification of fungi

A total of 50 berries (7 – 8 berries per bunch) from each sample were plated in Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) and incubated at 25 ±1 °C in the dark for one week. The detection of fungi in grape samples was also made by plating methods with surface disinfection. The whole 50 grapes were surface-disinfected in 1% NaClO for 1 min according methods of **Magnoli et al. (2003)**. After thorough washing with sterile distilled water (3 times, total amount 1L), and drying were inserted on the agar surface DRBC as a whole fruit and incubated at 25 ±1 °C in the dark for 5 – 7 days.

The identification of fungal taxa based on macroscopic and microscopic features, with guidelines by **Pitt and Hocking (2009)**.

Media used for isolation of fungi were:

a) Dichloran Rose Bengal Chloramphenicol agar medium (DRBC, MERCK, Germany) to which rose bengal (25 $\mu g.mL^{-1}$) was used to inhibit rapidly growing fungi and chloramphenicol (100 $\mu g.mL^{-1}$) was used as bacteriostatic agents.

Penicillium strains were isolated and cultivated on these medias:

b) Malt extract agar (MEA),
c) Czapek yeast agar (CYA),
d) Creatine-Sucrose agar (CREA),
e) Yeast Extract agar (YES) **(Samson et al., 2010)**.

Genus *Penicillium* was identified to species level based on morphological characters according to special mycological literature of **Pitt and Hocking (2009), Samson and Frisvad (2004)** and **Samson et al. (2002a, 2010)**.

Mycotoxins analysis

A total of 15 different isolates of filamentous fungi from different samples belonging to *Penicillium* spp. were examined for their ability on mycotoxins production by thin layer chromatography (TLC) according to **Samson et al. (2002b)**, modified by **Labuda and Tančinová (2006)**. Extracellular metabolites – citrinin, griseofulvin and patulin were carried out on YES agar and intracellular roquefortine C and cyclopiazonic acid on CYA agar. A few pieces of mycelium with approximate size 5 x 5 mm were cut from colonies and placed in an Eppendorf tube with 500 µL of chloroform:methanol – 2:1 (Reachem, Slovak Republic). The content of the tubes was stirred for 5 min by Vortex Genie ® 2 (MO BIO Laboratories, – Carlsbad, CA, USA).

Mycotoxins detection

Mycotoxins extracted from fungal isolates cultures were determined by thin layer chromatographic technique on pre-coated silica gel plate (Alugram ® SIL G, Macherey – Nagel, Germany). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on TLC plate. The plate was put into TEF solvent (toluene:ethyl acetate:formic acid – 5:4:1, toluene – Mikrochem, Slovak Republic; ethyl acetate and formic acid – Slavus, Slovak Republic). After elution the plate was air-dried. Mycotoxins were identified by comparison with appropriate reference standards of mycotoxins.

Table 1 Fungi identified in Slovak wine grapes from 2018 by the direct plating method.

Fungal taxa	No.	Fr (%)	RD (%)
Alternaria	868	100	87
Aspergillus	5	14	<1
Aureobasidium	1	7	<1
Botrytis	18	43	2
Cladosporium	4	21	<1
Epicoccum	7	36	<1
Mucor	3	14	<1
Penicillium	5	21	<1
from this			
P. citrinum	3	7	
P. expansum	1	7	
P. glabrum	1	7	
Rhizopus	49	93	5
Sordaria	13	71	1
Trichoderma	1	7	<1
Mycelia sterilia	19	71	2
Total isolates	**1001**		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

Table 2 Fungi identified in Slovak wine grapes from 2018 by the direct plating method with surface disinfection.

Fungal taxa	No.	Fr (%)	RD (%)
Alternaria	414	100	81
Aspergillus	1	7	<1
Botrytis	1	7	<1
Cladosporium	49	79	10
Epicoccum	3	21	<1
Fusarium	2	14	<1
Penicillium	14	43	3
from this			
P. citrinum	6	21	
P. griseofulvum	1	7	
P. hordei	4	7	
P. chrysogenum	3	21	
Rhizopus	7	29	1
Trichoderma	1	7	<1
Mycelia sterilia	17	64	3
Total isolates	**509**		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

Table 3 Toxinogenity of selected *Penicillium* strains, isolated from exogenous and endogenous mycobiota of wine grapes.

Species/Exo	C	G	P	CPA	RC
P. citrinum	3*/3**				
P. expansum	1/1		0/1		0/1
Species/Endo					
P. citrinum	6/6				
P. griseofulvum		1/1	1/1	0/1	0/1
P. hordei					0/1
P. chrysogenum					0/3

Note: * - number of isolates with ability to produce mycotoxin, ** - number of tested isolates, C – citrinin, G – griseofulvin, P – patulin, CPA – cyclopiazonic acid, RC – roquefortin C.

Roquefortine C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange spot. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Patulin by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH), (Merck, Germany) in methanol and heating at 130 °C for 8 min and then detectable as a yellow-orange spot. Directly under UV light with a wavelength of 365 nm were visualized citrinin as a yellow-green-tailed spot and griseofulvin as a blue spot.

Statisic analysis

The obtained results were evaluated and expressed according to relative density (RD) and isolation frequency (Fr). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam et al., 2009)**. These values were calculated according to **González et al. (1999)** as follows:

$$RD\ (\%) = (ni / Ni) \times 100$$

where ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. These values were calculated according to **González et al. (1999)** as follows:

$$Fr\ (\%) = (ns / N) \times 100$$

where: ns – number of samples with a species or genus, N – total number of samples.

RESULTS AND DISCUSSION

A survey study was conducted on 14 samples of fresh grape which collected from Vrbove, Slovakia. Isolation of fungi contaminated fresh grape resulted in collecting of 1001 fungal isolates. Data in Table 1 show that, eleven fungal genera namely *Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Epicoccum, Mucor, Penicillium, Rhizopus, Sordaria, Trichoderma* and *Mycelia sterilia* (unidentified fungus without creation fruiting bodies) were identified from fresh grape samples.

Data in the same Table 1 showed that, *Alternaria* was the most frequently occurring genus (100 %) which record 868 isolates, with the highest relative density 87%. *Rhizopus* was the second predominant genus which recorded frequency of 93%, but low relative density (5%), followed by *Sordaria* (71% Fr, 1% RD). *Botrytis, Epicoccum, Penicillium, Cladosporium, Aspergillus, Mucor Aureobasidium* and *Trichoderma* were less fungal frequency occurred in all grape samples.

Khashaba et al. (2018) reported that prevalence of *Alternaria* was moderate fungal frequency (50%) and *Aspergillus* was the most frequently occurring genus (100%) in Egypt. *Penicillium* was the second predominant genus which recorded frequency of 92.5%. In our study *Aspergillus* and *Penicillium* were less fungal frequency occurred in all grape samples. Similar results that *Aspergillus* is the main fungal genus were obtained by several studies **(Alisa et al., 2007; Fredj et al., 2007)**. *Alternaria, Botrytis* and *Cladosporium* were three of the most frequent genera in all four winemaking regions in Portuguese **(Serra et al., 2006)**, representing 16, 17 and 24% of the total identified strains, respectively.

Figure 1 *Penicillium expansum.*

Figure 2 *Penicillium chrysogenum.*

According to the region considered, other frequent fungi were *Aureobasidium pullulans, Aspergillus niger, Epicoccum nigrum, Penicillium brevicompactum, P. thomii* and *Rhizopus*. *Aspergillus* and *Penicillium* were also an important part of the mycobiota representing 15 and 24%, respectively, of all the fungi found in the regions. *Aspergillus* was more frequent than *Penicillium* in almost all regions. In our samples *Botrytis* represented 2% of the total identified strains and *Cladosporium* less than 1%, but *Alternaria* was the most frequent genus, too. **Serra et al. (2006)** described that the most frequent *Penicillium* species were *P. brevicompactum, P. thomii* and *P. glabrum* which together accounted for approximately 71% of the strains identified in the genus. Five species of *Penicillium* including *P. brevicompactum*, *P. citrinum, P. echinulatum, P. expansum* and *P. solitum* reported from grapes in Korea **(Won et al., 2007)**. **Serra and Peterson (2007)** described two new species of *Penicillium*, namely, *P. astrolabium* and *P. neocrassum* from contaminated grapes in Portugal. The reports on *Penicillium* species occurring on grape and raisin from Iran are very rare **(Rahmani et al., 2012; Maulani et al., 2012)**, mainly *P. expansum*, *P. brevicompactum* and *P. glabrum* **(Houbraken et al., 2014)**. Five species represent new records for the mycobiota of Iran are *Penicillium crocicola*, *P. olsonii, P. sumatrense, Talaromyces atroroseus* and *T. minioluteus* **(Khodaei et al., 2016)**. During our survey, five isolates belonging to three *Penicillium* species (*P. citrinum, P. expansum* and *P. glabrum*) were isolated and identified from exogenous colonisation (Table 1). Their occurrence was very sporadically. On the other hand, *Penicillium* were between the most common species identified in grape from Small Carpathian area during harvesting 2011 to 2013 **(Felšöciová et al., 2015)**. From 13 different *Penicillium* species with high frequency (93%) were 4 main species: *P. chrysogenum, P. crustosum, P. expansum* and *P. griseofulvum*. *Penicillium citrinum* was less common species identified in grape samples.

By the endogenous (surface-disinfected) plating method were identified nine different genera from the 509 fungal strains (Table 2): *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Penicillium, Rhizopus, Trichoderma* and *Mycelia sterilia*. The three most abundant genera found by descending order and with the highest relative density were *Alternaria, Cladosporium* and *Penicillium*. Most of all genera had relative density less than 1%.

Most of the fungi found are ubiquitously distributed, such as the field fungi *Alternaria, Cladosporium* and *Epicoccum*, which occur commonly in the air, plant surfaces, debris and soil **(Serra et al., 2006)**. Fungal species capable of causing rot in grapes (e.g. *Aspergillus niger, Botrytis cinerea, Rhizopus*) were not common inhabitants of the berry surface.

Four *Penicillium* species were found: *Penicillium citrinum, P. griseofulvum, P. hordei* and *P. chrysogenum* (Table 2). According **Felšöciová et al**. **(2013)** some similar species but in lower number of abundances were isolated from the Nitra wine growing region in 10 analysed samples, namely *P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum, P. chrysogenum* and *Penicillium* spp. The occurrence of most was also very sporadically, except *P. crustosum, P. chrysogenum* and *P. expansum*. Nine *Penicillium* species (*P. canescens, P. citrinum, P. crustosum, P. expansum, P. funiculosum, P. glabrum, P. griseofulvum, P. chrysogenum* and *P. variabile*) were found from grapes grown in the Central Slovak wine region, but there occurrence was also very sporadically except two *P. expansum* and *P. chrysogenum* **(Felšöciová et al., 2015)**.

As shown in Table 3, thin layer chromatographic analysis of 15 tested isolates representing 5 potentially toxigenic species showed that 11/15 (73%) produced at least one mycotoxin. Positive toxigenity was detected in *P. citrinum* (9 out of 9 strains screened). *Penicillium expansum* (Figure 1) produced only citrinin, did not produce patulin and roquefortin C, *Penicillium griseofulvum* produced griseofulvin and patulin, the production of cyclopiazonic acid and roquefortin C was not confirmed. Negative toxigenity were detected in *Penicillium hordei* and *P. chrysogenum* (Figure 2) on roquefortin C.

Penicillium spp. in our samples was generally low (3% RD). On the other hand, *Penicillium* was a common component of the grapes mycobiota from 2011 to 2013 in the Small Carpathian area **(Felšöciová et al., 2015)**. Ninety three percent of samples were colonies by the genus *Penicillium*. During the survey, 251 isolates belonged to 14 *Penicillium* species. Out of 124 strains, 84% produced at least one mycotoxin by TLC method. The most frequent was *P. chrysogenum*. Interesting was, that almost all tested strains on roquefortin C were producted (100 out of 102). No mycotoxin (RC) was formed by the examined four species (*P. expansum, P. griseofulvum, P. hordei* and *P. chrysogenum*) isolated from our grape samples.

CONCLUSION

The present work indicated that the examined grape fruits were contaminated with several fungi especially members of *Alternaria*. Members of *Aspergillus* and *Penicillium*, fungi capable of producing mycotoxins such as aflatoxins and ochratoxin A, were very rare. These findings indicate that strict hygiene microbiological must be applied during different stages of harvest, transport, storage and handling to avoid the harmful effects on human health.

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THE INFLUENCE OF SELENIUM ON SELECTED HEAVY METALS CUMULATION IN OYSTER MUSHROOM FRUITING BODIES

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ABSTRACT

Food safety is a very frequent topic. The article deals with the problems of fortification of the most grown mushroom in Slovakia, and the 3rd most grown mushroom in the world, *Pleurotus ostreatus*. Due to the high environmental pollution of soils and air, there is a risk of the production of dangerous fruiting bodies with high heavy metals content. It is known that these substances can promote serious health effects on human body, such as bone weakness or kidney damages (cadmium) and negative process of cognitive developing (lead). The experiment was focused on biofortification with selenium to reduce the accumulation of selected heavy metals (lead, cadmium) in oyster mushroom, grown with intensive cultivation under artificial conditions. This work confirms that the application of sodium selenate to the growing substrate with straw as the main component can reduce the accumulation of cadmium (by 22.45%) and lead (by 64.81%). Research by various authors reported the ability of the oyster mushroom to embed selenium from the substrate into the fruiting bodies. Based on the results of the experiments, we propose to fortify the growing substrate for the production of oyster mushroom by selenium. This way we produce a food with a high antioxidant potential.

Keywords: oyster mushroom; *Pleurotus ostreatus*; heavy metals; food safety; mushrooms; lead; cadmium

INTRODUCTION

Oyster mushroom is popular especially because of its delicious taste. Its composition is also very important. Oyster mushroom contains high amounts of proteins and carbohydrates, minerals such as calcium, phosphorus, iron and other, vitamins like thiamine, riboflavin and niacin as well as low fat **(Sturion and Oetterer, 1995; Justo et al., 1998; Manzi et al., 1999)**.

According to **Silveira et al. (2006)** energy value of *P. ostreatus* is between 139.36 and 213.05 kcal.100 g^{-1} fresh mushrooms. Hyphae are composed of cell wall components such as chitin, other hemicelluloses and β-glucans, which often play a key role in the pharmacological use of mushrooms. For example, in enhancing macrophage function, resisting many bacterial, viral, fungal and parasitic infections, activating non-specific immune stimulation, lowering blood cholesterol levels and blood glucose levels **(Cheung, 2009)**. Although the mushrooms are not plants, they are often included between vegetables in terms of dietary properties. Most species of the genus *Pleurotus* are known for their healing potential.

Selenium (Se) is an essential element with antioxidant effects. It is an important component of several major metabolic reactions, including synthesis of thyroid hormones, antioxidant defence systems and immune functions **(Köhrle and Gärtner, 2009; Gandhi, Nagaraja and Prabhu, 2013)**. The essentiality of selenium was demonstrated in 1957 **(Hegedűs et al., 2006; Hegedűs, Hegedűsová and Šimková, 2007)**. In 1976, the necessity of selenium for humans was proven, despite the fact that previously were highlighted his negative effects **(Hegedűs et al., 2008; Jakabová et al., 2008; Jakabová et al., 2009)**.

It is therefore clear that mushroom enriched with inorganic forms of selenium can produce functional foods with positive effects on human health, anti-inflammatory and anti-tumour effects **(Clark et al., 1996)**.

Cultivation of saprophytic fungi on substrates rich in selenium may be an effective means of producing food with built-in selenium. The content of selenium in mushrooms is generally higher than most vegetables **(Rayman, Infante and Sargent, 2008)**, but this indication is very variable. In contrast with biologically available selenium concentrations in the soil and the related content of selenium in wild mushrooms, in cultivated mushrooms is the content of the embedded selenium variable depending on the species and their maturity. Its content is also dependent on the type and quality of the substrate **(Kalac, 2009)**. The concentration of selenium in the fruiting bodies of the favourite edible mushroom is in the range from <1 – 20 μg $Se.g^{-1}$ dry weight of the fruiting bodies.

The concentration of selenium in the fruiting bodies of mushroom *P. djamor* produced on high selenium substrates

compared to the control substrate without selenium contamination exceeds 800 times the amount, i.e. 141 μg $Se.g^{-1}$ weight of dried mushroom. It has been confirmed that as well as other species of genus *Pleurotus* are able to cumulate selenium **(Wang et al., 2005; Estrada et al., 2009; Da Silva et al., 2012)**.

Scientific hypothesis

Application of sodium selenate into the substrate will reduce the lead and cadmium content in oyster mushroom fruiting bodies.

MATERIAL AND METHODOLOGY

Establishment of experiments to produce oyster mushroom fruiting bodies

In the experiment was used the production strain of oyster mushroom *(Pleurotus ostreatus)* KRYOS B. Mushroom production was based on controlled cultivation conditions, in the premises of The AgroBioTech Research Centre, in two cultivation periods. The production of mushrooms proceeded in the following phases:

- inoculum preparation,
- preparation of the substrate,
- inoculation by inoculum and incubation at 25 °C about 2 weeks,
- initiation of the fruiting bodies at 11 °C for one day,
- fructification (12 hours, 16 °C in dark and 12 hours, 16 °C under light).

The experiment consisted of 4 variants, each variant had 10 repetitions. The whole experiment was carried out in two cultivation periods in the following terms:

- First cultivation period (1. CP) – from 04. April 2016 to 26. May 2016.
- Second cultivation period (2. CP) – from 09. June 2016 to 27. July 2016.

Created variants of experiments:

Preparation of all substrates consisted of wetting of dry straw pellets in water in a weight ratio of 1:2.6.

C – control – without selenium;

X – 0.5 $mg.dm^{-3}$ Se – with addition of 0.5 $mg.dm^{-3}$ Se in the form of sodium selenate aqueous solution;

Y – 1.0 $mg.dm^{-3}$ Se – with addition of 1.0 $mg.dm^{-3}$ Se in the form of sodium selenate aqueous solution;

Z – 2.0 $mg.dm^{-3}$ Se – with addition of 2.0 $mg.dm^{-3}$ Se in the form of sodium selenate aqueous solution.

After the end of the incubation, the containers were evenly bleached, overgrowth with mushroom mycelium, and no visible signs of contamination. In the next step the cultivation containers were covered with transparent plastic layers with a 3 cm hole in the middle of the lid. The microthene foil was cut in the shape of a cross in the place of hole. The initiation of fruiting bodies production was carried out by repeated substrate cooling in two cycles.

The fruiting following after the phase of fruiting bodies germ initiation was similar in 2 cycles from 12 May (1. CP) and from 17 July (2. CP).

Collection and processing of oyster mushroom fruiting bodies

Oyster mushroom fruiting bodies were harvested in the optimal growth phase. Fruiting bodies were lyophilized on a LYOVAC GT2 (Germany) for 24 – 30 hours. The lyophilizate was then homogenized by grinding to a fine powder and further used to determine the selected qualitative parameters.

Analytical methods for determining selected qualitative factors

Quantitative determination of the content of selected risk metals except mercury was carried out in the mineralized samples by AAS (AAS Varian AA Spectr DUO 240 FS/240Z/UltrAA) flame technique. The results were evaluated by the calibration curve method.

Ready-made CertiPur stock calibration solutions (Merck, Germany) was used with well-known concentrations of the heavy metals to be monitored. The final values of the measured parameters were subsequently obtained by software translating the calibration curve with the absorbance of the monitored analyte in the sample.

Statistic analysis

The statistical evaluation of the results was carried out by standard methods using statistical software Statgraphics Centurion XVII (StatPoint, USA) – multifactor analysis of variance (MANOVA), LSD test.

RESULTS AND DISCUSSION

It is generally known that different species of mushrooms tend to accumulate different substances from the substrate and from the environment, with no exception for risk metals. We evaluated the content of selected risk metals (lead and cadmium) during our experiment. For each cultivation period 40 samples of lyophilized fruiting bodies grown on selenium fortified substrates were analysed. Average values for individual variants are given in Table 2. Dehydrated samples of the control substrate without sodium selenium application were also analysed for the presence of metals (Table 1).

In terms of contamination of the oyster mushroom fruiting bodies by risk metals, currently applicable regulations and decrees set the highest limits only for two risk elements - cadmium and lead. According to **Commission Regulation (EU) 2015/1005** of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs, the maximum allowed limit for oyster mushroom fruiting bodies intended for consumption is till 0.30 $mg.kg^{-1}$ Pb of fresh matter of the fruiting bodies. As regards **Commission Regulation (EU) No 488/2014** of 12 May 2014 amending Regulation (EC) No 1881/2006, as regards maximum levels of cadmium in foodstuffs, they were determined at levels of up to 0.20 $mg.kg^{-1}$ Cd of fresh fruiting bodies. Since the samples analysed by us were lyophilized, we carried out a conversion to fresh matter of the fruiting bodies. The results are shown in Table 3.

After the calculation we can state, that the critical limits of heavy metals set by the applicable food quality regulations were not exceeded.

Table 1 Average content of metals in samples of dehydrated substrate.

Variant	Pb (mg.kg^{-1})	Cd (mg.kg^{-1})
C	2.20	0.21

Table 1 Average content of metals in lyophilized samples of fruiting bodies*Pleurotus ostreatus* KRYOS B fortified by selenium.

Variant	Pb (mg.kg^{-1} ±SD)	Cd (mg.kg^{-1} ±SD)
C	1.62 ±0.28^{b}	0.25 ±0.05^{b}
X	1.94 ±0.64^{b}	0.26 ±0.03^{b}
Y	1.59 ±1.05^{b}	0.21 ±0.06^{ab}
Z	1.05 ±0.41^{a}	0.19 ±0.04^{a}

Note: C – 0.0 mg.dm^{-3} Se; X – 0.5 mg.dm^{-3} Se; Y – 1.0 mg.dm^{-3} Se; Z – 2.0 mg.dm^{-3} Se; the values in the columns with different letters are significantly different from each other.

Table 3 Lead and cadmium content in the mass of fresh fruiting bodies*Pleurotus ostreatus* KRYOS B and dependence on applied selenium doses.

Variant	Dry matter (%)	average periods
	Pb (mg.kg^{-1})	
C	11.44	0.18
X	10.46	0.19
Y	10.69	0.16
Z	11.69	0.11
	Cd (mg.kg^{-1})	
C	11.44	0.02
X	10.46	0.02
Y	10.69	0.01
Z	11.69	0.02

Note: C – 0.0 mg.dm^{-3} Se; X – 0.5 mg.dm^{-3} Se; Y – 1.0 mg.dm^{-3} Se; Z – 2.0 mg.dm^{-3} Se.

Therefore, these fruiting bodies can be considered as a safe food product for the consumer also after application of selenium. In the case of the interaction of selenium and selected heavy metals, it was statistically proven in both cases that the increased content of selenium in the substrate reduces the accumulation of lead and cadmium. Average for both cultivation periods pointed out that in the variant with 2.0 mg.dm^{-3} Se was the lead accumulation statistically significant reduced by 64.81%. Reduction in accumulation occurred after application of 1.0 mg.dm^{-3} Se (2.47%) too, but it was not statistically significant. In a variant with 0.5 mg.dm^{-3} Se, lead accumulation was increased by 19.75%, but it was not statistically significant (p >0.05).

Similar results were also found in the case of cadmium accumulation in fortified oyster mushroom fruiting bodies, on average for both cultivation periods. In variant with 2.0 mg.dm^{-3} Se was statistically proven lower cadmium accumulation by 24%, while in a variant with 0.5 mg.dm^{-3} Se, its accumulation grew by 4%, but statistically not significant. In variant with 1.0 mg.dm^{-3} Se, accumulation of cadmium was statistically not significant decrease by 16%. Based on the above results it can be stated, that the fortification of substrates with selenium not increases the accumulation of selected risk metals statistically significantly in any of the experimental variants. On the other hand, in the variant with 2.0 mg.dm^{-3} Se, was statistically proven decreased accumulation of lead and cadmium in the fruiting bodies of edible oyster mushroom. **Lepšová (2001)** argues that the oyster mushroom cultivated in the wood can accumulate only a small amount of heavy metals from the environment; therefore, there is no need to fear their increased level in the harvested fruiting bodies. The opposite phenomenon can occur in the intensive production of edible mushrooms, where the grain is used as a substrate, which is a by-product of intensive agricultural production.

Stihi et al. (2011) in their study found that wild oyster mushroom in Romania, distant 0.5 km from the source of pollution contained Cr (1.81 mg.kg^{-1}), Mn (12.4 mg.kg^{-1}), Fe (387.00 mg.kg^{-1}), Ni (1.85 mg.kg^{-1}), Cu (12.5 mg.kg^{-1}), Zn (41.30 mg.kg^{-1}), Se (2.64 mg.kg^{-1}), Cd (0.95 mg.kg^{-1}) and Pb (0.64 mg.kg^{-1}) in lyophilized samples. Fruiting bodies in a location 10.5 km distant from the same source of pollution contained only slightly lower concentrations of

the monitored elements, namely Cr (1.08 mg.kg^{-1}), Mn (11.8 mg.kg^{-1}), Fe (284.00 mg.kg^{-1}), Ni (1.29 mg.kg^{-1}), Cu (10.20 mg.kg^{-1}), Zn (37.90 mg.kg^{-1}), Se (2.57 mg.kg^{-1}), Cd (0,87 mg.kg^{-1}) and Pb (undetectable) in lyophilizates. These results confirm the ability of oyster mushroom to accumulate risk metals. In samples of fruiting bodies fortified with selenium, which were analysed in our work, many lower concentrations of the elements were found. The findings can be explained by a better quality of the production substrate.

Siwulski et al. (2017) on the basis of their research indicate that the lowest levels of Al, Fe, Mn, P and Se were observed in the *P. ostreatus* 930 strain. The lowest concentrations of Ca, Cr, K, Nd, Te and Zn were determined in *P. florida*, while in *P. pulmonarius*, were observed trace amounts of Er, Rh, Sc, Tm and Zr. On the other hand, *P. ostreatus* strain 930 contained the highest content of Cu and Lu, whereas *P. florida* of the Rh element. Significant differences were observed in *P. ostreatus* K 22, *P. citrinopileatus* and *P. eryngii*. The highest content of Al, Cr, Er, Fe, Pt, Th, Ti and Tm was determined in *P. ostreatus* K 22, the elements Mg, Mn, P, Re, Se and U were most represented in *P. Citrinopileatus,* and *P. eryngii* accumulated the highest concentration of As, B, Ca, In, Na, Nd and Sr. *P. ostreatus* strain K 22 contained the lowest concentration of In, *P. citrinopileatus* lowest concentration of Na and *P. eryngii* the lowest concentration of Th. From other tested species and strains contained *P. ostreatus* HK 35 lowest content of As and highest level of Te, *P. ostreatus* H 195 lowest content of Cd, Pb and U and highest level of Sc. The authors emphasize that the highest concentration of Pb and Cd was determined in fruiting bodies of *P. ostreatus* 80. The high cumulative potential of the said strain can be specifically used in the soil myco-remediation processes. *P. djamor* was rated as the most efficient K and Zn accumulator. We confirm the fact that the individual strains of edible oyster mushroom are different from each other in the ability to accumulate selected compounds.

Quarcoo and Adotey (2013) monitored the accumulation of selected heavy metals by oyster mushroom fruiting bodies in Ghana. They found that the fruiting bodies contained on average 0.04 mg.kg^{-1} Pb, 0.04 mg.kg^{-1} As, 43.77 mg.kg^{-1} Fe, 0.35 mg.kg^{-1} Cd and 0.04 mg.kg^{-1} Hg in a lyophilized mass. Our samples of the fortified fruiting bodies contained lower concentrations of these elements.

Kaya and Bag (2010) analysed 24 species of mushrooms occurring in Turkey. In the case of *Pleurotus ostreatus*, significant levels of heavy metals were found, namely 20.87 mg.kg^{-1} Al, 0.41 mg.kg^{-1} B, 2.11 mg.kg^{-1} Cd, 0.90 mg.kg^{-1} Co, 0.21 mg.kg^{-1} Cr, 39.36 mg.kg^{-1} Cu, 40.57 mg.kg^{-1} Fe, 4.22 mg.kg^{-1} Mn, 1.23 mg.kg^{-1} Ni, 2.14 mg.kg^{-1} Pb and 86.83 mg.kg^{-1} Zn in lyophilizate. Again, we conclude that the concentration of selected elements was lower in our experiment.

Demirbas (2001) found in the dry matter fruiting bodies of the edible oyster mushroom Pb (3.24 mg.kg^{-1}), Cd (1.28 mg.kg^{-1}), Hg (0.42 mg.kg^{-1}), Cu (13.6 mg.kg^{-1}), Mn (6.27 mg.kg^{-1}), Zn (29.8 mg.kg^{-1}) and Fe (81.6 mg.kg^{-1}).

Tuzen, Ozdemir and Demirbas (1998) determined in the dry matter of edible fruiting bodies Pb (0.11 mg.kg^{-1}), Cd (0.55 mg.kg^{-1}), Hg (0.31 mg.kg^{-1}), Fe (48.6 mg.kg^{-1}), Cu (5.0 mg.kg^{-1}), Mn (10.3 mg.kg^{-1}) and Zn (19.3 mg.kg^{-1}).

Lasota, Florezak and Karmanska (1990) prove Cd (11.2 mg.kg^{-1}), Hg (1.2 mg.kg^{-1}), Zn (0.8 mg.kg^{-1}) and Pb (0.0 mg.kg^{-1}) in lyophilized fruiting bodies of oyster mushroom.

All of the above-mentioned findings of a large number of authors point to the fact that edible mushrooms are able to accumulate heavy metals from the environment and the from the substrate into the fruiting bodies, which can greatly worsen the quality of the production. Our argument explains the different concentrations of the rated elements compared to the cited authors, as the content of the selected elements in the concrete fruiting bodies is directly related to the content of the analysed element in the growing substrate. For the intensive production of edible mushroom is important to use only high-quality lignocellulosic material analysed for the content of risk metals. From the point of view of the accumulation of selected metals, we recommended to fortify the cultivation substrate of oyster mushroom with the selenium in order to produce a functional and uncontaminated foodstuff.

CONCLUSION

At work we monitored the accumulation of risk elements – lead and cadmium in the oyster mushroom fruiting bodies. We found that the limit set by the European Commission Regulations, which concern about the content of lead and cadmium in oyster mushroom fruiting bodies has not been exceeded. Fruiting bodies are of satisfactory quality and are suitable for daily consumption.

In the experiment, the reduction of lead and cadmium accumulation was statistically confirmed after application of 2.0 mg.dm^{-3} Se. Cadmium content decreased by 22.45% and lead by 64.81% compared to the control variant. From the results it is clear that by fortification of the substrate (the main component of which is straw) by selenium, we produce not only a high-quality food with a high antioxidant potential, but we can also prevent the accumulation of risk elements of lead and cadmium from the substrate.

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QUALITY OF BISCUITS AS AFFECTED BY ADDITION OF FIBRE

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ABSTRACT
The aim of the study was to propose formulas with addition of bamboo and hemp fibre and grape seed flour in an amount of 3, 6 and 9% and then assess the effect of added fibre on the quality of biscuits. A total of 20 samples were baked, in half of the samples the basic ingredient was wheat flour, and in the rest of samples spelt flour was used. During a baking experiment, it was found that the best effect on the product volume and the weight after baking had bamboo fibre. Minor baking losses occurred in biscuits with wheat flour. The best sensory results were attained with the sample having 3% of grape seed flour with a spelt flour base. The crispiest was the sample made from spelt flour with 3% of hemp fibre and the wheat samples with bamboo fibre were of the lightest colour. Control samples had the highest nutritional values and biscuits with added bamboo fibre contained the lowest energy in both formulas.

Keywords: fibre; biscuit; baking experiment; sensoric assessment; nutritional value

INTRODUCTION
Biscuits are very popular in the food industry. **Kumar et al. (2015)** indicate in their study that annual consumption of biscuits per person is 10 to 15 kg in developed countries. The biscuits are popular owing to their sensory attributes, long shelf life, relatively low price, suitable packaging sizes and availability **(Vitali, Dragojević and Šebečić, 2009; Brownlee et al., 2017)**. Due to competition in the market and increased demand for healthy, natural and functional products, the aim is to improve the nutritional value and functionality of biscuits by changing their nutritional composition. The beneficial properties and positive effect of fibre on the human organism are known for decades. Many studies show its association with body weight and insulin levels in the blood. Foods rich in fibre are digested more slowly and absorption of nutrients takes longer, which increases the feeling of satiety and consequently longer breaks between meals and lower energy intake. Although the energy gain from fibre is small, its main function is to protect and act as prevention against many non-infectious diseases of mass occurrence, such as heart and blood vessel diseases, colon cancer and tumours, obesity, and diabetes. It affects the excretion of hormones by glands with internal secretion, including cholecystokinin, which is secreted by cells in the small intestine, stimulates pancreatic secretion, and controls the central feeling of satiety. It helps to reduce postprandial glucose levels, unsaturated fats, blood triacylglycerols, and LDL cholesterol **(Marko, Rakická and Šturdík, 2015; Van Der Kamp et al., 2010)**.

Enriching biscuits with different types of fibre not only improves viscosity, texture, sensory properties and product durability with its physicochemical properties, but also reduces the energy value of the product **(Vitali, Dragojević and Šebečić, 2009; Brownlee et al., 2017)**. Through the formula adjustment and fortification, they are made to suit different uses, such as for sick, children, athletes or army **(Kadlec, Melzoch and Voldřich, 2012)**.

Scientific hypothesis
We are expecting the significant effect of added fibre on the quality of biscuits. Based on spectrophotometric color measurement it was assumed that the lightest colour would have the samples with bamboo fibre and the darkest color would have samples with grape seed flour.

According to sensoric analysis we assumed that the best evaluated sample would be the sample containing 6% of bamboo fibre even if both flours were used.

Based on the strength measurement we assumed that the smallest strength would be measured at samples enriched with hemp fiber.

MATERIAL AND METHODOLOGY
Biscuits of two kinds of flour were baked. The base consisted of wheat and spelt flour to which bamboo and hemp fibre and grape seed flour were added in an amount of 3, 6 and 9%. Subsequently, the quality of durable bakery products affected by the fibre was examined. Biscuits were baked and assessed in the pilot plant and laboratory of the Institute of Food Technology at Mendel University in Brno.

Two formulas were proposed (Table 1 and Table 2). The standard mixture consisted of wheat and spelt flour to which the selected fibre was added, always in the same proportion – 3, 6 and 9%.

Table 1 Proposed formula with a wheat flour base.

Sample no.		1	2	3	4	5	6	7	8	9	10
Wheat flour[g]		350	339.5	329	318.5	339.5	329	318.5	339.5	329	318.5
Powdered sugar [g]		120	120	120	120	120	120	120	120	120	120
Butter [g]		130	130	130	130	130	130	130	130	130	130
Vanilla sugar [g]		4	4	4	4	4	4	4	4	4	4
Lemon zest [g]		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Eggs* [pcs]		2	2	2	2	2	2	2	2	2	2
Fibre	**[g]**	0	10.5	21	31.5	10.5	21	31.5	10.5	21	31.5
	[%]	0	3 (2.91)**	6 (5.82)	9 (8.73)	3 (1.8)	6 (3.6)	9 (5.4)	3 (1.14)	6 (2.28)	9 (3.42)

Note: *Eggs were of size M (53 – 63 g). **The numbers in brackets indicate the actual fibre content declared by the manufacturer on the individual fibre packaging, converted to the percentage of the given formula.
Sample no. 1: control without fibre.
Samples no. 2 – 4: bamboo fibre in an amount of 3, 6, and 9%.
Samples no. 5 – 7: hemp fibre in an amount of 3, 6, and 9%.
Samples no. 8 – 10: grape seed flour in an amount of 3, 6, and 9%.

Table 2 Proposed formula with a spelt flour base.

Sample no.		11	12	13	14	15	16	17	18	19	20
Spelt flour [g]		350	339.5	329	318.5	339.5	329	318.5	339.5	329	318.5
Powdered sugar [g]		120	120	120	120	120	120	120	120	120	120
Butter [g]		130	130	130	130	130	130	130	130	130	130
Vanilla sugar [g]		4	4	4	4	4	4	4	4	4	4
Lemon zest [g]		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Eggs*[pcs]		2	2	2	2	2	2	2	2	2	2
Fibre	**[g]**	0	10.5	21	31.5	10.5	21	31.5	10.5	21	31.5
	[%]	0	3 (2.91)**	6 (5.82)	9 (8.73)	3 (1.8)	6 (3.6)	9 (5.4)	3 (1.14)	6 (2.28)	9 (3.42)

Note: **Eggs were of size M (53 – 63 g). **The numbers in brackets indicate the actual fibre content declared by the manufacturer on the individual fibre packaging, converted to the percentage of the given formula.
Sample no. 11: control without fibre.
Samples no. 12 – 14: bamboo fibre in an amount of 3, 6, and 9%.
Samples no. 15 – 17: hemp fibre in an amount of 3, 6, and 9%.
Samples no. 18 – 20: grape seed flour in an amount of 3, 6, and 9%.

The technology of production started with sifting flour and then mixing it with other loose ingredients (powdered sugar, vanilla sugar, fibre, and lemon zest). In the next step, softened butter and eggs (both yolks and whites) were added. All ingredients were worked together and kneaded for 7 minutes to develop smooth dough that was left to restin a cool place (10 °C) for 2 hours; meanwhile other doughs were prepared.

After the maturation phase, the dough was rolled with a special rolling pin having an adjustable dough sheet thickness set to 4 mm, so that all biscuits were of even height. Biscuits were cut out from all the doughusing round stamps with embossed patterns of 5.5 cm in diameter; the edges of shaped biscuits were higher due to the embossing pattern used. The cut-out biscuits were placed on a baking tray lined with parchment paper and put in a preheated hot air rotary oven at 160 °C for 15 minutes.

The baked biscuits were laid on stainless steel tables and left to cool freely. After cooling, on the same day, the physical measurement was carried out, followed, on the next day, by sensoric assessment using the proposed questionnaire and colour measurement using a spectrophotometer.

Sensoric assessment of biscuits

All samples were assessed the next day after baking. A panel of 10 trained judges completed the sensory questionnaire. A total of 20 samples with different fibre added were assessed. The questionnaire was composed of 10 questions and biscuit samples were assessed for colour, hardness (break strength), aroma, surface, shape and homogenity, taste, ease of bite, adhesion to palate, bite swallowing, and overall impression.

Hardness measurement

The biscuit strength was measured using the TIRATEST 27025 (TIRA Maschinenbau GmbH, Germany) on the day of their technological production. A penetration test at a rate of 100 mm.min^{-1} was used to determine the brittleness of biscuits, with a stick of 3 mm diameter used as extension and a path of 5 mm. The strength was measured in [N].

Colour measurement using a spectrophotometer

The colour of biscuits was measured the day after the technological production, using the Konica Minolta CM-3500d spectrophotometer with a 30 mm measuring aperture and D65 illumination mode, in the reflectance mode with SCE (specular component excluded). The samples were measured from the bottom of the biscuit, each twice by attaching.

Determination of nutritional value

To determine the nutritional value of baked biscuits, the nutritional values per 100 g of ingredient used as declared by the manufacturer on the package were needed. The measured values were recalculated to the amount of ingredient used in the formula. The final data was converted to the weight of biscuits after baking and the results were again indicated per 100 g of baked biscuits.

Statisic analysis

Statistical comparison of samples using the Duncan's test was done separately for each kind of flour. Dependencies between measurements of individual sensory characteristics were assessed by factor analysis, with factor rotation according to a simple VARIMAX method Statistical test results were calculated using the STATISTICA CZ 12 software, the chosen significance level was 0.05. Statistical test results were calculated using the STATISTICA CZ 12 software, the chosen significance level was 0.05.

RESULTS AND DISCUSSION

Baking experiment

From the results of baking experiment measurements the following summary of characteristics was evaluated: weight of biscuits after baking [g], baking loss [%], product volume (mL.100 g^{-1}) and index number [-].

The weight of individual samples after baking differed based on the fibre used and its physicochemical properties. In the wheat-based formula, the control sample weighed 596 g, the highest weight showed the biscuits with addition of bamboo fibre (no. 2, 3, 4) and the sample no. 10 (with 9% of grape seed flour); other samples weighed under 600 g.

Among the samples no. 11 to 20, the highest weight had the control sample (608 g). The weight of samples no. 12, 13, and 14 was higher than 590 g, other samples weighed less. In both formulas, the percentage weight of biscuits increased with the increasing amount of bamboo fibre and grape seed flour added. On the contrary, the weight of hemp fibre samples decreased with the decreasing amount added. In contrast, **Gómez et al. (2010)** found no significant influence of the fibre type on the weight of the products, and no significant differences were foundas regarded the percentage of added fibre.

The highest value of the weight lost by baking in samples no. 1 to 10 showed sample no. 8 (with the addition of 3% of grape seed flour) with a loss of 15.41% and the lowest loss (10.51%) was also calculated for the addition of grape seed flour in sample no. 10. In samples no. 11 to 20, sample no. 17 (with 9% of hemp fibre) had the greatest loss in baking (21.01%), while sample no. 14 (with 9% of bamboo fibre) had the smallest (11.69%). In both wheat and spelt-based formulas, the lowest value of baking loss showed samples to which bamboo fibre was added; the loss in baking diminished with its enrichment. This is due to the binding capacity of bamboo fibre of up to 700%. At higher binding capacity, the dough was tough and poorly workable.The same result was obtained by **Gómez et al. (2010)**. Thus, with adding more fibre, it is necessary to improve the technological development which incorporates into the dough other ingredients inhibiting the water absorption **(Raymundo, Fradinho and Nunes, 2014)**. In the case of hemp fibre, on the other hand, the losses increased with higher percentage added due to the higher fat content in fibre. In general, smaller baking losses occurred in samples no. 1 to 10, due to the wheat flour that contains less fibre as compared to the spelt flour, which is whole grain. As also stated in study by **Frakolaki et al. (2018)**, higher fibre content in spelt flour negatively affects the dough rheology and product quality**.** Losses higher than 18% were recorded only in samples with spelt flour, samples no. 16, 17 and 18, and higher losses were also obtained in sample no. 15 (17.41%) which may mean that hemp fibre in an amount of 3, 6 and 9% and the addition of 3% of grape seed flour has a negative effect on the physical properties of the dough.

The smallest volume in samples no. 1 to 10 was observed for sample no. 10 (with 9% of grape seed flour) and the largest volume in sample no. 4 (with 9% of bamboo fibre). In the case of spelt flour biscuits, the sample no. 17 (with 9% of hemp fibre) had the lowest volume, i.e. 11.2% less compared to the control, while the sample no. 14 with the addition of bamboo fibre had the largest volume, 14% more than the sample no. 11, which was the control sample without fibre.

In both formulas, the largest volumes were observed in biscuits enriched with bamboo fibre that increased their volume with the addition of fibre. By contrast, in the case of hemp fibre and grape seed flour, the volume of samples decreased with their addition. The volumes of spelt flour-based samples (no. 11 to 20) were generally smaller.

Contrary to this, **Hrušková and Švec (2016)** found that biscuits with the addition of hemp seed flour, both fine and wholemeal, had their shape and volume unchanged.

Regarding the index number, arching slightly increased in wheat and spelt flour formulas (samples no. 1 to 20) with the addition of bamboo fibre. In samples no. 1 to 10, the most flattened was the fibre-free control (no. 1) with an index number of 0.1. In samples where the basic ingredient was spelt flour, all samples had the value of 0.09, except samples no. 13 and 14 with the addition of bamboo fibre (0.1 points) which were the most arched. Index numbers ranged from 0.09 to 0.12, meaning that fibre does not affect the shape and arching of biscuits too much.

Sensoric assessment of biscuits

The best impression from the wheat-based samples gave the sample no. 3 enriched with 6% of bamboo fibre (21 points), while the sample no. 10 with 9% of grape seed flour was the worst. In the second formula, the sample no. 18 was the best with 18.7 points and the worst overall impression was given by sample no. 14 with 28.1 points. The samples of both formulas were classified into one homogeneous group according to the Duncan's

test, i.e. no statistical differences were found between them ($p > 0.05$).

In grape seed flour and hemp fibre samples, the colour was the worst from the sensoric properties, and the surface was the best rated, while in the bamboo fibre samples the surface was the third worst rated property and the colour was the best rated descriptor. Unlike the wheat flour samples, the colour of all spelt flour samples was the worst rated of all of the selected attributes. It is, however, noticeable that the colour in the bamboo fibre samples was rated better than that in the hemp fibre and grape seed flour samples. Another observed difference is that samples with grape seed flour and bamboo fibre have the ease of bite as the second worst-rankedin the displayed properties, while in hemp fibre, rating of this property is comparable to that of the surface, shape and taste.

The relationships between the ratings of individual sensoric properties were determined using the factor analysis with a simple VARIMAX method of factor rotation (Table 3).

Based on the method, three significant factors were identified. The first factor was based on the close relationship of taste, adhesion, swallow ability, and overall impression. From a connection with the overall impression it can be concluded that taste, adhesion and swallow ability can be the main sensoric properties upon which the judge assesses the food in question. The second factor was based on an assessment of hardness, ease of biting and aroma. The third factor revealed the continuity of surface and shape assessments.

Measuring the colour of biscuits on a spectrophotometer

The results were expressed in accordance with the CIE L*a*b colour scheme with respect to D65 illumination. The higher the L* values in the graph, the lighter the colour of the sample.

In terms of the L* variable, which determines lightness from black to white, Duncan's test found that samples no. 5 to 10 had significantly lower values ($p < 0.05$) and the sample no. 4 had statistically significantly higher ($p < 0.05$) values, which statistically differs from all other samples from group 1 to 10. As the bamboo fibre amount increased, the biscuits were lighter in colour. Among the spelt flour samples, it was found that the samples no. 15 to 20 had statistically significantly lower values ($p < 0.05$) of the L* variable. The lightest sample (59.2%) was the sample with 6% bamboo fibre added.

For both formulas, the Duncan's test divided samples into five homogeneous groups with alpha significance level = 0.05. The darkest samples were the result of grape seed flour – sample no. 9 with 51.3% and sample no. 20 with 43%. Also **Maner, Sharma and Banerjee (2015)** found that grape seed flour gave biscuits their brown colour.

Biscuit strength assessment

For samples with wheat flour (1 – 10), the Duncan's test found that samples no. 3, 5 and 10 had statistically significantly lower ($p < 0.05$) hardness values compared to the control and the sample no. 8 had statistically significantly higher value than the control.

According to **Cappa, Lucisano and Mariotti (2013)**, the fibre binds water and retains it in the product during baking, so the fortified products are softer compared to the control, which corresponds to samples no. 2, 3, 4, 5 and 10 with the wheat flour base and samples no. 15, 16, 17, 18 and 20 with the spelt flour base. If the water content in the product is small, as with durable baking products, the ingredients compete with each other for water, and the fibre cannot perform its function as well as hydrocolloids that increase the dough's ability to bind water.

When comparing samples no. 11 to 20 in terms of hardness N, samples no. 15 and 17 showed statistically significantly lower values of hardness compared to the control and samples no. 12, 13 and 14 statistically significantly higher ($p < 0.05$) values. Samples of both formulas (1 to 20) were classified in five homogeneous groups using the Duncan's test with alpha significance level of 0.05. Furthermore, it was demonstrated that in both groups the smallest strength was measured in samples containing 3% of hemp fibre (no. 5 and 15) with average values of 23.9 and 20.8 N respectively. **Mancebo et al. (2017)** found that all fibre-enriched biscuits showed greater strength than the control and the biscuits with bamboo fibre added were the hardest.

Table 3 Factor analysis with a simple VARIMAX method of factor rotation.

Variable	Factor 1	Factor 2	Factor 3
Colour	0.35	0.14	-0.45
Hardness	-0.29	**0.79**	-0.07
Aroma	0.26	0.66	-0.08
Surface	0.12	-0.08	**0.84**
Shape and homogeneity	0.28	0.09	**0.81**
Taste	**0.81**	0.11	-0.11
Ease of bite	0.34	0.62	0.20
Adhesion	**0.74**	-0.07	0.33
Swallowability	**0.86**	-0.10	0.18
Overall impression	**0.75**	0.14	0.31

Table 4 Nutritional values of biscuits with wheat flour converted to 100 g.

Sample no.	Fibre amount [%]	Energy value [kJ/kcal]	Fats [g]	Saturated fatty acids [g]	Carbohydrates [g]	Sugars [g]	Proteins [g]	Salt [g]	Fibre [g]
1	0, control	1980.83/473.12	20.91	12.65	63.63	21.62	8.85	<0.01	1.76
2	3, bamboo	1904.84/455.13	20.23	12.28	60.32	20.88	8.38	<0.01	3.31
3	6, bamboo	1899.47/453.6	20.21	12.27	59.27	20.85	8.22	<0.01	4.99
4	9, bamboo	1887.72/451.35	20.18	12.24	58.03	20.89	8.03	<0.01	6.53
5	3, hemp	1962.55/468.85	20.92	12.67	62.03	21.46	8.91	<0.01	2.75
6	6, hemp	1957.97/467.86	20.97	12.59	60.83	21.46	9.04	<0.01	3.87
7	9, hemp	1952.50/466.74	21.21	12.69	59.68	21.45	9.16	<0.01	4.77
8	3, grape seed flour	1978.11/472.11	20.96	12.67	63.06	21.79	8.87	<0.01	2.47
9	6, grape seed flour	1975.64/472.27	21.01	12.69	62.49	21.96	8.89	<0.01	3.04
10	9, grape seed flour	1973.08/472.44	21.07	12.71	61.91	21.14	8.91	<0.01	3.61

Table 1 Nutritional values of with spelt flour converted to 100 g.

Sample no.	Fibre amount [%]	Energy value [kJ/kcal]	Fats [g]	Saturated fatty acids [g]	Carbohydrates [g]	Sugars [g]	Proteins [g]	Salt [g]	Fibre [g]
11	0, control	1929.64/459.18	20.61	12.44	56.04	21.36	10.4	<0.01	5.18
12	3, bamboo	1915.06/457.91	20.54	12.42	54.88	21.29	10.15	<0.01	6.75
13	6, bamboo	1903.65/454.81	20.5	12.42	53.82	21.25	9.91	<0.01	8.36
14	9, bamboo	1892.23/452.18	20.47	12.41	52.75	21.21	9.67	<0.01	9.87
15	3, hemp	1925.00/458.21	20.7	12.45	55.07	21.38	10.56	<0.01	7.17
16	6, hemp	1923.52/458.00	20.83	12.48	54.19	21.37	10.64	<0.01	7.48
17	9, hemp	1922.18/457.30	20.96	12.51	53.3	21.35	11.24	<0.01	8.29
18	3, grape seed flour	1927.52/459.18	20.67	12.48	55.67	21.54	10.38	<0.01	6.15
19	6, grape seed flour	1925.40/459.18	20.71	12.47	55.3	21.69	10.33	<0.01	6.71
20	9, grape seed flour	1923.27/459.18	20.75	12.49	54.93	21.85	10.3	<0.01	6.96

Nutritional values of biscuits

The calculated nutritional values per 100 g of wheat-based biscuits are shown in Table 4. The highest energy value, in addition to the control sample no. 1 (1,980.83 kJ), contained sample no. 8 (1,978.11 kJ) with 3% of grape seed flour. The least energy was contained in sample no. 4 (1,887.72 kJ) with the addition of 9% of bamboo fibre.

Table 5 shows the nutritional values per 100 g of spelt-base biscuits. The highest calorific values were calculated for the control sample no. 11 (1,929.64 kJ) and for sample no. 18 (1,927.52 kJ) with 3% of grape seed flour and the lowest energy values were reported for samples with bamboo fibre added (no. 11 to 14).

In both formulas, the control sample (no. 1 and no. 11) without the addition of fibre had the highest energy value because the formulas contained 100 percent of the flour amount. Samples no. 11 to 20 contained more fibre due to the spelt flour in which the amount of dietary fibre per 100 g is higher than in the wheat flour.

Bamboo fibre samples contained the most fibre due to the amount of fibre contained (97%) per 100 g of fibre. Biscuits enriched with grape seed flour contained the least fibre, but data from the study by **Maner, Sharma and Banerjee (2015)** clearly indicate that products enriched with grape seed flour have a positive effect on consumer health. Flour made from grape seeds and press cakes is nutritious and has antioxidant properties. It can be deduced from the results of the study by **Bilgiçli, İbanoglu and Herken (2007)** that fibre as a substitute for wheat flour reduces energy intake, but also the digestibility of proteins.

CONCLUSION

The aim of the study was to create formulas and subsequently to determine the effect of fibre additions on the quality of biscuits. Bamboo and hemp fibre and grape seed flour were used in the amounts of 3, 6 and 9 %. The basis of the first formula was wheat flour, while the second formula contained the spelt flour as the primary ingredient.

We performed a baking experiment, sensory evaluation, hardness measurement by TIRA test, spectrophotometric color measurement and a nutritional value was calculated.

It can be concluded from the results that the fibre as a substitution for the flour used reduces the energy intake, but also the digestibility of proteins, improves the functionality and nutritional value of the biscuits by changing their composition.

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FUNCTIONAL PROPERTIES OF MUFFIN AS AFFECTED BY SUBSTITUING WHEAT FLOUR WITH CAROB POWDER

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ABSTRACT

Carob (*Cerationa soliqu*a L.) pod is the good source of dietary fiber, minerals and polyphenolic substances. The aim of this study was to prepare muffin where wheat flour was substituted with carob powder, and determine some physicochemical properties. Carob powder was prepared by milling dry carob pods to particles smaller than 600 μm. Then wheat flour in muffin dough was replaced by carob powder in 5, 10, 15 and 20% (w/w) and subsequently baked at 180 °C for 20 min. It was found that the height of the muffin fortified with carob powder decreased in comparison with that in control muffin sample. Although the height of muffins decreased with the increase in level of carob powder, the differences were not statistically significant. Weight loss was similar for all the muffin samples in this study. Moisture content of muffins with carob powder was significantly higher than that in control. Addition of carob powder had also effect on water activity of muffin. While 0.905 a_w was observed in control sample, significantly higher a_w values were determinated in fortified muffins (0.912 – 0.923 a_w). The antioxidant characteristics were determinated using spectrophotometric assays for total phenolics (TPC), total flavonoids (TFC), radical scavenging activities (DPPH, ABTS) and hydrogen peroxide scavenging (HPS). TPC values gradually increased with the increase in level of carob powder from 348.1 to 829.1 μg gallic acid.g^{-1} dry matter but TFC values significantly increased in muffin with 15 and 20% (w/w) of carob powder. All the antioxidant assays showed strong and positive association with the increase in level of carob powder. Addition of carob powder resulted in the increase of browning index and FAST index as a metrics of the formation of Maillard products.

Keywords: fortification; phenolics; antioxidant; browning

INTRODUCTION

Carob powder or flour is the product of the fruit of *Ceratonia siliqua L.* Carob powder is usually prepared from mature, dried carob pod (without seed) after milling to desired particle size. Carob powder is a good source of sucrose and other simple sugars (maltose, mannose), unsaturated fatty acids and minerals such as calcium, potassium and iron **(Ayaz et al., 2009)**. High content of dietary fibre, both soluble and non-soluble, is the most important parameter, which makes the carob powder applicable in various food products such as bread **(Durazzo et al., 2014)** and cookies **(Roman et al., 2017)**. Carob powder is also used as a replacer of cocoa in cocoa and chocolate-based products decreasing the content of caffeine and theobromine but keeps the cocoa-like aroma, particularly when roasting **(Loullis and Pinakoulaki, 2018)**. In addition, carob powder is the rich source of polyphenolic substances exhibited promising pharmacological actions such as antioxidant, antibacterial, anti-inflammatory and anti-diabetic activities **(Rtibi et al., 2017)**.

In a recent work of **Pawlowska et al. (2018)**, the effect of substitution of cocoa powder for carob powder at 5% (w/w) level in muffin dough was examined. Improved antiradical activity and higher content of phytosterols have been observed in their study. However, their research was focused on substitution of cocoa powder at a single level. Replacing wheat flour for carob flour/powder may lead to both cocoa-like aroma and gluten-free products **(Roman et al., 2017; Lauková, Kohajdová and Karovičová, 2016)**.

Scientific hypothesis

Increasing content of polyphenolic substances and increasing antioxidant status of muffins prepared by partial substitution of wheat flour for carob powder are expected.

MATERIAL AND METHODOLOGY

All the solvents (methanol, ethanol and acetone) and acids (sulphuric and acetic acids) were purchased from Lach-Ner s. r. o. (Neratovice, Czechia). Folin-Ciocalteau reagent solution, gallic acid, quercetin hydrate, (+)-catechin, vanillin, aluminium chloride, 2,2-diphenyl-1-

picrylhydrazyl (DPPH), 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, hydrogen peroxide, ferrous ammonium sulphate, 1,10-phenanthroline and (±)-6-hydroxy-2,5,7,8-ramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA). All the chemicals were of analytical grade.

Sample preparation and baking procedure

Dry carob pods (*Ceratonia silique* L.) without seed were purchased in local supplier. Pods were milled using knife mill Grindomix GM 200 (Retsch®, Haan, Germany) to obtain powder. Particles smaller than 600 μm were separated by passing through analytical sieve and used for subsequent experiments. Carob powder was stored in evacuated plastic bag at room temperature until used.

Muffin formulation dough was adopted from the study of **Ambigaipalan and Shahidi (2015)** The control sample (without carob powder) contains 65 g of wheat flour and 37.5 g of sugar (Castello, Lidl Stiftung, Germany), 3.0 g of baking powder (Dr. Oetker, Bielefeld, Germany), 0.62 g of salt, 60.0 mL of 2.5% fat milk (Pilos, Louny, Czechia), 20.0 mL of canola oil (Promienna, Lidl Stiftung, Germany) and 1 egg (large). A portion of wheat flour was substituted for carob powder at 5, 10, 15 and 20% (w/w) levels to obtain fortified muffin. When carob powder content had been 25% and 30% (w/w), unacceptable taste was observed by three panelists. A dough was prepared by mixing all the ingredients, a batter (30 g) was transferred into muffin paper cups and baked in pre-heated oven at 180 °C for 20 min. Muffins were baked in quadruplicate, cool to room temperature and moisture content, water activity, weight and height loss were immediately determined. Thereafter, muffins were mixed and stored in evacuated plastic bag at -20 °C.

General chemical and physical analysis

Moisture content of carob powder samples and muffins was determined in moisture analyser (Kern MLB 50-3, Kern & Sohn GmbH, Balingen, Germany) at 103 °C to a constant weight. Water activity was measured at 25 °C in Aw Sprint TH 500 (Novasina AG, Lachen, Switzerland). Weight loss was calculated from the weight difference between dough and muffin using balance (sensitivity 0.001 g). Height loss was measured using digital caliper (sensitivity 0.01 mm) in three random positions. For measurement of those parameters, muffin samples were cooled down after baking for 1 h at ambient temperature.

Preparation of extracts

Aqueous-organic extracts of muffin or carob powder were prepared according to **Durazzo et al. (2014)** with slight modification. About 3 – 4 g of muffin or carob powder was placed in plastic tube with 20 mL of methanol/water (1:1, v/v). The tubes were placed in vertical shaker for 60 min followed by centrifugation at 5000 rpm for 10 min. The supernatant was removed and 20 mL of acetone/water (70:30, v/v) was added to pellet. After shaking for another 60 min, both methanolic and acetonic extracts were combined, centrifuged (5000 rpm, 5 min) and stored at -20 °C.

Determination of phenolics

Total phenolic content (TPC) was determined using Folin-Ciocalteau procedure as was provided in our previous study **(Brožková et al., 2018)**. The methanol/acetone extract (1.0 mL) was mixed with 0.5 mL of Folin-Ciocalteau reagent and 5.0 mL of distilled water followed by addition of 1.0 mL of 5% Na_2CO_3. After 60 min of incubation in a dark cabinet, the absorbance was measured at 765 nm. The same volume of distilled water was added instead of sample extract to obtain control measurement. The results were expressed as gallic acid equivalent (mg GA.g^{-1}) of dried matter (DM).

Total flavonoid content (TFC) was measured after formation of flavonoids-$AlCl_3$ complex in acetate solution followed by recording absorbance at 415 nm **(Brožková et al., 2018)**. The results were expressed as quercetin equivalent (mg QE.g^{-1} DM).

Determination of antioxidant capacity

Three various methods were used to assess antioxidant capacity of carob powder and muffin. Two assays are based on both electron and hydrogen atom transfer mechanisms involving the reaction of antioxidants with stable chromogen radical DPPH or ABTS. The procedures were adopted from our previous study **(Brožková et al., 2018)**. Briefly, 5.0 mL of DPPH methanolic solution (25 mg.L^{-1}) was mixed with 0.5 mL of methanol/acetone extract and the decrease of absorbance was observed after 35 min at 517 nm. ABTS radical cation (ABTS•+) was prepared from ABTS solution (5.0 mL, 50 mg.L^{-1}) and 100 μL of potassium persulphate (64 mmol.L^{-1}). The mixture was stored in a dark for 12 – 16 hours at laboratory temperature before use. Diluted ABTS•+ solution (3.0 mL) was mixed with 0.5 mL of methanol/acetone extract and the decrease of absorbance was recorded at 734 nm after 50 min of incubation. For both DPPH and ABTS assays, the results were expressed as Trolox equivalent antioxidant capacity (TEAC) in μg Trolox.g^{-1} DM.

Hydrogen peroxide scavenging (HPS) assay represents the method describing the effect of antioxidant against reactive oxygen species **(Mukhopadhyay et al., 2016)**. Extract (0.5 mL) was mixed with 0.25 mL of 1.0 mM ferrous ammonium sulphate and 62.5 uL of 5.0 mM H_2O_2. After 5.0 min of incubation in a dark cabinet at laboratory temperature, 1.5 mL of 1.0 mM 1,10-phenanthroline was added and subsequently incubated for 10 min. The absorbance was red at 510 nm against blank. Distilled water was added to control sample instead of extract and H_2O_2 (total volume 1.562 mL). The increase in HPS activity was reflected by the decrease of absorbance. The % of H_2O_2 scavenging ability was calculated as $(A_{test}/A_{blank}).100$, where A_{test} is the absorbance of the solution containing sample extract, ferrous ammonium sulphate, H_2O_2, 1,10-phenanthroline and A_{blank} is the absorbance of solution containing only ferrous ammonium sulphate and 1,10-phenanthroline.

Determination of Maillard products

The reaction of reducing sugars and tryptophan during roasting of carob powder and baking of muffin may result in formation of advanced Maillard products. Those

products were determinated according to the FAST (Fluorescence of Advanced Maillard products and Soluble Tryptophan) method of **Birlouez-Aragon et al. (2001)**. Briefly, sample extract was prepared by sonication (30 min) of 1.0 g of sample in 40 mL 0.1 M borate buffer solution (pH 8.2). The extract was filtered through filter paper (Whatmann no. 2) and fluorescence of advanced Maillard products (F_{amp}) was recorded at excitation and emission wavelengths of 353 nm and 438 nm, respectively. The decrease of tryptophan amount (F_{trp}) was monitored at excitation and emission wavelengths of 353 nm and 438 nm, respectively. Acryl cuvettes and fluorimeter Fluorat® 02 Panorama (Lumex Instruments, Mission, Canada) were used. FAST index was calculated as (F_{amp}/F_{trp}).100 and the results were expressed in %.

The formation of brown pigment resulted in Maillard reaction was measured in 80% of ethanol at 420 nm against distilled water according to **Krishnan et al. (2010)**. The results were expressed as browning index (BI) in absorbance unit (A_{420}). UV/VIS Spectrophotometer DU 530 (Beckman Coulter Inc., Brea, CA, USA) was used for all colorimetric measurements.

Statistic analysis

The results were expressed as the average means with standard deviations of 4 replicates (n = 4). Non-parametric statistical tests were used throughout this study. *i.e.* Tukey´s multiply comparison method was to find differences between means. Association between carob powder levels and selected variables were determined using Spearman´s correlation coefficient (*r*). Statistical treatment was performed on the probability level of p = 0.05 (Statistica CZ, StatSoft CR s. r.o., Prague, Czechia).

RESULTS AND DISCUSSION

The main composition of carob powder was as followed (in g.100g^{-1} DM): crude protein (7.37 ±0.80), crude fat (0.43 ±0.05), reducing sugar (13.12 ±0.4), crude fiber (26.30 ±0.25), ash (3.29 ±0.22) and moisture (7.20 ±2.00), similarly to those found by **Mohamed, Hamed and Al-Okbi (2008)** for carob pods. Concerning fibre content, lower amount was found in our study (~26%) in comparison with carob powder dried in microwave oven (~50%) **(Tounsi et al., 2017)**. Those differences may be attributed to the different methods, which were used for fibre determination (enzymatic or chemical) or it may be caused by the variability in processing technology and particle size of powder **(Benković et al., 2017)**. Nevertheless, the crude fibre content found in this study fits to the range of 7.6 – 38% previously presented in a review article of **Loullis and Pinakoulaki (2018)** for carob pulp composition.

Weight loss and height of muffins fortified with carob powder

The height and weight loss of the control sample was 3.57 cm and 5.69 g, respectively. The general decrease in height was presented in Table 1. As can be seen, the addition of carob powder caused the reduction of height in comparison with the control. The height of muffin fortified with carob powder significantly decreased from 3.38 to 3.03 cm (p <0.05) with the increase of carob powder level. The decrease in height may be attributed to the dilution of gluten and disruption of gluten network as was previously described by **Lauková, Kohajdová and Karovičová (2016)**, who observed the decrease in diameter and volume of biscuits enriched with apple powder. Similarly, addition of chicory syrup to muffin dough significantly reduced the loaf volume of the products **(Zacharová et al., 2018)**. Incorporating of dietary fibre into dough formulation also exhibited reduction in volume and height of muffin fortified with red capsicum pomace powder **(Nath et al., 2018)**. Although the content of crude fibre in fortified muffins was not evaluated in this study, the content of carob powder with 25% of crude fibre should not be omitted and it requires further investigation. Weight losses of fortified muffins were not statistically significant for all the carob powder samples in comparison with the control muffin sample in the present study.

Moisture content and water activity of muffins fortified with carob powder

Weight loss occurs usually due to the evaporation of water during baking and reflects the changes in moisture content. As can be seen from Table 1, moisture content of muffin samples with carob powder (except of muffin with 15% (w/w)) was significantly higher than this in control. Some authors also observed small but significant increase in moisture content of bread fortified with 10% and 20% of carob flour **(Salinas et al., 2015)**. In a study of **Karaca, Saydam and Guven (2012)**, addition of carob molasses in yogurt significantly increased water-holding capacity of samples. The addition of carob powder to muffin formulation significantly increased water activity from 0.905 (control) to 0.912 – 0.923 a_w for muffins with carob powder (Table 2). Despite those findings, a_w values still fell within the safe water activity range.

Table 1 Weight loss, height, moisture content and water activity of muffin with different levels of carob powder.

Level (%, w/w)	Height (mm ±SD)	Weight loss (g ±SD)	Moisture content (g.100 g^{-1} ±SD)	Water activity
control	3.57 ±0.1^{d}	5.69 ±0.17^{a}	30.19 ±0.84^{a}	0.905 ±0.001^{a}
5	3.38 ±0.11^{bd}	5.71 ±0.18^{a}	31.52 ±0.55^{b}	0.914 ±0.002^{b}
10	3.23 ±0.16^{c}	5.62 ±0.15^{a}	32.41 ±0.19^{b}	0.923 ±0.001^{c}
15	3.22 ±0.05^{c}	5.51 ±0.14^{a}	29.76 ±0.43^{a}	0.918 ±0.001^{b}
20	3.03 ±0.13^{c}	5.58 ±0.16^{a}	31.12 ±0.41^{b}	0.912 ±0.002^{b}

Note: Average mean ± standard deviation (n = 4); significant difference between means in column is indicated by different small letters (p <0.05).

Table 2 Antioxidant properties of muffin with different levels of carob powder.

Level (%, w/w)	TPC	TFC	$TEAC_{DPPH}$	$TEAC_{ABTS}$	HPS
control	193.2 ±18.1[a]	35.7 ±7.1[a]	ND	198.2 ±4.4[a]	5.5 ±0.4[a]
5	348.1 ±6.9[b]	49.6 ±3.3[a]	222.9 ±6.1[a]	1048.8 ±58.5[b]	19.8 ±0.3[b]
10	435.7 ±4.9[c]	49.1 ±2.0[a]	393.5 ±8.6[c]	1683.4 ±18.7[c]	30.6 ±0.2[c]
15	670.0 ±1.2[d]	73.0 ±7.8[b]	1099.5 ±23.9[e]	3137.5 ±4.9[d]	45.8 ±0.2[d]
20	829.1 ±8.8[e]	81.7 ±1.7[b]	1228.3 ±65.6[e]	3599.4 ±21.7[e]	74.6 ±0.2[e]

Note: TPC, Total phenolics content (μg gallic acid.g^{-1} DM); TFC, total flavonoids content (μg quercetin.g^{-1} DM); TEAC, Trolox equivalent antioxidant capacity (μg Trolox.g^{-1} DM); HPS hydrogen peroxide scavenging (%); significant difference between means in column is indicated by different small letters (p <0.05).

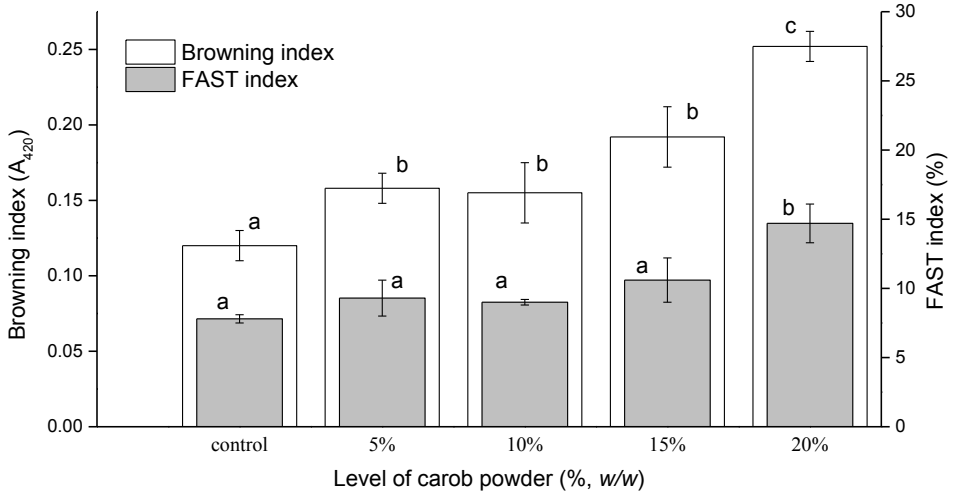

Figure 1 The effect of substitution of carob powder for wheat flour on browning index (white bars) and FAST index (gray bars) in muffin. Average mean ±standard deviation (n = 4).

Total phenolic, total flavonoid and condensed tannin contents in muffins fortified with carob powder

Total phenolic content of fortified muffins significantly increased in comparison with that in control (Table 2). While 193.2 μg GAE.g^{-1} DM was found in control sample, the substitution of wheat flour for carob powder resulted in gradual increase in TPC values from 348.1 to 829.1 μg GAE.g^{-1} DM with the increase of carob powder levels from 5% to 20% (w/w) (p <0.05). **Seczyk, Swieca and Gawlik-Dziki (2016)** also demonstrated that increase addition of carob flour to pasta resulted in the increase of total phenolic content. TFC values were significantly higher than in control (35.7 μg QE.g^{-1} DM; p <0.001) for all fortified muffins without regard of carob powder level. However, the increase in TFC values was not proportional to addition of carob powder. The addition of 5% and 10% (w/w) of carob powder led to the similar TFC values 49.6 – 49.1 μg QE.g^{-1} DM and to 73.1 – 81.7 μg QE.g^{-1} DM when 15% and 20% (w/w) of powders were added.

Antioxidant capacity of muffins fortified with carob powder

Antioxidant characteristics are presented in Table 3. While control muffin sample did not exhibit antioxidant capacity in terms of DPPH assay, the addition of carob powder at 5% (w/w) level to muffin recipe resulted in sharp increase to 222.9 μg Trolox.g^{-1} DM. Then, $TEAC_{DPPH}$ values increased with the increase in carob powder level. The highest $TEAC_{DPPH}$ values were obtained for muffin with 15% and 20% (w/w); *i.e.* 1099.5 and 1228.3 μg Trolox.g^{-1} DM (p <0.01), respectively. Antioxidant capacity measured by ABTS assay is based on similar principle as DPPH assay. Both radicals are able to accept hydrogen atoms or electrons provided by phenolic compounds in the extract. ABTS assay gave higher TEAC values than those in DPPH assay, probably due to the steric hindrance or different solubility of active substances in solutions, which were used for the preparation of stable radicals **(Apak et al, 2016)**. As can be seen from Table 3, ABTS assay always exhibited higher TEAC values at the same level of carob powder in comparison with those determined using DPPH assay (pairwise comparison test was not provided) in current study. It is known that each phenolic constituent contributes in different manner to total antioxidant capacity. We have found that rutin exhibited pro-oxidative effect during drying of buckwheat-based products **(Brožková et al., 2018)** and the same pattern was observed for quercetin, rutin or chlorogenic acid during processing of tomato paste **(Jacob et al., 2010)**. The addition of carob powder significantly increased the ability to scavenge H_2O_2 from initial 5.5% (control) to 19.8% followed by further increase with the increase of carob powder level (Table 3).

Table 3 The correlation analysis of increasing carob powder level *vs.* selected variables in muffin.

Parameters	*r*
Height	-0.756**
Weight loss	-0.006
Moisture content	-0.454
Water activity	-0.486
TPC	0.974***
TFC	0.824**
$TEAC_{DPPH}$	0.971***
$TEAC_{ABTS}$	0.971***
HPS	0.972***
Browning index	0.892***
FAST index	0.692**

Note: TPC, total phenolic content; TFC, total flavonoid content; TEAC, trolox equivalent antioxidant capacity; HPS hydrogen peroxide scavenging; * p <0.05, ** p <0.01, *** p <0.001.

Maillard reaction products in muffins fortified with carob powder

The higher content of reducing sugars and specific amino acids in food matrix may lead to the formation of various Maillard reaction products, which can be measured, in general, at specific wavelength or determination of compounds can be provided using HPLC techniques. Since carob powder is rich in reducing sugars (fructose, glucose) and reactive amino acids such as lysin or proline **(Ayaz et al., 2009; Benkovic et al., 2017)**, the formation of Maillard reaction products should be under control. With the increase in roasting temperatures and roasting times (130 – 150 °C for 5 – 30 min), the coloured Maillard reaction products, fluorescent compounds and hydroxymethylfurfural significantly increased in carob powder as was described in a study of **Cepo et al. (2014)**. The browning and FAST indices were determined in the present study. Addition of carob powder resulted in significantly higher BI when compared with the control muffin sample (p <0.05). BI values increased with the increase of the amount of carob powder up to 0.252 (A_{420}) for muffin with 20 % of carob powder (Figure 1). Surprisingly, FAST index did not differ in muffins fortified with carob powder from that found in control muffin sample (p >0.05) except in muffin with 20% level of carob powder, where significantly higher value was found (p <0.01).

The results of correlation analysis

The correlation analysis revealed that addition of carob powder instead of wheat flour to muffin recipe in the range from 5% to 20% (w/w) did not have effect on the weight loss, moisture content and water activity (Table 3). Strong and positive correlation coefficients ranged from 0.824 to 0.974 for antioxidant properties in terms of TPC, TFC, both TEAC values and hydrogen peroxide scavenging assay were obtained. While browning index showed strong positive correlation with the increase of carob powder level (r = 0.892, p <0.001), FAST index exhibited weak but significant association with the carob powder level (r = 0.692, p <0.01). However, this association is rather statistical than practical. It was evident from multiply comparison (see Figure 1) that FAST index values were not statistically different for control sample and muffins with 5 – 15% (w/w) of carob powder.

CONCLUSION

The additions of carob powder significantly affect the height of the muffins but not weight. Significant differences in moisture content of muffin samples were observed but it was not proportional to the level of carob powder. The addition of carob powder significantly increased water acitivity of muffins in comparison with the control sample. It was found that TPC gradually increased with the increase level of carob powder while TFC significantly increased in muffins fortified with higher levels of carob powder. Antioxidant capacity has increased with the increase level of carob powder in terms of DPPH, ABTS, and hydrogen peroxide scavenging assays. On the other hand, the addition of carob powder was associated with the formation of Maillard products. Browning index was significantly higher for muffin with the lowest carob powder level than that in control sample, but FAST index was significantly higher in muffin with the highest level of carob powder.

The results of this study showed that the replacement of wheat flour with carob powder increased the antioxidant status of muffins, however the higher the level of carob powder, the higher the potential to the formation of Maillard products.

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COMPARISON OF ANTIOXIDANT ACTIVITY, CONTENT OF POLYPHENOLS AND FLAVONOIDS IN LITURGICAL AND COMMON WINES

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ABSTRACT

This article deals with the comparison of biologically active substances (antioxidant capacity, content of polyphenols and flavonoids) in samples of common and liturgical wines. For determination were chosen these varieties Pinot Noir, Red Traminer and Chardonnay. The total content of polyphenols and flavonoids were found by visible diode array spectrophotometer. For determination of total antioxidant capacity was used the DPPH test. Results of this paper did not prove a general difference between liturgical and common wines, although between individual samples a statistically significant difference was found. Furthermore, the results show considerably higher values of biologically active substances in red wines Pinot Noir against white wine Red Traminer and Chardonnay – the total antioxidant capacity was considerably in excess of values up to 30 times, the total content of polyphenols up to 50 times and the total content of flavonoids up to 50 times. From the content of biologically active substances point of view, the red wine is recommended for human health.

Keywords: altar wine; kosher wine; antioxidant activity; polyphenols; flavonoids

INTRODUCTION

Wine is a very popular beverage over the world. The traditional production of wine is already known several thousands of years. For example, the ancient Romans knew wine and popularised wine consumption for its health benefits **(Lukacs, 2012)**. Since time immemorial, wine is also used at various religious ceremonials to pay tribute to gods at rituals of corresponding church. In Christian religion wine symbolizes Jesus Christ blood **(Bible, 1991)**. The production of altar wine using as „blood of the Lord" at Eucharist is followed the rules, that are set down by Czech Bishop Conference for the Czech Republic **(Koudelka, 2010)**. According to instructions of **Congregation for Worship and the Discipline of the Sacraments (2004)** the altar wine must be natural from grapevine, without other additives and chemically treated. First of all, Czech Bishop Conference requires that wine was made only from grapes coming from Bohemia and Moravia. It is possible to give another rule, that used grapes must have 20 degrees of sugar content at least **(Koudelka, 2010)**.

Not only in Christianity, but also in other religions the liturgical wine, which is used for religious purposes, must meet the requirements of relevant religion and must be approved by a relevant religious authority. For example, at Jewish religious ceremonies wine is used much more than in Christian religion and rules for its production are more stringent. In Jewish religion Tóra looks at wine and bread as at „good things of life" and „things for pleasure of gods and people" **(Torah, 2012a,b,c; Divecký, 2005; Bondyová and Sliva, 2008)**. Wine is divided into several groups **(Mlček et al., 2018)**. Apart from boiled wine, non-Jew cannot touch kosher wine or open it due to the maintenance of kosher quality. Strict rules, that are apply to the manipulation with wine, are valid even for its growing and processing. For example, according to the commandment in the third book of Moses it is necessary to let a vineyard fallow and rest every seventh year (sabbatical year).

The wine grape is a basis for the production of grape wine. During processing of wine, the large amount of substances convenient for human health arise or get directly into final beverage from grapes. That is why wine is a significant source of biologically active substances such as antioxidants, polyphenols, flavonoids or mineral substances **(Mlček et al., 2018)**. The content of biologically active substances in grapes and wine depends on the variety of wine, locality, climate conditions, used agrotechnology and technology of processing and storage. Important biologically active substances are antioxidants, which prevent or reduce the oxidative destruction of substances, in which are contained in small amount. The substances with high antioxidant capacity have especially plant origin. Wine is the rich source of substances with antioxidant capacity, that can prevent damaging of DNA, peroxidation of lipids and formation of free radicals. So, it can be one of prophylaxis instruments before lifestyle diseases, especially cardiovascular **(Anastasiadi et al., 2010; Snopek et al., 2018b)**. Another biologically active substances in wine are

polyphenols, that arise as secondary metabolites. They have an important role in reducing the risk of cardiovascular diseases similarly to antioxidants **(Snopek et al., 2018a)**. Flavonoids are classified to group of polyphenols according to type of reaction. They are very reactive and affect the wine oxidation. In case of gentle processing of grapes and of careful pressing the polyphenols content ranges under 200 $mg.L^{-1}$ in white wine. In red wine the polyphenols content is 3 – 10 times higher. The total daily intake of polyphenols is estimated at 1 g **(Pavloušek, 2010)**. Due to positive effects of biologically active substances in wine on human health it is necessary to observe their content **(Valášek et al., 2014)**. Due to stricter conditions at the production of liturgical wine it can come about the variability of these biologically active substances content and difference from common wines.

Scientific hypothesis

Scientific hypothesis is: The total antioxidant capacity and the content of polyphenols and flavonoids in Czech liturgical and common wines are differed.

The aim of the study was a comparison of the antioxidant capacity and the content of polyphenols and flavonoid in Czech liturgical and common wines. Wine samples were selected with respect to their comparability of vintage, subregion and attribute.

MATERIAL AND METHODOLOGY

Wine samples

For analysis of total antioxidant capacity and the total content of polyphenols and flavonoids in sacramental wines were selected wines Pinot Noir and Red Traminer, Chardonnay was used for comparison in kosher wines. For each variety, 2 samples of common wine and 2 samples of liturgical wine were tested. Samples were chosen to ensure the highest comparability possible (vintage, sub-area and attribute), but different producers. Due to the difficulty of acquiring the comparable samples, their gathering took 15 months.

Samples were bought gradually in the common market, specialized wine shops and directly from the producers. Two bottles of each wine were bought and analysed. The samples in Table 1 were tested.

Table 1 Wine origin and category.

Sample	Category	Vintage	Sub-area, village, track	Quality
			Pinot Noir	
PN1	M	2012	Znojemská, Stošíkovice na louce, U tří dubů	VB
PN2	M	2012	Velkopavlovická, Havraníky, Staré vinice	VH
PN3	B	2012	Znojemská, Miroslavské Knínice, Stará hora	VH
PN4	B	2012	Velkopavlovická, Velké Bílovice	VH
			Red Traminer	
TR1	M	2013	Velkopavlovická	VH
TR2	M	2013	Znojemská, Stošíkovice na louce, U tří dubů	VH
TR3	B	2013	Znojemská, Bzenec	PS
TR4	B	2013	Znojemská, Sedlec, Nad Nesytem	PS
			Chardonnay	
CH1	K	2010	Izrael, Samson	Q
CH2	K	2011	Slovácká, Hýsly / Moštěnsko	PS
CH3	B	2010	Mikulovská, Perná, Purmice	PS
CH4	B	2011	Znojemská, Bzenec	VH

Note: K – kosher wine, M – communion wine, B – common wine, VB – special selection of berries, VH – special selection of grapes, PS – Late harvest, Q – quality.

Table 2 Results of the total antioxidant activity, the content of polyphenols and flavonoids in czech liturgical and common wines of varieties Pinot Noir (RM), Red Traminer (TR) and Chardonnay (CH).

Wine	Use	Total antioxidant activity [$mg.L^{-1}$ AAE]		Total content of polyphenols [$mg.L^{-1}$ GAE]		Total content of flavonoids [$mg.L^{-1}$]	
		M	SD	M	SD	M	SD
RM1	Liturgical	7160.2	57.0	12118.8	274.3	8689.3	476.1
RM2	Liturgical	2684.7	99.6	6349.1	291.4	5273.9	452.8
RM3	Common	4704.2	79.2	8146.7	393.3	5907.9	686.7
RM4	Common	3750.0	81.3	8246.7	806.7	6526.3	706.7
TR1	Liturgical	228.5	2.9	228.0	2.4	148.7	11.0
TR2	Liturgical	775.0	50.0	416.7	4.4	612.0	395.6
TR3	Common	894.0	5.7	440.2	6.9	250.0	163.3
TR4	Common	683.3	37.5	325.0	41.7	377.6	237.2
CH1	Kosher	359.2	40.5	298.6	8.8	591.8	290.8
CH2	Kosher	750.0	158.3	395.8	62.5	357.1	7.7
CH3	Common	890.0	10.0	453.4	11.8	906.7	442.1
CH4	Common	375.0	262.5	304.2	12.2	349.0	11.0

Total antioxidant capacity assay

To determine total antioxidant capacity (TAC) DPPH (1,1- diphenyl -2 picrylhydrazyl) assay was used according to the study by **Brand-Williams et al. (1995)**. The stock solution was prepared by dissolving 24 mg of DPPH with 100 mL of methanol and then stored at -20 °C until needed. The absorbance of DPPH radical without wine was measured daily. The sample solution was obtained by mixing 10 mL of the stock solution with 45 mL of methanol to obtain the absorbance of 1.1 ±0.02 units at 515 nm using the spectrophotometer LIBRA S6 (Biochrom, Cambridge, UK). The wine (210 µL) was allowed to react with 4 mL DPPH solution for 1 hour in the dark. Then, the absorbance was taken at 515 nm. Antioxidant capacity was calculated as a decrease of the absorbance value using the formula:

Antioxidant capacity (%) = (A0 - Ai/A0) x 100%, where A0 is the absorbance of a blank (without the sample) and Ai is the absorbance of the mixture containing the sample. Calculated antioxidant capacity was converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE) **(Rupasinghe, Jayasankar and Lay, 2006)**.

Total phenolic content assay

To measure total phenolic content (TPC) Folin-Ciocalteau reagent was used. 0.1 mL of wine was taken and mixed with water in a 50 mL volumetric flask. There-after, 0.5 mL of Folin-Ciocalteau reagent and 1.5 mL of 20% solution of Na_2CO_3 were added. The resulting absorbance was measured by LIBRA S6 spectrophotometer (Biochrom, Cambridge, UK) at the wavelength of 765 nm. Water was used as reference **(Thaipong et al., 2006)**. The results were expressed as grams of gallic acid (GAE) per kg of fresh mass (FM).

Total flavonoid content assay

Total flavonoid content (TFC) was determined by using 0.85 mL of juice mixed with 8.5 mL of 30% ethanol, 0.375 mL of $NaNO_2$ (c = 0.5 mol.dm^{-3}) and 0.375 mL of $AlC1_3.6H_20$ (c = 0.3 mol.dm^{-3}) as is described by **Park et al. (2008)**. The mixture was measured at the wavelength of 506 nm by LIBRA S6 spectrophotometer (Biochrom, Cambridge, UK). Total flavonoid content was calculated from a calibration curve by using rutin as the standard. The results were expressed in mg.kg^{-1} of FM.

Statistic analysis

The data were analysed using Excel 2013 (Microsoft Corporation, USA) and STATISTICA Cz version 12 (StatSoft, USA). Results were expressed by average ± standard deviation. Comparison of the results was performed using by Kruskal-Walllis test (α = 0.05). The samples of individual varieties were compared to each other. Furthermore, all samples of individual varieties of liturgical wines were compared against common wines.

RESULTS AND DISCUSSION

Total Antioxidant Capacity

In the study basic biologically active substances were determined in wine of these varieties – Pinot Noir, Red Traminer and Chardonnay. The basic measured results of the total antioxidant capacity, the content of polyphenols and flavonoids are given in Table 2.

The highest values of total antioxidant capacity were determined for variety Pinot Noir and these ones significantly exceed values of another observed varieties (up to 30x between sample RM1 a TR1). For variety Pinot Noir average values were measured over 2600 mg.L^{-1} AAE for every sample, while for another two varieties Red Traminer and Chardonnay were found below 900 mg.L^{-1} AAE. This finding confirms the fact, that antioxidant capacity is much higher for red wines than for white wines. This is due to the fact that in red wines substances with antioxidant capacity, for example phenolic substances, are in a significantly higher amount. In these more general facts results are in accordance with **Rupasinghe and Clegg (2007)** and **Szajdek and Borowska (2008)**. **Špakovská (2012)** in her study presents the higher antioxidant activity in red wines than in white wines. Average values were measured for each sample for variety Pinot Noir.

Results, presented in Table 2, were used for a comparison between each sample for given wine variety. Results of the comparison of total antioxidant capacity are shown in Table 3. Even though between many compared samples the statistically significant difference (p <0.05) was found out for each variety, the difference between liturgical and common wines was not generally proven. For example, for variety Pinot Noir the statistically significant differences were calculated for all samples.

However, total antioxidant capacity in common and altar wine is in the same range of values (Table 2) so the statistically significant difference between these varieties of wine was not confirmed.

Total Phenolic Content

Similarly, to total antioxidant capacity, the values of polyphenolic substances are significantly higher than for other observed varieties. For varieties Pinot Noir the total phenolic content was above 6300 mg.L^{-1} and for varieties Red Traminer and Chardonnay, average values of total phenolic content were set to 500 mg.L^{-1}. Statistical results of comparison for the total content of polyphenols are shown in Table 4. Again, the statistically significant difference (p <0.05) was found between many samples of each variety, but the difference between liturgical and common wines was not generally proven.

The phenolic substances are mainly contained in a grape peel. White wines are not macerated with peels in the production process, but peels are immediately removed and for this reason white wines contain fewer phenolic substances. In this aspect, the results are consistent with **Rupasinghe and Clegg (2007)** and **Faitova et al. (2004)**. **Špakovská (2012)** presents the content of polyphenols in the range of 299 to 407 mg.L^{-1} for white wines and for red wines in the range of 2130 to 650 mg.L^{-1}, which is consistent with our measured values.

Jančářová et al. (2013) states that the total polyphenol content is gradually decreasing with increasing time. Similarly, **Andjelkovic, Radovanović and Radovanović (2013)** also states, that the total polyphenol content grows during maturation and then decreases, ranging from 74.04 to 315.45 mg GAE.g^{-1}. The result of the above studies is finding, that the total polyphenol content varies with time.

Table 3 Comparison of total antioxidant activity between each samples for each variety. *p*-values were calculated by Kruskal-Walllis test.

Sample	RM1	RM2	RM3	RM4
RM1	-	**0.00**	**0.00**	**0.00**
RM2	**0.00**	-	**0.00**	**0.00**
RM3	**0.00**	**0.00**	-	**0.00**
RM4	**0.00**	**0.00**	**0.00**	-
	TR1	**TR2**	**TR3**	**TR4**
TR1	-	**0.00**	**0.00**	**0.00**
TR2	**0.00**	-	**0.00**	0.05
TR3	**0.00**	**0.00**	-	**0.00**
TR4	**0.00**	0.05	**0.00**	-
	CH1	**CH2**	**CH3**	**CH4**
CH1	-	**0.00**	**0.00**	0.89
CH2	**0.00**	-	0.56	**0.01**
CH3	**0.00**	0.56	-	**0.00**
CH4	0.89	**0.01**	**0.00**	-

Table 4 Comparison of total phenolic content between each samples for observed varieties. *p*-values were calculated using Kruskal-Walllis test.

Sample	RM1	RM2	RM3	RM4
RM1	-	**0.00**	**0.00**	**0.00**
RM2	**0.00**	-	**0.00**	**0.00**
RM3	**0.00**	**0.00**	-	0.79
RM4	**0.00**	**0.00**	0.79	-
	TR1	**TR2**	**TR3**	**TR4**
TR1	-	**0.00**	0.00	**0.00**
TR2	**0.00**	-	0.00	**0.00**
TR3	**0.00**	**0.00**	-	**0.00**
TR4	**0.00**	**0.00**	0.00	-
	CH1	**CH2**	**CH3**	**CH4**
CH1	-	**0.00**	**0.00**	0.38
CH2	**0.00**	-	0.51	**0.01**
CH3	**0.00**	0.51	-	**0.00**
CH4	0.38	**0.01**	**0.00**	-

Table 5 Comparison of total flavonoid content between each samples for observed varieties. *p*-values were calculated using Kruskal-Walllis test.

Sample	RM1	RM2	RM3	RM4
RM1	-	**0.00**	**0.00**	**0.00**
RM2	**0.00**	-	0.09	**0.00**
RM3	**0.00**	0.09	-	0.16
RM4	**0.00**	**0.00**	0.16	-
	TR1	**TR2**	**TR3**	**TR4**
TR1	-	**0.02**	0.16	**0.04**
TR2	**0.02**	-	0.07	0.24
TR3	0.16	0.07	-	0.30
TR4	**0.04**	0.24	0.30	-
	CH1	**CH2**	**CH3**	**CH4**
CH1	-	0.08	0.18	0.07
CH2	0.08	-	**0.01**	0.17
CH3	0.18	**0.01**	-	**0.01**
CH4	0.07	0.17	**0.01**	-

Due to the method of agrotechnical processing of altar wines, which should have the sugar content 20 °NM and more, later harvesting is supposed. Harvest time may be affected by the amount of polyphenols, their content can be lower.

On the other hand, the content of fragrances can increase, and organoleptic properties can improve.

Total flavonoid content

The last observed group of biologically active substances was group of flavonoids and their total content. Similarly,

to the two previous observed parameters, values of total flavonoid content for variety Pinot Noir were significantly higher compared to other varieties.

For variety Pinot Noir the content of flavonoids ranged above 5200 mg.L^{-1}, but for varieties Red Traminer and Chardonnay values were measured below 1000 mg.L^{-1}. Comparison between each sample for observed varieties is given in Table 5. However, one can conclude here (to take account of total values), that the statistically significant difference between liturgical and altar wines cannot be established, but the difference between the individual samples can be established.

As stated by **Rupasinghe and Clegg (2007)**, the nature and concentration of flavonoids in wine samples could influence both the wine variety and damaged grapes during harvest and the differences in processing methods. **Sandler and Pinder (2003)** states, that flavonoid content in red wines can exceed 1200 mg.L^{-1}. This reality is in line with our obtained results. In case of white wines, the content of flavonoids is up to several times lower due to their technological processing.

CONCLUSION

This study deals with the content of biologically active substances in samples of liturgical and common wines and their comparison, which did not show the difference between liturgical and common wines, although the statistically significant difference between the individual samples was found. The total content of polyphenols and flavonoids and the total antioxidant capacity of samples were measured. The biologically active substances are important for the impact on human health. Results documented significantly higher values of biologically active substances in red wine for variety Pinot Noir against white wines for varieties Red Traminer and Chardonnay. The article also mentions the fact, that the content of individual biologically active substances changes and it is necessary to balance these quantities according to the requirements of the producer and the customer.

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CHICKEN SKIN GELATINE AS AN ALTERNATIVE TO PORK AND BEEF GELATINES

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ABSTRACT

Poultry meat-processing industry produces considerably large amounts of by-products (such as chicken skins, heads, feathers, viscera, bones and legs) containing significant volumes of proteins, particularly collagen. One of the possibilities of advantageous utilization of these under-used by-products can be their application as a raw material rich in collagen for preparation of gelatine, a partial hydrolysate of collagen. In the present study, chicken skins obtained as a by-product from the chicken-breast processing were purified from non-collagen proteins, pigments and fats. Collagen was treated with proteolytic enzymes and the gelatine extraction was performed in distilled water at temperatures of 40, 50, 60, 70 and 80 °C during the constant extraction time of 60 min. The influence of the technological conditions on gelatine functional properties including viscosity, clarity, water holding and fat binding capacity, emulsifying and foaming properties was explored. Certain functional properties of prepared gelatines were significantly affected by the extraction temperature, while on some other properties the extraction temperature had no significant effect. Viscosity of prepared chicken skin gelatines was in the range from 3 to 5.7 $mPa.s^{-1}$, clarity from 1.5 to 2%, water holding capacity from 3.8 to 5.6 $mL.g^{-1}$, fat binding capacity from 0.9 to 1.3 $mL.g^{-1}$, emulsion capacity from 35 to 50%, emulsion stability from 73 to 88%, foaming capacity from 18 to 61% and finally foaming stability was from 4 to 39%. Chicken skin gelatines were compared with commercial food grade pork and beef gelatines. Prepared chicken skin gelatines showed better viscosity, fat binding capacity and foaming stability than mammalian gelatines, while water holding capacity, emulsifying stability and foaming capacity were not as good as in beef and pork gelatines. Emulsifying capacity was comparable with commercial gelatines. Therefore, chicken skin gelatine has the potential as an alternative to traditional gelatines from mammalian sources, such as pork or beef bones and skins.

Keywords: chicken skin; collagen; food grade gelatine; functional properties; poultry by-products

INTRODUCTION

Extensive manufacture of poultry meat produces large amounts of by-products such as viscera, feet, heads, bones, blood, feathers or skins **(Zhu et al., 2010)**. These by-products are normally composted or used for the production of livestock feed. Unfortunately, a common practise in some developing countries is unfortunately to landfill or incinerate them. On the other hand, in some countries poultry by-products, such as heads, paws and stomachs, are cooked, fried and consumed as traditional meals **(Toldra, Mora and Reig, 2016)**. Poultry by-products are rich in proteins, enzymes and lipids **(Ockerman and Hansen, 2000; Raju, Rose and Rao, 1997)** and thus possess nutritional and economic potential **(Salminen and Rintala, 2002)**.

Traditional sources of collagen for the production of gelatines are skins, connective tissues and bones from beef or pork origin **(Morrison et al., 1999)**. Gelatine gained from pig skin accounts 46% of the production, from beef skin 29%, from bones 23% and 2% accounts gelatine made from other sources **(Ahmad and Benjakul, 2011)**. Pork gelatine is prohibited to use in Kosher and Halal foods, whereas beef gelatine cannot be consumed by Hindus **(Kaewruang et al., 2013)**. That is why, alternative sources of collagen, such as fish bones, skins and scales, are becoming more important. Another alternative source can also be poultry by-products including chicken, turkey or duck skin. It is estimated that chicken skins represents about 15% of live weight of the animal **(Sheu and Chen, 2002)**. Thus, chicken skins should be considered as by-products with significant economic potentional. One of the further options of the application of chicken skins, by-products from the conversion of chicken meat to chicken breast, is to use it as a raw material for gelatine extraction.

Gelatine is a partial hydrolysate of collagen with a wide range of potential functions based on its specific structure

(Norziah et al., 2009). This unique biopolymer supply elasticity, viscosity and stability in foods **(Zhou, Mulvaney and Regenstein, 2006)**. Gelatine gel has the ability of "melt in the mouth" which gelatinous agents of plant origin, such as starch, alginate, pectin, agar and carrageenan lack **(Bazawine and He, 2003)**. If the concentration of gelatine solution is suitable for network forming, transition from sol to gel occurrs **(Kaur et al., 2002)**. Gelatine is, due to its unique properties, used as a food ingredient in various types of products to modify e.g. elasticity, slicability and cohesion, (for example in desserts, lunch meats, aspics, marshmallows, ice creams, coating, puddings, sauces, yogurts), in the biomedical field (e.g. wound dressing and three-dimensional tissue regeneration products) or in numerous non-food applications (e.g. photography, paper manufacture, matches, coating, sizing) **(Chatterjee and Bohidar, 2005; GMIA, 2012; Petrášová et al., 2016)**. Gelatine also finds its application in the pharmaceutical industry in the production of soft and hard capsules **(Karim and Bhat, 2008)**. The global consumption of gelatine in 2011 was 348,000 tons and in 2018 it was expected to be as much as 450,000 tons **(Sheela, 2014)**.

Gelatine quality is determined mainly by gelatine gel strength expressed in the Bloom value **(Binsi et al., 2009)**. Further functional characteristics of gelatine including viscosity, clarity, water holding and fat binding capacity, emulsifying and foaming properties are also important, mainly in the food industry.

Viscosity of gelatine and other protein solutions depends on internal characteristics, such as molecular weight, amino acid content or surface charge **(Masuelli, 2011)**. Several studies devoted to viscosity of gelatine solutions have been published recently. **Masuelli and Sansone (2012)** studied intrinsic viscosity of gelatine. **Qiao et al. (2013)** determined viscosity of gelatine in solutions of monovalent and divalent salts.

Clarity of gelatine gel may be a significant feature in products which are required to be transparent **(Bower et al., 2006)**. It is an important organoleptic property and determines mainly acceptability of final products **(Zarai et al., 2012)**. Clarity is the opposite of turbidity. Turbidity is influenced by inorganic, protein and mucosubstance contaminants which remain in gelatine unless they have been completely removed during the gelatine preparation.

Water holding capacity (WHC) is the elemental gelatine characteristic and desirable property in food products including sausages, custards and dough because it is supposed to draw water without dissolving proteins and thus attaining products thickening and viscosity. The ability of gelatine to bind water is one of the most significant properties, which is benefitial in numerous food applications. WHC is an important feature for reducing water losses and juiciness of frozen fish or meat products while they are being cooked **(Rawdkuen, Thitipramote and Benjakul, 2013)**. Better WHC may be connected with a higher quantity of hydrophilic groups and affected by many factors, such as a protein concentration and ionic strength **(Kinsella, 1976; Li, Jia and Yao, 2009; Ninan, Joseph and Aliyamveettil, 2014)**. Higher WHC is also related with desired rheological and textural characteristics and reduction in dehydration during the storage **(Simões et al., 2014)**.

Fat binding capacity (FBC) is required property in minced meat formulations helping retain flavour and palatability and prolong the shelf life of baked goods, soups and meat products **(Rawdkuen, Thitipramote and Benjakul, 2013)**. FBC is a significant functional property specifically important in the production of meat and confectionary products **(Souissi et al., 2007)** as it determines the ability of collagen to bind fat through non-polar chains of macromolecules **(Bhaskaracharya, Kentish and Ashokkumar, 2009)**. FBC of proteins is related to hydrophobicity of the surface and to the level of exposure of hydrophobic residues inside gelatine molecule. It may be influenced by various factors, such as a type of protein and degree of hydrolysis **(George, Joseph and Zynudheen, 2010; Kristinsson and Rasco, 2000)**.

Emulsifying capacity (EC) and emulsifying stability (ES) of gelatine are especially utilized in the cosmetic industry during the preparation of ointments and creams. **Kinsella (1976)** defined EC of gelatine as the volume of oil that can be emulsified by gelatine and hydrolysates. Gelatines and hydrolysates are surface active substances and encourage to form oil-in-water emulsions since they are soluble in water and have functional groups both hydrophilic and hydrophobic **(Wilding, Lilliford and Regenstein, 1984)**. It is generally presumed that emulsifying properties of gelatines/hydrolysates are probably affected by difference in their peptide composition, molecular size and lipophilic–hydrophilic arrangement **(Li, Jia and Yao, 2009)**. Gelatines with high gelatine gel strength improves emulsifying properties if they are added to final products **(Gómez-Guillén et al., 2011)**.

The capability of forming stable gelatine foam is crucial in the preparation of confectionery products including marshmallows or other whipped products. This may be explained by probable presence of a large molecules of peptides in chicken skin collagen which can form stable films around gas bubbles **(Souissi et al., 2007)**. In order to create a stable foam on water-air interface, molecules must contain hydrophobic regions that appear during the unfolding of proteins **(Gómez-Guillén et al., 2011)**. Foaming properites of gelatine may be important in the bakery industry as they help to stabilize foaming products, such as pies, breads and cakes **(Djagny, Wang and Xu, 2001)**.

The aims of this study

This paper continues in the previous research of authors focusing on designing of the proper technological conditions for the chicken skin gelatine preparation, testing the effects of the extraction temperature on gelatine gel strength.

The aims of this paper are as follows:

1. Preparation of chicken skin gelatines at different extraction temperatures according to the method described in the previous work **Mrázek et al. (2019 – in press)**.
2. Testing of functional properties of chicken skin gelatines in relation to food applications: viscosity, clarity, water holding and fat binding capacity, emulsifying capacity and stability and foaming capacity and stability.

3. Comparison of functional properties of gelatine prepared from chicken skin with commercial food grade pork and beef gelatines.
4. Evaluation of extraction conditions affecting the functional properties of prepared gelatines.

Scientific hypotheses

There were presumptions that technological conditions during the extraction of gelatines (e.g. temperature) affect the functional properties of prepared gelatines and that functional properties of chicken skin gelatines will be comparable with functional properties pork and beef gelatines.

MATERIAL AND METHODOLOGY

Appliances, tools and chemicals

Stevens LFRA Texture Analyser for measuring gelatine gel strength (Leonard Farnell and Co ltd., England), SPAR Mixer SP-100AD-B meat grinder (TH Industry RD, Taiwan), Rotina 35 centrifuge (Hettich, Germany), IKA T 25 digital Ultra-Turray desintegrator (IKA-Werke, Germany), Memmert ULP 400 drying device (Memmert GmbH+Co. KG, Germany), LT 43 shaker (Nedform, Czech Republic), Kern 440-47 electronic scale, Kern 770 electronic analytical balance (Kern, Germany), A 10 labortechnik analytical mill (IKA-Werke, Germany), ULP 400 drying oven (Memmert GmbH+Co. KG, Germany), Samsung fridge-freezer (Samsung, South Korea), Thermo Haake C 10 thermometer (Thermo Fisher Scientific, USA), Helios Epsilon spectrophotometer (Thermo Fisher Scientific, USA), Whatman No. 1 paper (Sigma Aldrich, UK), a metal filter sieve with the size of pores 1 and 2 mm (Labor-komplet, Czech Republic). Chemicals: NaCl, NaOH, petroleum ether, ethanol and chloroform (Verkon, Czech Republic); all chemicals were of analytical grade. Proteolytic enzyme Polarzyme 6.0 T-serine endoprotease manufactured by fermentation of microorganisms that are not present in the final product (Novozymes, Denmark) with declared enzyme activity of 6 KPU.g^{-1} (kilo protease unit.g^{-1}). Commercial gelatines, pork DO12119 260 Bloom (type A) and beef D529 260 Bloom (type B) of the grain size of 2 mm. Virgin sunflower oil (Via Naturae, Czech Republic).

Preparation of chicken skin gelatines

Chicken skins were purchased from Raciola (Uherský Brod, Czech Republic). The composition of chicken skins was as follows: dry matter: 53.6 ±1.5%; in dry matter: proteins: 16.5 ±1.3, collagen: 92.6 ±0.1, fats: 85.0 ±2.4, inorganic solids: 0.9 ±0.3 **(Davídek et al., 1981; ISO 3496-1994)**. The raw material was processed into gelatines according to the method described in **Mrázek et al. (2019 – in press**). The raw material was ground to the size of particles of 3 mm and separation of non-collagen parts was performed using 1 M NaCl and 0.5% NaOH. After filtration and rinsing with water, raw material was dried at 35 °C. Separation of fats was performed using the mixture of solvents of petroleum ether and ethanol at the ratio of 1:1 (w/w). The filtration process was followed by proteolytic enzyme pre-treatment using 0.5% Polarzyme 6.0T in distilled water at pH 7.5. After filtration and rinsing with water, 5 experiments of gelatine extraction were realized in distilled water at 40, 50, 60, 70 and 80 ±0.5 °C for 60 min. After filtration of gelatine solution using Whatman no.1 paper (Sigma Aldrich, UK) and drying it in a thin layer at 45 °C ±0.3 °C. Gelatine powder was prepared by grinding of the gelatine film to the size of particles of 1 – 2 mm using A 10 labortechnik analytical mill (IKA-Werke, Germany). Samples of gelatines were then subjected to further analysis.

Testing of functional properties of gelatines

Prepared gelatine samples were analysed in order to compare the functional properties of gelatines produced under different extraction temperatures. Results were compared with the analyses of two types of commercial food grade gelatines (pork and beef).

Viscosity

Viscosity of gelatine solution was measured according to the method described at **GMIA (2013)**. 6.67% gelatine solution was prepared as follows: 7.5 g of gelatine was mixed with 105 mL of distilled water and maintained at room temperature for 2 h in order to swell. The sample was afterwards dissolved in 65 °C water bath for not more than 10 min. Gelatine solution was transferred to the viscosity pipette placed inside thermometer Thermo Haake C 10 (Thermo Fisher Scientific, USA). The temperature of 60.00 ±0.05 °C was maintained. Time required for 100 mL of gelatine solution to pass through the capillary tube of the pipette by draining gelatine solution was measured. Viscosity of gelatine sample was calculated from the following equation:

$$\nu = k \cdot t - \frac{B}{t}$$

ν – kinematic viscosity [mm^2.s^{-1}]
k – the viscosity constant detected by calibration fluid (0.5)
t – arithmetic mean of measured flow times [s]
B – correction constant for kinetic energy determined from dimensions of the viscometer (2.8)

Kinematic viscosity was converted to dynamic viscosity according to the following equation:

$$\eta = \nu \cdot \rho$$

η – dynamic viscosity [mPa.s]
ρ – gelatin solution density [g.cm^{-3}]

Density of gelatine was 1.003 g.cm^{-3} ±0.005 and it was determined by pycnometric method.

Clarity

Clarity of gelatine solution was determined according to the method described at **GMIA (2013)**. The same gelatine solution as for viscosity measurement was used. It was heated at the temperature of 45 °C in water bath and transmittance value at λ = 640 nm using Helios Epsilon spectrophotometer (Thermo Fisher Scientific, USA) was recorded.

Water holding capacity

Water holding capacity (WHC) was determined in conformity with the method described by **Nasrin, Noomhorm and Anal (2015)**. Gelatine sample (1 g) was weighed and dispersed in 25 mL of distilled water in test tube by vortexing for 5 min at room temperature. After that, it was centrifuged using Rotina 35 centrifuge (Hettich, Germany) at 3,000 rpm for 30 min. Supernatant was filtered with Whatman no. 1 paper and the sample was then weighed again.

Water holding capacity was calculated using a formula:

$$WHC = \frac{w_1}{w_0}$$

WHC – water holding capacity [mL.g^{-1}]
w_1 – weight of sample after analysis [g]
w_0 – weight of sample before analysis [g]

Fat binding capacity

Fat binding capacity (FBC) was determined according to the method by **Li, Jia and Yao (2009)**. Gelatine sample (0.1 g) was weighed and dispersed in 10 mL sunflower oil in test tubes and properly mixed by vortexing for 1 min and allow to stand for 30 min at room temperature. Afterwards, gelatine was dispersed in oil and centrifuged at 3,000 rpm for 30 min. Free oil was decanted and FBC was calculated using the following formula:

$$FBC = \frac{w_1}{w_0}$$

FBC – water holding capacity [mL.g^{-1}]
w_1 – weight of sample after analysis [g]
w_0 – weight of sample before analysis [g]

Emulsifying properties

Emulsifying capacity and stability were determined according to the method by **Neto et al. (2001)**. 5 mL of gelatine solution (prepared by heating at 45 °C) at concentration of 10 mg.mL^{-1} was homogenized with 5 mL of sunflower oil for 1 min. Thereafter, the mixture of gelatine and oil was centrifuged at 1,100 rpm for 5 min. Emulsifying capacity was determined using the following formula:

$$EC = \frac{H_1}{H_0} \cdot 100$$

EC – emulsifying capacity [%]
H_1 – height of emulsion layer [mm]
H_0 – height of the total content [mm]

After that the emulsion of fat and gelatine was heated in 55 °C water bath followed by centrifugation at 1,100 rpm for 5 min. Emulsifying stability was calculated using the formula:

$$ES = \frac{H_1}{H_0} \cdot 100$$

ES – emulsifying stability [%]
H_1 – height of emulsion layer after heating [mm]
H_0 – height of emulsion layer before heating [mm]

Foaming properties

Foaming capacity and foaming stability were determined according to the method by **Sathe, Deshpande and Salunkhe (1982)**. 0.6 g of gelatine and 30 mL distilled water was mixed and heated at 60 °C. Foam was prepared by homogenization at 10,000 rpm for 5 min using IKA T 25 Digital Ultra-Turray desintegrator (IKA-Werke, Germany).

Foamed gelatine solution was poured into 250 mL measuring cylinder and foaming capacity was calculated using the formula:

$$FC = \frac{V_1 - V_0}{V_0} \cdot 100$$

FC – foaming capacity [%]
V_1 – volume of foamed liquid [mL]
V_0 – initial volume of liquid [mL]

Thereafter foaming stability was determined. The principle was based on measuring the volume of foamed gelatine solution after 30 min; foaming stability was calculated according to the following formula:

$$FS = \frac{V_2 - V_0}{V_0} \cdot 100$$

FS – foaming stability [%]
V_2 – volume of foamed liquid after 30 min
V_0 – initial volume of liquid [mL]

Statistical analysis

All analyses were performed in triplicate; linear regression, 1-sample and 2-sample t-test testing on the significance level of p 0.05 were applied to all results using Minitab 18 statistical software for Windows (Minitab 213 Inc., USA).

RESULTS AND DISCUSSION

Testing of functional properties of chicken skin gelatines

Tables 1 and 2 show obtained values of viscosity, clarity, water holding capacity (WHC), fat binding capacity (FBC) emulsifying capacity (EC), stability (ES), foaming capacity (FC) and stability (FS) of gelatines prepared at different extraction temperatures.

Viscosity

The relationship between viscosity and extraction temperature is not statistically significant ($p>0.05$). Viscosity moderately decline with an increasing extraction temperature as can be seen in Figure 1. It plummet from 50 °C to 60 °C. At 60 °C the values reached the minimum.

Table 1 Viscosity, clarity, water holding capacity (WHC) and fat binding capacity (FBC) of prepared chicken skin gelatines at different extraction temperatures. CSG – chicken skin gelatines.

CSG (°C)	Viscosity (mPa.s ±SD)	Clarity (% ±SD)	WHC ($mL.g^{-1}$ ±SD)	FBC ($mL.g^{-1}$ ±SD)
40	5.2 ±1.51	1.51 ±0.51	3.85 ±0.30	0.97 ±0.20
50	4.4 ±1.87	1.95 ±0.75	3.99 ±0.15	1.15 ±0.25
60	2.7 ±0.14	1.45 ±0.35	4.59 ±0.19	1.26 ±0.22
70	3.0 ±0.15	1.61 ±0.31	5.00 ±0.19	1.06 ±0.07
80	5.7 ±0.12	1.95 ±0.55	5.58 ±0.18	0.87 ±0.08
p-value	0.939	0.558	0.002	0.622

Table 2 Emulsifying capacity (EC) and stability (ES) and foaming capacity (FC) and stability (FS) of prepared chicken skin gelatines at different extraction temperatures. CSG – chicken skin gelatines.

CSG (°C)	EC (% ±SD)	ES (% ±SD)	FC (% ±SD)	FS (% ±SD)
40	50.00 ±7.86	72.50 ±3.54	48.89 ±1.92	38.89 ±9.91
50	43.27 ±1.65	87.50 ±0.85	35.56 ±3.85	33.33 ±3.25
60	37.50 ±5.89	81.67 ±2.36	17.78 ±5.09	8.89 ±6.94
70	36.84 ±0.87	85.71 ±0.91	20.00 ±5.77	4.44 ±5.09
80	35.09 ±2.48	84.52 ±1.68	61.11 ±9.62	5.56 ±9.62
p-value	0.019	0.290	0.904	0.030

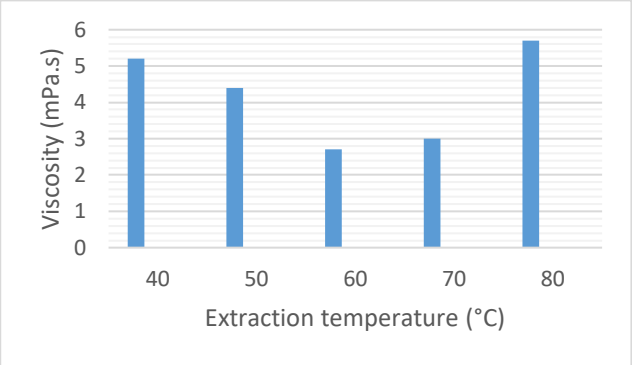

Figure 1 Viscosity of chicken skin gelatines prepared at different extraction temperatures.

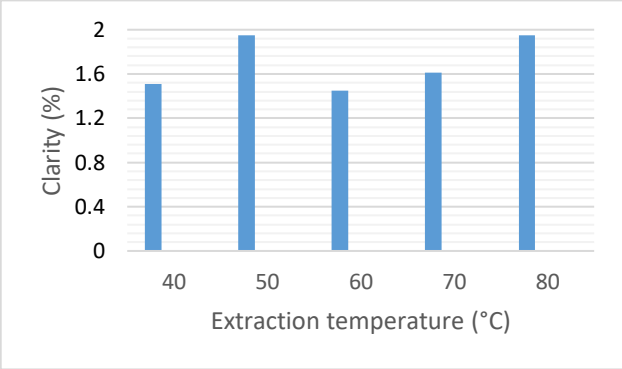

Figure 2 Clarity of chicken skin gelatines prepared at different extraction temperatures.

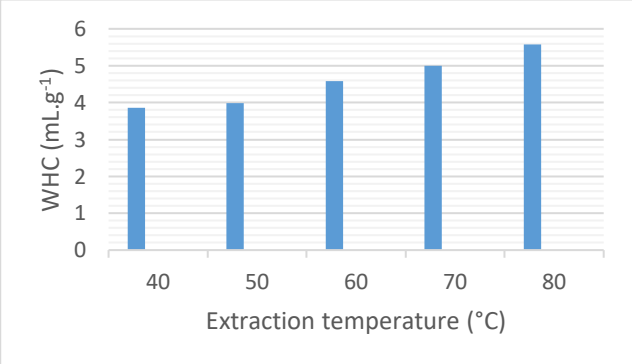

Figure 3 Water holding capacity (WHC) of chicken skin gelatines prepared at different extraction temperatures.

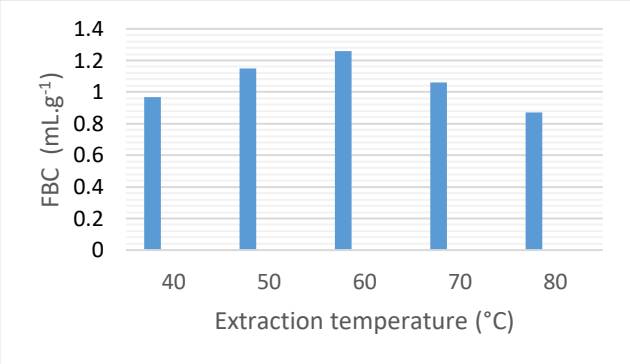

Figure 4 Fat binding capacity (FBC) of chicken skin gelatines prepared at different extraction temperatures.

An upward trend is observed between the extraction temperatures of 60 °C and 70 °C. However, at 80 °C viscosity soar slightly above the level registered at 40 °C. Viscosity of gelatine solutions is the highest at the extraction temperature of 80 °C and lowest at 60 °C. This may be explained by the fact that at the temperature of 60 °C the level of hydrolysis is the highest and collagen chains have the lowest molecular mass resulting in lower viscosity. This assumption was proved by the highest

gelatine yield recorded at this extraction temperature in the previous study **Mrázek et al. (2019 – in press)**.

Ninan, Joseph and Aliyamveettil (2014) anounced viscosity of grass carp skin gelatine of 7.07 mPa.s. **Rafieian, Keramat and Kadivar (2011)** reported viscosity of chicken gelatine from deboner of 5.85 mPa.s. **Bichukale et al. (2018)** stated that viscosity of poultry skin and bone ranged from 3.83 to 9.10 mPa.s. Therefore, viscosity of prepared chicken skin gelatines is comparable with data obtained in other studies.

Clarity

No significant influence of the extraction temperature on clarity has been observed (p >0.05). As depicted in Figure 2, clarity values is in a range from 1.5 to 1.9% which represents very low level of clarity. This may be attributed to residual impurities in gelatine. **Mad-Ali et al. (2017)** reported turbidity of gelatine solution from 1.8 to 2% depending on drying method.

Water holding capacity (WHC)

The effect of extraction temperature on WHC is statistically significant (p <0.05). WHC increases almost linearly with an increasing extraction temperature as Figure 3 depicts (R^2=97.23). WHC of gelatine has been extensively examined during the last few years. **Omar and Sarbon (2016)** studied the effect of drying method on functional properties and antioxidant activities of chicken skin gelatine hydrolysate and recorded WHC values from 8.4 mL (vacuum oven dried) to 63.7 mL.g^{-1} (freeze dried) depending on drying method and pH of gelatine. **Dhakal et al. (2018)** investigated optimal conditions of collagen extraction from chicken feet by papain hydrolysis and synthesis of chicken feet collagen based biopolymeric fibres and determined WHC of 1.9 mL.g^{-1}. **Surangna and Anal (2016)** discussed the optimization of extraction of functional protein hydrolysates from chicken egg shell membrane (ESM) by ultrasonic assisted extraction (UAE) and enzymatic hydrolysis and reported values of WHC varying from 1.9 to 2.9 mL.g^{-1} depending on the type of pre-treatment. Therefore, prepared gelatines analysed in this study are similar to these results.

Fat binding capacity

The relationship between FBC and extraction temperature is not statistically significant (p >0.05). As can be seen in Figure 4, FBC rises with an increasing extraction temperature until reaches the peak at 60 °C; then it decreases to a slightly lower value than it was observed at 40 °C. This may stem from the fact that at the extraction temperature of 60 °C the rate of hydrolysis is the highest resulting in more hydrophobic residues exposed for bonding with fat molecules. Several studies have been proceeded in order to determine FBC of gelatine. **Li, Jia and Yao (2009)** examined amino acid composition and functional properties of collagen polypeptide from Yak (Bos grunniens) bone and reported FBC of only 0.21 to 0.29 mL.g^{-1}. **Surangna and Anal (2016)** determined FBC from 2.5 to 4.4 mL.g^{-1} and **Dhakal et al. (2018)** reported FBC of 5.3 mL.g^{-1} which is in accordance with the results of this study.

Emulsifying capacity and stability (EC and ES)

The influence of extraction temperature on EC is statistically significant (p <0.05). Figure 5 shows that there is a decrease of EC between the extraction temperatures of 40 °C and 60 °C. However, EC remains nearly steady from 60 °C to 80 °C. This trend may be caused by changes in gelatine structure affected by the temperature rise.

The mean of ES were significantly higher than the mean of EC (p <0.001). ES soars between the extraction temperatures of 40 °C and 50 °C and fluctuate from 50 °C to 80 °C. The highest emulsifying capacity and stability was recorded at the extraction temperatures of 40 °C and 50 °C, respectively. Several studies have been conducted to determine emulsifying properties. **Li, Jia and Yao (2009)** studied amino acid composition and functional properties of collagen polypeptide from Yak (*Bos grunniens*) bone and stated EC of yak bone collagen of 57.3% which is slightly higher than EC of chicken skin gelatine extracted at 40 °C. **Shahidi, Xiao-Qing, Synowiecki (1995)** investigated production and characteristics of protein hydrolysates from Capelin (*Mallotus-villosus*) and reported EC of lyophilized capelin protein hydrolysates of 50.9% and ES of 92% which is comparable with the present study. **Omar and Sarbon (2016)** examined the effect of drying method on functional properties and antioxidant activities of chicken skin gelatin hydrolysate and registered EC and ES of chicken skin gelatine of approx. 56% which is very similar to the results by **Li, Jia and Yao (2009)**.

Foaming capacity and stability (FC and FS)

The relationship between FC and extraction temperature is not statistically significant (p >0.05). Figure 6 shows that between the extraction temperatures of 40 and 60 °C there is a steep decrease of FC. From 60 °C to 70 °C it remained steady followed by a dramatic soar between 70 °C and 80 °C. This thermal behaviour can be explained by the fact that the level of hydrolysis is probably the highest at the temperature of 60 °C (as it was mentioned previously); therefore, collagen molecules contain shorter chains and are unable to form a stable foam.

The mean of FS is not significantly different from the mean of FC (p =0.141); however, the effect of extraction temperature on FS is statistically significant (p <0.05). FS values were slightly lower at 50 °C compared to FC; however, decrease of FS is more obvious at 40, 60 and 70 °C in comparison with FC and the extreme difference was recorded at 80 °C. It is obvious that an increasing temperature causes a decline in FS. The most appropriate extraction temperature for the best foaming properties seems to be 40 °C due to the significantly high FC value and highest FS value. Several studies have been performed in order to investigate foaming properties.

Haddar et al. (2011) studied physicochemical and functional properties of gelatin from tuna (*Thunnus thynnus*) head bones and reported FC from 64 to 80% and FS from 41 to 60% depending on the concentration of gelatine. **Jain and Anal (2016)** investigated optimization of extraction of functional protein hydrolysates from chicken egg shell membrane (ESM) by ultrasonic assisted extraction (UAE) and enzymatic hydrolysis and reported FC of protein hydrolysate prepared from eggshell

Table 3 Comparison of viscosity, water holding capacity (WHC), fat binding capacity (FBC), clarity of chicken skin gelatine extracted at 40 °C with commercial food grade pork and beef gelatines.

	Viscosity (mPa.s ±SD)	WHC (mL.g^{-1} ±SD)	FBC (mL.g^{-1} ±SD)	Clarity (% ±SD)
CSG	5.2 ±1.51	3.85 ±0.30	0.97 ±0.20	1.51 ±0.51
PG	2.4 ±0.05	4.43 ±0.26	0.42 ±0.11	65.33 ±0.47
BG	3.5 ±0.17	6.42 ±0.26	0.71 ±0.06	86.17 ±4.31

Note: CSG – chicken skin gelatine extracted at 40 °C; PG – commercial food grade pork gelatine; BG – commercial food grade beef gelatine.

Table 4 Comparison of emulsifying capacity (EC), emulsifying stability (ES), foaming capacity (FC) and foaming stability (FS) of chicken skin gelatine extracted at 40 °C with commercial food grade pork and beef gelatines.

	EC (% ±SD)	ES (% ±SD)	FC (% ±SD)	FS (% ±SD)
CSG	50.00 ±7.86	72.50 ±3.54	48.89 ±1.92	38.89 ±9.71
PG	30.67 ±4.04	94.44 ±9.62	62.23 ±3.87	14.40 ±1.91
BG	57.67 ±4.04	88.89 ±9.91	55.10 ±1.71	13.17 ±0.23

Note: CSG – chicken skin gelatine extracted at 40 °C; PG – commercial food grade pork gelatine; BG – commercial food grade beef gelatine.

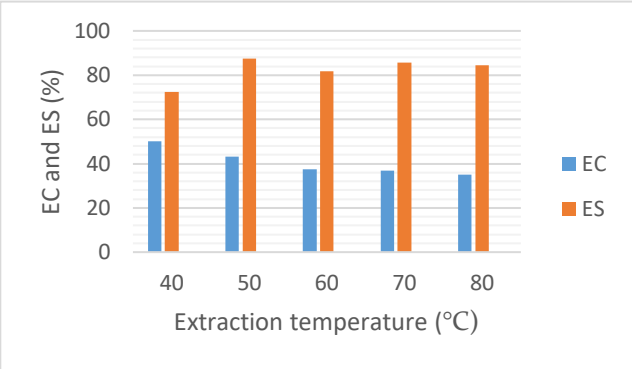

Figure 5 Emulsifying capacity (EC) and stability (ES) of chicken skin gelatines prepared at different extraction temperatures.

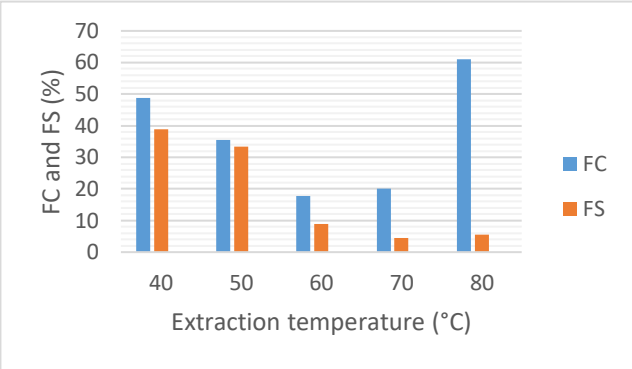

Figure 6 Foaming capacity (FC) and stability (FS) of chicken skin gelatines prepared at different extraction temperatures.

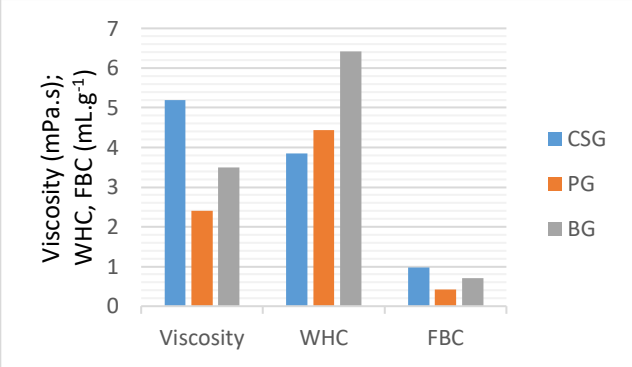

Figure 7a Comparison of viscosity, water holding capacity (WHC) and fat binding capacity (FBC) of chicken skin gelatine (CSG) prepared at 40 °C with commercial pork (PG) and beef (BG) gelatine.

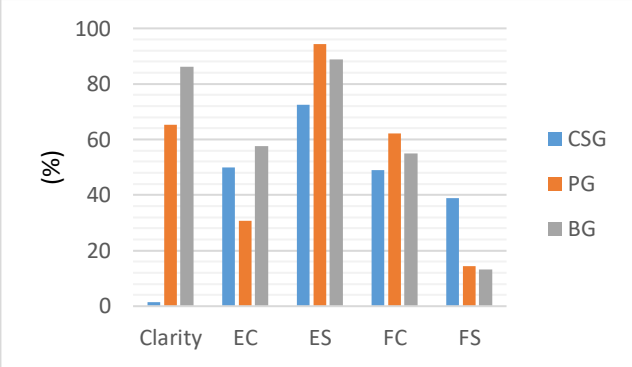

Figure 7b Comparison of clarity, emulsifying capacity (EC), emulsifying stability (ES), foaming capacity (FC) and foaming stability (FS) of chicken skin gelatine (CSG) prepared at 40 °C with commercial pork (PG) and beef (BG) gelatine.

membrane in the range from 21.7% to 28.3% and FS from 8.3 to 25% depending on the applied method of preparation. **Dhakal et al. (2018)** examined optimization of collagen extraction from chicken feet by papain hydrolysis and synthesis of chicken feet collagen based biopolymeric fibres and reported FC of 16.7% and FS of 11.7% which is in accordance with the results of this study.

Comparison of functional properties of chicken skin gelatines with commercial food grade pork and beef gelatine

The mean of viscosity of chicken skin gelatines (CSG) is significantly higher than the viscosity of pork gelatine (p <0.05), whereas it is not significantly different in comparison with viscosity of beef gelatine (p >0.05). The mean of clarity of CSG is significantly less than the viscosity of pork and beef gelatines (p <0.05). The mean of WHC of CSG is not significantly different from WHC of pork gelatine (p >0.05); however in contrast to beef gelatine it is significantly less (p <0.05). The mean of FBC of CSG is significantly greater than WHC of pork and beef gelatines (p <0.05). The mean of EC of CSG is significantly greater than EC of pork gelatine (p <0.05), while the mean of EC of CSG is significantly less than EC of beef gelatine (p <0.05). The mean of ES of CSG is significantly lower than ES of pork gelatine (p <0.05), however the mean of ES of CSG is not significantly different from ES of beef gelatine (p >0.05). The mean of FC of CSG is significantly less than FC of pork and beef gelatines (p <0.05). The mean of FS of CSG is not significantly different from FS of pork and beef gelatines (p >0.05).

Gelatine extracted at the temperature of 40 °C was chosen for the illustration of comparison with commercial food grade beef and pork gelatine because this gelatine has the highest gel strength as described in the previous study, **(Mrázek et al., 2019 – in press)**, emulsifying capacity, foaming capacity and stability, and significantly high viscosity of all prepared samples. Tables 3 and 4, Figures 7a and 7b display obtained data. Viscosity of prepared chicken skin gelatine is higher by 53% and 31% than viscosity of pork and beef gelatine, respectively. WHC of chicken skin gelatine is lower by 67% and 15% than WHC of beef and pork gelatine, respectively. FBC of prepared gelatine is higher by 57% and 27% than FBC of pork and beef gelatine, respectively. On the other hand, clarity of prepared gelatin is considerably lower than clarity of pork and beef gelatine. This may be attributed to difficulty in the cleaning process in laboratory conditions. EC of chicken skin gelatine is 15% lower than EC of beef gelatine whereas it is by 39% higher than the value of pork gelatine, which are comparative results. ES of chicken skin is 30% lower than ES of pork gelatine and 23% lower than beef gelatine. FC of prepared gelatine is lower by 27% and 13% than FC of pork and beef gelatine, respectively; while FS is higher by 63% and 66% than FS of pork and beef gelatine, respectively, which are excellent results. In addition, FS is 4.3 times lower than FC in commercial gelatines, whereas only 1.3 times lower in prepared chicken skin gelatine. This may be ascribed to the difference in intrinsic properties and composition of proteins in various gelatine sources **(Damodaran, 2005)**.

CONCLUSION

Chicken skin gelatines were prepared by extraction in distilled water at 5 different temperatures of 40, 50, 60, 70 and 80 °C at constant extraction time of 60 min. Functional properties of gelatines (viscosity, clarity, water holding capacity, fat binding capacity, emulsifying capacity/stability and foaming capacity/stability) were investigated. Results revealed that the extraction temperature has an influence on the properties of gelatine. With respect to the highest emulsifying capacity, foaming stability and high viscosity of gelatine, the extraction temperature of 40 °C appears to the most appropriate; in addition, this gelatin has the highest gel strength. The most suitable extraction temperature for the highest viscosity, water holding capacity and foaming capacity was 80 °C; however, for the highest fat binding capacity it was 60 °C and for emulsion stability 50 °C.

Functional properties of chicken skin gelatine extracted at 40 °C were compared with those of commercial food grade pork and beef gelatine. Viscosity, fat binding capacity and foaming stability of chicken skin gelatine were higher in comparison with mammalian gelatines. Water holding capacity, emulsifying stability and foaming capacity of chicken skin gelatine were lower than those of mammalian gelatines. Clarity of chicken skin gelatines were significantly lower than clarity of mammalian gelatines; this will be a subject of the following research. All tested gelatines showed comparable emulsifying capacity.

The results of experiments have proven that it is possible to prepare chicken skin gelatine with comparable functional properties to food grade beef and pork gelatine. Chicken skin gelatine has a promising potential to be an alternative to mammalian gelatines.

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THE IMPORTANCE OF MILK AND DAIRY PRODUCTS CONSUMPTION AS A PART OF RATIONAL NUTRITION

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ABSTRACT

The paper is focused on the issue of consumption of milk and dairy products as an important part of the rational nutrition of the population of the Slovak Republic. The aim of the paper is to highlight the development of consumption of drinking milk and selected dairy products, including cheese and acid-based products, in the last 20 years in the conditions of the Slovak Republic. Furthermore, the paper focuses on comparing current consumption of milk and dairy products with recommended intakes resulting from the rationalization of diet. Based on the results, it can be stated that the consumption of milk and dairy products is insufficient at the level of 70% of the recommended consumption intakes of the selected food group. In connection with this, it is important to note that the consumption of drinking milk is low. The results obtained by processing the secondary data were supplemented with the primary data obtained from the questionnaire survey. Based on the results, we have conclude that most consumers are trying to maintain a healthy lifestyle and rational diet, which is just the consumption of milk and dairy products. Consumers especially prefer drinking milk, cheese and yoghurt, whose consumption is still low, which the respondents attribute to the high prices of the monitored products as compared to their income. On the other hand, the results showed the main factors determining the consumption of milk and dairy products, among which we can include quality, composition, price, durability and nutrition data. Based on the results obtained by processing secondary data and primary research, we suggest informing and educating consumers about the positive health effects and highlighting the recommended benefits to a greater extent.

Keywords: milk; dairy product; consumption; recommended intake; rational nutrition

INTRODUCTION

The aim of rational nutrition is to ensure the necessary intake of nutrients that are relevant to the health of the human organism **(Golian et al., 2018; Holienčinová et al., 2013)**. However, at present consumers do not have enough information to make reasonable decision when buying the food. Important food, which consumption is inadequate in the conditions of the Slovak Republic and which are essential for health, includes milk and dairy products. For this reason, the aim of the paper is to highlight the basic issues and issues related to milk and dairy products, with an emphasis on the importance of consumption and its sufficiency in relation to the recommended intakes resulting from the rationalization of eating.

Milk and dairy products represent one of the most elemental foods for all age categories of the population because of its biological component, which is the basis for the promotion and maintenance of rational nutrition of the population, regarding to which it has got a significant position **(Michaelidou, 2008; Pereira, 2014)**. Milk is a multicomponent blend, with its primary components, which are proteins, fat, lactose, vitamins and minerals, involved in the formation of a complex food **(Keresteš, 2016; Čuboň, Haščík and Kačániová, 2012; Nicklas, O'Neil and Fulgoni, 2009)**.

Milk proteins present in the milk are made of casein and whey proteins, which are nutritionally and technologically essential and represent an irreplaceable amino acid intake for humans. Another milk constituent is carbohydrates that do not form a significant portion, but lactose is the primary ingredient. Lactose is a hydrocarbon that can only be found in milk and has a significant role in energy production. Milk fat, representing an easily digestible emulsion containing lecithin and a relatively low level of cholesterol, is another important ingredient in milk. According to **Beck and Coad (2017)** the main minerals are calcium, phosphorus and potassium, with iron and copper having a less pronounced presence. In the context of this, there is an important ratio of calcium and phosphorus, which should be 1:1.3, and the magnesium content for the optimal skeleton development in child and juvenile consumers. In addition to the ingredients, milk also consists vitamins that can be diversified into two categories, water-soluble vitamins and fat-soluble vitamins. In cow´s milk, there is enough of vitamin A, B1, B2, B6 and

B12, biotin and lower amount of vitamin C **(Zeleňáková and Golian, 2008)**.

On the basis of the mentioned above, we can conclude that milk is an important food because milk and dairy products take an irreplaceable place in the intake of nutrients that are essential for the growth of the human organism and can be considered as a medicine for all age categories of consumers with different levels of their health condition **(Košičiarová, Nagyová and Holienčinová, 2017; Herian, 2006)**.

Nouzovská (2007), based on studies by several experts who regard milk as an exceptional food for its ingredients, highlights its need for body building. She also points to substances that provide the human body with energy in the required amount and essential elements that body cannot produce by itself, vitamins, minerals, hormones, enzymes with a positive aspect of non-stressing the body. In the context of the individual components of milk and their beneficial effects, we can define the basic benefits of consuming dairy products for the health of consumers **(Koca et al., 2017)**.

Regular consumption of milk and dairy products has, in the first place, beneficial effects on health of bones and teeth. We state that cow´s milk is an important source of calcium and minerals **(Prentice, 2014)**. Furthermore, the milk also contains vitamins that positively influence the health of the bones. An inadequate amount of calcium and vitamin D can lead to negative health consequences, in particular can cause osteoporosis and osteopenia. For this reason, it is beneficial and necessary for people to include milk and dairy products in their daily diet. On the other hand, it is important to note that cow's milk consumption is not sufficient to prevent osteoporosis, it is important to maintain also the physical activity, strength training, absence of smoking and drinking alcohol, complex healthy eating with a focus on diet containing low sodium and a significant amount of potassium **(International Osteoporosis Foundation, 2015)**.

Consumption of milk and dairy products as part of a rational, healthy diet also has positive effects on optimal blood pressure. Cow's milk, which is an important source of potassium and contains a low sodium content, is effective in preventing and promoting the elimination of the risk of cardiovascular disease. However, milk and dairy products should be consumed according to recommended intakes, since their over-consumption may have adverse health effects for consumers. As we have already mentioned, cow's milk contains not only high amounts of potassium but also saturated fat and cholesterol, whose excessive consumption can cause an increased risk of heart disease. If the consumers have the high blood pressure and cardiovascular disease reported for the longer time, they should consider cholesterol intake, when milk and dairy products with low fat and salt content are recommended **(Ware, 2016)**.

Ware (2016) consider as a significant positive of cow's milk its content of a wide range of proteins, including all essential amino acids that have a beneficial effect on the growth and recovery of human muscle tissue. Cow's milk and high-fat milk products contain a sufficient amount of saturated fat, which can be considered as the energy source needed to increase the muscle mass in the body. Maintaining a healthy amount of muscle in the body is one of the basic prerequisites for promoting metabolism and ensuring optimal body weight **(Geng, Qi and Huang, 2018)**. It can be said that by consuming of milk, consumers can increase their muscular mass and receive the energy they need to exercise and promote a healthy lifestyle.

Several studies confirm that cow's milk has, in addition to the above-mentioned benefits, other aspects contributing to the positive health of humans. Cow's milk and dairy products due to its high content of minerals act preventively against the development of colon cancer. Experts also point to a sufficient amount of vitamin D in cow's milk, which has a beneficial effect on hormones associated with mood, appetite and sleep, which can lead to the elimination of depression of consumers. Various studies aimed at examining the positive effects of cow's milk on the human organism also emphasize its importance in brain and nerve tissue development, regulation of body temperature, bowel movement, bile acid reduction, good vision, and resistance to infections of various kinds **(Herian, 2006)**.

Analysis of individual milk components leads us to the conclusion that cow´s milk and dairy products have demonstrated beneficial effects on human health, therefore are set the recommended intake of consumption.

Table 1 Composition of cow´s milk %.

Ingredient	%
Water	87.0
Dry matter	13.0
Protein	3.2
Lactose (carbohydrate)	4.8
Fat	4.2
Minerals	0.8

Note: own processing based on **Obermaier and Čejna, 2013**.

Table 2 Recommended intake for milk and dairy products consumption.

Milk and dairy product	Recommended annual intake in kilograms per person	Recommended daily intake in grams per person
Milk and dairy products	220.0	602.7
of which: Cheese and curd	10.1	27.7
Drinking milk	91.0	249.3
Sour-milk products	14.0	38.3

Note: own processing based on **Kajaba et al., 2012**.

Recommended intake of milk and dairy products are formulated with regard to recommended dietary intake to give the consumer daily basic nutrients, minerals and vitamins. In the context of the above, recommended intake of milk and its consumption are set at 220 kg per capita per year, with an intake for adequate consumption between 206 kg and 240 kg. Receipt of the analysed commodity is necessary due to its positive effects on consumer health. The following table shows the recommended annual and daily consumption of individual dairy products.

Milk and dairy products can be generally considered as recommended food for all age categories of consumers that are very difficult to substitute, and with regard to children's consumers, their intake is irreplaceable **(De Pelsmaeker, Schouteten and Gellynck, 2013)**. For this reason, it is important to draw attention to children and the need to include milk and dairy products into their daily meals. Recognizing the need for milk by children of low age can lead to a positive relationship and a regular consumption of dairy foods even in adulthood.

As already mentioned, consumption of milk and dairy products is beneficial for consumers and can be considered as a form of prevention against various diseases. **Lecerf (2013)** considers milk as a food from which the child obtains 51% of calcium, 31% of phosphorus, 27% of zinc, 41% of iodine, 15% of selenium, 39% of vitamin B2, 42% of retinol, 20% of vitamin B12, 24% of protein and 18% of magnesium from the recommended daily dietary intake. In the case of adults, the proportion of milk and dairy products is different from the recommended daily dietary intake. Adult consumers obtain, by consuming the recommended daily dietary intake of milk and dairy products, 45% of calcium, 24% of phosphorus, 20% of zinc, 32% of iodine, 11% of selenium, 29% of vitamin B2, 30% of retinol, 20% of vitamin B12, 18% of protein and 11% of magnesium.

Scientific hypothesis

The aim of the paper is to point out the consumption of milk and dairy products in the conditions of the Slovak Republic, with an emphasis on the development of consumption of individual types of dairy products compared to the recommended intake and the identification of factors affecting the level of consumption.

For a deeper analysis of the research objectives, the following hypotheses were formulated:

Hypothesis 1: We assume that there is a dependence between respecting health principles in rational nutrition and selected demographic characteristics of respondents (age, gender, education).

Hypothesis 2: We assume that there is a difference in the evaluation of higher prices as a main reason of low consumption of milk and dairy products between respondents from households with different monthly incomes.

Hypothesis 3: We assume that consumers assess the importance of the individual criteria for choosing milk and dairy products differently.

MATERIAL AND METHODOLOGY

Regarding the development of the consumption of milk and dairy products in comparison with the recommended intake, the secondary data had been obtained from the Statistical Office of the Slovak Republic and the Research Institute of Agriculture and Food Industry.

The primary data was obtained from questionnaire survey, implemented in an electronic version and conducted on a sample of 516 respondents from April to December 2018 in the Slovak Republic.

Respondents participating in the survey were diversified into 9 categories by gender (women 64.1%, males 35.9%), age (up to 25 years 43.0%, 26 – 35 years 23.3%, 36 – 50 years 19.2%, 51 – 60 years 8.5%, more than 61 years 6.0%), education (basic 1.7%, secondary school 45.9%, university 52.3%), permanent residence (countryside 48.6%, city 51.4%), economic status (student 36.2%, employed 48.3%, the self-employed person 4.7%, unemployed 1.2%, maternity leave 2.9%, retired 6.8%), monthly household income (less than 1,000 Euro 19.8%, 1,001 – 2,000 Euro 54.7%, 2,001 – 3500 Euro 23.1%, 3,501 – 4,500 Euro 2.1%, more than 4,501 Euro 0.4%) and by the number of members of the household (1 member 3.7%; 2 members 18.8%; 3 members 22.9%; 4 members 37.6%; 5 members 12.0%; more than 5 members 5.0%).

Statistic analysis

Collected data was processed by using Microsoft Excel and then evaluated in the statistical program XL Stat. The formulated hypotheses were tested by applying the following statistical methods:

- Contingency table chi-square test,
- Fisher's exact test,
- Cramer's coefficient,
- Mann-Whitney test,
- Friedman test,
- Nemeny's method.

In hypothesis testing, if the *p*-value is lower than significant level, in case of XL Stat software, it is 0.05, the null hypothesis is rejected, and the alternative hypothesis is confirmed **(Witek, 2016)**.

RESULTS AND DISCUSSION

In the Slovak Republic, the development of consumption of milk and dairy products per capita was accompanied by a fluctuating trend in the period 1997 – 2017 (Figure 1) **(Statistical Office of the Slovak Republic (SO SR), 2018)**. In 2003, the consumption of milk and dairy products began to decline. In 2010 there was an increase in consumption of the monitored food, but the average consumption did not reach the recommended intake level resulting from rationalization of meals. This trend persisted until 2017, so it is necessary to point out the possible reasons for the relatively low consumption of milk and milk products that could be caused by the living conditions of the population of the Slovak Republic, by unconfirmed information about the negative effects of milk consumption on human health or by the diagnosis of lactose intolerance **(Zingone et al., 2017)**. At present, the consumption of milk and dairy products is 176.1 kg per capita, which represents lower consumption by 20.0% compared to the recommended intake. The consumption of milk and dairy products was mainly due to drinking milk, cheese and sour-milk products.

Development of consumption of drinking milk is not proportional to the total consumption of milk and dairy products in the analysed period 1997 – 2017 (Figure 2). We predict this on the basis of the downward trend in milk consumption in the long run. In the first reference year, consumption was 75.8 kg per capita and in 2017 only 46.3 kg. Of course, the fluctuations in milk intake by Slovak consumers were also recorded. The largest increase was registered in 2010, which was determined by the state's support activities for milk consumption. However, in the context of the above, it is also important to note the elimination of consumption from 2011 to the present. Consumption of drinking milk does not cover the recommended intake and falls by almost 50%, which we consider to be a very significant lack of rational nutrition. In the next two years, we expect an even lower consumption of drinking milk based on the determinant coefficient (R^2), which should reach an annual level of about 40 kg per capita in the Slovak Republic. Low milk consumption contributes to increasing milk prices and to constantly expanding supply of other dairy products such as cheese, yoghurt, or other sour-milk products **(Kubicová, 2008; Kubicová and Habánová, 2012)**.

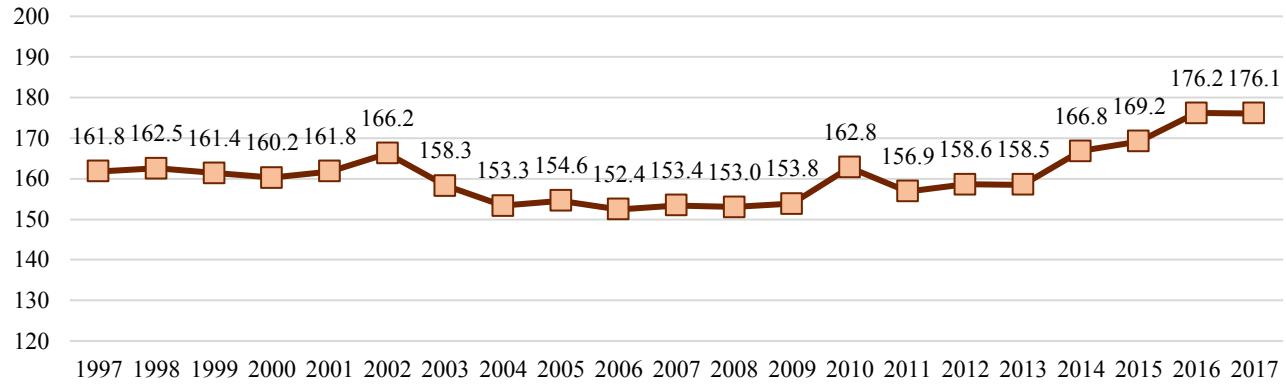

Figure 1 Average consumption of drinking milk and dairy products in kilograms per inhabitant of the Slovak Republic. Note: own processing according to SO SR, 2018.

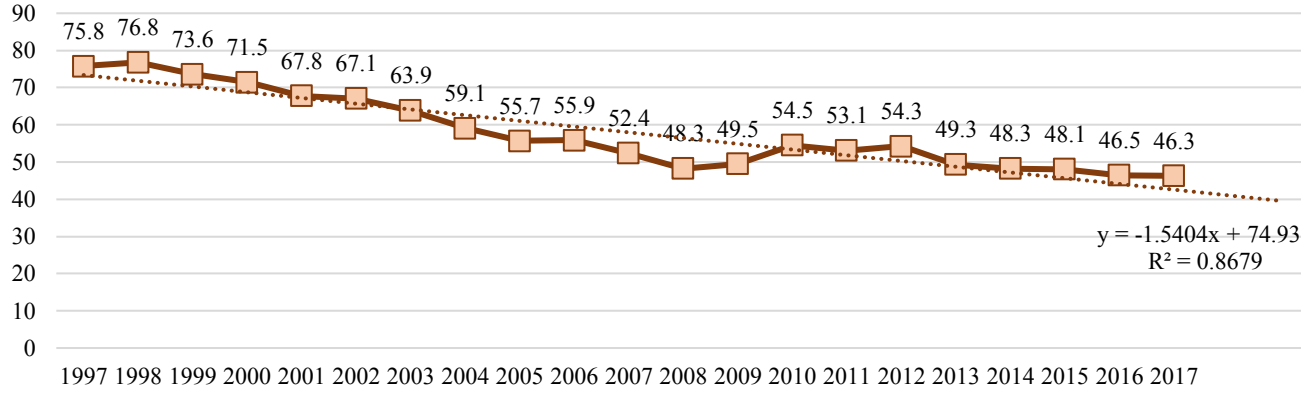

Figure 2 Average consumption of drinking milk in kilograms per inhabitant of the Slovak Republic. Note: own processing according to SO SR, 2018.

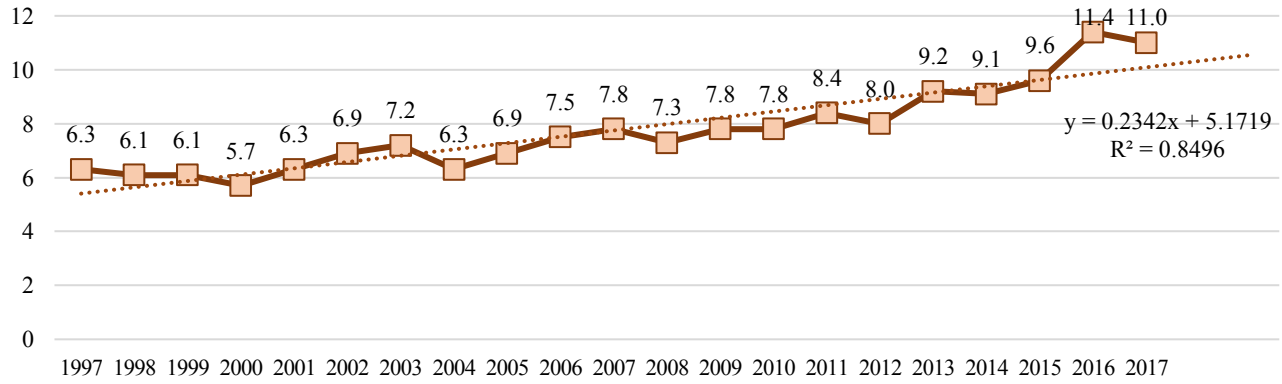

Figure 3 Average consumption of cheese in kilograms per inhabitant of the Slovak Republic. Note: own processing according to SO SR, 2018.

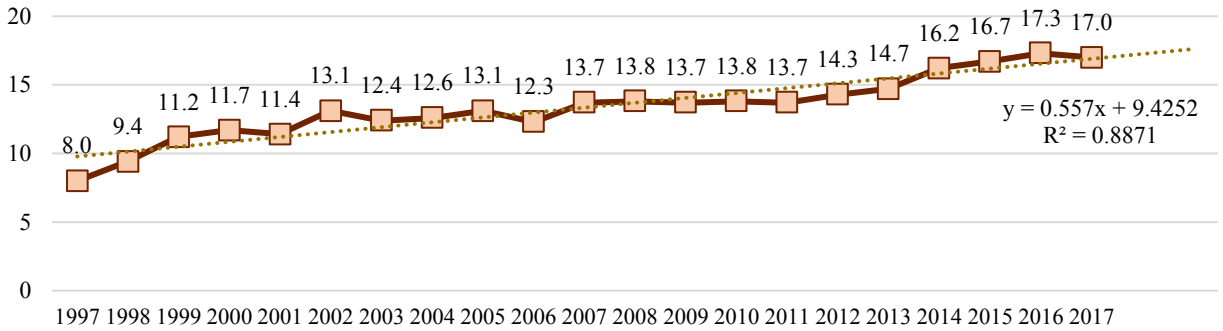

Figure 4 Average Consumption of sour-milk products in kilograms per inhabitant of the Slovak Republic. Note: own processing according to SO SR, 2018.

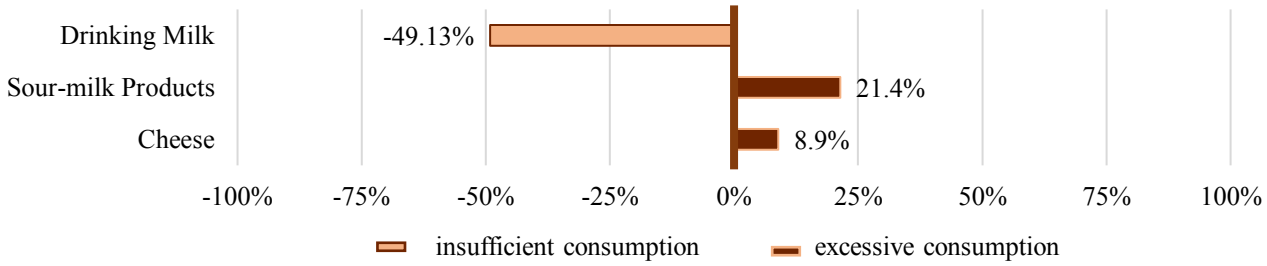

Figure 5 Comparison of recommended and real consumption of milk and dairy products in %. Note: own processing according to SO SR, 2018.

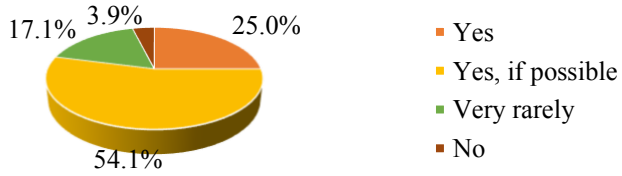

Figure 6 Respecting health principles in rational nutrition. Note: questionnaire survey, 2018.

Table 3 Influence of selected demographic factors on respecting health principles in rational nutrition.

Factors	*p*-value	correlation	Cramer's V-coefficient
Respecting Health Principles and Gender	0.008	yes	0.151
Respecting Health Principles and Age	0.024	yes	0.123
Respecting Health Principles and Education	0.001	yes	0.152

Note: questionnaire survey, 2018.

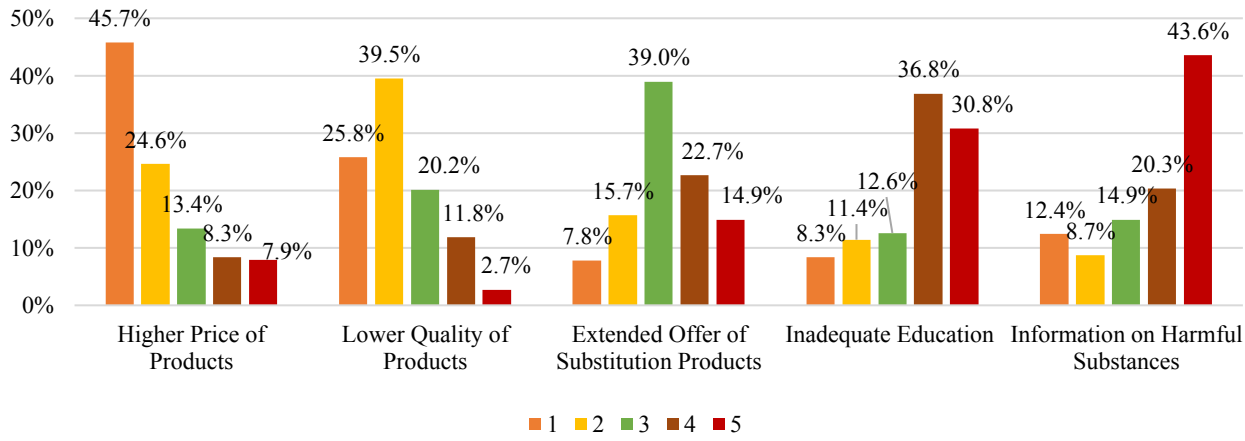

Figure 7 Reasons for low consumption of milk and dairy products compared to recommended intakes. Note: questionnaire survey, 2018.

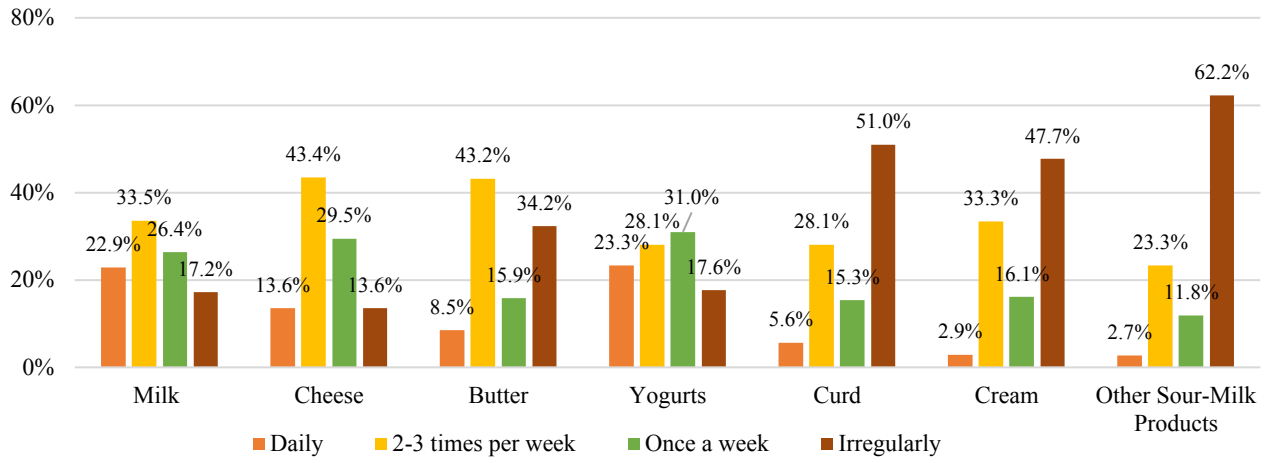

Figure 8 Preference of selected types of dairy products and milk according to regularity of their consumption. Note: questionnaire survey, 2018.

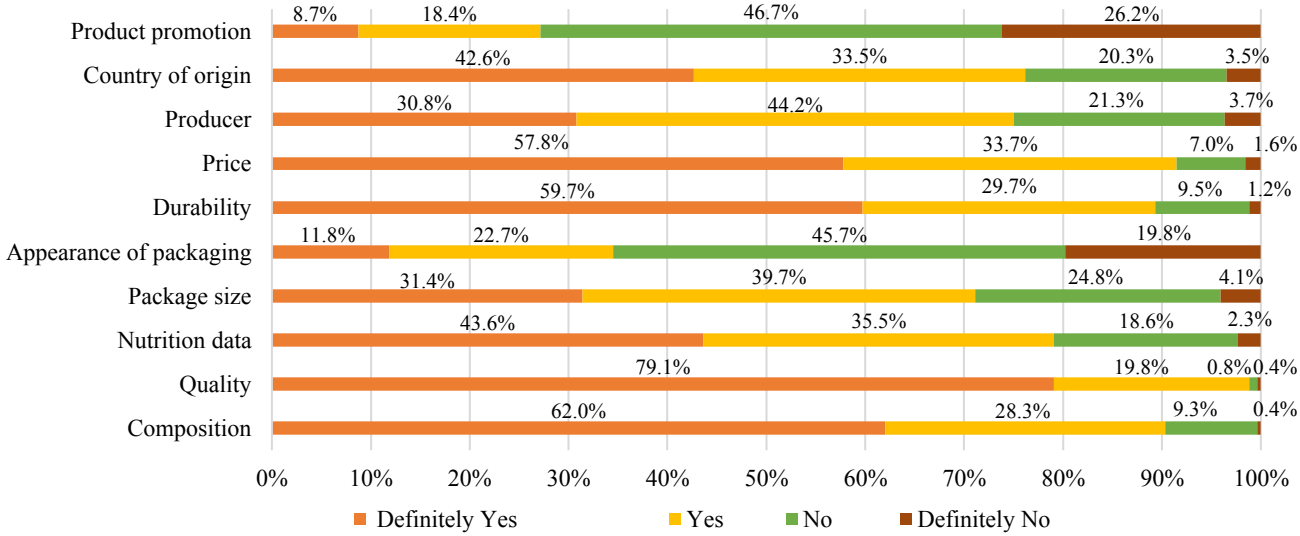

Figure 9 Factors determining the consumption of milk and dairy products. Note: questionnaire survey, 2018.

Table 4 Differences in factor evaluation when choosing milk and dairy products by applying the Friedman Test and Nemeny's Method

Sample	Frequency	Sum of ranks	Mean of ranks	Groups				
Quality	516	1,722.500	3.338	A				
Composition	516	2,162.500	4.191		B			
Durability	516	2,262.000	4.384		B			
Price	516	2,265.000	4.390		B			
Nutrition data	516	2,711.000	5.254			C		
Country of origin	516	2,785.000	5.397			C	D	
Producer	516	3,025.000	5.862				D	
Package size	516	3,077.500	5.964				D	
Appearance of packaging	516	4,078.000	7.903					E
Product promotion	516	4,291.500	8.317					E

Note: questionnaire survey, 2018.

The consumption of cheese is directly proportional to the total milk and dairy consumption. In the monitored period, a gradual increase in the consumption of cheese was recorded (Figure 3). In 1997, consumption was 6.3 kg and in the last analysed year up to 11 kg, which represented an increase in consumption by 74.6%. On the basis of the determinant (R^2), a slight decrease in cheese consumption is estimated to approximately 10.5 kg per person in the following two years, but with a view to rationalizing the diet

and the recommended intake, cheese consumption will still be at a sufficient level.

Consumption of sour-milk products is similar to consumption of cheese. In the analysed period 1997 – 2017 there was a gradual increase in the intake of sour-milk products by Slovak consumers (Figure 4). In connection with mentioned, it is important to highlight the favourable consumption growth of 212.5% from 8.0 kg in 1997 to 17 kg in the last reference year.

By 2011, the consumption of dairy food was below the recommended dose of 14 kg, but it is slightly higher than in 2012 and currently stands at around 21.4% above the rational diet recommendations.

In view of the above, the consumption of sour-milk products is expected to increase and in 2019 it should reach an annual level of approximately 17.5 kg per inhabitant of the Slovak Republic. **Kubicová and Dobák (2012)** emphasize that the increase in consumption of sour-milk products was mainly determined by the increased and varied range of products of domestic and foreign production associated with marketing communication and a wider range of price levels.

The question of the adequacy of current consumption of milk and dairy products can be judged by the level of recommended intake. Excessive consumption, i.e. consumption higher than the recommended intake, is recorded in the case of cheeses and sour-milk products. The consumption of sour-milk products is 21.4% higher compared to the recommended intake and the consumption of cheeses is 8.9% higher compared to the recommended intake. On the other hand, inadequate consumption, i.e. consumption lower than the recommended intake, is recorded in a case of drinking milk, which is 49.13% lower compared to the recommended intake (Figure 5).

Consumption of milk and dairy products is influenced also by the level of retail prices in relation to the average income of the population **(Bousbia et al., 2017)**, the gross domestic product and its distribution among the population, the standard of living, structure of the market, intensity of international trade or the individual consumer behaviour **(Matošková and Gálik, 2016; Skořepa, 2009)**. Consumer behaviour can be considered as one of the main factors determining the consumption of milk and dairy products. We identified these on the basis of a questionnaire survey focused on consumers of milk and dairy products, aimed at identifying consumers' attitudes towards rational eating, the positive health effects of milk consumption from the point of view of consumers, preferences in milk and dairy products and factors determining consumer behaviour when choosing milk and dairy products. In relation to health and a healthy lifestyle resulting from rational diets, it was necessary to point to the percentage of consumers adhering to this trend. Based on the research results (Figure 6), it can be stated that 25% of respondents adhere to regular health principles in rational nutrition and 54.1% of consumers follow rational diets on an irregular basis. As a result, almost 80% of respondents are nutritionally focused on the health aspect and try to follow recommended intakes.

Regarding to this question several dependences between the selected socio-demographic variables of respondents and respecting the health principles in rational nutrition (Hypothesis 1). Applying the statistical tests Contingency table chi-square test and Fisher's exact test, statistically significant dependency has been proved between respecting health principles in rational nutrition and gender (p-value = 0.008), age (p-value = 0.024) and education (p-value = 0.001). From the aspect of dependence tightness, there is a slight dependence demonstrated by the calculation of the Cramer coefficient (Table 3).

Recommended intake of milk consumption is also result from its positive effects on consumer health. The positive health effects of drinking milk and dairy products are recognized by 88.4% of respondents involved in consumer survey. Respondents have highlighted milk as a very good food, containing calcium, protein, iron and zinc, which greatly affects bones, teeth, nails or skin. In addition, the polls evaluated milk and dairy products as an excellent source of vitamins and minerals. The positive effect of milk consumption is also perceived by respondents who consume milk, especially because of the proven effects related to the growth of the organism, the support of the immune system. Other beneficial effects assessed by consumers include support for better digestion, energy intake, preventive effects against osteoporosis, memory support and brain function, prevention of high blood pressure, elimination of sleep problems as well as ensuring proper functioning of the thyroid gland. Several studies show that the consumers were made aware of the good health benefits and reducing risk for exposing to diseases **(Peng, West and Wang, 2006; Alwis, Edirisinghe and Athauda, 2011)**. On the other hand, **Krešić et al. (2010)** emphasize, that consumers do not have adequate knowledge about the health benefits of milk and dairy products consumption.

As most consumers involved in the questionnaire survey are aware of the positive effects of drinking milk and dairy products and are trying to keep their consumption in line with the recommended intakes, we were interested in reason of the lower consumption in the Slovak Republic in comparison with the requirements for rationalization of meals. Consumers should rank 5 reasons of low consumption, 1 being the most important reason and 5 the least important reason and the reasons were ranked according to the average order. On the basis of the results of the survey (Figure 7), consumers consider the higher price of the monitored products as the main reason for lower consumption of milk and milk products compared to the past (1.62) followed by a lower quality (2.00), an extended offer substitution product (3.13), inadequate education (3.62), and unconfirmed information on harmful substances in milk (3.62). Regarding to this question the difference in the evaluation of higher prices as a main reason of low consumption between respondents from households with different monthly incomes was examined (Hypothesis no. 2). Applying the statistical test of Mann-Whitney U test it could be concluded that the calculated p-value (0.901) is more than significance level $\alpha = 0.05$, which means, that the null hypothesis was accepted and there is not statistically significant difference in evaluation of higher prices as a main reason of low consumption between respondents from households with the monthly income less than 2,000 euro and respondents from households with the monthly income more than 2,000 euro In connection with the questionnaire survey, up to 23.4% of respondents are aware of insufficient consumption of the selected food group.

The questionnaire survey shows that all respondents consume milk and dairy products at least in a minimum amount, mainly because of rational diet (20.3%) and nutritional content (14.0%).

In the context of the above, the objective of the questionnaire survey was to identify which dairy products are most preferred among consumers. The results of the consumer survey (Figure 8) proved that the respondents engaged in the questionnaire survey purchased all kinds of dairy products, with milk and cheese as the most preferred ones. This is confirmed by the fact that milk (56.4%), cheese (57.0%), butter (51.7%) and yogurt (51.4%) are most commonly bought and consumed by consumers in regular intervals. **De Graaf et al. (2016)** found out that Most participants consumed milk and dairy products at least once per day (54.1%), or multiple times per week (27.3%). **Kapsdorferová and Nagyová (2005)** and **Pinto et al. (2016)** identified milk, yoghurt and cheese as the most commonly preferred dairy food among consumers. On the other hand, other sour-milk products are least preferred, which was confirmed by their purchase by 70% of respondents in irregular intervals, followed by curd (66.3%) and cream (63.8%).

Based on the results (Figure 9) can be concluded that quality (98.8%), price (91.4%), composition (90.3%), durability (89.3%) and nutrition data (79.1%) are the most important factors for consumers when choosing the specific dairy products. **Kumar and Babu (2014)**, **Paraffin, Zindove and Chimonyo (2018)** and **Kurajdová et al. (2015)** confirm our results and identify quality, nutritional value and price as the main factors determining the purchase of milk and dairy products. **Zajác et al. (2012)** highlights the quality and safety of raw cow´s milk as a factor that consumers of dairy products increasingly focus on. **Nagyová et al. (2019)**, concluded within their research that consumers consider as healthy food products those with a positive effect on human organism and food, which is subject to rigorous control requirements for quality of food products. On the other hand, the promotion of the product (72.9%), the appearance of the packaging of the product (65.5%) and the size of the packaging (28.9%), consumers do not consider as important criteria when deciding to buy a given product. **Bytyqi et al. (2008)** concludes that consumer behaviour in the purchase of milk and dairy products is not affected by factors connected with the packaging of milk and dairy products. The **GfK survey (2017)** has shown that for the Slovak consumers is still a very important price when deciding about purchasing chosen food, including milk and dairy products, but the emphasis on quality is clearly rising. 59% of consumers say they closely monitor the prices of food in different stores and shop where the best deal is offered. As a result, consumers are focusing on quality, but at the same time they are looking for quality for the best price.

In relation to the assessment of the various factors influencing the choice of milk and dairy products by consumers, we found differences in the assessment of these criteria among the respondents (Hypothesis No. 3). Based on the Friedman test, it is possible to identify differences in factor evaluation confirmed by the statistical calculation of the p-value (<0.0001), which is lower than the alpha significance level (0.05). By using Nemeny's method and based on the data in the following Table 4, we conclude that quality is the most important criterion when choosing milk and dairy products (Group A), another group of significant factors is created by price, durability and composition (Group B), followed by a set of criteria created by the nutrition data and country of origin (Group C), the other group of factors consisted from the country of origin, the manufacturer and the package size (group D), and the last group of factors is the appearance of packaging and the promotion of the product (Group E). By dividing the factors determining consumer behaviour when choosing milk and dairy products into these groups, it is possible to point to the differences in the assessment of individual criteria (groups) by consumers. The country of origin criterion is placed in two groups (Group C and Group D), which can be explained by the fact that there is no statistically significant difference in their ratings among Group C and Group D factors. However, between groups C and D there is a difference in the assessment of factors by consumers.

CONCLUSION

Paper focuses on the consumption of milk and dairy products and its importance within the rational nutrition of the population of the Slovak Republic. Based on the results, it can be stated that at present the consumption of milk and dairy products is at the level of 70% of the recommended consumption of the given food group. Consumption of cow´s milk is alarmingly low, covering only about 50% of the recommended milk consumption, while consumption of cheese, curd and sour-milk products has risen in recent years and slightly exceeds the recommended consumption intakes. Other dairy products, cream, powdered milk and condensed milk or butter do not represent a significant share of total consumption. Regarding previous, a consumer survey was conducted, in which we identified that 80% of consumers are aware of a healthy lifestyle that also includes consumption of milk and dairy products in view of their proven positive effects. In the context of this question, there the formulated hypothesis was proved, and it means that there exists the dependence between the respecting of the health principles in rational nutrition and selected demographic characteristics of respondents (gender, age and education). Consumers attribute importance to milk consumption, especially due to calcium, protein, iron and zinc content, which greatly affect bone, teeth, nails or skin, and the overall positive effect on the human body. However, consumers are aware of the inadequacy of consumption of the monitored food, which is justified by the high prices of milk and dairy products. In the context of this question there was formulated hypothesis, which was rejected and there is no statistically significant difference in the evaluation of higher prices as a main reason of low consumption of milk and dairy products between respondents from households with monthly incomes of less than 2,000 euro and respondents from households with monthly incomes of more than 2,000 euro. Despite above mentioned, consumers consume the milk and dairy products at regular intervals, with the most preferred milk, cheese and yogurt. The choice of specific dairy products is determined by different criteria, which respondents evaluate differently, and it means the proved hypothesis related to consumer assessment of the importance of individual criteria for choosing milk and dairy product. The most important criteria for milk and dairy products consumption are quality, composition, price,

durability and nutrition data. Nutrition information is becoming more and more noticeable to consumers when choosing dairy products, which we consider to be an important aspect in the direction of rational nutrition. Due to the obtained results, we note that it is essential to address consumers in particular with regard to information and education in the selection of cow´s milk and dairy products. Consumers need to be permanently aware of recommended doses of consumption of a given commodity in terms of its beneficial effects due to the strengthening of bones, teeth, muscle mass, prevention of cardiovascular diseases, colon cancer and other positive effects.

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ASSESSMENT OF LIPID PEROXIDATION IN DAIRY COWS WITH SUBCLINICAL AND CLINICAL MASTITIS

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ABSTRACT

Mastitis is still one of the major causes of economic losses in dairy sector. The routine application of bacteriologic examination of milk samples is often insufficient and for this reason, alternative parameters are used to identify trends in the development of the udder health. Therefore, the objectives of this study were to determine the relationship of oxidative product levels, using malondialdehyde (MDA) as a marker on occurrence of mastitis and its causing pathogens. Dairy herd of 223 Slovak spotted cattle were tested for etiology and occurrence of mastitis based on assessment of clinical signs, abnormal udder secretions, Californian Mastitis Test (CMT) with subsequent collecting of milk samples for bacteriological examination. From 892 quarter milk samples were selected for MDA detection 51 subclinical (SM) and 26 clinical mastitis (CM) quarters with positive CMT score and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp. as well 40 healthy quarters. Results showed that among the current pathogens of the mammary gland belong CNS, *S. aureus, S. sanguinis, S. uberis* and *E. coli,* which were the most frequently isolated from SM and CM. The highest MDA level was observed from clinical cases of mastitis however, increased MDA levels were detectable from subclinical cases. Bacterial isolates from subclinical quarter milk samples are different levels of MDA. In this study, we found that quarter milk samples infected with *S. uberis* were higher compared to other pathogens. In conclusion, differences in both severity of mastitis and mastitic pathogens were associated with differences of oxidative products in infected udders.

Keywords: cows; lactation; mastitis; lipid peroxidation; *S. uberis*; coagulase negative staphylococci

INTRODUCTION

The quality and quantity of milk obtained from the cows is important for the dairy sector. Infection of mammary gland - mastitis is one of the biggest problems of dairy producers causes great losses every year in the livestock economy. The disease is usually local but may become systemic, although rarely, in immunocompromised animals **(Tančin et al., 2006; Shari, Umer and Muhammad, 2009; Andrei et al., 2010; Vršková et al., 2015)**.

Mastitis is characterized by several physical and chemical alterations of the milk and corresponding pathological changes in the mammary tissue depending on the type of the disease. Intramammary infection (IMI) is caused by interaction of various factors associated with the animal, pathogens and the environment, so nature and duration of the disease varies accordingly **(Taponen et al., 2006; Suriyasathaporn et al., 2006; Zajác et al., 2012)**.

More than 140 different microorganisms are recognized to cause mastitis. Infectious agents like bacteria, viruses, fungi and algae are mostly the primary causes of the disease.

Authors **Vasil' et al. (2009)** and **Tenhagen et al. (2006)** indicate that up to 95% IMI is caused by bacterial pathogens. At routine bacteriological examination of milk from suspect cows, or secretion of the cow udder with clinical mastitis we can detect *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, CNS, *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., *Pseudomonas* spp. and others.

During a bacterial infection the number of somatic cells in milk increases, especially that of polymorphonuclear cells (PMN). The antibacterial activity of PMN cells is partially mediated through reactive oxygen species (ROS); an excess of these species correlating with the absence of optimal amounts of antioxidants can lead to oxidative stress. It was shown that the occurrence of oxidative stress in cattle may contribute to some periparturient disorders (retained fetal membranes, udder edema, mastitis) or metabolic diseases **(Castillo et al., 2006; Sharma et al., 2011)**.

Oxidative stress in veterinary medicine and particularly in ruminant health is a relatively young field of research. Early identification of udder health problems is essential for dairy farmers and veterinarians to ensure not only the animal well-being but also the milk quality and dairying productivity. Economic aspects interfere with the routine application of bacteriologic examination of quarter milk samples. For this reason, alternative indicators of oxidative stress are used to identify trends in the development of the udder health in a dairy herd. Although these parameters belong detection of lipid peroxidation reactive aldehydes such as malondialdehyde (MDA) **(Suriyasathaporn et al., 2012; Sharma et al., 2016)**.

MDA is one of the several low molecular weight end products formed during the radical induced decomposition of polyunsaturated fatty acid. MDA modifies the physical structures of cell membranes and is indirectly involved in the synthesis of protein, DNA, and RNA. In addition, it has mutagenic and carcinogenic properties **(Turk et al., 2017; Kapusta et al., 2018)**.

Scientific hypothesis

Lipid peroxidation reactive aldehydes have been identified as a key factor in numerous pathologies, including udder inflammation. Detection of MDA may be helpful in the health management of cows. Therefore, the goal of this study was to evaluate the etiology of mastitis and to assess of lipid peroxidation by measuring milk malondialdehyde level in raw cows' milk.

MATERIAL AND METHODOLOGY

Animals and milking

The experiment was carried out in dairy herd of 270 Slovak Pied cattle in north of the Slovakia. Dairy cows from monitored herd were kept in a free housing system with a separate calving barn and equipped with individual boxes with bedding and were allowed *ad libitum* access to water. Their diet was formulated according to international standards **(NRC, 2001)** to meet the nutritional requirements of a 600 kg cow, yielding 15 - 25 kg of milk per day. The cows were milked twice a day at 4:30 a.m. and 4:30 p.m. in the fishing-milking parlour (FarmTec) 2x10. Milking and pulsation vacuum were set at 42 kPa. Pulsation ratio was 60:40 at a rate of 52 c.min^{-1}.

Examination of health status

The examination of health status included clinical examination of the mammary gland, examination fore-strip of milk, with CMT reaction, subsequent collecting of milk samples for bacteriological examination, subsequent cultivation and identification of pathogenic bacteria. Clinical examination consisted of heart rate, body temperature, respiratory rate, udder health including the mammary gland palpation, evaluation of macroscopic changes in milk, and evaluation of somatic cell count in milk using the CMT (Indirect diagnostic test, Krause, Denmark). CMT was performed from the 223 milked cows on 892 quarter milk samples and the score was evaluated (Table 1) according to **Jackson and Cockcroft (2002)**. Following washing and drying the mammary teats, 70% ethanol was sprayed, and a few streams of milk were discarded. Afterwards, quarter milk samples of the secretion (10 mL) were then collected with aseptic techniques in accordance with **National Mastitis Council guidelines (2001)**. The samples were cooled and immediately transported to the laboratory.

Laboratory analyses

Bacteriological examinations were performed according to commonly accepted rules **Malinowski et al. (2006)**. Milk samples (10 μL) were cultured at the respective veterinary practice according to their routine procedures, usually employing Columbia Blood Agar Base with 5% of defibrinated blood, Staphylococcal medium N° 110, Baird-Parker agar, Edwards Medium, Mac Conkey Agar (Oxoid, (OXOID Ltd., Basingstoke, Hants, UK) incubated at 37 °C for 24 h (Figure 1).

Figure 1 Bacteriology analysis cultured on selectives medium. Note: *S. aureus* (1A - 1B), *S. warneri* (2A - 2B), *S. uberis* (3A - 3B), *E. coli* (4), *A. viridans* (5).

Beside evaluation of bacterial growth characteristics another assays were used to bacterial species determination: pigment and coagulase production, catalase activity, haemolysis, Gram staining and other virulence factors. Bacteria *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriaceae* spp. were isolated on blood agar and cultivated at 37 °C for 24 h and detailed identified biochemically using the Staphy test, Strepto test, resp. Entero test and identification by software TNW Pro 7.0 (Erba-Lachema, CZ) according to the manufacturer's instructions. Identification of typical *Trueperella pyogenes* colonies derived from pure or mixed culture were distinguished by colony morphology, post incubation haemolysis, Gram staining, catalase test. Specific colonies were plated on defibrinated sheep blood agar (5%) and incubated aerobically at 5% CO_2 atmosphere at 37 °C for 72 hours. Colonies morphologically compatible with *T. pyogenes* were subjected to a conventional phenotypic assay API Coryne strips (BioMe'rieux, France).

MDA determination from quarter milk samples

For milk MDA detection were selected three groups from all monitored cows. The first group of 10 healthy cows (40 quarters milk samples) without clinical signs, negative score of CMT and negative bacteriological examination. From the 29 cows (51 quarters milk samples) on the basis of positive CMT score, without clinical signs and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp. were selected second group with subclinical mastitis.

The third group of 11 cows (26 quarters milk samples) was selected on the basis of clinical signs, positive CMT score and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp.

Milk MDA level from selected milk samples was measured by the photometric method based on a reaction with thiobarbituric acid (TBA) described by **Andrei et al. (2010)**.

Briefly, one hundred microliters of milk sample were properly mixed with 1 mL of trichloroacetic acid with a vortex mixer. Afterwards 400 mL of TBA was added. The mixture was boiled for 30 min and subsequently cooled down by tap water. The solution was duplicate analysed by UV spectrophotometry at 532 nm against its blank reaction mixture. The results were expressed in $nmol.mL^{-1}$ milk.

Statistical analysis

The data of milk MDA level from selected groups of cows and selected mastitis pathogens are presented as the mean (M) ±standard error of the mean (SEM). Difference between groups and pathogens causing subclinical mastitis were analysed by using analysis of variance (ANOVA) followed by Tukey comparison test and minimum criteria for statistical significance was set at $p \leq 0.05$ for all. Approximate probabilities were evaluated with Post Hoc Test for the statistical analysis of MDA level between selected pathogens.

RESULTS AND DISCUSSION

Table 1 shows the health status udder of 223 lactating dairy cows. From (892 quarter milk samples were recorded in 161 positive samples (18.1%) with CMT score trace or 1 - 4. Negative CMT score were recorded in 731 quarter milk samples (81.1%). A total of 127 infected quarter milk samples from 67 mastitis cows were the most commonly diagnosed form of SM 8.7% from all investigated quarters. At quarter levels, 16 (1.8%), 34 (3.8%) quarters were classified as latent and clinical mastitis, respectively. Numbers and percentages of isolates separated for their severity of mastitis are shown in Table 2. CNS gave the highest percentages (37%) representation on the etiology of mastitis in the monitored herd. SM were represented by up to 61.4% from all mastitis samples. The most common pathogens in CM were *S. aureus*, *S. uberis* and *S. sanguinis*. Other bacteria and *S. intermedius* had very few data and were excluded from analyses.

Table 1 Evaluation of CMT in monitored herd.

	All exanimated quarters*	Healthy quarters	Quarters with positive CMT score*	Infected quarters
n	892	731	161	127
%	100	81.1	18.1	14.2

Evaluation of CMT score				
CMT score	**n**	**%**	**SCC x 10***	**Interpretation***
0 (negative)	731	81.1	0 - 200	Healthy quarters
T (trace)	29	3.3	200 - 400 (±50)	Healthy or LM[1]
1	61	6.8	400 - 650 (±150)	SM[2]
2	40	4.4	850 - 1,200 (±200)	SM or CM[3]
3	23	2.6	1,500 - 5,000 (±300)	CM
4	8	0.9	Over 5,500	CM

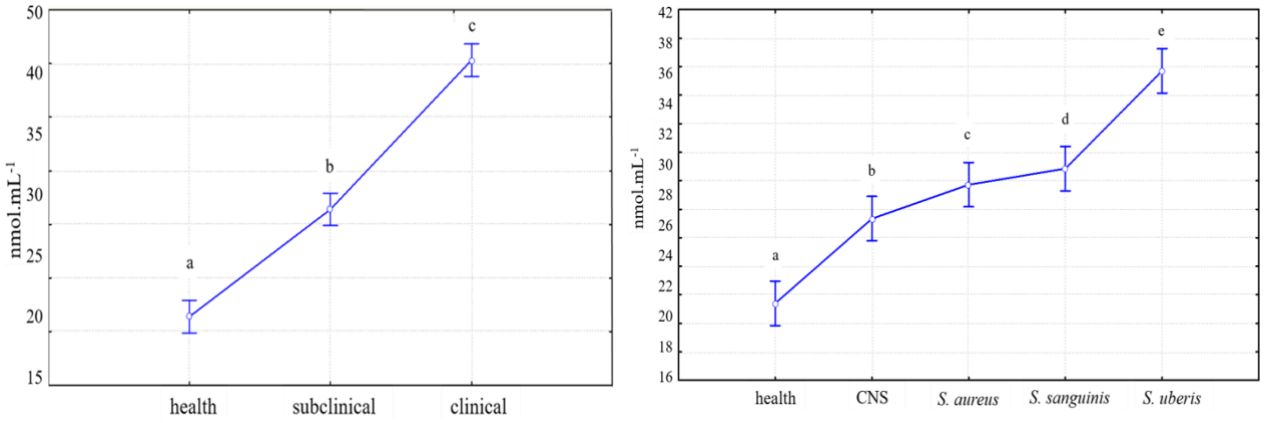
Note: n - number of tested quarters, All exanimated quarters* - quarters with milk secretion, 8 quarters were rejected, CMT score* - quarters with positive evaluation of Californian Mastitis Test with score trace, 1, 2, 3 or 4, SCC*- Somatic cell count and interpretation of severity mastitis according to **Jackson and Cockcroft (2002)**.

LM[1] - Latent mastitis are characteristic only with the presence of bacterial pathogens in samples of milk without changing its consistency and SCC. SM[2] - Subclinical mastitis are characteristic with positive CMT score, bacteriological cultivation, increased SCC, reduced milk yield without clinical signs. CM[3] - Clinical mastitsare characteristic with positive CMT score, bacteriological cultivation, high level of SCC, changing the consistency of the milk, reduced or loss of milk production with clinical signs.

Table 2 Isolated microorganisms from infected quarters in monitored herd.

Isolated microorganisms	n	%	Latent		Subclinical		Clinical	
			n	%	n	%	n	%
***Staphylococcus* spp.**								
S. aureus	16	1.8	-	-	7	0.8	9	1.0
S. haemolyticus	18	-	4	0.4	12	-	2	0.2
S. chromogenes	15	1.7	-	-	8	8.9	7	0.8
S. warneri	11	1.2	2	0.2	9	1.0	-	-
S. xylosus	9	1.0	3	0.3	6	0.7	-	-
S. intermedius	5	0.6	1	0.1	2	-	2	0.2
***Streptococcus* spp.**								
Str. sanguinis	9	1.0	-	-	4	0.4	5	0.6
Str. uberis	8	0.8	-	-	5	0.6	3	0.3
Str. spp.	6	0.7	2	0.2	4	0.4	-	-
Other bacteria								
E. coli	7	0.8	1	0.1	5	0.7	1	0.1
Aerococcus viridans	7	0.8	-	-	7	0.8	-	-
Trueperella pyogenes	5	0.6	1	0.1	4	0.4	-	-
Mixed infection*	11	1.2	2	0.2	4	0.4	5	0.6
Total	**127**	**14.2**	**16**	**1.8**	**78**	**8.7**	**34**	**3.8**

Note: n - number of isolated bacteria from exanimated quarters; Mixed infection* - mixed infection caused two or more bacteria.



A B

Figure 2 Milk malondialdehyde level (MDA) in raw cows' milk. Note: (A) MDA concentration separated by healthy quarters (n = 40), quarters with subclinical mastitis (n = 51) and quarters with clinical mastitis (n = 26); (B) MDA concentrations separated by isolates (n = 51) from quarters with subclinical mastitis. [a-e]Overall MDA values without common superscript differ significantly (p <0.05).

Table 3 MDA concentrations separated by isolates (n = 51) from quarters with subclinical mastitis.

	Groups	Normal	CNS	*S. aureus*	*Str. sanguinis*	*Str. uberis*
		21.40	27.34	29.72	30.85	37.71
1	Normal		**0.000159**	**0.000134**	**0.000134**	**0.000134**
2	CNS	**0.000159**		0.206953	**0.019734**	**0.000134**
3	*S. aureus*	**0.000134**	0.206953		0.838401	**0.000134**
4	*Str. sanguinis*	**0.000134**	**0.019734**	0.838401		**0.000134**
5	*Str. uberis*	**0.000134**	**0.000134**	**0.000134**	**0.000134**	

Note: Tukey HSD test; variable MDA approximate probabilities for Post Hoc Tests Error: Between MS = 5.9733, df = 45.000.

Averages of milk MDA for the selected groups and for the selected pathogens causing SM were shown in Figures 2(A) and (B), respectively. Averages, values of milk MDA in this study from health, subclinical and clinical group were 19.6, 28.8, and 41.3 nmol.mL^{-1}, respectively. From univariate analyses, results show that differences between selected groups and pathogens causing SM were related to MDA concentrations (p <0.001). Milk from SM and CM quarters had highest MDA concentrations and were statistically different (p <0.001) compared to healthy quarters (Figure 2(A)). MDA from milk samples with *S. aureus*, CNS, *Str. sanguinis* and *Str. uberis* was

significantly higher compared to MDA from health milk samples (Figure 2(B)). Results from Post Hoc probabilities showed, that MDA from milk samples infected with *Str. uberis* were statistically higher (p <0.001) compared to other pathogens (Table 3).

Bacteria are the most common cause of mastitis and hence bacteriological culture is routinely used in the laboratory diagnosis. The bacterial pathogens isolated from 127 affected quarters were CNS (41.7%), followed by CPS (16.5%), *Str. sanguinis* (7.0%), *Str. uberis* (6.3%) and other bacteria with mixed infections (8.6%) (Table 2).

The present findings are in accordance with the findings of **Kivaria and Noordhuizen (2007)** isolated *Staphylococcus* spp. followed by *Streptococcus* spp., *E. coli* and *Klebsiella*.

Tenhagen et al. (2006) isolated *Staphylococcus* spp. followed by *E. coli*, streptococci and *Pseudomonas*.

In many countries, CNS have been acknowledged more and more frequently as a cause of intramammary infections in dairy cattle **(Pyörälä and Taponen, 2009; Lange et al., 2015)**.

In the case of CNS, there has been not only an increasing prevalence of such infections but also an expanding list of species reported to be involved in the process. Results of studies from different countries and continents revealed that more than 20 CNS species have been isolated from milk samples of mastitic cows, the most common being *S. chromogenes, S. haemolyticus, S. epidermidis, S. simulans* and S. *xylosus* **(Zadoks et al., 2011; Lange et al., 2015; Khazandia et al., 2018)**.

In our study the most common CNS species such as *S. haemolyticus, S. chromogenes, S. warneri* and *S. xylosus* have been isolated from infected quarter milk samples of cows.

According to **Vasil' et al. (2016)** staphylococci and streptococci are the main etiological agents of ruminant IMI. *Staphylococcus aureus* with CNS are the most frequent isolates from subclinical and clinical cases of IMI.

Monday and Bohach (1999) present in their study that CNS are the most prevalent pathogens causing subclinical mastitis in dairy ruminants. Although less pathogenic than *S. aureus*, CNS can also produce persistent SM as well as producing thermostable enterotoxins. Nevertheless, despite the accepted role of these bacteria as major IMI causing pathogens in dairy ruminants, the pathogenicity of the different CNS species varies widely.

In our study, CNS, *S. aureus* and streptococci were most common isolated from SSM what is generally seen as an increase in the SCC in milk of the infected quarter with positive CMT score.

Increase in SCC during the inflammatory process in mammary glands indicates increased neutrophils in milk which result in oxidation reactions and increase MDA levels. In cows with mastitis, serum lipid peroxidation levels were increased, and the level of blood glutathione peroxidase was decreased compared to the levels in healthy cows **(Atroshi et al., 1996)**.

According to our previous study **Zigo et al. (2014)** infected lactating cows are more sensitive to oxidative stress than cows without IMI. An imbalance between increased production of ROS and the availability of antioxidant defenses needed to reduce ROS accumulation during the infection and may expose cows to increased oxidative stress.

Lipid peroxidation is one of the important consequences of oxidative stress. MDA is generated as a consequence of lipid peroxidation and, as such, is assayed as a biomarker of oxidative stress. The significant higher changes in milk MDA concentration of cows with subclinical and clinical mastitis in the present study (Figure 2(A)) are in accordance with the study **Suriyasathaporn et al. (2012)** that showed a significant increase of milk MDA levels in cows with SM and CM.

According to our expectation, CM quarters had highest MDA concentrations. This might be caused by the higher levels of udder defence mechanism. With the perspective of early diagnosis have shown increased MDA levels in SM quarters. This study showed that MDA level was different among pathogens causing subclinical mastitis (Figure 2(B)). Characteristics of pathogens may be the reason for differences in oxidative environment in udders but MDA from milk samples wit *Str. uberis* were statistically higher compared to other pathogens (Table 3).

CONCLUSION

In the study, we confirmed that the current pathogens mammary gland includes CNS, *S. aureus, S. sanguinis* and *S. uberis*, which were most often isolated from SM and CM. The highest MDA level was observed from clinical cases of mastitis however, elevated levels of MDA were detectable from SM quarters. Bacterial isolates from subclinical quarter milk samples are different levels of MDA. In this study, we found that *S. uberis* was statistically higher compared to other pathogens.

Subclinical mastitis is difficult to detect because of a lack of clinical signs that can be easily identified by visual inspection and palpation of the udder. As can be seen from our study, one of the additional methods for detecting SM can be measurement of MDA level in raw cows' milk.

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FRUIT AS A SOURCE OF ANTIOXIDANTS AND TRENDS IN ITS CONSUMPTION

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ABSTRACT

The positive effects of fruit on human health are mainly attributed to their antioxidant activity. The aim of this work was to observe public awareness about antioxidants consumed in fruit, to analyze their preferences and the frequency of fruit consumption in selected population groups. Preferences were assessed by questionnaire, which was attended by 220 respondents. Information about the presence of antioxidants in fruit showed 85% of respondents. Temperate zone fruit is prefered by 48% of respondents and 52% of respondents prefer fruit of southern zone. Fresh fruit is consumed by 54% of respondents, 18% of respondents prefer fruit juices, compotes are consumed by 12% of respondents, fruit spreads by 11% of respondents, and 5% favour the dried fruit. Fruit is consumed by 31% of respondents once to three times a week, 26% of respondents consumed fruit once a day, 23% occasionally and 20% of respondents more times a day. In terms of sex, higher fruit consumption was recorded at women who consume fruit mostly once a day, while men only once to three times a week. The relationship between place of residence and the possibility to grow their own fruit as well as preference between home and the consumption of fruit by country of origin was confirmed.

Keywords: consumer behavior; purchase; Chi-Square test of Independence; fruit consumption; research

INTRODUCTION

Consumer behavior regarding the food market is changing and is affected by several factors. Significant part of the consumer's decision to buy fruit takes visual marketing, mainly the shape of fruit and color as well as some other sensory characteristics such as aroma or flavor. Fruit purchase is also affected by dietary habits, which are created mainly during childhood. Consumer decision-making process is affected by own experience, the brand, as well as the reputation of the producer, the country of origin and others. Identifying of customer needs and examining their buying behavior is the basis for developing strategies and advertising campaigns. These activities can increase product sale.

Horská and Berčík (2014) examined the effect of light on the purchasing decisions of consumers and the perception of lighting on the food market. Consumer attitudes to food safety was investigated by **Nagyová et al. (2018), Serenčéš a Rajčániová (2007)**, perception of food safety in Slovakia by **Golian et al. (2018)**, differences in consumer behavior at organic product purchase by **Vietoris et al. (2016)**; **Kádeková et al. (2017)**.

Epidemiologic evidence of a protective role for fruit and vegetables in cancer prevention is substantial. The strength of this scientific base guides US national policymaking in diet and health issues and facilitates community and local programs that address national dietary goals to increase fruit and vegetable consumption **(Duyn, Duyn and Pivonka, 2000)**.

Positive effects of fruit have been attributed to their antioxidant activity, which depend mainly on the content of polyphenols and vitamins in fruit **(Lamien-Meda et al., 2008)**. The most important antioxidants include vitamin C, vitamin A and its precursor beta-carotene, vitamin E, trace elements – zinc, copper, selenium and iron, ascorbic acid, tocopherol, lycopene **(Lenucci et al., 2006)**. The most frequently occurring elements are potassium, sodium, magnesium, calcium, chlorine, sulfur and phosphorus. Fruit contain also a number of important minerals, organic acids, vitamins, tannins, enzymes, flavonoids, aromatic substances. Cohort studies demonstrates that increased consumption of fruit and vegetables from less than 3 to more than 5 servings per day is related to a 17% reduction in coronary heart disease (CHD) risk **(He et al., 2007)**.

A diet rich in fruit and vegetables significantly reduces the risk of lifestyle diseases such as cardiovascular disease, atherosclerosis, cancers, diabetes mellitus **(WHO, 2004)**. Based on several epidemiological studies it was shown that the intake of foods rich in antioxidants, especially fruit and vegetables, cereals and some natural oils increases the concentration of antioxidants in the blood, and is associated

with a decrease in mortality and some chronic diseases such as the obesity **(Bes-Rastrollo et al., 2006)**.

Several healthy foods such as olive oil, fruit, vegetables, nuts, legumes, have been inversely associated with depression risk and even have been postulated to improve depressive symptoms **(Lang et al., 2015)**. Polyphenol content and antioxidant activity in varieties of apple and pear were observed by **Mendelová et al. (2011)**, in bilberry **Habánová et al. (2013**), in cranberries and blackberries **Vollmannová et al. (2014)**. Changes in the composition of orange juice have been examined by **Sádecká et al. (2014**), in a strawberry juice by **Predná et al. (2016)**, in grapefruit juice by **Belajová et al. (2017)**. Degradation of ascorbic acid in orange juice observed **Aguirre et al. (2019)**; **Kopuncová et al. (2018)**; **Aguilar et al. (2017)**; **Lu et al. (2018)**.

The aim of this work was to analyze behavior of consumers at fruit consumption, to search for knowledge awareness/level about antioxidants in foods, to examine which kinds of fruit people prefer and the form(state) of fruit most commonly consumed by Slovak consumers, and if depending on their place of residence.

Scientific hypothesis

We investigated the validity of the following hypotheses as a part of our research:

Hypothesis H1: We suppose that the place of fruit purchase is affected by the economic activity of the consumer.

Hypothesis H2: We expect that consumers who originate from the village will prefer fruit of Slovak origin.

Hypothesis H3: We suppose that fruit of temperate zone will be more preferable by consumers who have the opportunity to grow this kind of fruit themselves.

Hypothesis H4: We suppose that gender, age, economic activity and the origin (village/city) of the consumer affect the frequency of fruit consumption.

MATERIAL AND METHODOLOGY

Preference of fruit and frequency of fruit consumption at Slovak consumers and their awareness of antioxidants importance in human nutrition were monitored by questionnaire technique. The survey was performed from March till May, 2018. The questionnaire consisted of 9 questions and 4 issues classificating age, sex, place of residence and economic activity of respondents.

Questions were as follows:

1. Have you heard about the presence and importance of antioxidants in fruit?
2. Where did you get information on antioxidants?
3. Which of the antioxidants do you know?
4. How often do you eat fruit?
5. What types of fruit do you most frequently eat?
6. In which form do you prefer to eat fruit?
7. Provide the most frequent place to buy fruit.
8. Can you grow your own fruit?
9. When buying a temperate zone fruit, you prefer fruit grown (in Slovakia, abroad)?

Sample group consisted of 220 respondents, women represented 50%, men 50%. Structure of respondents by age was as follows: young people aged 18 – 25 years accounted for 53%, age group 26 – 40 years was represented by 16%, age group 41 – 55 years up 21%, respondents aged over 56 years accounted for 9% of the group. Structure of respondents according to their economic activity: 45% of students, 37% employed, 11% unemployed and 7% retired. The place of residence was at 40% respondents living in the town and 60% in the village.

Statisic analysis

Basic approaches of descriptive statistics was used, as well as methods of association measurement. Results were analyzed by Chi-Square statistic. Statistical significance has been tested based on the *p*-values. Correlations were proved by the Cramer's V coefficient. Statistical analysis was performed in SAS Enterprise 5.1 software.

RESULTS AND DISCUSSION

Antioxidants present in fruit protect the human body against undesirable chemical reactions, increase the resistance of the body, support the immune system, contribute to healthy skin and act against aging of cells. Antioxidants affect nervous system function, particularly the functioning of the brain **(Kindersley, 2001)**. **Jennings et al. (2012)** state that the increased frequency of total flavonoid intake significantly reduces blood pressure and contributes to the overall reduction in the risk of cardiovascular diseases.

We monitored respondent awareness about the importance and presence of antioxidants in fruit. 85% of respondents had information about the presence of antioxidants. For the most used sources of information on antioxidants, respondents identified the internet (29% of respondents) and media (28% respondents) school 18%, magazines 17% (Figure 1). As antioxidants they recognise vitamins (47% of respondents), minerals (29%), 20% of coenzyme Q10, and only 4% of flavonoids and polyphenols. People should consume over 1 g of flavonoids and phenolic acids daily **(Rui, 2003)**. The fruit can be consumed fresh as well as frozen, canned or otherwise modified. We determined that consumers prefer to have fresh fruit (54% of respondents), 18% of respondents prefer fruit juices, compotes 12%, fruit spreads reported 11% of respondents and 5% of respondents prefer dried fruit (Figure 2).

Consumption of fresh fruit and fruit products per capita and year (in terms of fresh fruit) in Slovakia observed **Meravá (2018)**. Consumption of fruit per capita and year reached 60.5 kg in 2017, consumption of fresh fruit reached 42.4 kg per person per year.

The qualitative properties of fresh garden fruit were analyzed by **Hegedüsová et al. (2016)**, **Mezey and Paulen (2003)**, the organoleptic characteristics of fruit and fruit juices during various storage conditions were evaluated by **Ozarda, Demirkoz and Özdemir (2015)**.

Modern trend knowledge in consumption and applying a healthy lifestyle also include consumer decision makers. By linking of current scientific knowledge and practice in the field of new fruit processing technologies, innovative multi-component products are developed, like smoothie.

Consumer preferences at fruit purchase

In terms of place of fruit purchase, respondents prefer supermarkets (48%). Directly from producers is fruit purchased by 19%, (Figure 3) in wholesale/retail stores by

17%. Similarly, 17% of respondents obtained the fruit directly from the small local markets.

We assumed that the purchase of fruit may be affected by economic activity of respondents (Hypothesis H1). Relationship between the place of fruit purchase and economic activity of the respondents is presented in Figure 3. Based on statistical evaluation there was not confirmed any existence of a statistically significant correlation. Place of fruit purchase is therefore influenced by other factors than the economic status of the respondents.

We also analyzed preferences of the respondents in relation to the selection and consumption of fruit according to its origin (Hypothesis H2). At 48% of respondents is prevalent fruit of temperate zone and 52% of respondents prefer fruit of southern zone. In our survey, the most commonly consumed fruit in the first order respondents indicated the pomes (51%), stone fruit (14%), tropical fruit (11%), small fruit (9%), grapes (9%) and nuts (6%).

In consumption of the temperate zone fruit in Slovakia per capita and year dominated apples (11.5 kg), grapes (4.8 kg) and peaches (2.5 kg). The consumption of small fruit per capita per year was 0.5 kg of currants, 1.0 kg of strawberries. Consumption of fresh fruit in fresh state on the Slovak market is increasing **(Meravá, 2018)**.

We also monitored the relationship between place of residence (village, city) and preference by country of origin (domestic versus imported fruit). Based on the *p*-value (*p*-value = 0.007), we can conclude that respondents who live in a village in its shopping behavior prefer fruit grown in Slovakia (Figure 5). Based on the Cramer coefficient (V = 0.18) this is only a weak correlation.

Fiľa and Tóthová (2013) state about the direct sale advantage in promoting the local economy, increasing employment in the region, reduction of transport costs as well as consolidation relations in the region. In relation farmer – consumer, there is a direct relationship between the seller and the buyer. Other benefits for producer are demand, development regarding consumers' needs, supporting social network. Food quality, transparent prices, contact with soil and animals belong to benefits for consumer **(Chreneková, Dirgasová and Fáziková, 2015)**.

Fruit originating from Slovakia is preferred by 86% of the respondents living in the villages and 72% of the respondents from the towns.

Ability to cultivate own fruit indicated 79% of respondents. In relation to the place of residence, it was found that 95% of respondents living in village can cultivate their own fruit. Among the respondents living in the city, the opportunity to cultivate own fruit have 55%. The results are shown in Figure 4.

We examined also the relationship (Hypothesis H3) between the ability to grow their own fruit and fruit preference according to zone, so we assumed that respondents who have the opportunity to grow their own fruit, will prefer more fruit of middle zone (Figure 4) but this relationship was not confirmed (*p*-value = 0.078).

Frequency of fruit consumption

To the question "How often do you eat fruit", 20% of respondents state consumption several times a day, 26% of respondents consume fruit once a day, 31% of respondents consume fruit once to three times per week, 23% of respondents consumed fruit only occasionally. By examining the frequency of fruit consumption (Hypothesis H4) by the age of respondents, we found that in the age category 18 – 24 years dominated consumption of fruit once to three times per week, aged 26 to 40 years consumption several times a day, in the age group 41 – 55 years dominated consumption of one fruit a day and in the age group over 56 years was prevalent once to three times per week. Following gender, we found that higher consumption of fruit is among women who consume fruit most common once a day, while men mostly consumed fruit only once to three times per week. At this point, we assumed the existence of a relationship among the frequency of consumption of fruit and gender, age of respondents, economic activity and provenances (place, residence) of respondents. It is not statistically significant difference in the frequency of fruitconsumption between men and women and it was not confirmed to be the difference in consumption of fruit among the age groups of respondents. Based on the relative frequencies it was shown that respondents who live in a village more often consume fruit, but the statistically significant difference between respondents from the village and the town was not confirmed (Table 1). Frequency of fruit composition was examined by several authors e.g. **Belej et al. (2016)**.

Fatrcová-Šramková, Schwarzová and Juríková (2017) examined the frequency of consumption of fresh as well as processed fruit and vegetables (canned products and vegetable and fruit juices) in adult females under the age of 26 and found that 1 – 2 portions of fresh fruit and 1 portion of vegetables predominated, that does not meet the recommendations. In the recommended doses of food it is stated that the consumption should be 96.7 kg for fresh fruit, of which fresh fruit of temperate zone is 57.7 kg. The World Health Organization **(WHO, 2004)** recommends consuming of five servings of fruit a day.

Eating habits of the population, food choice at purchase, choice of appropriate processing of fruit and its storage and even the popularity of certain foods are derived from the acquired habits from early childhood. Ongoing changes in the diet of consumers are affected by several factors such as globalization of markets, global trends in diet, developing tourism. Preferences of consumers can be affected by acquired eating habits and to a large extent, household income.

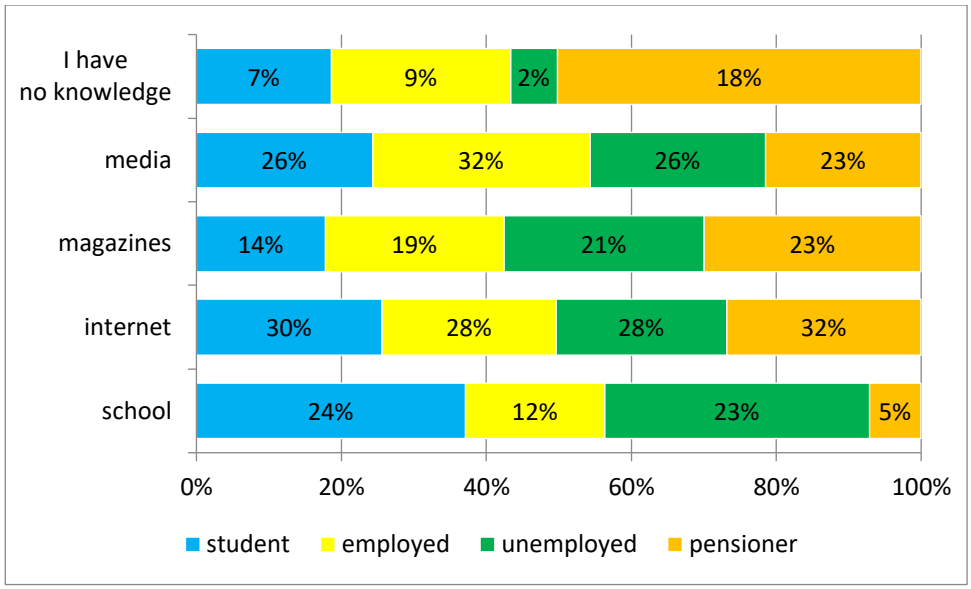

Figure 1 Respondents' answers to the question: "Where did you get information about antioxidants?" in relation to the economic activity of respondents.

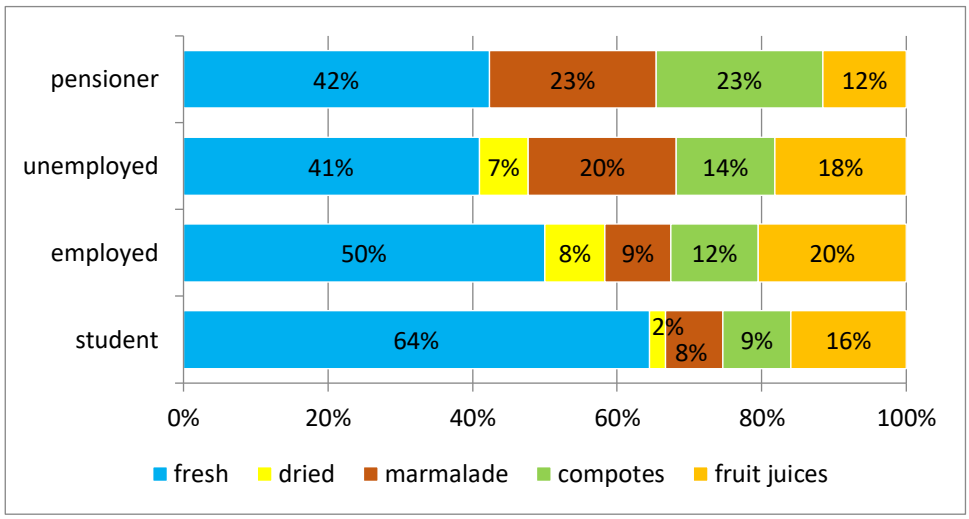

Figure 2 Respondents' answers to the question: "In which form do you prefer to consume fruit?" in relation to the economic activity of respondents.

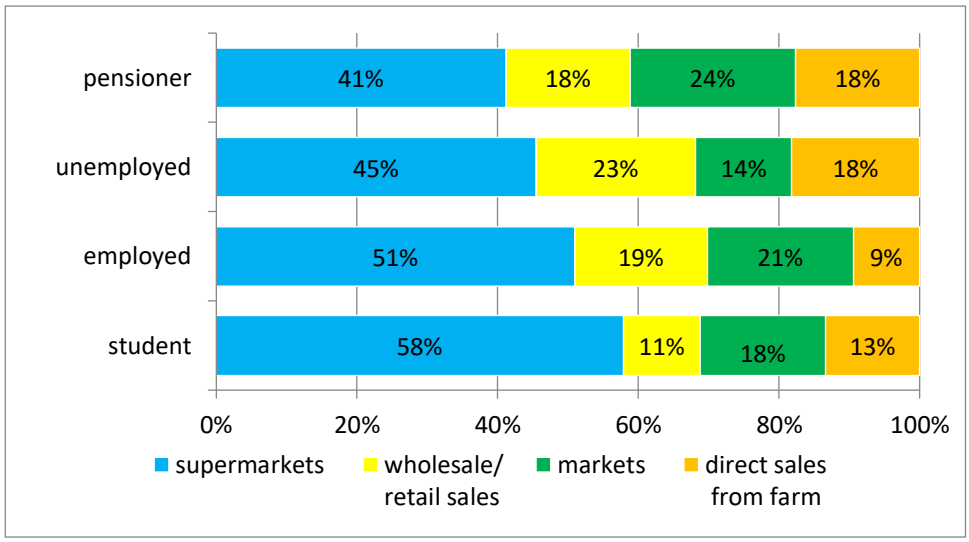

Figure 3 Most oftenplaceoffruitpurchase in relation to theeconomicactivityofrespondents.

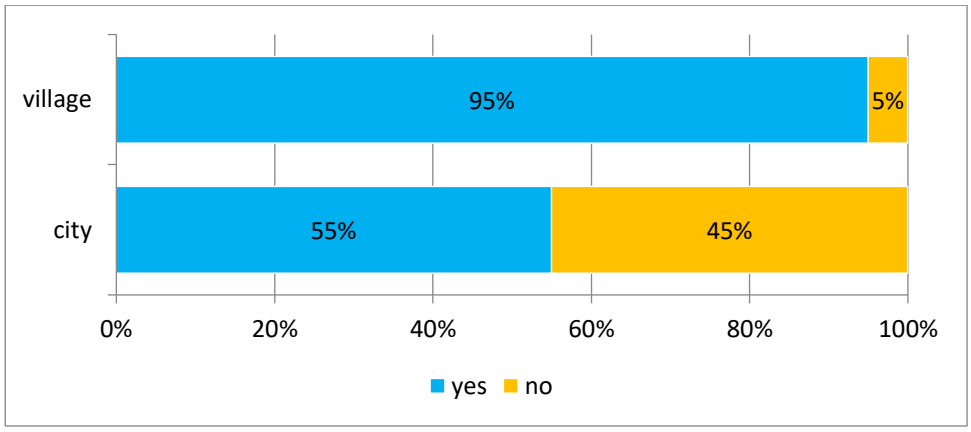

Figure 4 Possibilityofrespondents to cultivatetheirownfruit in relation to theplaceofresidence.

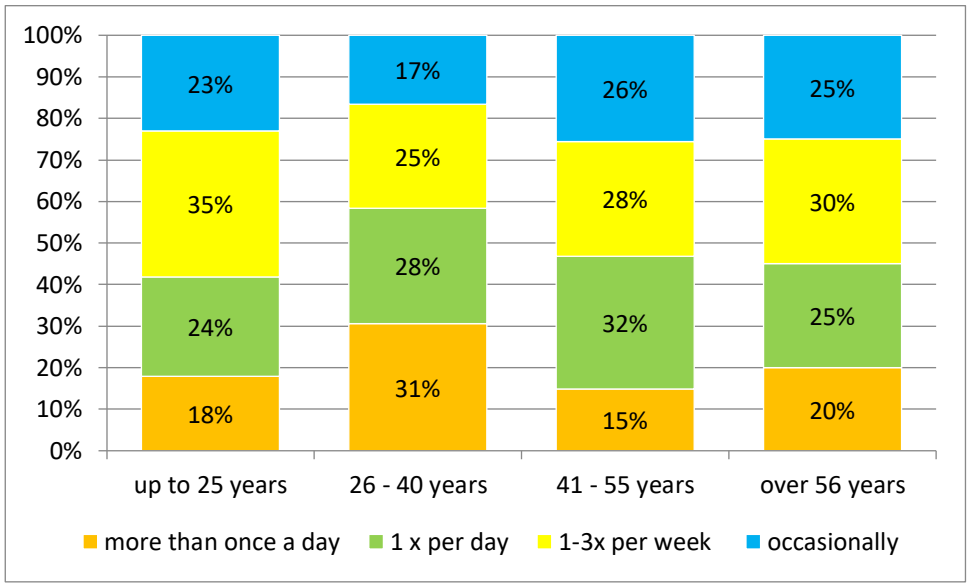

Figure 5 Frequencyoffruitconsumption in relation to theageofrespondents.

Table 1 The results of the statistical testing to the hypotheses.

Hypothesis:	Statistics:	Chi-Square	Phi Coefficient	Contingency Coefficient	Cramer's V
H1	Value	7.706	0.176	0.173	0.101
	Prob.	0.564			
H2	Value	7.328	0.183	0.180	0.183
	Prob.	0.007			
H3	Value	3.107	0.119	0.118	0.119
	Prob.	0.078			
H4 (gender)	Value	1.938	0.094	0.094	0.094
	Prob.	0.585			
H4 (age)	Value	5.745	0.162	0.16	0.093
	Prob.	0.765			
H4 (economic activity)	Value	12.398	0.237	0.231	0.137
	Prob.	0.192			
H4 (placeofresidence)	Value	4.932	0.15	0.148	0.15
	Prob.	0.177			

CONCLUSION

We can conclude that 85 % of respondents have some information on the presence ofantioxidants in fruit. Almost one-third of respondents eat fruit once to three times per week, 26 % respondents consume fruit once a day, 23% occasionally and 20 %respondents several times a day. Consumers prefer to consume fresh fruit (54% of respondents), 18% of respondents prefer fruit juices, compotes are prefered by 12%, fruit spreads reported 11% of respondents and 5% of respondents prefer dried fruits. Most often is fruit purchased in supermarkets (48%). Directly from producers is fruit obtained by 19%, in wholesale/retail stores 17%. Similarly, 17% of respondents obtain the fruit directly at the small local markets. Respondents living in the village often consume fruit, but statistically significant difference between respondents from village and town was not confirmed. Information obtained suggest that the importance of fresh fruit and juices containing natural antioxidants is quite well known, but their annual consumption is in fluctuating character.

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OCCURENCE AND ANTIMICROBIAL RESISTANCE OF COMMON UDDER PATHOGENS ISOLATED FROM SHEEP MILK IN SLOVAKIA

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ABSTRACT

The aim of this work is to identify the spectrum, frequency and antimicrobial resistance of bacterial pathogens occurring in sheep dairy herds in Slovakia. Of a total of 310 samples of sheep's milk coming from three breeds during two seasons (2017 and 2018), at least one potential pathogen was isolated from 102 samples (32.9%). A total of 131 microbial isolates were isolated. The most represented species were coagulase negative staphylococci CoNS (75.6%), followed by *Streptococcus agalactiae* (10.7%), *Staphylococcus aureus* (6.9%), *Streptococcus dysgalactiae* (4.6%), *Escherichia coli* (1.5%), *Enterococcus faecium* (1.5%), and others (*Streptococcus uberis*, *Streptococcus parauberis*, *Candida* sp., *Klebsiella* sp., moulds) below 1%. A total of 99 isolates of CoNS were tested for antimicrobial resistance. Of these, 63.6% were resistant to at least one antibiotic. A total of 24.2% of the tested isolates were resistant to 3 groups of antimicrobials simultaneously (multi-drug resistance). The highest resistance was observed to lincomycin (57.6%) and neomycin (36.4%), the lowest to sulfamethoxazolum+trimethoprim (0%) and enrofloxacin (3.0%). Based on the results of this work, it is possible to assume a similar spectrum of pathogens and their antimicrobial resistance described in the literature also within the Slovak sheep farms focused on milk production.

Keywords: sheep; milk; mastitis; pathogen; antibiotic

INTRODUCTION

Public health problems associated with consumption of unpasteurized cow's milk and raw-milk products have been well documented **(De Buyser et al., 2001; Harrington et al., 2002)**. There is no evidence that the risk from unpasteurized ewe's milk is any lower **(Allerberger et al., 2001; McIntyre et al., 2002)**. Pathogenic microorganisms can gain access to milk either by faecal contamination or by direct excretion from the udder into the milk.

The importance of subclinical mastitis as a limiting factor in sheep's milk production is well-known. In addition to decreased milk yield, decreased viability of lambs, subclinical mastitis also greatly reduces the hygienic quality of milk as well as its technological properties.

Current knowledge of mastitis in small ruminants has been reviewed by some authors **(Bergonier et al., 1999; Bergonier and Berthelot, 2003; Lafi et al., 1998; Contreras et al., 2007)**. The causative organisms of mastitis are categorized as major or minor pathogens **(Harmon, 1994)**. The most common major pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, coliforms and enterococci, while other pathogens such as *Streptococcus* spp., *Pseudomonas aeruginosa*, *Mannheimia hemolytica*, *Corynebacteria*, Coagulase negative staphylococci and fungi, are considered to be minor pathogens which can produce intramammary infection in small ruminants, but occurrence rates are lower **(Contreras et al., 2007)**.

Differences in climatic conditions, production patterns, breeding management and breeding practices influence the different epidemiology and clinical manifestation of sheep milk. The aim of this work is to identify the spectrum, frequency and antimicrobial resistance of bacterial pathogens occurring in sheep dairy herds in Slovakia.

Scientific hypothesis

Staphylococci and streptococci are the most common cause of mastitis in milk sheep and their occurrence in milk is frequent. Antibiotics are not used in sheep as often as in dairy cows, but antimicrobial resistance can occur as the environmental impact to farm animals.

MATERIAL AND METHODOLOGY

Sampling and culture

During two seasons, samples of milk from 3 sheep farms were withdrawn repeatedly in the number 160 (season 2017) and 150 (season 2018). The animals for collection were selected by random selection. The breeding

organization was the following: farm 1 – Slovak Tsigaya, farm 2 – Lacaune, farm 3 – Slovak Walachian/Lacaune. Milk samples of volume 10 mL were collected into sterile tubes from both halves after disinfection and two streaks. After sampling, the samples were cooled to 5 – 10 °C, then frozen and transported to the laboratory. Bacteriological examination was performed within 5 days after collection. Milk samples (10 µL inoculum) were cultured on a selective diagnostic PM test (Lab-Media-Servis, CZ) at 37 °C for 24 hours. Isolated strains of pathogens were subsequently verified by typing with BBL Crystal® (Becton, Dickinson & Co., New Jersey, USA). A milk sample was classified as positive if at least one colony-forming unit (CFU) of *S. aureus* or *Streptococcus* (*Str.*) *agalactiae* was isolated. For other agents, the presence of at least three CFUs was needed for positive classification. Samples were classified as contaminated if three or more bacterial types were isolated from one milk sample and growth of a major udder pathogen was not identified. If growth of a major udder pathogen was found in combination with contaminating species, the sample would be diagnosed as positive for growth of the major udder pathogen.

Susceptibility testing

In vitro susceptibility of the isolates against antimicrobial agents was determined by the standard disk diffusion procedure **(CLSI 2008; 2013)**.

Coagulase-negative staphylococci (n = 99) were tested for susceptibility to 6 antimicrobial agents from the following groups: penicillin (amoxicillin+clavulanic acid 2:1 AMC), tetracyclines (tetracycline TET), aminoglycosides (neomycin NEO), lincosamides (lincomycin LCM), sulphonamides (sulfametoxazolum-trimethoprim SXT), and quinolones (enfofloxacin EFX). The isolates were tested by disc diffusion method according to the CLSI manual **(CLSI 2008; 2013)** using the following antimicrobial discs (Oxoid, Basingstoke, England): AMC (30 µg), NEO (30 µg), TET (30 µg), LCM (2 µg), SXT (25 µg), EFX (5 µg). The diameters of the inhibition zones were evaluated (susceptible, intermediate, resistant) according to CLSI breakpoints. Appropriate quality control tests were performed using reference strains of *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212.

Statistic analysis

Simple descriptive statistics was used. The results of cultivation were processed into a percentage of individual microbial species. The results of antimicrobial resistance were also expressed as the percentage of resistant isolates in each type of antimicrobial.

RESULTS AND DISCUSSION

Of a total of 310 samples of sheep's milk coming from three farms, at least one potential pathogen was isolated from 102 samples (32.9%). The results are shown in the Table 1. The most represented pathogens were coagulase negative staphylococci CoNS, namely *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus xylosus* (75.6%), followed by *Streptococcus agalactiae* (10.7%), *Staphylococcus aureus* (6.9%), *Streptococcus dysgalactiae* (4.6%), *Escherichia coli* (1.5%), *Enterococcus faecium* (1.5%) and others (*Streptococcus uberis*, *Streptococcus parauberis*, *Candida* sp., *Klebsiella* sp.). The cause of the sheep's mastitis may be a number of microorganisms, according to the literature, they are mainly representatives of the genus *Staphylococcus* **(Bergonier and Berthelot, 2003; Zigo et al., 2011)**. The results of this work also confirm this bacterial genus as dominant in the farming conditions included in our observation, in both consecutive seasons.

Several authors state that CoNS are most frequent pathogens responsible for subclinical mastitis of dairy sheep **(Fthenakis, 1994; Burriel, 1997; Lafi et al., 1998; Pengov, 2001; Ariznabarreta, Gonzalo and San Primitivo, 2002; Gonzalo et al., 2002)** and *Staphylococcus aureus* is more frequent in meat sheep **(Jones, 1991; Watson et al., 1990; Hariharan et al., 2004; Mork et al., 2007)**. The farms included in this work are focused on the production of sheep's milk and CoNS represent a significant prevalence of all isolated pathogens. *Staphylococcus aureus* represented less than 7% of isolates.

Streptococci are probably the second most common cause of ovine mastitis **(Bergonier et al., 1999)**. The most commonly isolated species are *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus dysgalactiae* **(Las Heras et al., 2002)**. In this work streptococci were mainly represented by *Streptococcus agalactiae*, but only in the first of the monitored seasons. In the second year of follow-up, the occurrence of streptococci in the samples taken decreased significantly. This difference could have been caused by certain breeding measures in the context of milk management and overall hygiene in the farm. The presence of other pathogens in the isolates obtained in this work was negligible.

Antibiotic resistance pattern for staphylococci isolated from subclinical mastitis refers mainly to cattle, and little is known about dairy sheep.

A total of 99 isolates of CoNS were tested for antimicrobial resistance. Of these, 63.6% were resistant to at least one antibiotic. A total of 24.2% of the tested isolates were resistant to 3 antimicrobials simultaneously. The highest resistance was observed to lincomycin (57.6%) and neomycin (36.4%), the lowest to sulfamethoxazolum+trimethoprim (0%) and enrofloxacin (3.0%). The results are shown in Table 2.

The high multidrug resistance rates observed in CoNS are in accordance with previous reports **(Kumar, Yadav and Singh, 2009; Sawant, Gillespie and Oliver, 2009; Vasil' et al., 2018)** and support the hypothesis that CoNS might play an important role as a source of genes resistant to *S. aureus* **(Taponen and Pyörälä, 2009)**. Considering such an assumption, studies about host specificity in *Staphylococcus aureus* and other pathogenic agents could provide useful information about the epidemiological importance of CoNS as reservoirs of genes resistant to pathogenic strains for humans. However, because CoNS are the major mastitis causing agents in small ruminants, the high frequency of resistant genes in such species reported here is worth noting.

Table 1 The numbers of microbial pathogens isolated from three sheep farms during two seasons in 2017 and 2018.

Pathogen	No. of isolates in the season						Total
	2017			2018			
	Farm 1	Farm 2	Farm 3	Farm 1	Farm 2	Farm 3	
Staphylococcus chromogenes	13	6	7	4	2	8	40
Staphylococcus epidermidis	3	4	3	3	4	3	20
Staphylococcus xylosus	8	14	5	4	3	5	39
Staphylococcus aureus	0	4	2	2	1	0	9
Streptococcus agalactiae	9	1	4	0	0	0	14
Streptococcus dysgalactiae	1	1	2	2	0	0	6
Streptococcus uberis	1	0	0	0	0	0	1
Streptococcus parauberis	1	0	0	0	0	0	1
Escherichia coli	0	2	0	0	0	0	2
Klebsiella sp.	0	0	0	0	1	0	1
Enterococcus faecium	1	1	0	0	0	0	2
Candida sp.	0			0	0	0	1
Micromycetes	0	0	1	0	0	0	1

Table 2 The results of antimicrobial resistance testing of coagulase negative staphylococci.

Antimicrobials	Zone diameters (mm) S – I – R	No. of isolates/percentage (n = 99)		
		Sensitive	Intermediate	Resistant
Amoxicillin+clavul.	≥30, 29 – 28, ≤27	81/81.8	3/3.0	15/15.2
Tetracycline	≥19, 18 – 15, ≤14	72/72.7	0/0	27/27.3
Lincomycin	≥20, 19 – 17, ≤16	33/33.3	9/9.1	57/57.6
Enrofloxacin	≥25, 24 – 21, ≤20	81/81.8	15/15.2	3/3.0
Sulphametoxazolum+trimet.	≥19, 18 – 16, ≤15	96/97.0	3/3.0	0/0
Neomycin	≥23, 22 – 20, ≤19	36/36.4	27/27.3	36/36.4

The drugs showing the lowest resistance rates were enrofloxacin and sulphonamides, which are in accordance with other studies on mastitis in several ruminant species **(Kumar et al., 2009)**. **Virdis et al. (2010)** reported high sensitivity to quinolones, but not to aminoglycoside in subclinical mastitis-causing staphylococci.

From the point of view of epizootology, CoNS are frequently found to be a pathogenic especially in the case of subclinical mastitis. They do not represent a major specific pathogen but can potentially cause infections that tend to have a mild clinical manifestation but cause losses in the milk production, in quantity and quality of milk.

CONCLUSION

Based on the results of this work, it is possible to assume a similar spectrum of pathogens and their antimicrobial resistance described in the literature also within the Slovak sheep farms focused on milk production. Coagulase negative staphylococci, as the most common pathogen, poses a risk in the form of a subclinical course of inflammatory changes in the mammary gland that often escape the attention of breeders, but can have a significant impact on the quality of milk production.

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APPLICATION OF ELECTRONIC NOSE FOR DETERMINATION OF SLOVAK CHEESE AUTHENTICATION BASED ON AROMA PROFILE

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ABSTRACT

Electronic nose with sensors is used in many industries and for various applications such as quality control, process monitoring, shelf life evaluation, origin or authenticity assessment. The aim of this work was to investigate the electronic nose with FID detectors applicability for characterization of steamed cheese and for the assessment of steamed cheese quality decay during storage. Samples of smoked and unsmoked steamed cheese varieties from 5 Slovak enterprises concerning different regions of Slovakia were analysed. Data from aroma profiles were processed by statistical technique PCA. Compounds like acetaldehyde, 1-propanal, propanoic acid, ethyl hexanoate, furfural, butan-2-one, isovaleric acid, 1-hexanol or α-pinene were determined as significant flavours in fresh steamed cheese samples. In the current study, no significant differences in aroma profiles between fresh and stored cheese samples were confirmed. Thus, differences in main odour substances composition of steamed cheese varieties, obtained from various producers in several geographic regions of Slovakia, were minor.

Keywords: Slovak steamed cheese; aroma profile; e-nose; authentication

INTRODUCTION

Electronic nose (e-nose) is an odour detection device using a sensor array **(Delgado-Rodríguez et al., 2012)**. Many industries use e-nose for diverse applications such as quality control **(Li et al., 2017; Xu et al., 2017; Gancarz et al. 2017; Chen et al., 2018; Buratti et al., 2018)**, process monitoring, durability assessment, origin ranking and originality **(Wilson and Baietto, 2009; Śliwińska et al., 2014; Li et al., 2017)**. Another implementation of e-nose in the food industry involve dairy products classification whether in terms of flavour, variety type, geographical origin, ripening stage (in case of cheese) or its shelf life prediction **(Ampuero and Bosset, 2003)**. E-nose can be used also to monitor volatile compounds that indicate female cattle fertile period, acknowledged by the study of **Manzoli et al. (2019)**.

Working principle of e-nose Heracles II is gas chromatography, detecting aroma compounds of very small concentrations in real-time of a few minutes and identifying them by comparing Kovats retention indices with the NIST library. To obtain vital information from the analysis of samples, multivariate statistics is applied most frequently **(Buratti et al., 2018)**. The use of e-nose analysis is a rapid, easy, reliable, accurate and non-polluting method practice.

Aroma perception occurs by olfaction, when olfactory receptors placed on the nasal cavity roof are stimulated by aroma active compounds. In terms of flavour recognition during food or beverage consumption, the aroma active compounds are perceived retronasally. Different combinations of large variety of compounds including short, medium chain fatty acids, alcohols, aldehydes, ketones, esters or sulphur compounds create a characteristic smell and flavour of each and every cheese product. Multiple cheese types may consist of the same aroma active compounds, yet they differ from each other by altered amount, and therefore the percentage content, of particular component in such product **(Niimi et al., 2014)**. According to **Commission Regulation (EC) No 656/2008**, the protected geographical indication "Slovenská parenica" is an official label of Slovak parenica type steamed cheese if it meets all characteristics and requirements defined within this Regulation (referring to Council Regulation (EC) No 510/2006). Here, Slovak parenica is defined as "a steamed, lightly smoked cheese wound into two rolls 6 – 8 cm in diameter and 5 – 8 cm high, connected in a 'S'- shape having yellow to brown colour on the outside after smoking; white to buttery yellow on the inside. The rolls are bound with cheese string or chain. Prior to being rolled up, the cheese strip is 2 – 3 mm thick, 5 – 8 cm wide and 4 – 6 m long. The ingredients used are fresh raw, unprocessed ewe's milk from grazing ewes of the Wallachian, improved Wallachian, Cigaja and East Friesian breeds or a mixture

of fresh raw, unprocessed ewe's milk and fresh raw, unprocessed cow's milk, containing at least 50% ewe's milk". In Slovakia, the production of parenica type cheese is very widespread, directed either as mechanized production for big dairy factories or as manual operation by small corporations.

In presented study, Slovak steamed cheese samples, produced by small and medium manufacturers from pasteurized raw cows' milk, were analysed. Being processed from cow's milk, they are not referred to as Slovenská parenica. For this work`s purposes, we will refer to the analysed samples as the steamed cheese in the following text. At the beginning of the steamed cheese production, a starter culture is added to the milk for the fermentation process and thereby to reduce the pH of the milk to the desired level. For such aim, mesophilic culture (*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*) and thermophilic culture (*Streptococcus salivarius* subsp. *thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus*) are the most commonly used ones **(Onipchenko et al., 2012)**. After the starter culture inoculation, the rennet is added, and the coagulation phase of milk begins. The optimum coagulation temperature is 30 to 35 °C. The created cheese curd is then treated by cutting, stirring and heating to a higher temperature, resulting in separating the whey out and thereby reducing the water content. The raw cheese block is then pressed with gentle pressure to promote the separation of the whey and such a cheese block is then an input raw material for the steaming process. The steaming process runs in two stages. In the first steaming stage, the shredded cheese curd is dosed into a hot water tank (65 – 85 °C). Due to the high temperature, the raw cheese dough gets a stretchable and mouldable consistency. In the second steaming stage, the raw dough is stretched and kneaded. After these phases, the next step in steamed cheese production is forming the cheese into desired shapes. Finally, the steamed cheese is subjected to a salt bath and in case of smoked cheese products the salted cheese undergoes smoking process **(Muliawan and Hatzikiriakos, 2008; Zimanová et al., 2016)**.

Scientific hypothesis

The aim of this study was to determine differences between steamed cheese products and to confirm the ability to evaluate the authentication of these products using the fast method of electronic nose with FID detectors.

MATERIAL AND METHODOLOGY

Sampling

Samples of smoked (S) and unsmoked (U) steamed cheese varieties from 4 Slovak enterprises classified as medium enterprises (samples no. 1, 2, 4, 5) and one small enterprise (sample no. 3) from different regions of Slovakia were collected. Samples were processed from pasteurized cow's milk. Sampling was performed on the day of production, and analysis of fresh samples was carried out on the following day. Subsequently, analyses of samples were performed again after storing the samples for 14 days at 4 – 8 °C. Samples were stored in original packaging bags sealed under protective atmosphere conditions in refrigerator at temperature 4 – 8 °C.

Sample preparation

Steamed cheese samples were sliced into small pieces, and by 4 g weighed into clean 20 mL headspace vials closed by magnetic cap with PTFE/Sil septum. Thus, prepared samples were stirred at 50 °C for 15 minutes on a shaker included as a part of the GC headspace autosampler (Combi Pal, Alpha M.O.S.).

Determination of aroma profiles

E-nose with two FID detectors (Heracles II, Alpha M.O.S.) was used for determination of steamed cheese samples aroma profiles. From the saturated air above the sample level, 5 mL volume was withdrawn using a headspace autosampler syringe and dispensed into the e-nose injector heated to 200 °C. The analysis itself lasted for 110 seconds and the separation of aroma compounds took place in two columns with following temperature program; for the 1st column: isotherm 80 °C, for the 2nd column: initial temperature 50 °C and temperature gradient 3 $°C.s^{-1}$ to 250 °C. Hydrogen was used as the carrier gas. Identification of the compounds was performed by matching the measured peaks with Kovats´retention indices with NIST library (The National Institute of Standards and Technology library) by software Alpha Soft V14 (Alpha M.O.S.).

Statistical analysis

Compounds with a discriminant >0.990 were selected, based on which the semi-qualitative evaluation was performed and PC analysis (Principal Component Analysis) was made by Alpha Soft V 14 (Alpha M.O.S.) software. Descriptors were analysed using single factor analysis of variance and significance was at $p < 0.001$.

RESULTS AND DISCUSSION

Thirteen specified aromatic compounds with a discriminant >0.990 were selected, based on which the semi-qualitative evaluation was performed and PCA analysis was made. Figure 1 displays results processed by PCA technique of the aroma profile of fresh steamed cheese samples. Among smoked and unsmoked fresh steamed cheese from the suppliers no. 1, no. 2 and no. 3 (negative scores), and no. 4 and no. 5 (positive scores) no statistically significant differences ($p < 0.001$) were evident in their aroma profiles (PC1 axis 95.94%). On the contrary, samples of smoked (negative scores) and unsmoked (positive scores) fresh steamed cheese from suppliers no. 2, no. 3, no. 4 and no. 5 were differed in their aroma profiles in the PC2 axis (3.34%). It is assumed that these manufacturers use a different way for cheese smoking in terms of wood used in combustion process.

Cheese types such as Italy mountain cheese, Camembert cheese, Mozzarella cheese, Cheddar cheese etc. are also the subject of number of studies **(Carafa et al., 2019; Batty, Waite-Cusic, Meunier-Goddik, 2019; Kim et al., 2014; Velasco et al., 2019)**, which are of a different cheese type as our investigated samples are, and therefore it is not possible to comapre the results of the Slovak steamed cheese samples with the results of the studies

mentioned above. The aroma profile of unripened steamed cheese produced from cows' milk is not fully investigated and reported, and therefore the results might not correlate with results of the authors of other studies.

Semi-qualitative evaluation was based on comparison of the Kovats` retention indices with the NIST library. Samples were divided into three groups, each consisting of samples with only small differences in their aroma components composition. As significant compound (discriminant >0.990) responsible for the fresh steamed cheese aroma, 1-propanal was detected in samples 1S, 1U, 3U and 2U. Differences causing compounds in aroma profiles of 2S and 3S fresh samples, compared to the other samples, were acetaldehyde, ethyl hexanoate, isovaleric acid, 1-hexanol, furfural, butan-2-one and α-pinene. **Bhandari et al. (2016)** determined the aromatic profile of ripened Italian Parmigiano Reggiano cheese from unpasteurized milk by SPME-GC-MS and detected of furfural, ethyl butanoate, ethyl hexanoate, ethyl octanoate, 2-nonanone, 2-heptanone, 3-methyl butanal, acetic, butanoic, hexanoic, octanoic and decanoic acids and benzeneacetaldehyde as the major compounds. On the contrary, carboxylic acids were the most abundant aroma compounds in the Torta del Casar cheese performing SPME-GC-MS analysis, specifically acetic, butanoic, hexanoic and octanoic acids **(Delgado-Martínez et al., 2019)**.

Smoked and unsmoked fresh steamed cheese samples from suppliers no. 4 and no. 5 differed from other samples by the presence of propanoic acid and 1-propanal in their aroma profiles composition. Identified compounds matched the NIST library with ≥50%. Therefore,

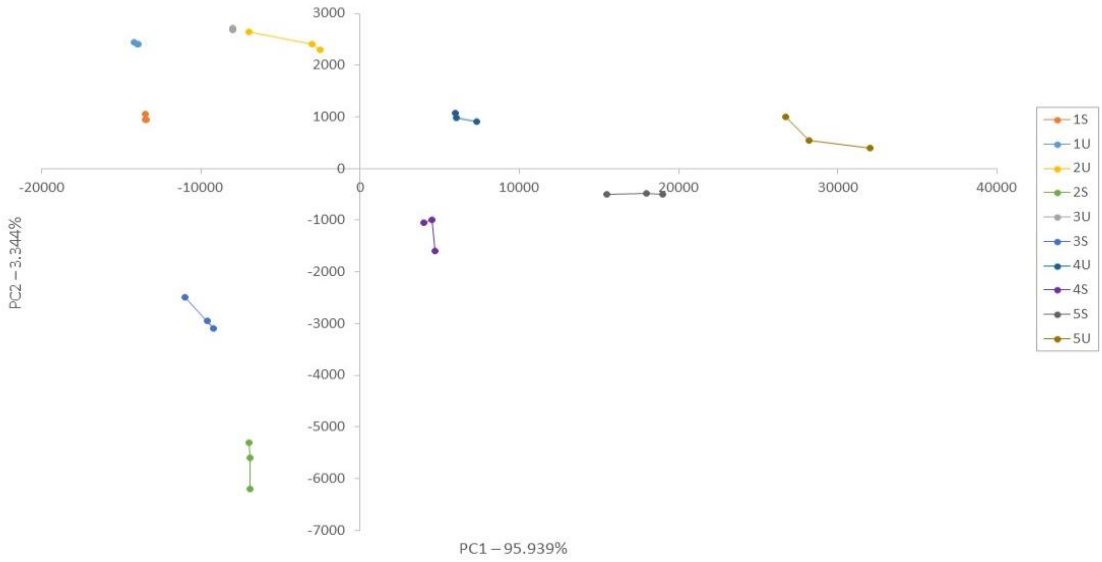

Figure 1 Projection of the samples onto the space defined by the first two principal components (PC1/PC2). Sample groups according to fresh: S (smoked steamed cheese) and U (unsmoked steamed cheese) of five Slovak producers (1 – 5).

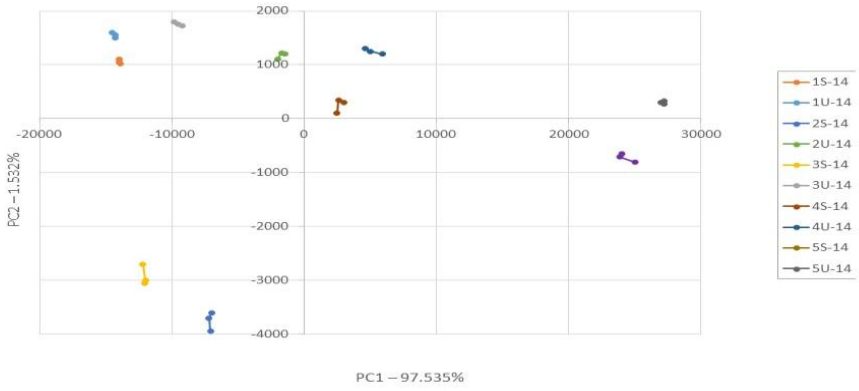

Figure 2 Projection of the samples onto the space defined by the first two principal components (PC1/PC2). Sample groups according to stored: S (smoked steamed cheese) and U (unsmoked steamed cheese) of five Slovak producers (1 – 5).

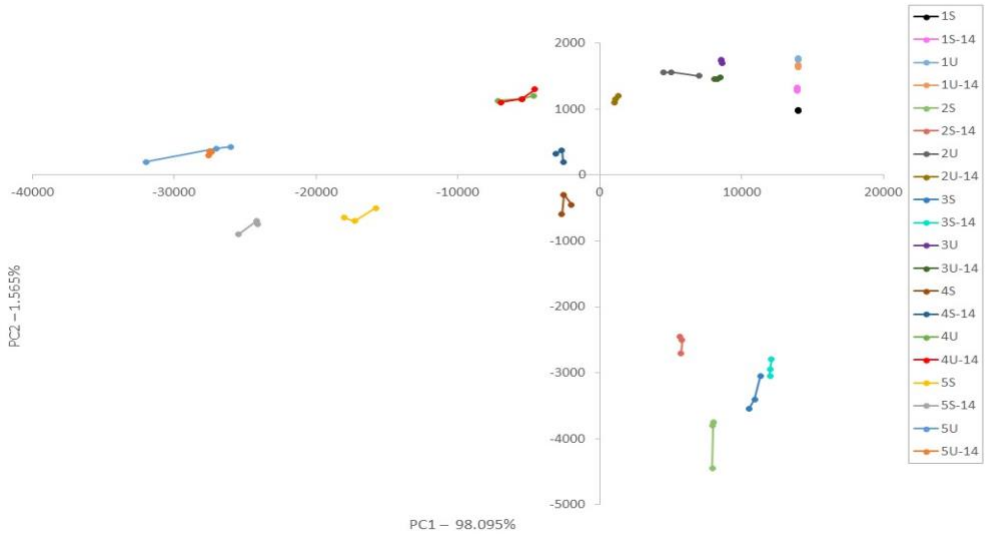

Figure 3 Projection of the samples onto the space defined by the first two principal components (PC1/PC2). Sample groups according to storage conditions: S (smoked steamed cheese) and U (unsmoked steamed cheese) of five Slovak producers (1 – 5).

verification of presented claims for comparison of aroma profiles by GC-MS or GC-FID-O analysis is highly recommended **(Sádecká et al., 2014; Šaková et al., 2015; Sádecká et al., 2016)**.

Samples were stored in the original sealed packages in refrigerator for 14 days, after which their aromatic profiles were determined the similar way as the fresh samples. The stored smoked steamed cheese samples 2S-14, 3S-14 nad 5S-14 were found in the negative scores of PC2 axis, while stored unsmoked samples from the same suppliers (2U-14, 3U-14 and 5U-14) were plotted in the positive PC2 axis 1.53%. There were no differences in the aroma profiles of the samples from the suppliers no. 1 and no.4 (1S-14 and 1U-14, 4S-14 and 4U-14) (PC2).

Evaluation was done by comparing Kovats` retention indices with NIST library (≥50%) and PCA analysis (see Figure 2). In case of stored steamed cheese samples, the number of compounds generating differences in the aromatic profile with discriminant >0.990 increased to 21 as oppose to the fresh ones. In analogy to the fresh samples, similar relationships between samples were observed, with propanal, acetaldehyde, isovaleric acid, 1-hexanol, furfural or α-pinene as predominating compounds. For smoked samples from suppliers no. 2 and no. 3 compounds such as diacetyl, 2-pentanone, 3-heptanone, 2-heptanone, ethyl hexanoate, hexanal and 2-methylpropanal. For stored samples no. 4 and no. 5, except propanoic acid and propanal, 2-methylpropanol was also a significant component of their aroma profiles, yet it was not recorded as significant in the equivalent fresh samples of the same suppliers.

For example, **Majcher et al. (2011)** studied a special type of Polish smoked (1 or 3 days) ewe cheese called Oscypek. Analysed smoked cheese consisted compounds from biochemical reactions (carboxylic acids, alcohols, aldehydes, ketones, esters, sulfur compounds), from smoking (furans/furanones, phenols) and from milk flavour (terpenes). The results of our research, analysing aroma profiles of Slovak steamed cheese by e-nose, confirm the presence of above mentioned compounds (furfural, propanoic acid, diacetyl, ethyl hexanoate, etc.) and compounds alike (2-butanone, propionaldehyde, etc.). **Thomsen et al. (2014)**, examined the aroma profile of seven commercial semi-hard cheese samples by GC-MS and their study is to some extent comparable to the aroma profile (2-propanol, 2-methyl-propanol, 3-methyl butanal, diacetyl, ethyl acetate, ethyl propanoate, ethyl butyrate, propanoic acid) of this work`s steamed cheese samples. **Guarrasi et al. (2017)** examined the effect of starter and non-starter lactic acid bacteria on the aromatic profile of Caciocavallo Palermitano cheese, traditional cheese in Western Sicily. The class of ketones represented a consistent percentage of the volatile compounds, followed by alcohols and esters. 2-butanol, butanoic and hexanoic acids and their esters, diacetyl and 3-hydroxy-2-butanone have been identified as significant compounds of volatile profiles of cheese by lactic acid bacteria. The identified α-pinene from terpenes group is usually introduced to cheese flavour as milk constituents **(Nogueira, Lubachevsky and Rankin, 2005; Majcher et al., 2011)**.

No statistically significant differences (PCA) (p <0.001) in aroma profiles of fresh and stored samples were detected when comparing samples from the same supplier, depicted in Figure 3, except smoked fresh (negative axis PC2) and stored (positive axis PC2) samples from the supplier no. 4.

CONCLUSION

This study for the first time proved possibility of steamed cheese quality evaluation in only a few minutes using e-nose. This simple and rapid method could be implemented also during sensory evaluation by tasters, allowing and granting a fast and inexpensive analysis of the controversial samples.

The results of this research have shown that odours of steamed cheese, obtained from several producers of different geographic Slovak regions, varied only a little in

terms of the main representative aroma substances by means of e-nose detection. Slight differences could be caused due to the use of cows' milk instead of ewe's milk, due to the different geographical area, and also due to the presence of various non-starter and starter dairy bacteria. The 2S and 3S samples had a different aromatic composition compared to other samples, thus opening up the space for evaluation of the cheese smoking effect on the aroma profile of the smoke vapours. For confirmation of submitted conclusions, it is appropriate to measure a larger number of samples and therefore obtain extensive data of the supplied products.

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EFFECT OF EUGENOL, NERIDOL AND PIPERINE FEED SUPPLEMENT ON THE THIGH MUSCLE FAT PROFILE OF BROILER CHICKENS

Mária Angelovičová, Michaela Klimentová, Marek Angelovič

ABSTRACT

The purpose of this study was to investigate of the broiler chicken thigh muscle fat profile after feeding a commercial supplement based on eugenol, nerolidol and piperine applied in feeding mixtures. Broiler chickens Ross 308 were reared in a pen equipped with a straw deep litter and placed into 2 groups. One group was designated as control and the second as experimental. Difference between control and experimental groups was in using of feed supplement in experimental feeding mixtures. Experimental supplement is a commercial powder product which was used in an amount of 10 g per 100 kg of feeding mixtures. Chickens of body weight of 1800.0 g were selected from each group, human killed and technologically processed to carcass. Samples were measured according to Fourier Transform Infrared Spectroscopy (FTIR) using the Nicolet 6700 instrument. Infrared area near middle was chosen for determining fat and fatty acids. Mean fat content was found slightly higher value 1.53 g.100g^{-1} in experimental group opposite 1.49 g.100g^{-1} in control group showing no statistically significant difference (p >0.05). Ratio among saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) was 4.24:5.89:1 in experimental group and 3.75:5.13:1 in control group. Omega-3 PUFAs content was reached 0.54% in experimental group and 0.58% in control group showing no statistically significant (p >0.05). Near-perfect correlation was found between total PUFAs and omega-6 PUFAs as well in the experimental group and control group showing linear, positive and statistically significant relation (p <0.01, p <0.001). Ratio between omega-3 and omega-6 PUFAs was statistically significant (p <0.05) closer in experimental group 1:14.65 opposite ratio 1:16.78 in control group. Conclusion: comparable fat profile in the thigh muscle was achieved, showing no statistically significant difference (p >0.05), in addition to the correlation between total PUFAs and omega-6 PUFAs, which was statistically significant in control (p <0.001) and experimental groups (p <0.01), and statistically significant (p <0.05) closer relation between omega-3 and omega-6 PUFAs in experimental group.

Keywords: feed supplement of eugenol; neridol and piperine; broiler chicken; thigh muscle; fat; fatty acid

INTRODUCTION

We have followed this study for ongoing research at the Slovak University of Agriculture in Nitra, published by **Tkáčová et al. (2015)**, which states, on the basis of the oxidative stability results of chicken meat, that the natural feed component has its justification. This issue requires however, further research. Natural-based products such as, for example, clams, oregano, mint, thyme and cinnamon have been used for many centuries as food preservatives and as medicinal plants, mainly because of their antioxidant and antimicrobial effects. At present, many studies confirm the wide range of properties of these natural resources for public health. Since the ban on the use of feed antibiotics has been the subject of many scientific teams' research. Of these plant products, the aroma of *Syzygium aromaticum* attracted attention mainly because of its strong antioxidant and antimicrobial activity **(Mbaveng and Kuete, 2017)**. **Kardum and Glibetic (2018)** examined natural resources richest on polyphenols. The results show that the spice is a food type with a higher content of polyphenols, followed by fruit, seeds and vegetables. The primacy of the spice is fragrant *Syzygium aromaticum*, which has a higher content of polyphenols and antioxidants. Essential oil of *Syzygium aromaticum* could also be used to influence lipid peroxidation and enzyme activities of catalase, glutathione-S-transferase, peroxidase, polyphenol oxidase and superoxide dismutase **(Afify et al., 2012)**.

Literature review conclusion on Syzygium aromaticum biological effects

On the basis of literary knowledge, *Syzygium aromaticum* is a very interesting plant with enormous potential to use as a food preservative as a rich source of antioxidant compounds. It is proved that its biological effects indicate the development of human and animal drugs and further use in agriculture. This information confirms the use of this plant over the centuries.

Lavender medicine (*Lavandula augustifolia, L. officinalis, L. vera*) contains essential oil, anthocyanins, phytosterols, sugars, minerals, kumaric acid, glycolic acid, valoric acid, ursolic acid, herniarin, coumarin and tallow **(Dănilă et al., 2018)**.

Essential oils from Lavender plants show a wide range of biological activities. The essential oil of *Lavandula dentata* has an inhibitory effect on the growth of bacteria including *Salmonella*, *Enterobacter*, *Klebsiella*, *Escherichia coli*, *Salmonella aureus* and *Listeria monocytogenes*. On the other hand, essential oil of *Syzygium bipinnata* has antibacterial properties against *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus* and *Bacillus subtilis* and antifungal properties against *Aspergillus niger*, *Penicillium notatum*, *Candida albicans* at concentrations from 0.5 to 2.0 $\mu g.mL^{-1}$ for bacteria and from 2.0 to 4.0 $\mu g.mL^{-1}$ for fungi **(Hanamanthagouda et al., 2010)**. Essential oils have antioxidant properties, which protect the cells against the harmful effects of free radicals. Lavender medical essential oil has proven antioxidant activity **(Blažeković et al., 2018)**. **Hui et al. (2010)** demonstrated the inhibitory effect of this essential oil on fat oxidation and lipid peroxidation in the linoleic acid model system. **Angelovič et al. (2015)** state that the mechanisms of oxidative degradation can be autoxidation in presence of atmospheric oxygen. **Chia-Wen et al. (2009)** used organic chemical compound 2.2-diphenyl-1-picrylhydrazyl (DPPH) to study the antioxidant properties of lavender essential oil and especially its ability to inactivate free radicals. The value of 15.18 ±0.009% at a concentration of 5 $g.L^{-1}$ indicated properties comparable to essential oils of lime and marjoram. **Viuda-Martos et al. (2011)** reported a significantly lower ability of essential oil to deactivate free radicals at a similar concentration (4.11%). Studies testing the ability of this essential oil to reduce 50% of DPPH residues resulted in different results ranging from 289 $\mu g.mL^{-1}$ to 48.7 $mg.mL^{-1}$. According to **Pereira et al. (2018)** some essential oils such as linalool and terpineol, have an effect on the central nervous system, weaken the physical activity of humans and animals, reduce anxiety, and ease sleep. In a study of brain waves, it turned out that 40 healthy adults had increased *beta* wave activity, and in mathematical tests they were more successful just after inhalation of essential oil lavender medical. On the other hand, it is reported that patients felt relaxed and showed a positive attitude towards life, but was accompanied by drowsiness **(Lee et al., 2017)**.

Literature review conclusion on *Lavandula augustifolia* biological effects

Experiences from scientific and scientific literature suggest that *L. angustifolia* and its secondary metabolites have a very good biological activity with many uses. Research has confirmed that lavender medicine grown in different regional regions is a valuable plant source with chemical and biological properties applicable in many areas of agriculture, medicine, etc. Current modern medicine should pay attention to the synergistic effect of secondary metabolites of plants and synthetic drugs as they can help solve many problems including microbial resistance to synthetic antibiotics.

Piper nigrum L., the genus *Piperaceae*, is one of the most widely used spices in the world, known for its pungent component piperine. Piperine is the major bioactive compound of *Piper nigrum* and *Piper longum*, which are reported to have immunomodulatory, anti-carcinogenic, antiasthmatic, stimulatory, hepatoprotective, anti-inflammatory, anti-viral and antimicrobial effects **(Abdulazeez et al., 2016)**. The method of storing and processing spices is particularly important because piperine is a key active compound and its effectiveness is easily lost due to exposure to heat, for example, in a variety of domestic culinary treatments. Piperine and its isomers pass light-induced isomerisation to isopiperine, chavidine and isochavine. Isomerisation increases with light intensity and exposure time. Chavicin slowly changes to piperine during storage, leading to a loss of pungency. Piperine inhibits many enzyme biotransformation drug responses, which is an important aspect for the metabolic activation of carcinogens and energy production of mitochondria **(Ezawa et al., 2017)**. Further studies confirm that piperine causes specific effects related to its concentration on mitochondrial bioenergy and energy metabolism enzymes **(Gorgani et al., 2017)**. **Mgbeahuruike et al. (2017)** they concluded that piperine significantly increased pancreatic lipase activity and stimulated pancreatic amylase, trypsin and chymotrypsin. Positive effects on pancreatic digestive enzymes developed by piperine consumed in the diet could be a contributing factor to the well-known digestive stimulating effect of black pepper. The bioavailability and bioactivity of many drugs effectively potentiate piperine. Piperine has the ability to inhibit several enzyme-mediated pathways and biotransformation reactions **(Mhaske, Sreedharan and Mahadik, 2018)**.

Literature review conclusion on *Pepper nigrum* L. biological effects

Black pepper is a rich source of many biologically active ingredients, such as monoterpenes, sesquiterphenes and other volatile compounds. Various health benefits of black pepper and black long spice have been confirmed by cell, animal and human tests. It has been found that it has many beneficial therapeutic applications. It is used as an immunomodulator, stimulant, hepato-protective, anti-inflammatory, anti-carcinogenic, contraceptive, fungicidal, antibacterial and anti-asthmatic material. It has also been found to cause increased bioavailability of nutrients from food, drugs, anti-carcinogenic and phytochemical substances, and stimulates the effect on enzymes metabolizing the drug. It accelerates metabolism and lipid peroxidation. Modern science has demonstrated the molecular basis of the pharmacological properties of black pepper and black long spice against human disease, and some clinical studies have demonstrated the safety and efficacy of the spice in humans.

Based on the above-mentioned issue, the aim of our work was experimental investigation of broiler chicken thigh muscle fat profile after feeding a commercial supplement based on eugenol, nerolidol and piperine applied in feeding mixtures.

Scientific hypothesis

Improving the fat profile of broiler chicken thigh muscles after feeding feed mixtures with the supplement of eugenol, nerolidol and piperine.

MATERIAL AND METHODOLOGY

Experiment was carried out at Poultry farm Zámostie, Slovakia. Broilers chickens were reared in a pen equipped with a straw deep litter. Broiler chickens Ross 308 were placed into 2 groups, each in 50 pcs. One group was designated as control and the second as experimental. The chickens in the pen had a permanent access to water, to the feed and enough place to move. They have secured welfare in accordance with **Council Directive 2007/43/EC**. Experimental period was 42 days divided into three stages from 1 to 14 days as a starter, from 15 to 35 days grower and from 36 to 42 days as a finisher. Difference between control and experimental groups was in using of feed supplement in experimental feeding mixtures. Experimental supplement is a commercial powder product based on eugenol, nerolidol and piperine which was used in an amount of 10 g per 100 kg of feeding mixtures.

Chemical analysis of broiler chickens thigh muscle on fat profile

Prepared of based samples for chemical analysis

Thigh samples were prepared for chemical analysis in accordance with **AOAC 983.18**. Chickens were slaughtered at 42 day of age. Chickens were selected from each group of body weight 1800.0 g, human killed and technologically processed to carcass. The left thigh was separated from each carcass. Each thigh was boneless and clear of skin. The thigh muscle was milled with a laboratory homogenizer. A 50.0 g sample was taken for chemical analysis from each homogenized thigh muscle.

Chemical analysis

Fourier Transform Infrared Spectroscopy (FTIR) was used for chemical analysis of broiler chicken thigh samples. Sample measurement was done using the Nicolet 6700 instrument. Infrared area near middle was chosen for determining fat, saturated fatty acids, polyunsaturated fatty acids, omega-3 polyunsaturated fatty acids and omega-6 polyunsaturated fatty acids.

Statistical analysis

All statistical analyses were computed using the ANOVA procedures of SAS software (version 9.3, SAS Institute, USA). Mean values $(\bar{x})$, standard deviation (SD) and variation coefficient (c_v) are reported in tables. Statistical significance was calculated using t-test. Differences between the treatments were considered significant at $p \leq 0.05$. The Pearson correlation coefficient (r_{xy}) was used to test the relation between the two variables.

According to **Cohen (1988)**, the calculated correlation coefficient value (r_{xy}) is interpreted as follows: r_{xy} over 0.5 is strong dependence, from 0.3 to 0.5 is middle dependence, and from 0.1 to 0.3 is weak dependence, less than 0.1 is trivial (simple) dependence. Correlation, relation between two variables, as near-perfect, is reached in the range of values from 0.9 to 1.0. A very strong correlation is characterized by values ranging from 0.7 to 0.9. The strength of correlation between two variables was statistically tested at a significance level $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

RESULTS AND DISCUSSION

Fat content in broiler chicken thigh muscle

Mean values and statistical analysis for fat content in broiler chicken thigh muscle shows Table 1. Mean fat content in broiler chicken thigh muscle was found slightly higher value 1.53 g.100g^{-1} in experimental group with supplement of eugenol, nerolidol and piperine opposite 1.49 g.100g^{-1} in control group. The difference in fat content in broiler chicken thigh muscle was not statistically significant between groups ($p > 0.05$). Based on the statistical evaluation of the results by standard deviation and coefficient of variation it was found that the measured values of the fat content in the broiler thigh muscle was more even in the control group compared to the measured fat content in the broiler chicken thigh muscle after feeding of the feeding mixtures with a supplement of eugenol, piperine and nerolidol ($SD = 0.33$, $c_v = 21.57\%$ opposite $SD = 0.37$, $c_v = 24.83\%$). Investigation and control of the total fat intake in the ingested food has its own justification. Total fat intake recommendations are based on evidence that indicates consumption outside of these ranges is associated with a greater intake of energy and saturated fatty acids (fat intake >35%) or greater intake in carbohydrate (fat intake <20%); higher intake of carbohydrate leads to increases in plasma triglyceride and reductions in high-density lipoprotein cholesterol levels **(Vannice and Rasmussen, 2014)**. The structure of each fatty acid differs. Individual fatty acids may have unique and specific impacts on health. The impact of specific fatty acids on disease incidence is difficult to elucidate. Chronic disease develops over many months to several years and is the culmination of many genetic and lifestyle factors. This complexity makes randomized controlled trials of dietary interventions largely impractical. Experimental trials, coupled with observational, epidemiological, and mechanistic studies, provide valuable evidence of the human health effects of dietary fat and specific fatty acids **(Mozaffarian, 2008)**.

Ratio among saturated, monounsaturated and polyunsaturated fatty acids in fat of broiler chicken thigh muscle

Mean values for ratio among saturated, monounsaturated and polyunsaturated fatty acids in fat of broiler chicken thigh muscle shows Table 2. Ratio among saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in fat of broiler chicken thigh muscle was 4.24:5.89:1 in experimental group with supplement of eugenol, nerolidol and piperine and 3.75:5.13:1 in control group. Fatty acid composition is a very important component of meat quality and has received considerable interest in view of its implications for human health **(De Smet, Raes and Demeyer, 2004)**.

It is well known that the higher intake of saturated fatty acids in the human diet increases the risk of the development of coronary heart disease, atherosclerosis and cancer **(Mensink and Katan, 1992)**, whereas monounsaturated fatty acid and polyunsaturated fatty acid, especially omega-3, have a number of associated health benefits **(Siriwardhana et al., 2012)**.

Table 1 Mean values and statistical analysis for fat content in broiler chicken thigh muscle.

Group	n	$\bar{x}$, g.100g^{-1}	*SD*	c_v, %	t-test
Control	6	1.49	0.33	21.57	
Experimental with supplement of eugenol, nerolidol and piperine	6	1.53	0.37	24.83	p >0.05

Note: n – multiplicity, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient *of* variation, p >0.05: no statistically significant difference between control and experimental group with supplement of eugenol, nerolidol and piperine.

Table 2 Mean values for ratio among saturated, monounsaturated and polyunsaturated fatty acids in fat of broiler chicken thigh muscle.

	Ratio		
	Saturated fatty acids	Monounsaturated fatty acids	Polyunsaturated fatty acids
Group	n = 6	n = 6	n = 6
Control	3.75	5.13	1
Experimental with supplement of eugenol, nerolidol and piperine	4.24	5.89	1

Note: n – multiciplity, ratio values are calculated from the average values of the fatty acid content.

Table 3 Mean values and statistical analysis for share of omega-3 and omega-6 polyunsaturated fatty acids from total fatty acids in fat of broiler chicken thigh muscle.

Group	n	$\bar{x}$, %	*SD*	c_v, %	t-test
Omega-3 polyunsaturated fatty acids					
Control	6	0.58	0.07	12.07	
Experimental with supplement of eugenol, nerolidol and piperine	6	0.54	0.03	5.55	p >0.05
Omega-6 polyunsaturated fatty acids					
Control	6	9.73	2.28	23.43	
Experimental with supplement eugenol, nerolidol and piperine	6	7.91	1.98	25.03	p >0.05

Note: n – multiplicity, $\bar{x}$ – mean, *SD* – standard deviation, c_v – coefficient *of* variation, p >0.05: no statistically significant difference between control and experimental group with supplement of eugenol, nerolidol and piperine.

Table 4 Correlation relation between polyunsaturated, omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle and statistically significant between two variables in control group.

Indicator	Omega-3 polyunsaturated fatty acids	Omega-6 polyunsaturated fatty acids
Polyunsaturated fatty acids	0.82, p >0.05	1.00, p <0.001
Omega-3 polyunsaturated fatty acids		0.73, p >0.05

Note: Numeric value – correlation coefficient (r_{xy}) between variables, p >0.05: no statistically significant difference between two variables, p <0.001: statistically significant difference between two variables

Table 5 Correlation relation between polyunsaturated, omega-3 a omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle and statistically significant between two variables in experimental group with supplement of eugenol, nerolidol and piperine.

Indicator	Omega-3 polyunsaturated fatty acids	Omega-6 polyunsaturated fatty acids
Polyunsaturated fatty acids	0.29, p >0.05	0.98, p <0.01
Omega-3 polyunsaturated fatty acids		0.28, p >0.05

Note: Numeric value – correlation coefficient (r_{xy}) between variables, p >0.05: no statistically significant difference between two variables, p <0.001: statistically significant difference between two variables.

Table 6 Mean values for ratio between omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle.

Group	Ratio		*SD*	c_v, %	t-test
	Omega-3 polyunsaturated fatty acids, n = 6	omega-6 polyunsaturated fatty acids, n = 6			
Control	1	16.78	2.91	17.34	p <0.05
Experimental with supplement of eugenol, nerolidol and piperine	1	14.65	3.72	25.39	

Note: n – multiplicity.

Share of omega-3 and omega-6 polyunsaturated fatty acids from total fatty acids in fat of broiler chicken thigh muscle

Mean values and statistical analysis for share of omega-3 and omega-6 polyunsaturated fatty acids from total fatty acids in fat of broiler chicken thigh muscle shows Table 3. Omega-3 polyunsaturated fatty acids content was reached 0.54% from total fatty acids in fat of broiler chicken thigh muscle in experimental group with supplement of eugenol, nerolidol and piperine and 0.58% in control group. These values are comparable without statistically significant (p >0.05). A slightly larger difference was found in the omega-6 polyunsaturated fatty acid content between the control group and the experimental group with the supplement of eugenol, nerolidol and piperine, which was not statistically significant (p >0.05). In recent years, there has been interest in the role of poultry meat as a dietary source of long chain omega-3 polyunsaturated fatty acids, including alpha-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:3) and docosahexaenoic acid (DHA, 22:6) **(Dalziel, Kliem and Givens, 2015)**. Observational studies randomized clinical trials, and *in vivo* experimental studies have established the likely magnitude and dose-response of benefits e.g. of fish and fish oil consumption as prevention of coronary heart disease and Sudden cardiac death as well as the effects of omega-3 fatty acids on a wide range of cardiovascular risk factors. The molecular mechanisms underlying these benefits are not well established, and continued experimental investigation is needed to clarify the effects of omega-3 fatty acids on different tissues on ion channels, other transmembrane protein receptors and lipid rafts, endoplasmic reticulum and mitochondrial function, and cytosolic nuclear receptors **(Mozaffarian, 2008)**.

Correlation relation between polyunsaturated, omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle

Correlation relation between polyunsaturated, omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle and its statistically significant between two variables in control group shows Table 4 and experimental group with supplement of eugenol, nerolidol and piperine shows Table 5. Near-perfect correlation relation was found between total polyunsaturated and omega-6 polyunsaturated fatty acids as well in the experimental group with supplement of eugenol, nerolidol and piperine, and control group. This correlation was linear, positive and statistically significant (p <0.01, p <0.001).

Ratio between omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle

Mean values of ratio between omega-3 and omega-6 polyunsaturated fatty acids in fat of broiler chicken thigh muscle shows Table 6. Ratio between omega-3 and omega-6 polyunsaturated fatty acids was statistically significant (p <0.05) closer in experimental group with supplement of eugenol, nerolidol and piperine 1:14.65 opposite ratio 1:16.78 between omega-3 and omega-6 polyunsaturated fatty acids in control group. Adequate intake of omega-3 polyunsaturated fatty acids, a balanced ratio between omega-3 polyunsaturated fatty acids and omega-6 polyunsaturated fatty acids, even a proper ratio between polyunsaturated fatty acids and saturated fatty acids, may reduce the risk of life-style diseases such as coronary artery disease, hypertension, diabetes, and inflammatory and immune disorders **(Zhou et al., 2012)**. There is evidence suggesting that saturated fatty acids have negative consequences on human health whereas polyunsaturated fatty acids have beneficial effects **(Gibbs, Rymer and Givens, 2013)**; polyunsaturated fatty acids should constitute 7% of total energy consumed **(Soriano-Santos, 2010)**.

CONCLUSION

Based on the statistical evaluation of our experimental results with the Ross 308 broiler chickens, we can conclude that by the effect of the biologically active substances of eugenol, nerolidol and piperine as a supplement in feed mixtures, compared to the control group, was achieved:

a) comparable fat profile in the thigh muscle, showing no statistically significant difference (p >0.05),in addition to the correlation between polyunsaturated and omega-6 polyunsaturated fatty acids, which was statistically significant in control (p <0.001) and experimental groups with eugenol, nerolidol, piperine (p <0.01),

b) statistically significant (p <0.05) and closer relationship between omega-3 and omega-6 polyunsaturated fatty acids.

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EFFECT OF WEANING SYSTEM AND TYPE OF MILK FLOW ON MILK PRODUCTION OF CROSSBRED EWES IMPROVED VALACHIAN AND TSIGAI WITH LACAUNE

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ABSTRACT

Improved Valachian (IV x LC; n = 41) and Tsigai (TS x LC; n = 44) crossbred ewes with Lacaune were used to study the effects of three weaning systems on milk production. Prior to parturition, ewes were assigned to one of the following three treatments for the first 53 day of lactation: 1) ewes weaned from their lambs at 24 h postpartum and afterwards machine milked twice daily (MTD), 2) ewes, beginning 24 h postpartum, kept during the daytime with their lambs and allowed them to suckle for 12 h, nights separated from their lambs for 12 h and machine milked once daily in the morning (MIX), and 3) ewes exclusively suckled by their lambs (ES). After the treatment period, lambs were weaned from MIX and ES ewes, and all three groups were machine milked twice daily. Furthermore, ewes were evaluated according to number of live-born and weaned lambs (with one (n = 35) or with two lambs (n = 50)). The measurements of milk yield and milk flow were performed on 110 ±5 day of lactation by the equipment for graduated electronic recording of the milk level in a jar in one-second intervals. No significant differences were observed in the measured values (total milk yield, machine milk yield, latency time, milking time, machine stripping, milk flow rate, and machine milk yield in 30 and 60 s) among weaning treatments and between ewes with one or two lambs and evaluated breeds too. The highest occurrence of one peak milk flow (milk flow without milk ejection) was found out in MTD ewes (50%) compared to MIX (19%) and ES (17%). In conclusion, the different systems of weaning did not influence the milk yield and milk flow parameters in the mid-lactation.

Keywords: ewe; weaning system; milk flow curve

INTRODUCTION

The mammary glands serve to nourish the new-born young in all mammalian species. However, in dairy animals such as the cows, the sheep, and goats, through genetic selection and breeding advances in milking technology, the mammary glands yield far more milk than a new-born young requirement for normal growth and far greater quantities than the original organ was designed to accommodate **(Marnet and Negrao, 2000; Nickerson, 2011)**. Approximately 25% of the total milk yield of a dairy ewe is produced during the first 30 day of lactation **(Folman, Volcani and Eyal, 1966)**; it is the period when lambs are typically allowed to suckle their dams. There are several lambs' weaning systems applied on dairy sheep farms. A mixed-management weaning system of suckling and milking (MIX, allowing suckling only during the day hours and performing once daily machine milking at mornings) is an option for the farmers to obtain milk, which lamb does not need for normal grow **(McKusick, Thomas and Berger, 2001; Dikmen et al., 2007)**. The main disadvantage of the MIX system is low milk fat content **(McKusick, Thomas and Berger, 2001)**. **McKusick et al. (2002)** observed the inhibition of milk ejection during machine milking (i.e. only cisternal milk was obtained) in ewes with MIX system. Moreover, also the inhibition of transfer milk fat was found out, whereas the transfer of milk protein from alveoli to cistern during the separation of MIX ewes from their lambs **(McKusick et al., 2002)**. Another system of weaning are exclusively milking (MTD; (where lambs are weaned at 24 h postpartum and the ewes are machine milked twice daily) and exclusively suckling (ES; during the first 30 to 60 d ewes are only suckled and no milking is performed) **(Marnet and Negrao, 2000; McKusick, Thomas and Berger, 2001; McKusick, 2002; Dikmen et al., 2007; Thomas et al., 2014)**. In Slovakia, the lambs are traditionally suckled until the weaning age of 40 to 60 days without any milking during this period. Due to intense crossing Tsigai and Improved Valachian with Lacaune, the milk production is growing **(Mačuhová et al., 2008; Tančin et al., 2011; Margetín et al., 2013)**. Therefore, it is necessary to optimize the weaning systems of lamb so that the market milk production is as high as possible while maintaining good milk quality. Whether the milk

ejection reflex during milking occurred can be found out by invasive detection of oxytocin release **(Bruckmaier et al., 1997; Marnet, Negrao and Labussière, 1998; Marnet and Negrao, 2000)** or by non-invasive method of recording milk flow during machine milking **(Bruckmaier et al., 1997; Marnet, Negrao and Labussière, 1998; Mačuhová et al., 2012)**. Milk flow kinetic could be a good indicator of stress load under different milking conditions **(Bruckmaier et al. 1997; Tančin et al. 2015)**.

The aim of the trial was to study the effect of three weaning systems on the milkability of ewes. Possible effect of number of lambs, mil flow type and breed was evaluated too.

Scientific hypothesis

In this study, we hypothesized that ewes, which were not suckled by lamb during the first 53 days of lactation, would have a higher milk production at the middle stage of lactation. The second hypothesis was that ewes with two lambs would have a higher production of milk at the middle stage of lactation than ewes with one lamb. The third hypothesis was that breed did not affect the production parameters. The milk flow type affects the production parameters was the fourth hypothesis.

MATERIAL AND METHODOLOGY

The experiment was conducted at the research farm of NPPC- Research Institute for Animal Production Nitra in Trenčianska Teplá. 85 animals of crossbreds Tsigai (50% TS x LC; n = 40) and Improved Valachian (50% IV x LC, n = 45) with Lacaune were included in the experiment. Prior to parturition, ewes were assigned to one of the following three weaning systems for the first day of lactation: 1) ewes weaned from their lambs at 24 h postpartum and afterwards machine milked twice daily (MTD), 2) ewes, beginning 24 h postpartum, kept during the daytime with their lambs and allowed them to suckle for 12 h, nights separated from their lambs for 12 h and machine milked once daily in the morning (MIX), or 3) ewes exclusively suckled by their lambs (ES). After the treatment period of 38 days, lambs were weaned from MIX and ES ewes, and all three groups were machine milked twice daily. Furthermore, the ewes were evaluated according to live - born and weaned lambs (with one (n = 35) or with two lambs (n = 50)). The measurements of milk yield and milk flow were performed on 110 ±5 day of lactation. The ewes were milked in one-platform milking parlour with 24 stalls. The milking machine was set to provide 160 pulsations per minute in a 50:50 ratio with vacuum level of 39 kPa. During each milking, ewes received 0.1 kg concentrate per head in the parlour. Ewes were milked routinely twice daily at 7:00 and 19:00 without any udder preparation. At the end of milking, machine stripping was performed (machine stripping started when milk flow rate declined to 0 $L.min^{-1}$, but not earlier than 70 s from the beginning of milking).

Milk flow recording and samples analysis

Milk flow kinetic was recorded using an electronic jar. Within the jar, there was a 2-wire compact magnetostrictive level transmitter (NIVO-TRACK, NIVELKO Ipari Elektronika Rt, Budapest Hungary) connected to a computer. The milk level was continuously measured by a transmitter that recorded the position of the float in the jar on a computer once per second. The milk flow patterns were drawn by using a formula by **Mačuhová et al. (2008)**. Milk flow rate ($L.min^{-1}$) = (L_n-L_{n-4}) x 15 (where L = recorded milk yield in L, n = time in s, 15 = coefficient to correct milk yield increase in 4 s to milk flow in $L.min^{-1}$). The following milking characteristics were evaluated: total milk yield (L), machine milk yield (L), machine stripping yield (L), milking time (i.e. time from attaching of clusters until the milk flow ceased before stripping; s), milk flow latency (i.e. time from attaching of cluster until start of milk flow 0.006 $L.min^{-1}$; s), peak flow rate ($L.min^{-1}$), machine milk yield in 30 s (l), and machine milk yield in 60 s (L). Milk flow curves were evaluated according to **Marnet, Negrao and Labussière, (1998)** and **Mačuhová et al. (2008)** into 4 milk flow types: one peak (no significant milk flow after 40 s of milking; 1P), 2 peaks (bimodal; 2P), plateau (represents milk flow by ewes with longer duration of steady phase and peak flow rate >0.4 $L.min^{-1}$ without clear differences between peaks 1 and 2; PLI), and plateau low (represents also milk flow curves with steady milk flow during milking but at peak flow rate ≤0.4 $L.min^{-1}$; PLII). In 6 animals, the curve of milk flow was not evaluated due to zero machine milk yield.

Statistic analysis

Data from evening milkings were available for statistical evaluation. The data set consisted of 85 measurements belonging to 85 ewes. Mixed model (Mixed procedure; SAS/STAT 9.1, 2002-2003) was applied to study the influence of the sources of variation in studied traits (milk production and milk emission parameters). The experimental measurements were performed during two years. Therefore, factor YEAR included 2 groups of ewes in model: data obtained during 2012 (n = 52) and 2013 (n = 33). Other factors included: FLOW represented 4 groups of ewes divided according to milk flow type (1P, n = 21 ewes; 2P n = 30 ewes; PLI n = 21 ewes; PLII n = 7 ewes), system of WEANING (only milking n = 22 ewes (MTD); milking/suckling n = 38 ewes (MIX); only suckling n = 25 ewes (ES)), number of LAMBs (single n = 35 ewes, twins = 50 ewes), BREED (Tsigai x Lacaune, (CxLC) n = 40 ewes, Improved Valachian x Lacaune, (IVxLC) n = 45 ewes).

$$y_{ijklm} = \mu + YEAR_i + LAMB_j + WEANING_k + FLOW_l + BREED_m + e_{ijklm}$$

where: y_{ijklm} – individual observations of studied parameters: total milk yield (L), machine milk yield (L), machine stripping (L), machine milking time (s), latency time (s), peak flow rate ($L.min^{-1}$), proportion of machine stripping from total milk yield (%), machine milk yield in 30 s and 60 s.

y_{ijklm} = the measurements of the studied parameters, μ = overall mean, $YEAR_i$ = the fixed effects of year (i = 2012, 2013), $LAMB_j$ = fixed effect of lambs (j = 1 to 2), $WEANING_k$ = fixed effect of weaning systems (k = 1 to 3), $FLOW_l$ = fixed effect of milk flow type (l = 1 to 4), $BREED_m$ = fixed effect of breed (m = 1 to 2.), e_{ijklm} = random error, assuming $e_{ijklm} \sim N(0, I\,\sigma_e^2)$.

Fixed effects of the model were estimated using the LSM (Least Squares Means) method. Statistical significance at the 5% level was tested by Fischer's F-test and differences between the estimated levels of effects were tested by Scheffe's multiple range tests.

RESULTS AND DISCUSSION

In Table 1, there are presented a basic statistics of studied traits. The year of measurement did not affect the evaluated parameters (Table 2, Table 3). The factor breeds did not influence the evaluated parameters except of the machine milk yield. The machine milk yield was significantly higher in IV x LC than TS x LC (0.217 ±0.015 and 0.170 ±0.016 l, resp.; p <0.0260). However, in previous studies **(Mačuhová et al., 2008, 2017)**, there were not found out any significant differences in the machine milk yield. Moreover, high proportion of the machine stripping from total milk yield was recorded in tested crossbreds IV x LC and TS x LC (50 ±3%; and 47 ±3%; resp.). 33% of the animals had a higher proportion machine striping yield from total milk yield than 50% (from 100 to 75% – 11% of animals; from 74.99 to 50% – 22% of animals; from 49.99 to 25% – 43.5% of animals; from 24.99 to 0% – 23.5% of animals).

Table 1 Characteristics of statistical file of studied traits.

Variable	N	Minimum	Maximum	Mean	Std Error
Total milk yield (TMY), L	85	0.055	0.710	0.362	0.015
Machine milk yield (MMY), L	85	0	0.504	0.216	0.013
Machine stripping (MS), L	85	0.03	0.511	0.146	0.010
MS/TMY, %	85	8	100	44	2.480
Milking time, s	85	23	132	66	2.678
Milk flow latency, s	85	8	113	22	2.252
Peak flow rate, L.min^{-1}	85	0	2.205	0.732	0.045
MMY in 30 s, L	85	0	0.42	0.102	0.009
MMY in 60 s, L	85	0	0.417	0.179	0.012

Table 2 Statistical significance (p-values) of tested factors on evaluated parameters.

	Year	Breed	Weaning system	Number of lamb	Milk flow type
Total milk yield (TMY), L	0.7081	0.1885	0.5495	0.4601	0.0007
Machine milk yield (MMY), L	0.5055	0.0260	0.4421	0.6102	<0.0001
Machine stripping (MS), L	0.8827	0.6682	0.5751	0.5982	0.2963
MS/TMY, %	0.3237	0.3277	0.7323	0.9608	<0.0001
Milking time, s	0.3772	0.7915	0.6748	0.9747	<0.0001
Milk flow latency, s	0.376	0.9438	0.6877	0.9116	<0.0001
Peak flow rate, L.min^{-1}	0.5913	0.1574	0.4631	0.2428	<0.0001
MMY in 30 s, L	0.9189	0.0812	0.8635	0.9927	<0.0001
MMY in 60 s, L	0.6053	0.0514	0.7991	0.8099	<0.0001

Table 3 Parameters of milkability of different year evaluation breed, weaning systems and number of lambs.

	Year		Breed		Weaning system			Number of lambs	
	2012	2013	TS x LC	IV x LC	MTD	MIX	ES	One	Two
N	52	33	40	45	22	38	25	35	50
Total milk yield (TMY), L	0.353 ±0.019	0.341 ±0.023	0.328 ±0.022	0.366 ±0.020	0.330 ±0.030	0.341 ±0.021	0.370 ±0.027	0.336 ±0.023	0.353 ±0.019
Machine milk yield (MMY), L	0.201 ±0.014	0.186 ±0.017	0.170 ±0.016^{a}	0.217 ±0.015^{b}	0.172 ±0.021	0.201 ±0.015	0.207 ±0.019	0.188 ±0.017	0.199 ±0.013
Machine stripping (MS), L	0.152 ±0.014	0.155 ±0.017	0.158 ±0.015	0.149 ±0.04	0.157 ±0.021	0.140 ±0.015	0.163 ±0.019	0.148 ±0.016	0.159 ±0.013
MS/TMY, %	46 ±3	50 ±3	67±3	47 ±3	50 ±4	46 ±3	49 ±3	48 ±3	48 ±2
Milking time, s	70 ±3	663	50±3	68 ±3	66 ±4	67 ±3	70 ±4	68 ±3	68 ±3
Milk flow latency, s	24 ±2	28 ±3	26±3	26 ±3	27 ±4	24 ±3	27 ±3	26 ±2	27 ±3
Peak flow rate, L.min^{-1}	0.659 ±0.049	0.618 ±0.059	0.587 ±0.055	0.690 ±0.051	0.573 ±0.075	0.688 ±0.053	0.654 ±0.067	0.600 ±0.058	0.682 ±0.047
MY30, L	0.088 ±0.010	0.089 ±0.012	0.076 ±0.011	0.101 ±0.010	0.083 ±0.015	0.089 ±0.011	0.094 ±0.013	0.088 ±0.012	0.089 ±0.009
MY60, L	0.165 ±0.018	0.154 ±0.015	0.141 ±0.014	0.178 ±0.013	0.150 ±0.019	0.166 ±0.014	0.163 ±0.018	0.157 ±0.015	0.162 ±0.012

Table 4 The effect of milk flow types on milkability of ewes

	Milk flow type			
	Bimodal	One peak	Plateau	Plateau low
N	30	21	21	7
Total milk yield (TMY), l	0.395 ±0.026^{a}	0.316 ±0.029^{ab}	0.424 ±0.030^{a}	0.252 ±0.035^{b}
Machine milk yield (MMY), l	0.266 ±0.019^{a}	0.172 ±0.021^{b}	0.271 ±0.021^{a}	0.064 ±0.052^{b}
Machine stripping (MS), l	0.129 ±0.019	0.144 ±0.021	0.153 ±0.021	0.188 ±0.025
MS/TMY, %	34 ±3^{a}	46 ±4^{a}	36 ±4^{a}	78 ±5^{b}
Milking time, s	72 ±4^{a}	39 ±4^{b}	65 ±4^{a}	94 ±5^{c}
Milk flow latency, s	14 ±3^{a}	17 ±4^{a}	20 ±4^{a}	53 ±4^{b}
Peak flow rate, L.min^{-1}	0.881 ±0.066	0.878 ±0.073	0.639 ±0.075	0.157 ±0.089
MMY in 30 s, l	0.141 ±0.013^{a}	0.129 ±0.014^{ab}	0.078 ±0.015^{b}	0.006 ±0.018^{c}
MMY in 60 s, l	0.220 ±0.017^{a}	0.155 ±0.019^{a}	0.229 ±0.019^{b}	0.034 ±0.023^{c}

Note: ^{a,b,c} The means in the same line without same letter were significantly different at $p \leq 0.05$.

Table 5 Frequency of milk flow types according to weaning system.

Weaning system		Type of milk flow				Total
		Bimodal	One peak	Plateau	Plateau low	
MTD	count	4	10	5	1	20
	% within group	20	50	25	5	
MIX	count	15	7	9	5	36
	% within group	41.67	19.44	25	13.89	
ES	count	11	4	7	1	23
	% within group	47.83	17.39	30.43	4.35	
Total	count	30	21	21	7	79
	%	37.97	26.58	26.58	8.86	

So high values of proportion of the machine stripping from total milk yield have not been detected in these crossbreds so far **(Mačuhová et al., 2008, 2017; Margetín et al., 2013)**. High machine stripping could be due to improper teat position of ewes **(Marnet et al., 1998)**, but this parameter was not evaluated in this study. The impact of the weaning system and the number of lambs on the performance parameters observed is shown in the Table 3. Unlike previous studies **(Dikmen et al., 2007; Thomas et al., 2014)**, it was not found any significant differences in the machine milk yield between different weaning systems. No machine milk yield was observed in 6 animals. Therefore, only 79 milk flow curves were evaluated. All four types of milk flow curves could be observed in the present study as in previous studies testing these crossbreds **(Mačuhová et al., 2012; Mačuhová et al., 2017; Tančin et al., 2011)**. The number and the frequency of occurrence of particular milk flow types are shown in Table 4. The highest occurrence of one peak milk flow was found out in MTD ewes (50%) compared to MIX (19%) and ES (17%). One peak milk flow curves are supposed to represent milk flow without alveolar milk ejection when only cisternal milk fraction is removed in response to machine milking **(Mayer et al., 1989; Bruckmaier et al., 1997)**. On the other side, the proportion of bimodal milk flow in MTD ewes was lower than in ewes of other systems. The milk flow curves with two peaks (bimodal) show alveolar milk ejection after the cisternal milk is removed. In consequence of the genetic selection for higher milk production or decreased average milk flow rate, the occurrence of bimodal milk flow curve has become rarer **(Marnet et al., 1998)** and a third type of milk flow with a plateau phase can be observed. Thus, the second peak is masked because at the time of milk ejection, the cistern fraction has not yet been completely removed from the udder when alveolar fraction descends into cistern for removal **(Marnet et al., 1998)**. Even the second peak is not observed, it is supposed that milk ejection occurs in ewes with this milk flow **(Marnet et al., 1998; Mačuhová et al., 2012; Tančin et al., 2011)**.

The proportion of this milk flow type was quiet similar in all weaning system. Ewes with bimodal and plateau milk flows had the highest machine milk yield (0.266 ±0.019, 0.271 ±0.021, 0.172 ±0.021, 0.064 ±0.052 L in bimodal, plateau, one peak, plateau low; p <0.0001; Table 5). According to **Labussiere (1988)** when ewes are not exclusively machine milked immediately post-partum, the longer they remain in contact with their lambs during the suckling period, the more difficult it is for them to adapt to exclusive machine milking following weaning. And whereas ewes with bimodal and plateau milk flow belong to well-adapted to machine milking **(Marnet et al. 2001)**, it is surprising that, ES ewes had the highest incidence of bimodal and plateau milk flows (Table 5). On the other hand, the release of oxytocin takes longer time during suckling compared to milking **(Marnet and Negrao, 2000)** what can support milk production, and probably the weaning took place at a time when there was no such great the mother-young bond. So, ewes were very well prepared for machine milking. When the milking machine parameters are optimized, and the ewes had time to adapt to the milking routine, oxytocin release patterns are similar during milking as during suckling **(Marnet and Negrao, 2000)**. The fourth, but least occurring milk flow type, was plateau low (9%). This type of milk flow was associated with the longest milking time (94 ± 5 in plateu low, 39 ±4 in one peak, 65 ±4 in plateau and 72 ±4 s in bimodal; p <0.0001, Table 5). According to **Bruckmaier et al. (1997)** this type of milk flow was obviously associated with extremely weak or totally absent oxytocin release during milking. This shape of milk flow in our study was probably due to uneven milk flow distribution in two udder halves, and it cannot be excluded that the milk ejection occurred also in ewes with this type of milk flow.

CONCLUSION

In conclusion, the application of different weaning systems (MTD, MIX, and ES) and the number of lambs had not effect on total milk yield, machine milk yield, machine stripping, and milking time in the middle of lactation. The relatively high ocurrence of bimodal and plateau milk flow curves was observed in ES system. Both milk flow types characterize better-adapted animals to machine milking, because it is assumed that the ewes with these milk flow types achieve milk ejection during milking.

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INVASIVE *SOLIDAGO CANADENSIS* L. AS A RESOURCE OF VALUABLE BIOLOGICAL COMPOUNDS

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ABSTRACT

The phytochemical characteristics of alien species have not yet been fully studied. Meanwhile, the reserves of their raw materials in the secondary distribution range are very large and can be used as new sources of functional ingredients for food, nutraceuticals, cosmeceuticals, and medicines. Particular attention is attracted by species which have closely related native plants that are included in the official pharmacopeia. *Solidago canadensis* L. in Slovakia has already formed powerful thickets, and a similar species *Solidago virgaurea* L. is used as a medicinal plant. The goal of our study is to examine biologically active compounds from leaves and inflorescences of *Solidago canadensis* collected in some invasive populations along the Nitra river and Gron river. Leaves and inflorescences of 3 populations have been taken for analysis. In addition, we tested herbal tea was made by a traditional procedure using 2 types of fermentation. The following parameters have been understudying: total dry matter, ash and protein content, total lipid, saccharides, vitamin C content, total carotenoid content, amino acids content, elemental analysis, and antioxidant activity. Mean values and variations of these parameters are given in the article. The results demonstrated that *S. canadensis* can be a valuable raw material resource for many sectors of the economy with the possibility of its wider application in the future.

Keywords: *Solidago canadensis*; goldenrod; phytochemical analyses; economic value

INTRODUCTION

Valuable phytochemicals with antioxidant, antimicrobial and other health benefits are synthesized by plants in the process of secondary metabolism. Therefore, medicinal and aromatic plants have been used in health care, food, and cosmetics since ancient times. The importance of herbal medicines in the XXI century increases significantly due to the rapid development of functional foods and nutraceuticals, which became the main trend in food science and technology, as well as in nutrition and disease prevention **(Nitrayová et al., 2014; Kraujaliene, Pukalskas and Venskutonis, 2017)**. Many plant species have not been sufficiently studied from the phytochemical point of view. This suggestion applies specially to alien plant species, the phytochemistry of which in the secondary distribution range may differ significantly, due to a change in the soil and climatic conditions during the expansion the secondary distribution range. Evaluation of widespread invasive species will, perhaps, reveal new sources of functional ingredients for food, nutraceuticals, cosmeceuticals, medicines, and other applications. Invasive species can be a valid resource and instead of eliminating them, there could be a great benefit obtained.

Canadian goldenrod (*Solidago canadensis* L.) is a highly aggressive alien plant native to North America. Once established, it can reduce biodiversity or locally out-compete all native plants. It is considered as the worst invasive alien weed in Europe **(Lambdon, 2008; Vinogradova et al., 2010)**. Nowadays it is widely spread in China, Russia (from Kaliningrad to the Far East), Japan, Taiwan, Europe and Australia **(Walck et al., 1999; Weber, 2000; Vinogradova, Mayorov and Choroon, 2010)**. Complex *Solidago canadensis* is a highly variable and its taxonomic status is not clear and difficult to assess **(Semple and Cook, 2006; Melville and Morton, 2011; Vinogradova, Ryabchenko and Mayorov, 2013; Semple et al., 2013; Semple et al., 2015; Vinogradova et al., 2017)**. Invasive *S. canadensis* may acquire spreading advantage in non-native habitat by using "novel weapons" to inhibit not only local plants but also soilborne pathogens **(Zhang et al., 2009)**. Thus, invasion success of *Solidago canadensis* is depended on allelopathic compounds which plant can release **(Abilasha et al., 2008)**. It has been found that acetone extracts of *S. canadensis* showed allelopathic effects on the growth of other weeds **(Solymosi, 1994)**.

Numerous organic compounds were reported for the genus *Solidago*, for example, flavonoids, phenolic acids and glucosides, polysaccharides, diterpenes, triterpenoid saponosides, tannins, and essential oils. These results highlighted the potential of using *S. canadensis* extracts as

a natural antimicrobial and antioxidant substances for food applications **(Deng et al., 2015)**. The flowers were used in traditional medicine as an analgesic, burns and ulcers treatment, febrifuge, gastrointestinal and liver aids **(Vinogradova and Kuklina, 2012; Zihare and Blumberga, 2017)**. This species has been used in traditional medicine as a urological and antiphlogistic medicament **(Apati et al., 2003)**. Many herbal preparations exploited in traditional medicine contain a significant number of polysaccharides or their glycoconjugates. It has been found that polyphenolic–polysaccharide–protein complexes isolated from medicinal plants of *Asteraceae* and *Rosaceae* families showed anticoagulant activity **(Pawlaczyk et al., 2009)**. Special medicals "Goldenrod" with antispasmodic, diuretic and anti-inflammatory effects is produced in Ukraine and Russia. Available for sale "Prostanorm" – for the treatment of prostate diseases, "Marelin" and "Fitolizin" – from urolithiasis **(Vinogradova and Kuklina, 2018)**.

In terms of bioeconomics, large populations of *S. canadensis* are important for honey production **(Botta-Dukat and Dancza, 2008)**. Our earlier data confirm the availability of *Solidago canadensis*: its aerial part containe from 0.1 to 0.7% of essential oil in the leaves and from 0.1 to 0.4% of essential oil in the inflorescens. A-pinene (1.3 – 61.2%), limonene (0.5 – 22.5%), bornyl acetat (3.4 – 29.8%) and germacrene D (1.8 – 39.2%) were the major compounds detected in oil samples of *S. canadensis*. Samples from inflorescences contained the maximal percentage of monoterpene hydrocarbons, while the leaves' samples showed the maximal cumulative percentage of sesquiterpene and monoterpene hydrocarbons **(Shelepova et al., 2018)**. The threats and benefits to food production from *S. canadensis* in the Nitra river basin are described in detail **(Fehér et al., 2016)**.

The aim of this article was to investigate the composition and structural features of the complex isolated from leaves and inflorescence of *S. canadensis*, growing in Slovakia for to assess the possibility of using this species for bioeconomics in the future.

Scientific hypothesis

Worldwide, works are being carried out the estimation of the content and accumulation of compounds during the growth of *Solidago*. The main research is done in the field of extracts. Essential oil is the next largest researches object. The scientific hypothesis of this study was to examine the leaves and inflorescences of *Solidago canadensis* due to its biologically active compounds. They may serve as potential sources of phytochemical compounds in foods and health promoting ingredients for humans.

MATERIAL AND METHODOLOGY

Biological material

Three populations from different regions of Slovakia have been observed: 1) nearby Nitra (N48.3355, E18.0468), 2) along the Gron river nearby Hronovce (N47.9915, E 18.6620) 3) along the Gron river nearby Zvolen (N48.5637, E19.1159). Populations of *Solidago* are very dense and occupy large areas (Figure 1). Thus, there is no shortage of this plant as a biological resource. Material has been dried in the shade, temperature

Figure 1 Thicket of *Solidago canadensis* L. in the Zvolen.

20 – 30 °C. Because of our earlier studies proved the minimal concentration of functional ingredients in stems **(Vinogradova and Kolesnikov, 2007; Akimova, Kolesnikov and Vinogradova, 2008)**, only leaves and inflorescences have been taken for analysis.

Preparation of herbal tea from S. canadensis leaves

The tested herbal tea was made by a traditional procedure. Herbal tea infusions were obtained by air-dried and freeze-drying leaves and activation using 2 types of fermentation – fermentation of leaves after deep freezing and fermentation of fresh leaves.

Chemicals

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and CentralChem (Slovakia).

Phytochemical analyses

Total dry matter, ash and protein content were determined according to EN method **(ČSN EN 12145, 1997)**. Total lipid content was determined according to methods specified in ISO method **(ISO 659:1998)**.

For determination of saccharides, 1 g of sample was extracted with 10 mL of extraction solution (ultrapure water and ethanol mixed in ration 4:1) in a 50 mL centrifugation tube placed on vertical shake table (GFL, Germany). After 1 h of extraction, samples were centrifuged for 4 min at 6000 rpm in a centrifuge (EBA 21, Hettich, Germany); the supernatant was filtered using a filter with 0.45 μm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water. An Agilent Infinity 1260 liquid chromatograph (Agilent Technologies, USA) equipped with ELSD detector was used for determination of saccharides. A Prevail Carbohydrates ES column (250/4.6 mm) was used

as a stationary phase and acetonitrile (VWR) mixed with water in 75:25 volume ratio was used as the mobile phase.

Total carotenoid content expressed as beta-carotene was analysed at a wavelength of 445 nm spectrophotometrically (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water, in purpose to the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained, and the absorbance was measured **(ČSN 560053, 1986)**. HPLC method of L-ascorbic acid (vitamin C) content estimation **(Stan, Soran and Marutoiu, 2014)** was used by the help of Shimadzu HPLC model LC2010 with PDA detector, for separation was used RP C18 column, mobile phase was methanol: water (5:95, v/v), PDA detector was adjusted to 243 nm.

Sample for elemental analysis was prepared using wet ashing method in a microwave oven (Milestone 1200, Milestone, Italy). Total of 0.25 g sample matrix was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha spol. s.r.o., Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha spol. s.r.o., Czech Republic). After the decomposition sample was filtered using a filter with 0.45 μm pore size and filled up to 25 mL in a volumetric flask with ultrapure water. Elemental analysis was performed using ICP-OES (Ultima 2, Horiba Scientific, France) according to the procedure described by **Divis et al. (2015)**.

Amino acids were determined by ion-exchange liquid chromatography (Model AAA-400 amino acid analyser, Ingos, Czech Republic) using post-column derivatization with ninhydrin and a VIS detector. A glass column (inner diameter 3.7 mm, length 350 mm) was filled manually with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size 12 μM and 8% porosity. The column was tempered within the range 35 to 95 °C. Elution of the studied amino acids took place at a column temperature set to 74 °C. A double-channel VIS detector with the inner cell volume of 5 μL was set to two wavelengths: 440 and 570 nm. A solution of ninhydrin (Ingos, Czech Republic) was prepared in 75% v/v methyl cellosolve (Ingos, Czech Republic) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. The prepared solution of ninhydrin was stored in an inert atmosphere (N_2) in darkness at 4 °C. The flow rate was 0.25 mL.min^{-1}. and the reactor temperature was 120 °C.

Antiradical activity

Biochemical analyse of antioxidant activity detection was conducted according to **Brand-Williams, Cuvelier and Berset (1995)**. Plant extracts were prepared in following solvents: methanol, ethanol and distilled water. 1 g of dry powder of plant sample was mixed with 25 mL of each solvent. Extraction was carried out during 12 hours at continuous stirring. Preparing of the radical solution was following: 25 mg of DPPH-radical (2,2-diphenyl-2-picrylhydrazyl) was solved in methanol (in 100 mL volumetric flask) and used for following dilution (1:10). 0.1 mL of investigated plant extract was added to 3.9 mL of a radical solution. The optical density of the radical solution was measured immediately and after 10 min of incubation in the dark after adding a sample. The measurement was conducted at 515 nm on the spectrophotometer (Genesis 20, Germany). Obtained data calculated using a formula:

$$\% \; Inh = \frac{A_0 - A_1}{A_0} x \; 100$$

Statistic analysis

Student *t*-test was used for the statistical analysis of the obtained results. Data are presented as mean ±standard error of the mean (SEM). $p < 0.05$ was considered statistically significant. Significance of $p < 0.05$ and $p < 0.01$ is shown by one and two asterisks, respectively.

RESULTS AND DISCUSSION

Phytochemicals possess various bioactivities and may serve as potential sources of antioxidants in foods and health promoting ingredients in humans. Chemical analyses of *Solidago canadensis* leaves and inflorescences revealed the presence of protein (12.2 wt/wt%), carbohydrates (4 wt/wt%)), lipids (5.1 wt/w %) and inorganic material (6.7 wt/wt%)) (Table 1a and 1b). Monosaccharide analysis of neutral carbohydrate part showed the presence of one main sugar – fructose (8.8 g.kg^{-1}), while other saccharides as maltose, sucrose, and lactose were found in low amounts only (<0.5 g.kg^{-1}). However, this may not always be the case. So, according to **Šutovska et al. (2013)**, *S. canadensis* complex (hot alkaline extraction of flowers) revealed the presence of rhamnose (~23 wt/wt%), arabinose (~20 wt/wt%), galactose (~17 wt/wt%) and glucose (~14 wt/wt%). In our study, we did not detect these monosaccharides.

S. canadensis contains vitamin C (L-ascorbic acid (1.53 μmol.g^{-1})) and beta carotene (93.9 mg.kg^{-1}) (Table 1a and 1b). The vitamin E family includes four tocopherols, α-tocopherol being the most potent member of this family **(Shahidi and Ambigaipalan, 2015)**. The major quantitative tocopherol in *S. canadensis* leaves and inflorescences was α-tocopherol (48.8 ±3.2 mg.kg^{-1} DWP). It is in agreement with **Kraujaliene, Pukalskas and Venskutonis (2017)**. Vitamin E is known as a reducing agent and may contribute to antioxidant activity by reducing the oxidized state of the phenolic antioxidant compounds. Thus, these compounds may be regenerated, allowing their antioxidant capacity to increase. Carotenoids have been reported to show chain-breaking antioxidant activity. These compounds are also effective singlet oxygen quenchers **(Demir, 2009)**.

Lipids are another important group of *S. canadensis* phytochemicals. The oil contents were 5.1% dry weight plant material (Table 1a and 1b). It is reasonable because the majority of botanical materials (leaves and inflorescences) contain low amounts of lipids. The fatty acids composition was comprised of saturated fatty, monounsaturated and polyunsaturated fatty acids (34.0; 14.3 and 44.8 g.100g^{-1} oil, respectively). The lipophilic fraction contains of 20 fatty acids; four acids (linoleic acid, C-18:2; oleic acid, C-18:1; palmitic acid, C-16, linolenic acid, C-18:3) is dominated. Of these acids, the first two, the unsaturated C-18 acids, amounted to 57.2% of the total (Figure 2).

Table 1a The contents of some phytochemical compounds in leaves and inflorescences of *Solidago canadensis* L.

Components	*mean ±SD*	Components	*mean ±SD*
Total dry matter (%)	91.07 ±1.14	**Saturated fatty acids (g.100g^{-1} oil)**	34.0 ±1.0
Total content of protein (%)	12.15 ±0.74	**Monounsaturated fatty acids (g.100g-1 oil)**	14.3 ±0.80
Total content of ash (%)	6.68 ±0.18	**Polyunsaturated fatty acids (g.100g^{-1} oil)**	44.8 ±2.10
Total content of lipids (%)	5.11 ±0.11	**Fructose (g.kg^{-1})**	8.8 ±0.50
Beta carotene (mg.kg^{-1})	93.9 ±5.30	**Maltose (g.kg^{-1})**	<0.50

Note: mean – arithmetic mean; SD – standard error of the mean.

Table 1b The contents of some phytochemical compounds in leaves and inflorescences of *Solidago canadensis* L.

Components	*mean ±SD*
Sucrose (g.kg^{-1})	<0.50
Lactose (g.kg^{-1})	<0.50
Vitamin A (retinyl acetate) (mg.kg^{-1})	<0.10
Vitamin E (α-tocopherol) (mg.kg^{-1})	48.8 ±3.20
L-ascorbic acid (vitamin C) (μmol.g^{-1})	1.53 ±0.01

Table 2 Mineral composition of air-dry leaves and inflorescences *Solidago canadensis* L. (mg.kg^{-1}).

Components	*mean ±SD*	Components	*mean ±SD*	Components	*mean ±SD*
P	4112 ±327	**Mg**	1387 ±71	**Se**	<0.2
K	21573 ±210	**Na**	8.0 ±0.4	**As**	<0.3
Ca	6665 ±78	**Al**	5.6 ±0.2	**Cd**	0.038 ±0.007
S	1585 ±61	**Cr**	<0.2	**Ni**	0.29 ±0.03
Fe	20.0 ±0.9	**Cu**	9.0 ±0.3	**Hg**	0.006 ±0.001
Mn	21.0 ±1.1	**Zn**	28.0 ±1.1	**Pb**	0.15 ±0.03

Note: *mean* – arithmetic mean; *SD* – standard error of the mean.

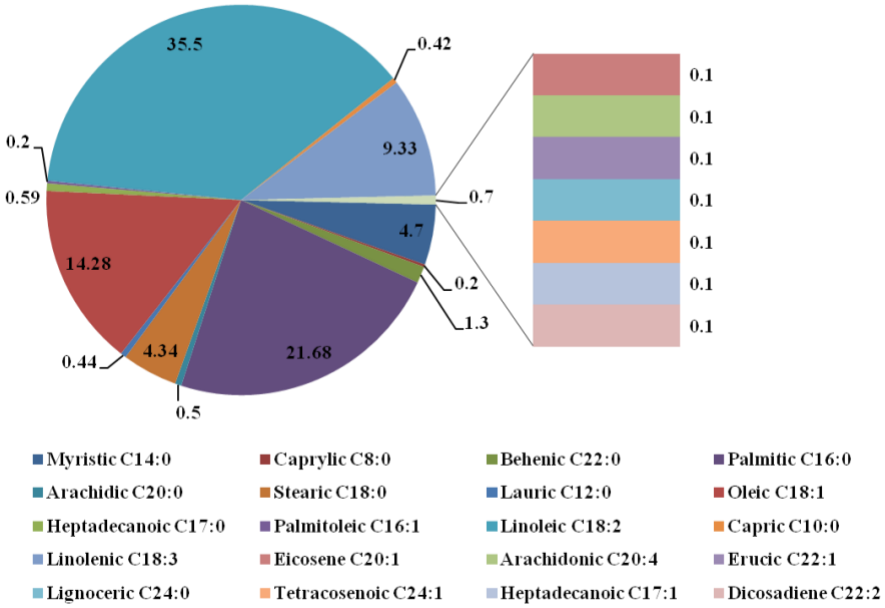

Figure 2 Fatty acid composition from leaves and inflorescences of *Solidago canadensis* L. (g.100g^{-1} oil). Note: Minor components (<0.1): Eicosene C20:1, Arachidonic C20:4, Erucic C22:1, Lignoceric C24:0, Tetracosenoic C24:1, Heptadecanoic C17:1 and Dicosadiene C22:2 are in the right column, their total amount is 0.7 g.100g^{-1} oil.

Table 3 Antioxidant activity of experimental herbal tea from *Solidago canadensis* L. leaves by DPPH method.

Specimen	Methanol extracts, %	Ethanol extracts, %	Water extracts, %
Freeze-drying leaves	54.66 ±1.09	35.67 ±0.48	34.92 ±1.04
Air-dried leaves	83.17 ±0.52	83.78 ±0.62	18.59 ±0.84
Fermentation of fresh leaves	76.32 ±1.15	55.55 ±1.25	66.91 ±2.52
Fermentation of leaves after deep freezing	74.78 ±2.83	56.80 ±0.65	58.20 ±1.05

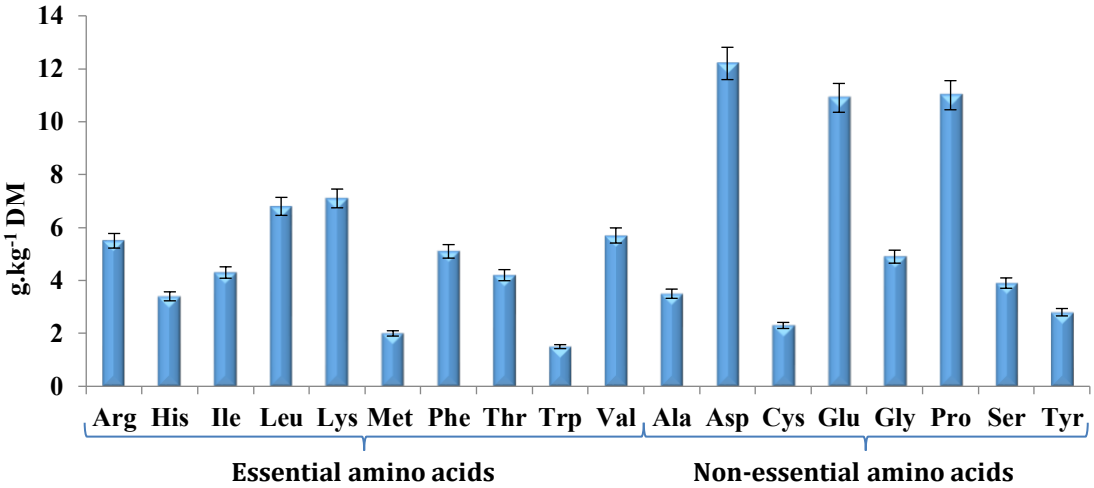

Figure 3 Amino acid composition from leaves and inflorescences of *Solidago canadensis* L. (g.kg^{-1} DM).

S. canadensis fatty acid profiles showed the presence of high amounts of linoleic acid (35.5%) (Figure 2). From linoleic acid is synthesized γ-linolenic acid and is the first intermediate in the conversion of linoleic acid to arachidonic acid. γ-linolenic acid prevents or alleviates a wide variety of human diseases, and it is important as a dietary and cosmetic component. Palmitic acid is the second quantitatively major compound (21.7 %).

Amino acid analysis has shown that the studied *S. canadensis* leaves and inflorescences contained 18 amino acids (10 essential and 8 non-essential) (Figure 3). Aspartic acid was found to be the dominant free amino acid (12.2 g.kg^{-1}) followed by a proline (11.0 g.kg^{-1}) and glutamic acid (10.9 g/kg), respectively. Proline content of *S. canadensis* inflorescences could be one of the parameters in the determination of *S. canadensis* honey authenticity and could be a good indicator of the botanical origin of honey. Amino acids found in high levels were: lysine, leucine, valine, arginine, phenylalanine – average content in from 7.1 to over 5.1 g.kg^{-1}. Lower but also important amounts of glycine, isoleucine, threonine, serine, alanine, histidine, tyrosine, cystine, methionine, and tryptophan (ranging from 1.5 to 4.9 g.kg^{-1}) were detected in the study. The total number of amino acids was 97.10 g.kg^{-1} DM, including total essential amino acids (45.60 g.kg^{-1} DM) and percentage of total essential amino acids (46.96%).

The results of the elemental analysis of *S. canadensis* leaves and inflorescences are summarized in Table 2; these values are averages of three independent measurements having ±SD. Concentration of various elements decreases in the order: K>Ca>P>S>Mg>Zn>Mn>Fe>Cu> Na>Al>Ni>As>Cr>Se>Pb>Cd>Hg.

Among the various elements As, Cr, Se, Pb, Cd, Hg are found to be present at the trace level. Zn, Mn, Fe, Cu, Na, Al, and Ni are at the minor level and K, Ca, P, S and Mg are at the major levels. This result is supported by the results of **Pytlakowska et al. (2012)**.

We note here that our focus is not to compare but rather to explore the mineral composition in the selected plants and to discuss their importance in human health. Due to a deficiency of these minerals in the human diet, most of these minerals are often taken as supplements **(Agarwal et al., 2011)** for their important role in human health.

In fact, the chemical constituents present in plants are responsible for their medicinal as well as toxic properties which include vegetable bases comprising of alkaloids and amines, glycosides, essential oils responsible for their characteristic odour, toxic substances known as toxalbumin, resins, and antibiotics. Whereby the trace elements play a very important role in the formation of these compounds.

One important factor for the formation of active constituents in plants are the trace elements because they are known to play an important role in plant metabolism and active constituents of medicinal plants are metabolic products of plant cells. Among the various elements estimated, the potassium and sodium ions play an important role in the diseases related to renal disorder. Potassium and sodium salts are partially responsible for the diuretic action of some drugs. Potassium is readily excreted by the kidneys both by glomerular filtration and by tubular excretion. Potassium salts act as osmotic diuretics and deficiency of potassium causes diabetic acidosis. Calcium ion concentration also plays an important role in the urinary tract system. Hypercalcemia causes renal failure and calcium stones in the urinary tract. Iron deficiency is common in uremic patients, it causes substantial blood losses. Iron may bind to the dialyzer membrane. Some reports indicate that dysgeusia, poor food intake, and impaired sexual function, which are common problems of uremic patients, may be improved by zinc supplements **(Rajurkar and Damame, 1998)**. Magnesium is reported to have a curative effect in more than 300 health disorders, including headache and fatigue **(Daur, 2015)**. Mn ameliorates some of the symptoms of diabetes and plays a role in the function of connective tissue, bones, and blood clotting factors Similarly Cr, Mn, Fe, Co, Cu, and Zn have been reported as essential or beneficial to human health **(Kozlowska et al., 2015)**.

The total antioxidant activity of extracts from freeze-drying leaves of *S. canadensis* was not high and amounted to 34.9% (aqueous extracts), 35.7% (ethanol extracts) and 54.7% (methanol extracts) (Table 3). While the total antioxidant activity of extracts from air-dry leaves was significantly higher for methanol and ethanol extracts and amounted to 83.2 and 85.8%, respectively. But it was lower for the water extract – 18.6%. The total antioxidant activity of the herbal tea samples from the 2 types of fermentation was generally similar and significantly higher than the

native samples for aqueous extracts (66.9 – 58.2 %). The indices of alcoholic extracts of herbal tea were lower than those for dry leaves but higher than those of native samples (55.6 – 56.8% (ethanol extract) and 74.8 – 76.6% (methanol extract)). **Deng et al. (2015)** determined antioxidant activity from leaves and bark of *S. canadensis* using DPPH radical. The capacity ranged from 25.3 – 37.2% (ultrasound-assisted extraction) to 18.8 – 21.1% (ethanol extraction) by DPPH method. **Demir et al. (2009)** reported results antioxidant capacity of young shoots with leaves for *S. virgaurea* using DPPH method. The antioxidant capacity ranged from 30.7% (aqueous extracts) to 64.3% (methanol extract) of DPPH inhibition.

CONCLUSION

The results demonstrated that *S. canadensis* can be a valuable raw material resource for many sectors of the economy. Many high added value products can be obtained from this species. This proves the value of *S. canadensis* for the bioeconomy and the possibility of its wider application in the future.

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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF SELECTED WILD LEAFY VEGETABLES GROWN IN BANGLADESH: A CHEAPEST SOURCE OF ANTIOXIDANTS

Mohammad Khairul Alam, Ziaul Hasan Rana, Sheikh Nazrul Islam, Mohammad Akhtaruzzaman

ABSTRACT

Nowadays, more attention has been paid on wild plants as new source of natural antioxidants. Therefore, methanolic extracts of 10 traditionally consumed wild leafy vegetables of Bangladesh were analyzed for their total phenolic content (TPC) and free radical scavenging activity. Folin–Ciocalteu method followed by spectrophotometric measurement was used to quantify the TPC of the selected wild leafy vegetables. Free radical scavenging activity was examined utilizing 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Different concentrations of the plant extract were applied to ascertain the dose response relationship in inhibiting DPPH free radical. The results revealed that the TPC ranged from 102.20 to 710.42 mg GAE.100g^{-1} dry weight (DW). The highest TPC was observed in *Bauhinia acuminata* (Shetokanchan) while *Leucas aspera* (Shetodhron) exhibited the lowest TPC among the undertaken vegetables. The studied samples proportionately inhibited DPPH with increasing concentrations. At high concentration (500 µg.mL^{-1}), the percentage inhibition of DPPH radical by plant extract ranged from 68.1 ±2.65% to 93.1 ±1.23%. The highest DPPH radical inhibition was observed in *Bauhinia acuminata* (Shetokanchan) (93.10 ±1.23%), followed by *Commelina benghalensis* (Bat baittashak) (91.97 ±1.31%), *Hydrocotyle sibthorpiodes L.* (Sakumubakla) (91.83 ±2.13%). The lowest DPPH radical inhibition among the studied samples was observed in *Leucas aspera* (Shetodhron) (68.1 ±2.65%). IC_{50} values measured by DPPH assay in this study ranged from 11.64 to 313.79 µg.mL^{-1}. The study findings indicated that the samples under study possesses strong activity against DPPH, and thus could be used as natural antioxidants in the food and/or pharmaceutical industry.

Keywords: antioxidants; Bangladesh; DPPH; wild vegetables; total phenolic content

INTRODUCTION

Antioxidant is termed as a compound whose major function is to inhibit the oxidation of biological molecules (lipids, proteins or other molecules) and hence provides a defensive effect against ROS (Reactive oxygen species) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and singlet oxygen ($_1O^2$) **(Moyo et al., 2013; Brindza et al., 2019)**. These ROS are produced in the body either as a by-product of normal cellular aerobic respiration or exposure to environmental factors such as pollution, radiation, cigarette smoke and herbicides **(Thomas et al., 2010)**.

The production of ROS in a healthy individuals is governed by the antioxidant defense mechanism **(Anahita, Asmah and Fauziah, 2015)**. Overproduction of ROS disrupts the antioxidant defense mechanism in the body and generates oxidative stress as a result of damaging effects on nucleic acids, proteins, enzymes and other biological molecules containing a lipid component of polyunsaturated fatty acids through oxidation **(Alam, Rana and Akhtaruzzaman, 2017a; Alam, Rana and Akhtaruzzaman 2017b; Škrovánková et al., 2018)**. Dietary antioxidant nutrients, which include phenolic acids, polyphenols, flavonoids vitamin E, vitamin C, and carotenoids are believed to scavenge free radicals and thus inhibit the oxidative mechanisms which are responsible for many disorders and diseases in humans such as infections, diabetes, arthritis, cardiovascular diseases, cancer, Alzheimer's diseases, AIDS etc. **(Mendoza-Wilson et al., 2016; Bystrická et al., 2017; Lenková et al., 2017)**.

Antioxidants are generally classified into two categories, synthetic and natural **(Gülçin, 2012)**. However, toxic effects of synthetic antioxidants (fx. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT)) has led to search for an alternative natural antioxidants which have no toxic effects **(Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**. Wild/traditional plants as a source of natural antioxidants

Figure 1 Photograph of selected wild leafy vegetables of Bangladesh.

are recently received much attention from the researchers **(Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**. These plants contain significant amounts of antioxidants, such as polyphenols, flavonoids, terpenoids, vitamin C, vitamin E, selenium, β-carotene, other carotenoids, which play an important role neutralizing free radicals **(Gülçin, 2012; Mendoza-Wilson et al., 2016)**. Several studies have indicated the inverse relationship between intake of traditional plants and chronic diseases **(Wang et al., 2011; Baba and Malik, 2015)**. Thus, increasing intake of these antioxidant rich plants can lower and/or prevent the generation of free radicals associated health problems **(Wang et al., 2011; Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**.

Bangladesh is blessed with a rich biodiversity of plant foods. Indigenous people residing in different areas of the country rely on local plant and plant products to meet their daily requirements for micro and macro nutrients **(Shajib et al., 2013)**. Despite widespread use of wild plants as staple and medicines in Bangladesh, little is known about the antioxidant potential of these plants. Therefore, in this study an effort has been made to study and report the total phenolic content **(**TPC**)** and antioxidant activity of some wild leafy vegetables of Bangladesh consumed by the indigenous community. The preliminary data from these vegetables could be incorporated into food composition database to enrich it and used for increasing awareness to preserve these and maintain biodiversity.

Scientific hypothesis

The content of total polyphenols and antioxidant activity were evaluated in different types of leafy vegetables consumed by specific community of Bangladesh. We presumed that there exist a significant difference with respect to total polyphenol content and antioxidant activity, measured by DPPH method, in different indigenous leafy vegetable species.

MATERIAL AND METHODOLOGY

Reagents

Folin-Ciocaltu reagent and gallic acid were obtained from Sigma–Aldrich Co. **(**St. Louis, MO, USA**)**. The analytical grade acetone, petroleum ether, and methanol were purchased from Merck **(**Darmstadt, Germany**)**.

Sample plan

Muli-region sampling plan was employed for the vegetable sampling. In order to conform the representative sample principle – "what the mass people consume' and from where they collect it"? **(Greenfield and Southgate, 2003)**, the vegetables were collected from the retail markets located at wholesale markets where the vegetables

are arrived from different geographical regions of the country and also from cultivation fields. It was, thus, ensured the representative sample.

Identification of vegetable sample

A taxonomist who also accompanied the collection team, confirmed the sample identity. The identified samples undertaken in this study were *Cajanus cajan, Stereospermum suaveolens, Calamus tenuis, Commelina benghalensis, Enhydra fluctuans, Albizia procera, Tamarindus indica, Hydrocotyle sibthorpiodes, Leucas aspera,* and *Bauhinia acuminate*. Photographs of the studied vegetables are also of given in the Figure 1.

Sample processing

The samples were collected fresh, packed into autoseal poly bags with little water spray and brought to the laboratory for processing and analysis. Two to three samples (250 – 500 g) were collected for each of the items from every market and growing fields, which were then mixed to make three analytes or composite test samples. Samples were first gently washed with tap water to remove sand and other extraneous material before being washed with distilled water. Surface water was removed with tissue paper, air dried, cut into small pieces. The sample was freeze-dried at -48 °C (il Shin lab.Co. Ltd., Korea) and stored in refrigerator for phenolics analysis.

Extraction of phenolics

Approximately two grams of freeze-dried powdered sample was taken into a 250 mL conical flask and 42.5 mL methanol and 7.5 mL 1N hydrochloric acid were added. It was soaked for 24 hours at room temperature with intermittent shaking. Extracts were filtered through No. 1 Whitman filter paper and the filtrate was concentrated using a rotary evaporator at low temperature under reduced pressure. Methanol was added to make a final concentration of 1 $mg.mL^{-1}$ to be used as stock solution.

Analysis of phenolic content

The total phenolics content was estimated by Folin-Ciocalteu colorimetric **(Alam, Rana and Islam, 2016)**. In brief,

150 µL diluted sample extract was added to 225 µL of x2 diluted Folin-Ciocalteu reagent and was kept for 5 minutes at room temperature. Then 1.125 mL of 2% Na_2CO_3 solution was added, mixed well and kept for 15 minutes at room temperature and finally, the absorbance was measured at 750 nm by UV-VIS Spectrophotometer (UV-1800, Shimadzu, Japan). Total phenol content was calculated using a standard curve made by standard gallic acid. Results were expressed as mg gallic acid equivalent (GAE) per 100 g dry weight (DW).

DPPH free radical scavenging assay

The antioxidant activity of the plant extracts was evaluated by utilizing 1,1-diphenyl-2-pycrylhydrazyl (DPPH) free radical according to **Piang-Siong et al. (2017)** with slight modification. Briefly, DPPH stock solution was prepared by dissolving 6.5 mg of DPPH in 5 mL of 100% methanol and protected from light. 100 µL of varying concentrations (100 – 500 $\mu g.mL^{-1}$) of the plant sample extracts were taken and the volume was made up to 200 µL using methanol. Each of the samples was then further diluted with methanol up to 4 mL and to each 200 µL of DPPH stock solution was added. The mixture was then shaken vigorously and allowed to stand at room temperature for 30 min in the dark. After 30 min, the mixture was measured spectrophotometrically at 520 nm (UV-1800, Shimadzu, Kyoto, Japan). Absorbance of the control (200 µL DPPH in 4 mL methanol) and blank (methanol without DPPH) were also measured. The DPPH free radical inhibition capacity was calculated according to the following equation:

$$\% \text{ DPPH inhibition} = ((1 - ((Abs_{sample} - Abs_{blank})/(Abs_{control} - Abs_{blank}))) \times 100$$

where, Abs_{blank} is the absorbance of the blank (containing only methanol), $Abs_{control}$ is the absorbance of the control reaction (containing all reagents minus plant extracts), and Abs_{sample} is the absorbance of the plant extracts. The plant extracts concentration required for 50% inhibition of DPPH free radical (IC_{50}) was estimated from the dose-response graph plotted with percentage inhibition and concentrations of plant extract.

Statistic analysis

Descriptive statistics were performed and values were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was employed to evaluate the differences among varieties for total polyphenol content and antioxidant activity and was declared significant when $p < 0.05$ at 5% level of significance. SPSS (version 20.0 SPSS Inc, IL, USA) was used to analyze the data.

RESULTS AND DISCUSSION

Total phenolic contents of selected wild vegetables

The TPC of selected wild vegetables was analyzed using the Folin–Ciocalteu method, and also presented in Table 1. Generally, these vegetables had high TPC. *Bauhinia acuminata* had the highest phenolic content (710.42 ±4.32 mg $GAE.100g^{-1}$ DW), followed by *Commelina benghalensis* L. (701.33 ±5.01 mg $GAE.100g^{-1}$ DW), *Hydrocotyle sibthorpiodes* L. (633.45 ±3.73 mg $GAE.100g^{-1}$ DW), *Albizia procera* (550.89 ±1.58 mg $GAE.100g^{-1}$ DW), *Cajanus cajan* Millsp. (491.51 ±2.71 mg $GAE.100g^{-1}$ DW). The lowest TPC was found in *Leucas aspera* (102.20 ±1.10 mg $GAE.100g^{-1}$ DW). The TPC in the wild leafy vegetables of the present study ranged from 102.20 to 710.42 mg $GAE.100g^{-1}$ DW. Previous study reported that the TPC of some common Indian leafy vegetables was in the range of 5 – 69.5 mg of tannic $acid.g^{-1}$ of extract **(Shyamala et al., 2005)**. Compared to other studies, the wild vegetables under investigation had higher TPC than some frequently consumed leafy and non-leafy vegetables **(Uusiku et al., 2010; Kaur and Kapoor, 2002; Gupta et al., 2005; Salvatore et al., 2005; Gupta and Prakash, 2009; Mohankumar, Uthira and Maheswari, 2018)**.

Table 1 Total phenolic content of the selected wild leafy vegetables.

Local Name	English Name	Scientific Name	Total phenolic content (mg GAE.100g^{-1} DW)
Orohorpata	Pigeon pea	*Cajanus cajan* Millsp.	491.51 ±2.71^{e}
Parul	Rose flower fragrant	*Stereospermum suaveolens*	405.64 ±2.97^{g}
Bet gach	Korok bet	*Calamus tenuis* Roxb.	340.53 ±2.85^{i}
Bat baittashak	Blue commelina	*Commelina benghalensis* L.	701.33 ±5.01^{b}
Helencha	Buffalo spinach	*Enhydra fluctuans*	435.12 ±3.48^{f}
Shada koroi	Labbec tree	*Albizia procera*	550.89 ±1.58^{d}
Tetul pata	Tamarind leaf	*Tamarindus indica*	380.25 ±2.05^{h}
Sakumubakla	Lawn marsh	*Hydrocotyle sibthorpiodes* L.	633.45 ±3.73^{c}
Shetodhron	Unavailable	*Leucas aspera*	102.20 ±1.10^{j}
Shetokanchan	white orchid-tree	*Bauhinia acuminata*	710.42 ±4.32^{a}

Note: different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Table 1 DPPH free radical scavenging activity of selected wild leafy vegetables.

Local Name	English Name	Scientific Name	% of DPPH$^{\cdot}$ Inhibition at different concentration				
			100	200	300	400	500
Orohorpata	Pigeon pea	*Cajanus cajan* Millsp.	48.24 ±1.05^{b,c}	62.03 ±0.18^{b,c}	75.1 ±2.45^{b,c}	84.66 ±4.37^{a,b}	90.79 ±5.81^{a,b}
Parul	Rose flower fragrant	*Stereospermum suaveolens*	45.55 ±6.08^{b,c}	56.03 ±0.06^{c,d}	70.72 ±0.18^{d}	82.41 ±0.88^{a,b}	89.31 ±1.11^{a,b}
Bat baittashak	Blue commelina	*Commelina benghalensis* L.	53.28 ±1.02^{a,b,c}	66.72 ±1.28^{a,b}	79.34 ±1.05^{a}	85.2 ±1.23^{a,b}	91.97 ±1.31^{a}
Helencha	Buffalo spinach	*Enhydra fluctuans*	49.66 ±2.28^{a,b,c}	63.45 ±1.10^{b,c}	73.48 ±0.88^{c,d}	81.07 ±1.93^{a,b}	90.10 ±0.89^{a,b}
Shada Koroi	Labbec tree	*Albizia procera*	48.10 ±3.50^{b,c}	60.34 ±0.71^{b,c,d}	71.55 ±0.92^{c,d}	87.76 ±3.50^{a}	91.21 ±4.90^{a}
Tetul pata	Tamarind leaf	*Tamarindus indica*	48.10 ±4.40^{b,c}	58.1 ±2.28^{c,d}	63.1 ±1.59^{e}	73.79 ±2.12^{b}	84.62 ±2.81^{a,b}
Sakumubakla	Lawn marsh	*Hydrocotyle sibthorpiodes* L.	56.03 ±0.67^{a,b}	67.38 ±1.05^{a,b}	77.59 ±1.23^{a,b}	84.31 ±1.28^{a,b}	91.83 ±2.13^{a}
Shetodhron	Unavailable	*Leucas aspera*	21.03 ±8.81^{d}	41.55 ±7.11^{e}	50.03 ±2.08^{f}	61.38 ±10.49^{c}	68.1 ±2.65^{c}
Shetokanchan	White orchid-tree	*Bauhinia acuminata*	60.24 ±2.27^{a}	72.66 ±1.58^{a}	80.21 ±0.35^{a}	88.76 ±0.95^{a}	93.1 ±1.23^{a}

Note: different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Secondary plant metabolites, such as aromatic phenolic compounds, are widely distributed throughout the plant kingdom and related with colour, sensory qualities and nutritional and antioxidant attributes of food. The antioxidant activity of phenolic compounds is mainly due to redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxy radical quenchers **(Kaur and Kapoor, 2002)**. Phenolic compounds such as flavonoids, phenolic acid and tannins exerts varied biological activities such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities and these effects could be attributed to their antioxidant activity **(Podsędek, 2007; Mertz et al., 2009)**.

However, it has been reported that the TPC of vegetables varies widely depending on the variety of vegetable, climatic conditions, stage of maturation, soil features, extraction methods, especially the extraction solvent, time and pH **(Kamffer, Bindon and Oberholster, 2010)** and thus makes it difficult for effective comparison, as different standard compounds have been used for their analysis.

Several studies have reported an inverse relation between flavonoid intake and risk of lung cancer, cardiovascular diseases **(Wang et al., 2011; Bystrická et al., 2017; Lenková et al., 2017; Brindza et al., 2019)** and biomarkers of inflammation **(Shaik et al., 2006)**. Hence, the consumption of such wild vegetables, rich in phenolic compound, could ameliorate or prevent the generation of chronic diseases.

DPPH free radical scavenging activity of selected wild vegetables

The DPPH free radical assay is an easy, reliable and quick way to evaluate antioxidant potential of various extracts **(Piang-Siong et al., 2017)**. This method is simple and do not call for a special reaction and device. In this method, the antioxidant activity is assessed on the basis of the ability of the antioxidant to donate hydrogen or electron to $DPPH^{\cdot}$ to produce a stable DPPH-H (diphenylhydrazine) molecule. Changes in the color occur as DPPH radicals in the environment decreases and this **(Piang-Siong et al., 2017)**. Hence the more potent antioxidant, more decrease in absorbance is seen and accordingly the IC_{50} value will be minimum.

$$DPPH^{\cdot} + A\text{-}H = DPPH\text{-}H + A^{\cdot}$$

In this study, the extracts of the undertaken vegetable samples were assessed for the antioxidant potential by utilizing the above principle of DPPH radical scavenging method. Table 2 represents the $DPPH^{\cdot}$ radical scavenging abilities of the wild vegetables used in this study. At 500 µg.mL^{-1}, *Bauhinia acuminata* (Shetokanchan) showed the highest DPPH inhibition (93.10 ±1.23%), followed by *Commelina benghalensis* (Bat baittashak) (91.97 ±1.31%), *Hydrocotyle sibthorpiodes L.* (Sakumubakla) (91.83 ±2.13%), *Albizia procera* (Shada Koroi) (91.21 ±4.90%), *Cajanus cajan* (Orohorpata) (90.79 ±5.81%). The lowest DPPH radical inhibition among the studied samples was observed in *Leucas aspera* (Shetodhron) (68.1 ±2.65%) compared to other samples studied. In this study, all extracts prepared from the vegetable leaves contain varying degrees of DPPH scavenging activity. At a higher concentration, these extracts exhibit more significant DPPH free radical scavenging activity. From Table 2, we can also observe that there is a dose response relationship in inhibiting $DPPH^{\cdot}$ free radical.

Fabaceae species are one of the several indigenous vegetables that have been reported to be rich in antioxidant compounds **(Gođevac et al., 2008)**. As stated, this was the first study to evaluate the antioxidant capacities of the undertaken wild vegetables, no reference data was available to compare. Thus, only few studies could be found examining the other members of the same species. In a study by **Gođevac et al., (2008)** using nine different Fabaceae species, they found that the Fabaceae species possess good antiradical activity. Similar results was also reported by **Srinivasan et al. (2016)**. Similar to our study, few other studies have also reported that the *Tamarindus indica*, *Commelina nudiflora* L., *Bauhinia variegata*, *Leucas aspera* exhibited potent DPPH free radical scavengers **(Rajani and Ashok, 2009; Das et al., 2011; Chew et al., 2012; Kuppusamy et al., 2015; Mbaye et al., 2017)**. It is speculated that the antioxidant activity of the extract is attributed to the presence of phenolic components **(Rajani and Ashok, 2009; Das et al., 2011; Chew, Jessica and Sasidharan, 2012; Kuppusamy et al., 2015; Mbaye et al., 2017)**.

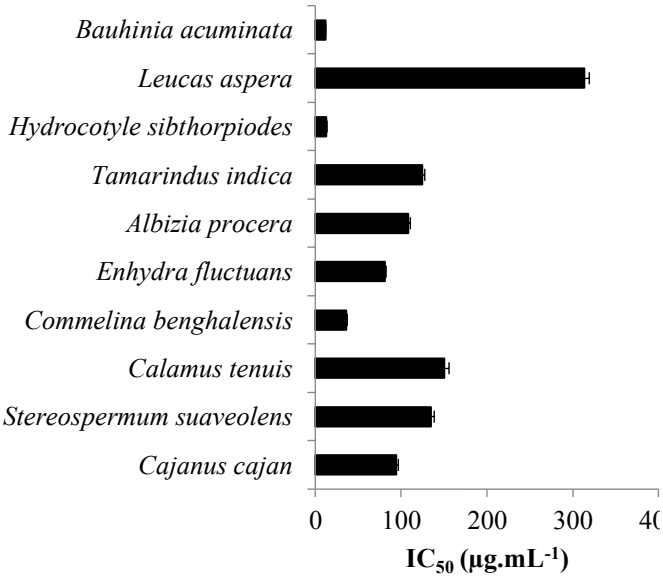

Figure 2 IC_{50} values of selected wild leafy vegetables of Bangladesh.

The DPPH radical scavenging activity was further expressed as the effective concentration (IC_{50}) at which antioxidant activity was 50% (Figure 2). The lowest IC_{50} values for the vegetables were recorded of *Bauhinia acuminata* (11.64 µg.mL^{-1}), *Hydrocotyle sibthorpiodes* L. (12.78 µg.mL^{-1}), *Enhydra fluctuans* (81.19 µg.mL^{-1}), *Cajanus cajan* (94.28 µg.mL^{-1}), whereas *Leucas aspera* (313.79 µg.mL^{-1}) exhibited the highest IC_{50} value in all the studied samples.

The IC_{50} values observed in this study were varying from other reports **(Gođevac et al., 2008; Rajani and Ashok, 2009; Das et al., 2011; Chew, Jessica and Sasidharan, 2012; Kuppusamy et al., 2015; Srinivasan et al., 2016; Mbaye et al., 2017)**. Ethanolic extract of *L. aspera* root exhibited a significant DPPH radical scavenging activity having an IC_{50} value of 7.5 µg.mL^{-1} **(Rahman, Sadhu and Hasan, 2007)** whereas we found 313.79 µg.mL^{-1} in methanolic extract of the leaves. Moreover, the ethanolic extract of whole *L. aspera* plant showed an IC_{50} value of 176.46 mg.mL^{-1} **(Das et al., 2011)**. This difference in scavenging activity could be due to the different solvent extract system, growing conditions, fractions of the plant and some other variables **(Rahman Sadhu and Hasan, 2007; Chew, Jessica and Sasidharan, 2012)**.

CONCLUSION

The selected wild plant extracts investigated in this study showed potent antioxidant activity and varying degree of phenolic content. Among the studied samples, *Bauhinia acuminata* (Shetokanchan) exhibited the highest phenolic content (710.42 mg GAE.100g^{-1}) and free radical scavenging activity (up to 93.1 ±1.23% inhibition). As the plant extracts are quite safe and the use of synthetic

antioxidant has been limited because of their toxicity, therefore, these wild vegetables could be employed as antioxidant additives or as nutritional supplements. However, further studies are required to isolate and characterize the individual components from these plants which are actually responsible for their antioxidant activities and develop their applications for food and pharmaceutical industries.

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GENETIC DIVERSITY IN TUNISIAN CASTOR GENOTYPES (*RICINUS COMMUNIS* L.) DETECTED USING RAPD MARKERS

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ABSTRACT

Castor (*Ricinus communis* L.) is a plant that is commercially very important to the world. It is produced in about 30 countries lying in the tropical belt of the world. It is an important plant for production of industrial oil. Assessment of genetic diversity of a crop species is a prerequisite to its improvement; hence it is important to identify the genetic diversity of castor genetic resources for development of improved cultivars. The present study is focused on estimation of genetic distance between 56 Tunisian castor genotypes, based on 18 RAPD markers. Seeds of castor were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. The ricin genotypes were obtained from 12 regions of Tunisia. The efficacy of the RAPD technique in this study is further supported by the obtained PIC values of the primers used in the analysis. PCR amplification of DNA using 18 primers for RAPD analysis produced 145 DNA fragments that could be scored in all 56 genotypes of Tunisian castor. The number of amplified fragments varied from 3 (OPE-07) to 13 (SIGMA-D-01), and the amplicon size ranged from 100 to 1500 bp. Of the 145 amplified bands, 145 were polymorphic, with an average of 8.11 polymorphic bands per primer. The lowest values of polymorphic information content were recorded for RLZ 9 (0.618) and the the highest PIC values were detected for OPD-08 (0.846) with an average of 0.761. A dendrogram was constructed from a genetic distance matrix based on profiles of the 18 RAPD primers using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 56 Tunisian castor genotypes were clustered into five main clusters. Genetically the closest were four genotypes from cluster 1 (BT-1 – S-5 and K-1 – N-3). Knowledge of the genetic diversity of castor can be used in future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

Keywords: castor; DNA; dendrogram; PCR; PIC

INTRODUCTION

Castor bean (syn. castorbean, castor, castor-oil-plant), *Ricinus communis* L. (2n = 20, X = 10), is a species of flowering plant in the spurge family, *Euphorbiaceae*. It is an oilseed crop cultivated mainly in India, Mozambique, Brazil, and China **(FAOSTAT, 2014)**.

Genetic diversity in a germplasm is the fundamental requirement for crop improvement programs. There are several genetic markers available for assessment of genetic diversity among the genotypes and accessions **(Kole and Rabinowicz, 2018)**. Though castor bean is a monotypic, it exhibits wide phenotypic diversity. In castor bean, genetic markers such as agro-morphological characters, biochemical and cytological markers were widely used in characterization of genetic variation in the germplasm **(Kole and Rabinowicz, 2018).**

Since 1990, random amplified polymorphic DNA (RAPD) markers have been successfully applied for identification of DNA polymorphism in various plant species **(Williams et al., 1990)**. They are often used for screening of a wide range of genetic stocks in order to find linkage with traits of agronomic significance **(Masojć, Myśków and Milczarski, 2001)**. Genetic diversity in castor bean was assessed by using both dominant and codominant molecular markers (random amplified polymorphic DNA, RAPD) **(Reddy, Nadigatla and Mulpuri, 2015; Vivodík et al., 2015a)**, inter-simple sequence repeats (ISSR) **(Wang et al., 2013; Vasconcelos et al., 2016)**, start codon targeted (SCoT)

(Kallamadi et al., 2015; Reddy, Nadigatla and Mulpuri, 2015), amplified fragment length polymorphism (AFLP) **(Allan et al., 2008; Quintero et al., 2013)**, simple sequence repeat (SSR) **(Gálová et al., 2015; Rukhsar et al., 2017)**, expressed sequence tag-simple sequence repeats (EST-SSR) **(Kanti et al., 2015; Wang et al., 2017)**, and random microsatellite amplified polymorphic DNA (RMAPD) **(Dong et al. 2012)**, and also advanced molecular markers, such as single nucleotide polymorphism (SNP) **(Foster et al. 2010)**, sequencerelated amplification polymorphism (SRAP) **(Lu et al., 2010)**, target region amplification polymorphism (TRAP) **(Simões et al., 2017a)**, and methylation-sensitive amplification polymorphism (MSAP) **(He et al., 2017)**. The polymerase chain reaction (PCR) has been used by many authors, such as **Žiarovská et. al., (2015)**; **Vyhnánek et. al., (2015)**; **Bošeľová and Žiarovská (2016)**; **Ražná et. al., (2016)**; **Žiarovská et. al., (2017)**; **Simões et. al., (2017b); Žiarovská et. al., (2018)**; **Ansari et al., (2018); Balážová et al., (2018); El-Fiki and Adly, (2019).**

Scientific hypothesis

The present study is focused on estimation of genetic distance between 56 Tunisian castor genotypes, based on 18 RAPD markers.

MATERIAL AND METHODOLOGY

Fifty-six castor (*Ricinus communis* L.) genotypes were used in the present study. Seeds of castor were obtained from the University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), Regional Station of Gabès, Tunisia. The ricin genotypes were obtained from 12 regions of Tunisia: S- Souassi (5 genotypes), BT- Bouthay (4 genotypes), GH- Ghomrassen (5 genotypes), BA- Sidi bou ali (5 genotypes), MT- Matmata (4 genotypes), AG- Mateur (5 genotypes), N- Nefza (4 genotypes), MD- Mednine (5 genotypes), M- Mornag (5 genotypes), G- Gabes (4 genotypes), K- Kebili (5 genotypes), KJ- Ksar jedid (5 genotypes). Genomic DNA of castor cultivars was extracted from leaves of 14-day old plantlets with GeneJET Plant Genomic DNA Purification Mini Kit according to the manufacturer's instructions. DNA concentrations were estimated by UV-Vis spectrophotometer Q5000, Quawell.

Figure 1 Photo of *Ricinus communis* L. (Spanishalex, Dreamstime.com)

Amplification of RAPD fragments was performed according to **Gajeraa et al. (2010)** using decamer arbitrary primers (Table 1). Amplifications were performed in a 25 μL reaction volume containing 100 ng of DNA, 12.5 μL of Master Mix (Genei, Bangalore, India) and 10 pmol of primer. Amplification was performed in a programmed thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified products were electrophoresed in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system.

Statisic analysis

A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed. For the assessment of the polymorphism between genotypes ricin and usability RAPD markers in their differentiation we used polymorphic information content (PIC) **(Weber, 1990)**.

RESULTS AND DISCUSSION

In the present study, the representatives of the genus *Ricinus communis* collected from 12 different parts of Tunisia were differentiated by the DNA fingerprinting patterns using 18 RAPD primers. The efficacy of the RAPD technique in this study is further supported by the obtained PIC values of the primers used in the analysis. PCR amplification of DNA using 18 primers (Table 1) for RAPD analysis produced 145 DNA fragments that could be scored in all 56 genotypes of Tunisian castor (Figure 2). The number of amplified fragments varied from 3 (OPE-07) to 13 (SIGMA-D-01), and the amplicon size ranged from 100 to 1500 bp. Of the 145 amplified bands, 145 were polymorphic, with an average of 8.11 polymorphic bands per primer. Results indicated the presence of wide genetic variability among different genotypes of Tunisian castor. To determine the level of polymorphism in the analysed group of Tunisian castor genotypes polymorphic information content (PIC) was calculated. The lowest values of polymorphic information content were recorded for RLZ 9 (0.618) and the the highest PIC values were detected for OPD-08 (0.846) with an average of 0.761.

A dendrogram was constructed from a genetic distance matrix based on profiles of the 18 RAPD primers using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 56 Tunisian castor genotypes were clustered into five main clusters (Figure 3). Cluster 1 contained 10 genotypes of castor from different regions of Tunisia and cluster 2 contained 10 genotypes of castor from different regions of Tunisia. Cluster 3 contained 6 genotypes of Tunisian castor and cluster 4 contained 17 genotypes of Tunisian castor and cluster 5 contained 13 genotypes of tunisian castor. Genetically the closest

were four genotypes from cluster 1 (BT-1 – S-5 and K-1 – N-3) (Figure 2).

Dhingani et al. (2012) used 25 RAPD primers in this study. Amplification of genomic DNA of 8 genotypes, using RAPD analysis, yielded 92 fragments, of which 72 were polymorphic, with an average PIC value of 0.29. Number of amplified fragments with RAPD primers ranged from 4 to 13, with the size of amplicons ranging from 100 to 2650 bp in size. The polymorphism ranged from 54.54 to 100.0, with an average of 79.54 percent. The objective of work **Machado et al. (2013)** was to identify genetically different cultivars of castor bean (*Ricinus communis*) using RAPD markers. A total of 58 RAPD primers were used to genotype 15 cultivars. The genetic dissimilarity between cultivars was calculated by Jaccard's index, using the unweighted pair-group method with arithmetic mean (UPGMA). Five hundred and fifty-two fragments were identified, of which 311 were polymorphic (56.3%). The cultivars were clustered in five groups, evidence that there is genetic difference among them. RAPD markers are efficient in the study of genetic dissimilarity in castor bean. The aim of the present study of **Lakhani et al. (2015)** was to study the molecular diversity for varietal identification and phylogenetic relationships among thirteen castor genotypes and identify those with distinct DNA profiles. Twenty-seven RAPDs primers were used, out of which 16 polymorphic primers revealed 100% polymorphism among the castor genotypes. Dendrogram was constructed using UPGMA method which revealed distinct clusters. Values of the polymorphic information content (PIC) value ranged from 0.423 to 0.883 with an average of 0.705. This work of **Tomar Rukam et al. (2014)** investigated the fingerprinting and phenotyping of 25 castor genotypes available in Gujarat and other States of India. An integrated approach based on the exploitation of morphological traits and molecular markers, such as RAPD and ISSR fingerprints was employed. Morphological trait analysis and statistical analysis of markers were useful for reconstructing a castor varietal dendrogram. The results of the morphological and molecular analyses allowed us to confirm a remarkable differentiation among castor genotypes. The UPGMA dendrogram obtained using morphological characters clearly separated the 25 genotypes of castor into three groups.

The aim of study **Vivodík et al. (2015a)** was to assess genetic diversity within the set of 111 ricin genotypes using 13 RAPD primers. For differentiation of 111 ricin genotypes 13 RAPD primers were used. Amplification of genomic DNA of 111 genotypes using RAPD analysis yielded 102 fragments, with an average of 7.85 polymorphic fragments per primer. Number of amplified fragments with RAPD primers ranged from 3 to 13, with the size of amplicons ranging from 100 to 1500 bp. The polymorphism information content (PIC) value ranged from 0.491 to 0.898 with an average of 0.764 and diversity index (DI) value ranged from 0.576 to 0.900 with an average of 0.776. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. In dendrogram separated unique genotype RM-32 from other 110 genotypes which were further grouped into 3 subclusters (1, 2, 3). Only four genotypes were not distinguished. The aim of work **Vivodík et al. (2015b)** was to detect genetic variability among the set of 32 castor genotypes using five random amplified polymorphic DNA (RAPD) markers. Amplification of genomic DNA of 32 genotypes, using RAPD analysis, yielded 41 fragments, with an average of 8.20 polymorphic fragments per primer. Number of amplified fragments ranged from 5 to 11, with the size of amplicons varied from 100 to 1200 bp. The polymorphic information content value ranged from 0.598 (RLZ 9) to 0.811 (RLZ 6) with an average of 0.746 and diversity index value ranged from 0.557 (RLZ 9) to 0.889 (RLZ 7) with an average of 0.784. The dendrogram based on hierarchical cluster analysis using unweighted pair group method with arithmetic average algorithm was prepared. The aim of work **Balážová, Vivodík and Gálová (2016)** was to detect genetic variability among the set of 30 castor genotypes using 6 RAPD markers. Amplification of genomic DNA of 30 genotypes using RAPD analysis yielded 50 polymorphic fragments with an average of 8.33 fragments per primer. Number of amplified fragments varied from 5 (RLZ7) to 11 (RLZ8) and the amplicon size ranged from 330 to 1200 bp. All 50 amplified bands were polymorphic. The polymorphic information content (PIC) values ranged from 0.774 (RLZ7) to 0.870 (RLZ8) with an average of 0.825 and index diversity (DI) value ranged from 0.786 (RLZ7) to 0.872 (RLZ8) with an average of 0.831. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared. Dendrogram separated ricin genotypes into three main clusters. Two genotypes (RM-72 and RM-73) were genetically the closest. Knowledge on the genetic diversity of castor can be used for future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production. **Vivodík et al. (2015c)** analyzed seventeen castor genotypes for genetic variability using Random Amplified Polymorphic DNA (RAPD) markers. Thirteen polymorphic RAPD primers amplified 102 DNA fragments, with an average of 7.85 fragments per primer. Number of amplified fragments ranged from 3 (OPE-07) to 13 (SIGMA-D-01), with the size of amplicons ranging from 100 to 1200 bp. The polymorphic information content (PIC) value ranged from 0.450 (OPE-07) to 0.892 (SIGMA-D-01) with an average of 0.771 and diversity index (DI) value ranged from 0.551 (OPE-07) to 0.894 (SIGMA-D-01) with an average of 0.787. The dendrogram based on hierarchical cluster analysis using UPGMA algorithm was prepared and analyzed genotypes were grouped into two main clusters and only two genotypes (RM-5 and RM-23) could not be distinguished.

Table 1 Statistical characteristics of the RAPD markers used in Tunisian castor genotypes.

Sr. no.	Primers	Primer sequence (5´-3´)	Molecular weight range (bp)	Total number of bands	PIC value
1.	OPA-02	TGCCGAGCTG	200 – 1000	7	0.729
2.	OPA-03	AGTCAGCCAC	100 – 800	9	0.652
3.	OPA-13	CAGCACCCAC	100 – 1500	7	0.780
4.	OPB-08	GTCCACACGG	200 – 800	8	0.715
5.	OPD-02	GGACCCAACC	200 – 1000	6	0.816
6.	OPD-07	TTGGCACGGG	100 – 900	8	0.714
7.	OPD-08	GTGTGCCCCA	200 – 600	7	0.846
8.	OPD-13	GGGGTGACGA	100 – 1500	12	0.810
9.	OPE-07	AGATGCAGCC	300 – 800	3	0.825
10.	OPF-14	TGCTGCAGGT	200 – 1200	5	0.812
11.	SIGMA-D-01	AAACGCCGCC	100 – 1200	13	0.731
12.	SIGMA-D-14	TCTCGCTCCA	200 – 1000	7	0.710
13.	SIGMA-D-P	TGGACCGGTG	200 – 1500	10	0.717
14.	RLZ 6	GTGATCGCAG	200 – 1500	12	0.794
15.	RLZ 7	GTCCACACGG	100 – 1000	10	0.790
16.	RLZ 8	GTCCCGACGA	200 – 1200	7	0.808
17.	RLZ 9	TGCGGCTGAG	200 – 1200	7	0.618
18.	RLZ 10	ACGCGCATGT	100 – 1500	8	0.832
		Total	-	146	
		Average	-	8.11	0.761

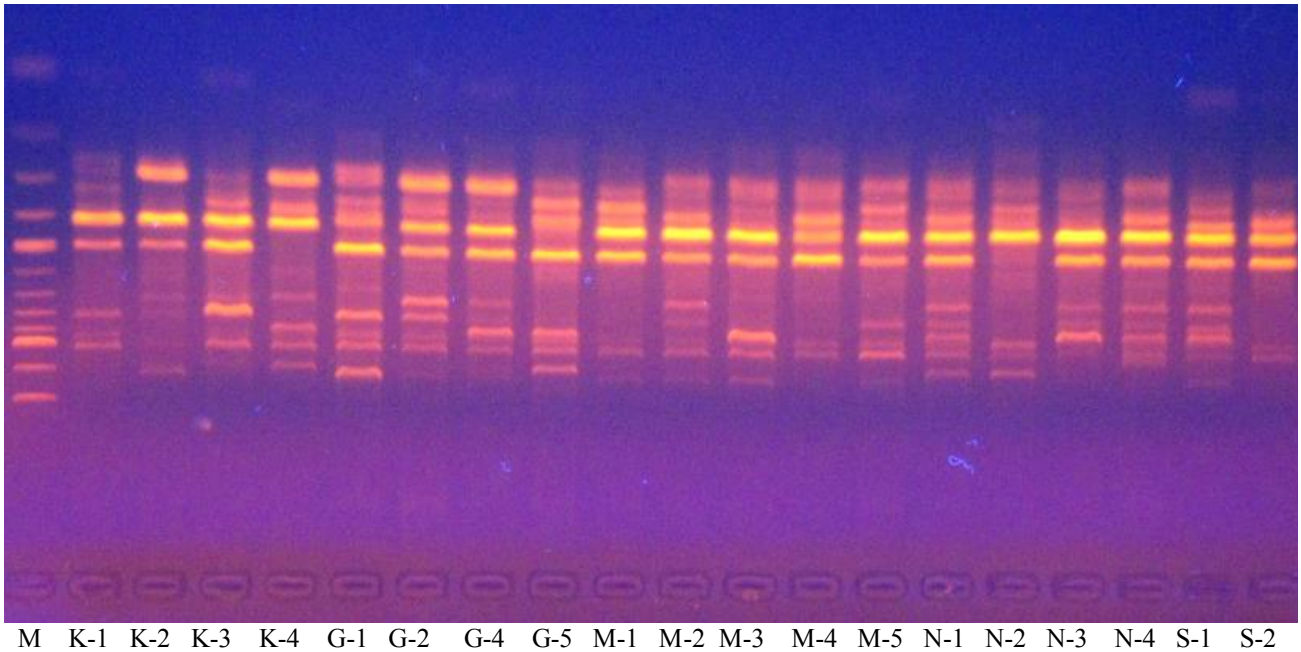

Figure 2 PCR amplification products of 19 genotypes of Tunisian castor produced with primer OPA-02.
Note: Lane M is Quick-Load® 100 bp DNA ladder and lanes 1-19 are Tunisian castor genotypes.

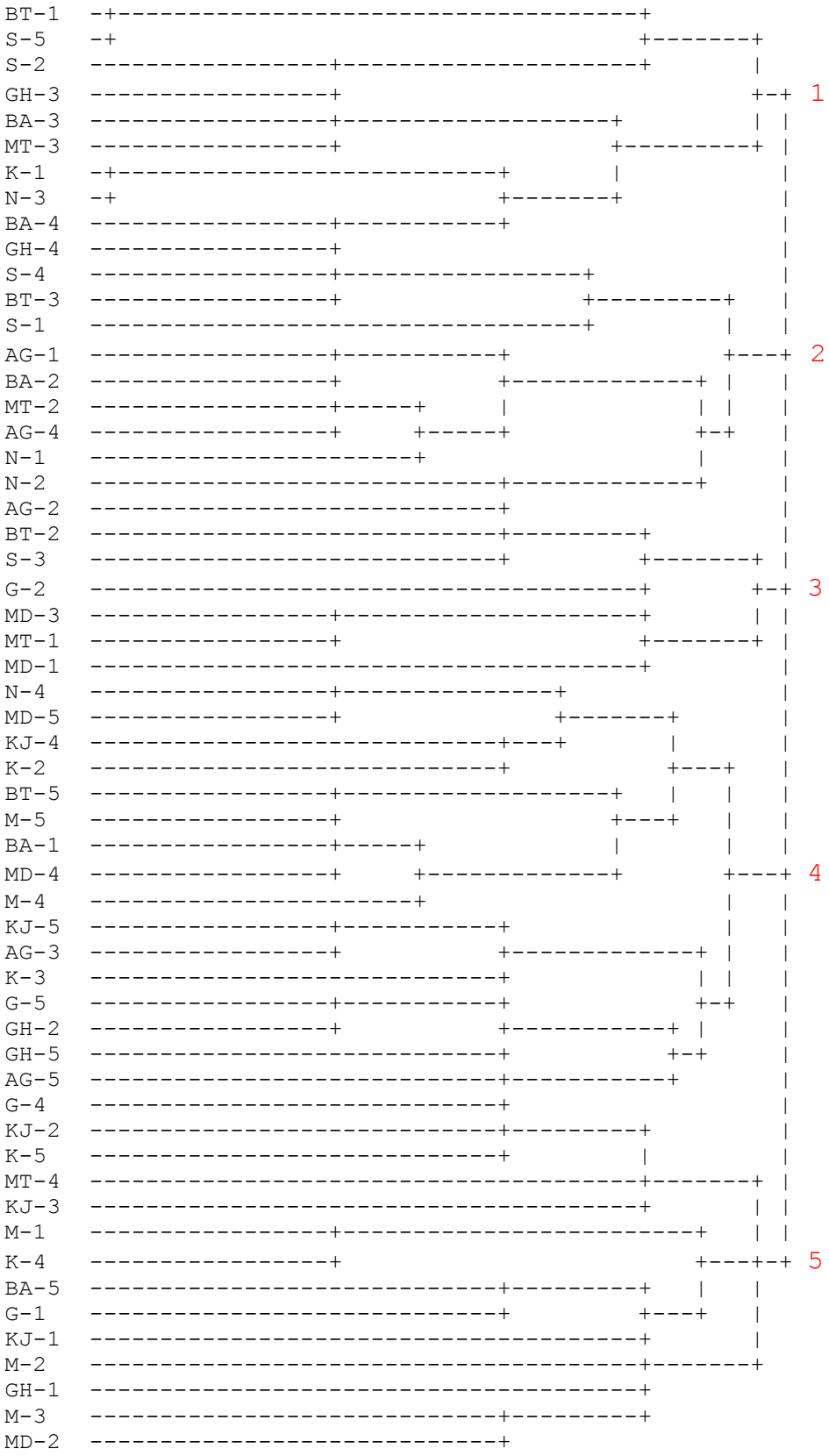

Figure 3 Dendrogram of 56 Tunisian castor genotypes prepared based on 18 RAPD markers.
Note: S – Souassi (5 genotypes), BT – Bouthay (4 genotypes), GH – Ghomrassen (5 genotypes), BA – Sidi bou ali (5 genotypes), MT – Matmata (4 genotypes), AG – Mateur (5 genotypes), N – Nefza (4 genotypes), MD – Mednine (5 genotypes), M – Mornag (5 genotypes), G – Gabes (4 genotypes), K – Kebili (5 genotypes), KJ – Ksar jedid (5 genotypes).

CONCLUSION

Genetic diversity in a germplasm is the fundamental requirement for crop improvement programs. PCR amplification of DNA using 18 primers for RAPD analysis produced 145 DNA fragments that could be scored in all 56 genotypes of Tunisian castor (Figure 1). The number of amplified fragments varied from 3 (OPE-07) to 13 (SIGMA-D-01), and the amplicon size ranged from 100 to 1500 bp. Of the 145 amplified bands, 145 were polymorphic, with an average of 8.11 polymorphic bands per primer. A dendrogram was constructed from a genetic distance matrix based on profiles of the 18 RAPD primers using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 56 Tunisian castor genotypes were clustered into five main clusters. Polymorphism revealed by RAPD technique was abundant and could be used for molecular genetics study of the castor accessions, providing high-valued information for the management of germplasm, improvement of the current breeding strategies, construction of linkage maps, conservation of the genetic resources of oat species and QTL mapping.

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RISK ASSOCIATED WITH FOREIGN BODIES IN FOOD IN THE CZECH REPUBLIC

Pavla Svrčinová, Hana Tomášková, Vladimír Janout

ABSTRACT
The food safety is the main concern of the politicians and inhabitants in whole Europe. According the currently valid legislation the food should be save. The food should be safe from all aspects: chemical, microbiological, physical and radiological. Physical hazard/foreign body in food is perceived by public as something to be very simply solved by food business operators. However, foreign body is the biggest single source of customer complaints received by food business operators, retailers and enforcement authorities. In even the best-managed processes, the accidental presence of unwanted items could occasionally occur. Foreign body in food is believed to be a matter of concern to all food business operators. However, the level of inclusion of physical hazards by Czech food business operators in the hazard analysis is still low. Consumers experience with foreign bodies in food or even health problems caused by foreign bodies is continuing high level. Consumer complaints regarding foreign bodies reported from food products should be an important question for the food industry that should implement corrective actions to prevent such unwanted events.

Keywords: physical hazards; foreign bodies; hazard analysis; health risk; HACCP

INTRODUCTION
People expect, that food they eat is hygienically and health safe. Mass consumption of food is the cause of a high risk to human health, but only in the case of harmful food. Protection of human, animal and plant health is one of the main economic priorities of each country. The political objective of the European Union is therefore to ensure that European Union citizens have access to safe and nutritious foods, so it must meet strict safety standards. In ensuring food safety, it is necessary to consider all aspects of the food production chain, because each subject can have a potential impact on food safety **(Nagyová et al., 2019)**. The issue of food safety and quality is very important in view of the growing globalization of economy, whose mission is to encourage food businesses to improve the production process and competitiveness **(Nagyová et al., 2018)**.

The aim of this study is to present results of a survey on the experience of the food business operators in the Czech Republic and consumers with the physical hazards/foreign bodies in food. According currently valid legislation namely **Regulation (EC) No 852/2004** on food hygiene Food business operators shall put in place, implement and maintain a permanent procedure or procedures based on the hazard analysis and critical control points (HACCP) principles. The HACCP principles include identifying any hazards that must be prevented, eliminated or reduced to acceptable levels. The HACCP requirements should take account of the principles contained in the *Codex Alimentarius* **(Regulation (EC) No 852/2004)**.

According the ***Codex Alimentarius*** **(1969)** Code of practice CAC/RCP1-1969 – General principles of food hygiene the HACCP should list all the hazards that may be reasonably expected to occur at each step according to the scope from primary production, processing, manufacture, and distribution until the point of consumption. In conducting the hazard analysis, wherever possible the following should be included: the likely occurrence of hazards and severity of their adverse health effects; the qualitative and/or quantitative evaluation of the presence of hazards; survival or multiplication of micro-organisms of concern and production or persistence in foods of toxins, chemicals or physical agents. However foreign bodies are in the Czech Republic still present in food on the market: RASFF annual report 2016 reported 106 notifications due the presence of foreign bodies. In 2017 there were 131 notifications **(European Union 2017; European Union 2018)**.

In the report prepared by Food and veterinary office is stated: "Better HACCP implementation/Final overview report the state of implementation of HACCP in the EU and areas for improvement "**(European Union, 2015)** identified as a major problem hazard analysis. There is a widespread lack of understanding of how to undertake a hazard analysis correctly and this process creates difficulties particularly for

small FBOs due to lack of available expertise. In many cases, the assessment of the likely occurrence of any hazard and the severity of their adverse health effects was not properly undertaken. In general, operators were better equipped to address microbiological hazards. Analysis of physical contaminants that is foreign bodies representing a food safety hazard, has not been so far reported. Gap analysis showed that for EU a comprehensive analysis of incidents of physical hazards is missing and that this information is available may provide clues on actual risks and possible contingency measures that can be related to type of physical hazards, type of food and regional specificities **(Djekic, Jankovic and Rajkovic, 2017)**.

This cross-sectional study tried to evaluate situation in the Czech Republic (CZ), three years later after publication of above-mentioned EU publication. Since then EU created on webpages platform for HACCP implementation wit aim to help small and medium size FBOs, however, no significant progress in CZ was not noted by our study. Quarter of the FBOs did not consider physical hazards/foreign bodies as a problem. To verify existence of this problem we carried out study among CZ population on their experience with foreign bodies in food and 67.91% of them had in past five year at least one experience with foreign bodies and four of them had health problem caused by foreign body in food. The results showed, that there is still gap in the hazard analysis carried out by the food business operators concerning physical hazards/foreign bodies and consumers still experience foreign bodies in food.

Scientific hypothesis

Hypothesis 1: We assume, that all CZ food business operators included in their hazard analysis risks associated with foreign bodies/physical hazards. During development their permanent procedure or procedures based on the HACCP principles .

Hypothesis 2: We assume, that all CZ food business operators correctly implemented during establishment of their permanent procedure or procedures based on the HACCP principles all steps as described in **Regulation (EC) No 852/2004** for physical hazards.

Hypothesis 3: We assume, that the average CZ citizen has no experience with foreign bodies in food.

MATERIAL AND METHODOLOGY

The cross-sectional study aimed at the experience of the food business operators (FBO) and consumers with foreign bodies/ physical hazards in food. The questionnaire for producers focused on hazard analysis done by FBOs and establishment of critical control points (CCPs) and critical limits (CL). The questionnaire was distributed by e-mail, or by post to 100 FBOs within whole CZ. The FBO were randomly selected from list of food producers registered in trade register. The second part of study was questionnaire for consumers. We distributed 200 questionnaires to randomly selected visitors of food festival, which took place in Moravia-Silesian region. Questionnaire focused on their experience with foreign bodies in food and adverse health effect of consumption of such food.

Statistic analysis

Chi-squar test was used to determine whether there is a significant difference between the the observed frequencies in two or more categories between men and women experience with foreign bodies. The level of statistical significance was set at 0.05. Statistical processing was performed using Stata v. 13 (StataCorp).

RESULTS AND DISCUSSION

The questionnaire to FBOs returned fully filled in 54%. Out of 54 questionnaires only 40 FBOs (75%) evaluated in their hazard analysis foreign bodies/ physical risks. Out of these FBOs, that included in their hazard analysis also physical hazards, assessed the most frequently these materials of foreign bodies: glass 31x (77%), hair, nails 28x (70%), metal, plastic, small bugs 22x (55%), stones and personal belongings 16x (40%). See also Table 1. Out of 40 FBO, who included in hazard analysis physical hazards, only 14 (35%) of them based on the hazard analysis identified critical production steps (CPS) in relation to physical hazards.

The identified CPSs were preparation 10x, reception of raw material nine times, expedition/delivery eight times, storage six times, personal hygiene twice, and cleaning one times. Some FBOs identified as critical more production steps. 14 (35%) FBOs identified CPSs in connection with physical hazards.

Out of them 10 (46%) only identified CPS, two (9%) established in these steps CCP but without CL. The last two (9%) established CPS with CCP and CL. Four (18%) FBO established CCP without CPS and CL, two (9%) defined CCPS with CL but without CPS. The same number of FBOs (2, 9%) established CL without CPS and CCP. See Table 2. For some materials as metal, glass, plastics, organic parts, small bugs, stones, wood or inner undesirable parts there were cases, when CCP was established without their assessment during hazard analysis. See Table 3.

In total 12 FBOs established CCP to manage/control physical hazards in their production, 10 of them carried out hazard analysis, two did not carried out hazard analysis for physical hazards at all. In one such case the CCP was established based on internal procedure and in the other case the CCP was established by supplier of the HACCP plan. The most frequently were CCPs established for hair, nails (12x), and glass (10x) metal (eight times). The only material for which any FBO decided to establish CCP was rubber. To prevent or eliminate a hazard or to reduce it to acceptable levels, the FBOs decided for stones (44%) hair, nails (43%) and inner undesirable parts 40% establish the CCP. For other materials it was lower percentage. See Table 4.

Only six FBO replied, that they do have established critical limits for CCPs in connection with physical hazards. In all cases they choose limit not present. Two of them established critical limit without establishing CPS, and two without defining CPS or CCP. The next question verified how the critical limit was validated and in total 25 FBOs replied, that they had their CL validated. 19 of them did not answered previous question “what is your critical limit“. Out of them 20 had CL established by supplier of the HACCP plan, three times it was done by FBO based on previous experience, in one case limit was based on internal procedure, ones it was chosen based on external cooperation.

In total 60 complaints concerning foreign bodies in food were received by FBOs during 2016. The highest number of complaints received by one producer was 20 and the lowest was one. The out of 54 participants on the study only 10 FBOs received consumers' complaint. Three of them had not included physical hazards in their hazard analysis. The FBO, that received highest number of complaints (20), did not evaluate physical hazard during hazard analysis and as corrective action this FBO choose the training of the staff. The most frequently compliant was due the presence hair or nails in food (nine times) or bugs (three times). Corrective action implemented all FBOs after compliant. In majority it included stricter control by supervisor during production (13x) or on reception of raw materials (seven times), providing personal with protective cloths (seven times), change of equipment (four times), stricter sanitation (four times) or installation of the x- ray (one times) into the production line. Three FBOs decide to provide staff with further training. The only one produces carried out reassessment of CCP established in the HACCP plan.

The fully filled in questionnaire returned 134 persons, out of them 85 females (63, 43%) and 49 men (36, 56%). The age of participants was mainly between 15 – 65 years – 123 participants, the rest were older people. The majority were with university degree 48% and high school 37%, the rest of participants had lower level of education, and the only person had no education. Out of 134 participants 91 (67, 91%) had experience with foreign body in food during the last five years. The females met foreign body in 58 cases, men in 33. Females met foreign body statistically more frequently than men (tested by ch^2 test on the level of 5%, $p \leq 0.0001$). Out of these 91 participants the majority – 40 persons (43%) discovered foreign body in food 2 – 5 times in the last 5 years, 37 (40%) persons only once and 13 people experienced foreign bodies more than 5 times. The results did not show the statistical difference between females and men concerning the frequency of foreign body discovery in food (tested by ch^2 test on the level of 5%, $p \leq 0.0001$).

Concerning material, the most frequently were notified stones 38x (28.3%), inner undesirable parts 37x (27%) organic parts 36x (26.8%), pests, hair and nails, each by 35 (26.11%) participants. The results show Figure 3.

The questionnaire included also questions on the solution of the discovery of the foreign body in food. Only nine persons solved the problem making complaint, out of them eight made complaint to food business operator and the only person to competent authority controlling food safety.

In the next step the comparison was made between experiences of consumers with foreign bodies founded in food with FBOs assessment done within their hazard analysis. The most frequently assessed material was glass- 31 FBOs and 10 (32%) decided to establish CCP to manage this hazard. The glass was notified only by 4 persons. For plastic 22 FBOs carried out hazard analysis and 6 (27.2%) of them established for this hazard CCP. This material was notified by 23 consumers. Small bugs were assessed by 22 FBOs, 7 (31.8%) managed that hazard by establishment of CCP. Small bugs were notified by 35 persons.

Out of 134 participants four (2, 9%) had adverse health Out of 134 participants four (2, 9%) had adverse health effect after consumption food with foreign body in it. One person had even two cases of health problem. Four times it was broken tooth and in one case it was wooden chip stacked in throat. All cases of health problem needed health care treatment.

For organic parts of food and inner undesirable parts of food only 9, respectively 10 FBOs carried out hazard analysis and 3 (33.3%) respectively 4 (40%) of them established CCP to manage this hazard. These two types of foreign bodies were frequently notified by consumers 36x, 37x. The most frequently notified foreign bodies were stones, while only 7 FBOs decided to manage this hazard by establishment of CCP, while 16 carried out hazard analysis. The hair and nail were assessed by 28 FBOs, 12 managed that hazard by CCP (42.85%).

Table 1 Number of FBOs in relation to material assessed in hazard analysis.

	Total	%*
Metal	22	55.0
Glass	31	77.5
Plastic	22	55.0
Organical parts	9	22.5
Small bugs	22	55.0
Stones	16	40.0
Wood	7	17.5
Textil	5	12.5
Hair, nails	28	70.0
Paper, carboard	12	30.0
Rubber	3	7.5
Inner undesirable parts	10	25.0
Personal belongings	16	40.0
Other	7	17.5

Note: *out of FBOs that assessed physical hazards (N = 40).

Table 2 Number of FBOs defining CPS, CCP or CL.

Established	Number	%
CPS	10	46
CPS.CCP	2	9
CPS. CCP. CL	2	9
CCP	4	18
CCP.CL	2	9
CL	2	9

Table 3 Materials for which FBO did not carried out hazard analysis however, CCP was established.

Material	Number of FBO
Metal	2
Glass	1
Plastic	1
Organic parts	2
Small bugs	1
Stones	1
Wood	1
Textil	1
Hair, nails	1
Paper, carboard	0
Rubber	0
Inner undesirable parts	4
Personal belongings	1
Other	1

Table 4 Material of foreign body and % of FBOs managing this hazards by CCP.

Material of foreign body	Assesed	CCP established	% of hazard managed by CCP
Metal	22	8	36.36
Glass	31	10	32.25
Plastic	22	6	27.27
Organic parts	9	3	33.33
Small bugs	22	7	31.81
Stones	16	7	43.75
Wood	7	1	14.28
Textil	5	2	40
Hair, nails	28	12	42.85
Paper, carboard	12	3	25
Rubber	3	0	0
Inner undesirable parts	10	4	40
Personal belongings	16	3	18.75
Other	7	1	14.28

Hair or nail were notified by 35 persons. The results did not show the statistical difference between FBO and consumers concerning the frequency of foreign body material assessment in hazard analysis and discovery in food (tested by ch^2 test on the level of 5%, $p \leq 0.0001$). Further details are presented in Figure 4.

A foreign body may be defined as something that the consumer perceives as being alien to the food. The perception of the consumer is important, since not all foreign bodies are in fact alien to the food, though all have the potential to give rise to a consumer complaint. Hence foreign bodies can range from items that are demonstrably alien to the food, such as pieces of glass, metal or plastic through items that are related to the food, such as fragments of bone in meat products to part of the food itself, such as crystals of sugar or salt that are mistaken for glass.

Foreign bodies may get into food at any stage from initial harvesting to final processing or even preparation and consumption by the consumer. Food processing should include procedures to remove foreign bodies incorporated during harvesting of the crop, but it can also give rise to foreign bodies itself, any foreign bodies can be traced back to pieces of food processing machinery **(Edwards M., 2014)**.

In the HACCP Annex, Hazard Analysis and the decision tree for determining CCPs focuses too much on microbiological hazards, while chemical and physical hazards are given less importance. This reflects the historic focus of HACCP when the initial guidelines were being developed, but chemical and physical hazards need to be addressed to cover issues such as, for example, the effective management of allergens with respect to food safety. In revising the GPFH text and the HACCP Annex, consideration should be given to how to incorporate additional guidance on chemical and physical hazards **(*Codex Alimentarius*, 2014)**.

Foreign matter is the biggest single source of customer complaints received by many food manufacturers, retailers and enforcement authorities. In even the best-managed processes, the accidental inclusion of unwanted items may sometimes occur. Foreign matter in foods is therefore quite rightly a matter of concern to all food manufacturers and retailers. Consumer complaints regarding foreign material reported from food products will continue to be a significant issue for the food industry. However, careful study of data from a wide range of foreign matter investigations demonstrates that in many cases the occurrence of foreign matter is far from random **(Edwards and Stringer, 2007)**. 16,878 foreign bodies injuries occurred in children aged 0 – 14 years have been recorded in the SUSY Safe databases. FB type was specified in 10,564 cases, among them 2,744 (26%) were due to a food item **(Van As et al., 2012)**.

Contrary to microbial and chemical hazards, physical contaminants are the most obvious evidence of contamination of product. Regarding types of foreign bodies notified the top three material were pest (54.6%), glass (17.4%) and metal (11.5%) **(Djekic at al., 2017)**. Consumer complaints about foreign bodies are a continuing problem for the food industry. Recent years have seen an increasing emphasis on consumer rights, with frequent encouragement in the media for consumers to complain to food companies about incidents that would in the past have been viewed as trivial **(Edwards, 2014)**.

The foreign bodies statistically were found more by women than men. This is due women are the main chefs at Czech homes. The results of study shoved, that the most frequently met foreign bodies by consumers were stones followed by organic foreign bodies (both inner and outer), followed by hair and small bugs. The difference could be caused by type of foreign bodies, when hair or inner organic parts are not seen by consumers as a problem. Therefore, these materials are not notified by them. The problems with foreign body in food were reported to FBOs only in minority of cases, even in the case of health problem caused by foreign body complaints was not made. This could be caused by no adverse health effect and by consumer's historical experience with their complaint's solution.

Food factory operatives are a major source of foreign bodies, from stray hairs not contained by hairnets or beard snoods to studs or sleepers from earrings. Personnel are a major potential source of foreign bodies in food premises of all kinds **(Edwards, 2014)**. The one of the most frequently founded foreign body by consumers involved in study was hair and nail. That is sign that staff is still one of the major sources of contamination. The root of this could be the staff itself, when the staff turnover in food industry is very high and staff has no specific background in food safety.

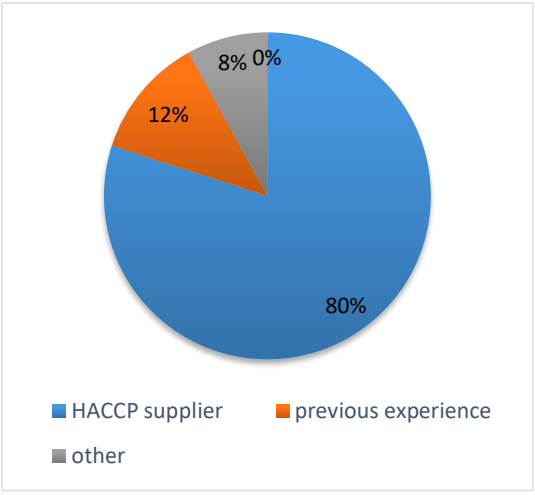

Figure 1 Validation of CL.

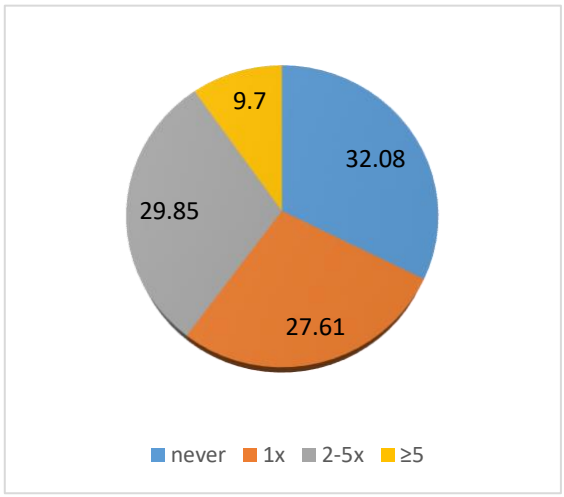

Figure 2 Frequency of foreign bodies founded in food.

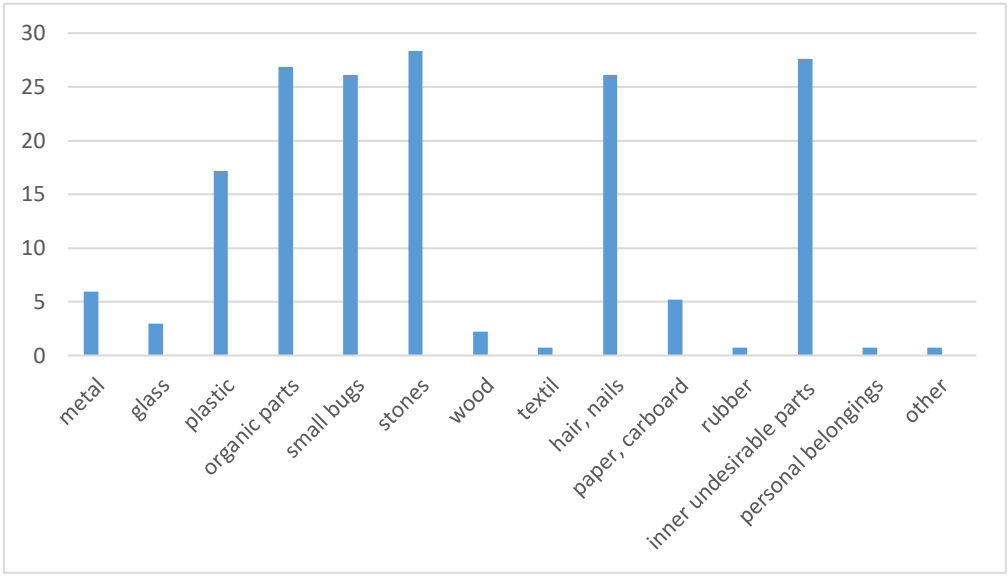

Figure 3 Foreign bodies discovered in food by material in %.

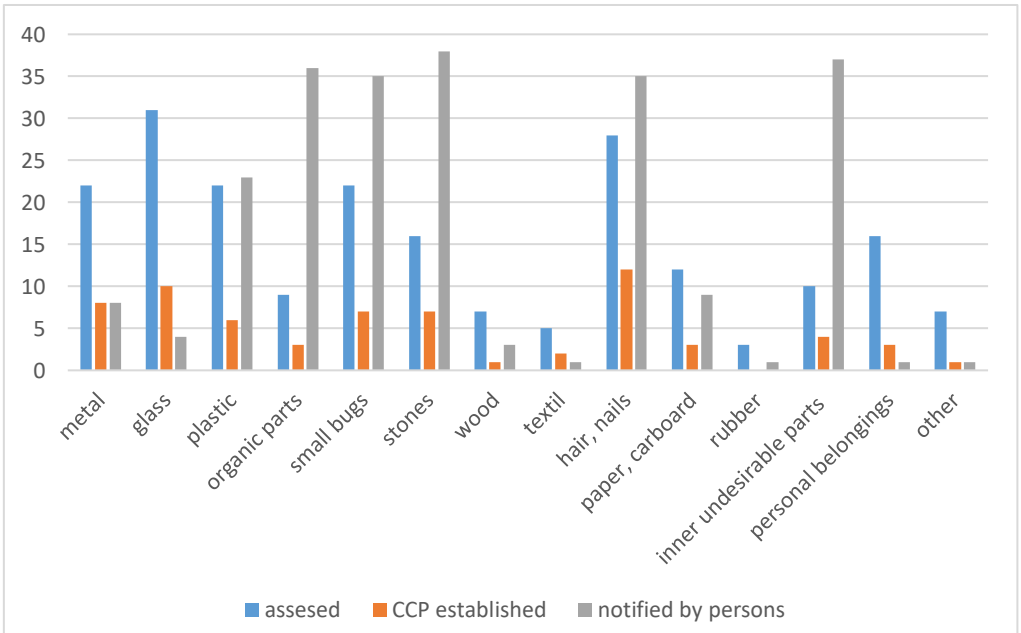

Figure 4 Comparison assessment carried out by FBOs, CCP establishment and consumers experience (total number).

Although, the FBOs provided staff by training as corrective measure in case of non-compliance, the training did not sufficiently prevent occurrence of hail or nail in food. Staff training should go together with thorough control by hierarchy directly on production site.

A good quality management system is vital to the effective prevention and control of foreign bogies in food manufacture. A structured preventive approach is likely to be the most reliable basis for such a system. The traditional approach of sole reliance on finished product analysis and factory inspection is nowadays unlikely to give acceptable assurance and consumer confidence that the process is under control on continuous basis. Hazard analysis is the approach which all companies, whatever their size, should use to identify the points in their manufacturing operations which critically affect product safety. Foreign body hazard analysis of a food product process starts with the identification of the sequential stages in the process from raw materials and packaging materials through to the dispatch, distribution and end use of the food product **(Edwards, 2014)**. Substantial part of the FBOs did not include in their hazard analysis physical hazards at all. The FBOs do not understand importance of hazard analysis, when CCPs or critical limits were in many cases established without carrying out hazard analysis. Some critical limits were established without having chosen critical control points. That is due the not understanding importance of hazard analysis for establishment of CCPs and CLs within the procedures based on HACCP principles.

The investigation of a foreign body incident involves a number of clear stages. The first essential step is to determine all the known facts in the case. It is important that precise details of the circumstances under which the foreign body was discovered are recorded. In particular, it is essential to know whether the foreign body was found when the pack was opened, during food preparation or whilst eating the product, and whether or not the foreign body could have been heated during preparation or mixed with other food products **(Edwards, 2014)**. In case of non-compliance caused by foreign body all FBOs implemented corrective action. However, only few of them applied further training for the staff, although large part of the complaints concerned hair or nail.

While the available technology may not eliminate all foreign bodies from food, the correct application of technology will assist in removing many of them **(Edwards, 2014)**. The critical point in the detection of physical contaminants is nearly always the large variability that it is observed in the distribution of impurities between the repeated determinations carried out on the same sample. This variability is due to the fact this kind of the contaminants do not have a uniform distribution within the sample, thus resulting in the need to transform the data into values that can express a normal distribution; alternatively, it may be necessary to increase the number of determinations in order to ensure a significant result **(Schiavo et al., 2015)**. There are many ways food processors can prevent physical hazards in food products **(CVO/Food Safety Knowledge, 2018)**. The presence of foreign bodies in food is of major concern to the producer. Mechanical separation techniques have been used for many years for foreign bodies in powdered and owing products based on size and weight. Optical inspection techniques extend the range of detectable foreign objects regarding shape and color in free materials. Metal detectors enable metallic particles inside the body of a product to be found. With advances in sensor technologies and computing power more advanced detection systems are becoming available **(Graves, Smith and Batchelor, 1998)**.

Our study has proven that there is still space for improvement from side of the FBOs; some of them do not implement all possible preventive measures in their establishments. There was one FBO did not including in hazard analysis physical hazards, although received 20 consumers complaints. It seems, that preventive measures applied by FBOs are not effective enough and do not prevent occurrence of some foreign bodies such as plastic, small bugs, stones, hair and nail. Organic parts and inner undesirable parts of food are not in focus of FBOs, while they are founded frequently by consumers.

Flour beetles are among the most common pest insects found in stored grain and milled products. Beetles have defensive glands which secret quinones such as 2-methyl-p-benzoquinone, 2-ethyl-p-benzoquionone, hydroquinone commonly referred to as benzoquinones. Benzoquinones have a carcinogenic effect, they are inhibitors of growth of various microorganisms, and they produce a self-defense mechanism in threat situations and affect population aggregation **(Lis et al., 2011)**. Stored product pest may be source of indirect contamination of stored commodities, by pesticide residues of chemical treatment by protectants. Some species of *Acarina, Blattodea, Coleptera, Lepidoptera* and *Psocoptera* may cause allergic reactions in humans exposed to remnants of their bodies. No critical levels are available for contamination of food agrocommodities by allergens of arthropods **(Mattos et al., 2016)**. The presence of pests in food was quite frequent among consumers, although quite substantial number of FBOs assessed hazard associated with them. There should be more focus on presence of pests in food as there is severe chemical risk associated with them.

1,309 complaints reported from 2000 to September 2002, 331 were related to foreign materials (25%), about 6% of those cases resulted in injury. The most common materials were identified as metal, glass and plastic **(Mattos et al., 2016)**. In our study the percentage of injuries was lower, the difference could be caused by the population under investigation. Our study included general population while above mentioned study investigated only cases, when foreign body was notified to the competent authority. However, the health effect of foreign body in food was severe and needed to be solved by health service providers.

CONCLUSION

All of our hypothesis were not proven to be truth. There is high number of FBOs not including physical hazards in hazard analysis or not following correctly all necessary steps in implementation of their procedures based on HACCP principles. As the result there is quite high number of consumers experiencing foreign bodies in food. The problem is also the quality of guidelines for hazard analysis, that do not include physical hazards and especially small FBOs do not have all necessary knowledge to carry out thorough hazard analysis. There should be more focus on physical hazards from competent authorities and producers

associations to develop guides to cover physical hazards in a future.

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ASSESSMENT OF ANTIMICROBIAL POTENTIAL OF SUBSTANCES ISOLATED FROM SOME WASTES OF MEAT PROCESSING INDUSTRY

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ABSTRACT

The slaughter of farm animals generates a large number of by-products. Meat waste management includes various methods, but cost-effective technologies are still in priority. This manuscript reports the results of the study of antimicrobial activity of substances isolated from such wastes of meat processing industry as bovine and pork mucous membranes and epithelial tissues. Proteomic study included two-dimensional electrophoresis with following mass spectrometric identification. Antimicrobial activity against *L. monocytogenes*, *P. aeruginosa* and *S. aureus* of neutralized native extracts and after enzymatic treatment as well as its ultrafiltrates was determined by flow cytometry with EvaGreen and PI dyes. It was shown that a large number of histones were found in bovine mucous membranes as well as several tissue-specific proteins, which would be a precursor of bioactive peptides. Bovine mucous membranes of the tongue and nasal cavity possessed the greatest activity in relation to *P. aeruginosa*, the rate of surviving cells decreased to 22.0%. Bovine mucous membranes of the rectum and the oral cavity, submandibular lymph nodes, pig mucous membranes of the larynx, tongue, lips, and rectum increased dead cells count up to 40% of all cells. Bovine nasal mucosa and pork mucous of labial cavity possessed the greatest activity against *S. aureus*, the rate of surviving cells did not exceed 10.0%. Determination of antimicrobial action against *L. monocytogenes* of native samples and treated with trypsin showed that bovine mucous membranes of the rectum and oral cavity, pork mucosa of the lips and submandibular glands were the most active. Treatment with trypsin or ultrafiltration demonstrated different effects on activity of samples. It was shown the perspectivity of recycling of such type of by-products into effective and demanded substances which can be used, for example, in the food industry as an alternative to chemical preservatives.

Keywords: AMP; antimicrobial activity; slaughter wastes; flow cytometry; mucous membranes

INTRODUCTION

The slaughter of farm animals generates a large number of by-products. The amount of such kind of wastes averages approximately about 10% to 15% of the value of the live animal in developed countries, in other countries this rate can reach for about two-third of the animal after slaughter **(Alao et al., 2017)**. The yield of animal by-products is high and ranges between 50-60% of the live weight **(Irshad and Sharma, 2015)**, including carcasses, hides, hoofs, and heads, offal, viscera, bones, fat and meat trimmings, blood **(Helkar, Sahoo and Patil, 2016)**. Non-carcass parts of animal (by-products) are divided into edible or inedible parts **(Barbut, 2015; Alao et al., 2017)**. Some internal organs, e. g. liver, kidney, hearts, tongue etc. could be used for humans food but it depends on traditions and religion **(Jayathilakan et al., 2012)**, as well as legacy regulations **(Jędrejek et al., 2016)**. Therefore, effective by-product utilization is a quite sharp problem.

Meat waste management included such methods as composting, aerobic and anaerobic digestion **(Banks and Wang, 2004; Arvanitoyannis and Ladas, 2008)**. Usage of by-products for animal feed and pet food and biofuel or solid fuel production is also widely developed **(Virmond et al., 2011; Jędrejek et al., 2016; Hamawand et al., 2017; Adhikari et al., 2018)**. On the other hand, for a safety reasons most produced feed materials are in severe restrictions in their use for feed farm animals, because slaughterhouse wastes are potentially contaminated by several pathogens **(Arvanitoyannis and Ladas, 2008; Jędrejek et al., 2016; Adhikari, Chae and Bressler, 2018)**.

Nevertheless, animal by-products are a good source of nutrients and bioactive substances, therefore its widely used in food industry, e.g. as functional ingredients, for technological applications and biopeptides production as well as for medical and pharmaceutical applications **(Toldrá, Mora and Reig, 2016; Alao et al., 2017; Chernukha et al., 2018)**. Some kind of by-products also recycled for fertilizer and chemical applications **(Irshad and Sharma, 2015; Toldrá Mora and Reig, 2016; Helkar Sahoo and Patil, 2016)**.

Concerning meat waste management, cost-effective technologies are in priority, therefore the developing progressive technologies, which can be based on by-

products as a source of certain bioactive substances is in demand.

This manuscript reports the results of the study of antimicrobial activity of substances isolated from some wastes of meat processing industry.

Scientific hypothesis

Most of the studied mammalian antimicrobial peptides (AMP) and proteins were isolated from neutrophils, but some such compounds were found in the small intestine, tongue, myeloid and epithelial cells **(Wang, Li and Wang, 2016)**. Therefore, not only the granular apparatus can be considered as a source of AMP, but also mucous membranes and epithelial tissues. These tissues due to its border position are constantly in contact with a wide range of pathogenic and opportunistic microorganisms and viruses, fungi, and therefore can potentially contain a set of substances with antimicrobial action.

MATERIAL AND METHODOLOGY

Pork mucous membranes of the larynx, tongue, labial and nasal cavities, rectum, and submandibular glands, bovine mucous membranes of the tongue, larynx, nasal and oral cavities, rectum and submandibular and lymphatic glands were selected as objects of study.

Proteomic study

Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). The subsequent detection of the proteins was carried out by staining with Coomassie R-250 (Applichem, USA) and silver nitrate (PanReac, Spain) as described previously **(Kovalyov et al., 2006)**. The resulting digital images were edited in a graphic editor and the quantitative protein content was calculated using ImageMaster 2D Platinum version 7 (GE Healthcare, Switzerland).

Protein fractions were excised from the gel, grinded and undergone trypsinolysis (Sigma, Germany) **(Zvereva et al., 2015)**. Obtained peptides were investigated by MALDI-TOF MS and MS/MS mass spectrometry on Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser(336 nm) in the positive ion mode in molecular weight range of 500 – 8000 Da with calibration according to known peaks of trypsin autolysis.

Bioinformatics analysis

Analysis of obtained tryptic peptides mass spectra was performed using Peptide Fingerprint option in Mascot software (Matrix Science, USA) with MH+ mass determination accuracy of 0.01%; search was performed in databases of the National Center for Biotechnology Information, USA (NCBI). Comparative analysis of obtained proteomic profiles was carried out with use of information module "Proteins of skeletal muscle of cows (Bos Taurus)" of the Database "Proteomics of muscle organs" (http://mp.inbi.ras.ru).

Preparing testing samples

Grinded mucous membranes were extracted with 10% acetic acid solution at ratio 1:5, stirring speed of 400 rpm, for 5 hours at 4 – 5 °C at Laboratory dispersing equipment (Labotex, Russia). Then extracts were centrifuged (Sigma 3K30, Germany) at 15,000 rpm and 4.0 °C for 5 min. The supernatant was neutralized to pH = 6 with a 4N sodium hydroxide solution. Neutralized extracts were subjected to trypsinolysis (PanReac, activity 328 USP $U.mg^{-1}$). Ultrafiltrates were obtained by centrifugation on centrifuge ultrafilters Amicon Ultra-4 (50kDA, Millipore). Native extracts and extracts after enzymatic treatment were subjected to a sterilizing filtration on syringe filters with a pore size of 0.22 μm (Nylon L. E., Teknokroma).

Antimicrobial activity study

The activity of antimicrobial substances contained in native extracts after neutralization and after enzymatic treatment, and ultrafiltrates were studied by flow cytometry. *L. monocytogenes* ATCC 13932, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 strains were obtained from the State Research Center for Applied Biotechnology and Microbiology (Obolensk, Moscow region, Russia). Suspensions with an approximate concentration of 1 × 106 $cells.mL^{-1}$ were used as positive controls. To obtain negative control of *L. monocytogenes* ATCC 13932, the resulting suspensions were heated at 100 °C for 10 min.

Neutralized native samples and ultrafiltrates analysis protocol

A total of 10 μL of tested sample was mixed with 90 μL *P. aeruginosa* ATCC 27853 or *S. aureus* ATCC 25923 (an approximate concentration of 1×10^6 $cells.mL^{-1}$) and incubated overnight in thermostat at 37ºC. A total of 20 μL overnight incubated mixture of tested sample and bacterial cells was mixed with 5 μL of EvaGreen (Synthol, Russia), 365 μL of deionized water, and 10 μL of DMSO (Biolot, Russia); then, samples were incubated in the dark for 15 min and green fluorescence signals corresponding to live cells were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events. A total of 20 μL overnight incubated mixture of tested sample and bacterial cells was mixed with 3 μL of PI (Logos Biosystems, Republic of Korea), 377 μL of 0.9% sodium chloride solution; then, samples were incubated in the dark for 15 min and red fluorescence signals corresponding to dead cells were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events. Survived cells were calculated in relation to control and expressed in percentage, dead cells were calculated in relation to the survived cells and expressed in percentage.

Native extracts and after enzymatic treatment analysis protocol

A total of 50 μL of tested sample was mixed with 50 μL *L. monocytogenes* ATCC 13932 (an approximate concentration of 1×10^6 $cells.mL^{-1}$) and incubated overnight in thermostat at 37 ºC. A total of 20 μL overnight incubated mixture of tested sample and bacterial cells was mixed with 5 μL of EvaGreen (Synthol, Russia), 365 μL of deionized water, and 10 μL of DMSO (Biolot, Russia); then, samples were incubated in the dark for 15 min and green and red fluorescence signals were measured on a Guava EasyCyte flow cytometer (Merck Millipore, Germany) up to 5000 events **(Kotenkova et al., 2019)**.

All cells corresponded to survive were taken as 100 percent, live and dead cells were calculated in percentage of survived cells.

RESULTS AND DISCUSSION

In previous study it was shown that a large number of histones were found in bovine mucous membranes as well as several tissue-specific proteins, which would be a precursor of bioactive peptides **(Kotenkova et al., 2019)**.

The proteomic study of porcine tissues was also carried out. A large number of histones were found too, such as H2B type 1-like, HIST1H2BB, H2B ½ and H2B type 1-N as well as proteins S100-A12 and AGR2, which were previously identified in bovine mucous membranes. Lysozyme C was identified in the mucous membranes of the tongue and rectum.

Bovine mucous membranes of the tongue and nasal cavity were the most active against *P. aeruginosa*, the proportion of survived cells decreased to 22.0%. In some cases, there was noticed an increase in the number of cells by almost 2 times when extracts of bovine lymphatic glands, mucous membranes of oral cavite and rectum, porcine mucous membranes of the larynx, tongue, labial cavity and rectum were added to the cell culture. However, a large amount of dead cells was noticed in these samples, the ratio of dead cells reached 40.0% of all cells. Presumably, this observation could be explained by the fact that AMP is "packed" into a protein molecule, which can be used by microorganisms initially as a substrate, and only after release demonstrate an antimicrobial effect **(Abaturov, 2011; Pasupuleti, Schmidtchen and Malmsten, 2012; Wang, 2014)**. The noted observation was confirmed by the fact that no such effect was observed while ultrafiltrates addition. In addition, addition of some ultrafiltrates with removed high molecular weight substances led to increase of antimicrobial activity. Native extracts of bovine mucous membrane of the larynx and submandibular glands salivary glands, pork mucous membrane of the nasal cavity did not have a significant antimicrobial effect against *P. aeruginosa*. Ultrafiltration of extracts of bovine mucous membranes of the larynx, oral cavity and rectum, pork mucous membranes of the larynx, tongue, nasal cavity, rectum and submandibular glands led to increasing of antimicrobial activity, in the case of bovine lymphatic glands and pork mucous membrane of labial cavity – did not affect on the activity, and in relation to bovine mucous membrane of the tongue and submandibular glands – on the contrary, reduced. Figure 1A shows the type of cytogram using Eva Green and PI dyes when antimicrobial activity was determined against *P. aeruginosa.*

Almost all native extracts were active against *S. aureus*. The greatest antimicrobial effect was observed when extracts of bovine mucous membrane of the nasal cavity and pork mucous membrane of the labial cavity were added to cell culture, the proportion of surviving cells did not exceed 10.0%. An increase of survived cells by more than 1.5 times was observed when pork rectum mucosa extract was added to cell culture. However, this sample showed a similar observation as in case *P. aeruginosa*: the proportion of dead cells reached 46.5% of all cells. It should be noted that the proportion of dead cells in all samples was significantly higher than in the experiment with *P. aeruginosa*. This observation indicated a higher activity of samples against gram-positive bacteria. Ultrafiltration of extracts in most cases did not lead to an increase in activity, except pork mucous membranes of the larynx, tongue and rectum – on the contrary, there was an increase in activity. Figure 1B shows the type of cytogram using Eva Green and PI dyes when antimicrobial activity was determined against *S. aureus.*

It was observed that EvaGreen dye, which is commonly used in PCR analysis, stained live cells of *L. monocytogenes* ATCC 13932 and fluoresced in green and red spectra; the dye also stained dead cells and only demonstrated red fluorescence **(Kotenkova et al., 2019)**. Bovine mucous membranes of the rectum and oral cavity, porcine mucous membranes of labial cavity and submandibular glands demonstrated the highest activity against *L. monocytogenes*, the proportion of living cells decreased to 2.7%. Enzymatic treatment with trypsin of extracts of bovine mucous membranes of the tongue, and submandibular and lymphatic glands, pork mucous membranes of the larynx, labial and nasal cavities, rectum resulted in increased activity, in the case of bovine mucous membranes of the larynx, nasal cavity and rectum, pork mucous membranes of the larynx, tongue and submandibular – did not effect on activity, and in relation to bovine mucous membrane of the oral cavity - on the contrary, reduced. Figure 2 shows the type of cytogram using Eva Green dye when antimicrobial activity was determined against *L. monocytogenes*.

In mammals, AMPs are most frequently found in blood, less often - in the saliva, mucous membrane of gingivae, tongue, cheeks and lips, submandibular gland and small labial glands, neutrophils, Paneth cells, tissues of small intestine, epithelial cells of nose and bronchi, and tracheae **(Kokryakov, 1995; Shamova, 1995; Jarczak et al., 2013; Shamova, 2013; Bosch-Marcé et al., 2014; Wang, 2014; Zhao and Lu, 2014; Wang et al., 2015; Zharkova, 2016)**. According to an analysis of the International UniProt Protein Database and Antimicrobial Peptide Database, porcine and bovine tissues have the high content of both AMPs and other substances with antimicrobial and antiviral action. Protegrins are determined in pigs; bovine tissues are characterized by cathelicidins and defensins. Different isoforms of lysozyme present in both animal species.

Nevertheless, in our pilot study we confirmed that mucous membranes and epithelial tissues of farm animals could be also a good source of such substances.

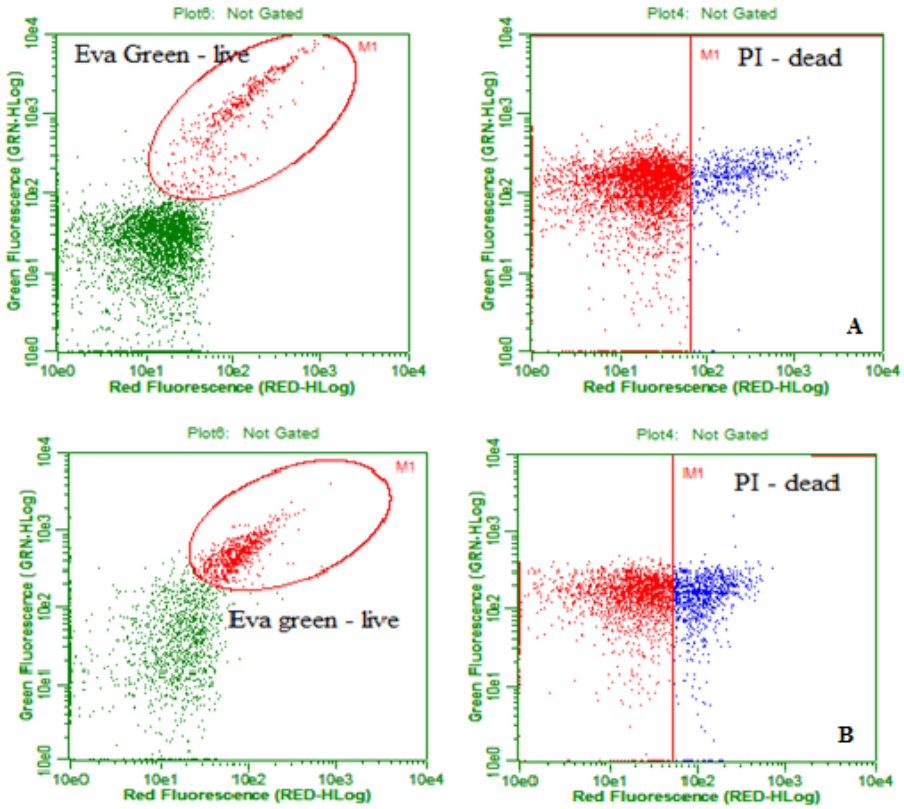

Figure 1 The type of cytogram using Eva Green and PI dyes when antimicrobial activity against *P. aeruginosa* (A) and *S. aureus* (B) of pork rectum was determined.

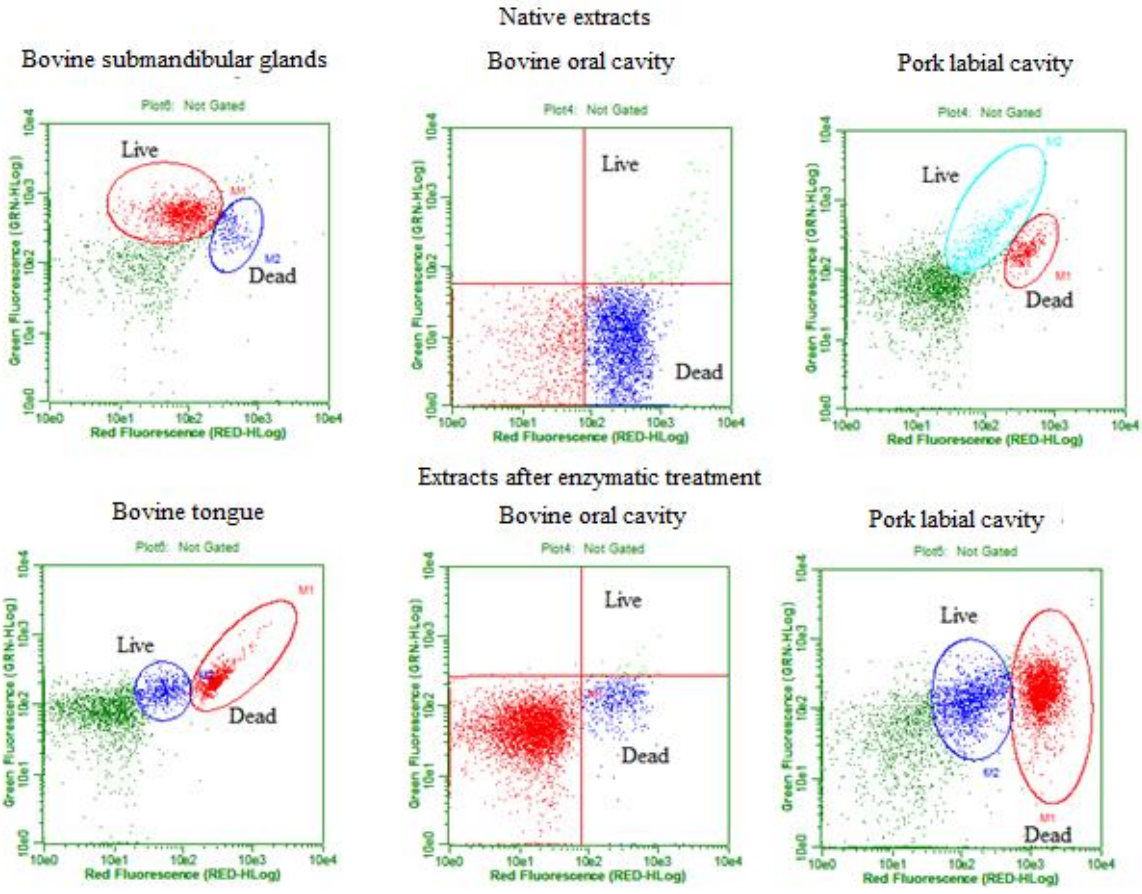

Figure 2 The type of cytogram using Eva Green dye when antimicrobial activity against *L. monocytogenes* of native extracts and after enzymatic treatment were determined.

CONCLUSION

Results of pilot study confirmed bovine mucous membranes of the tongue and nasal cavity, pork mucous membrane of labial cavity as a most promising source of antimicrobial compounds. The samples were the most active against gram-positive bacteria. It was also interesting observations found in respect of bovine submandibular lymph nodes and pork mucosa of the rectum. In connection with the revealed observation of "unpacking" of AMP from a high molecular weight protein molecule by a culture or trypsin, it is planned to consider in more detail the expediency of removal of high molecular weight substances from extracts, as well as the effects of enzyme treatment.

Moreover, it was shown the perspectivity of recycling of such type of by-products into effective and demanded substances which can be used, for example, in the food industry as an alternative to chemical preservatives.

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PROTEOMIC STUDY OF PIG'S SPLEEN

Ekaterina Romanovna Vasilevskaya, Anastasiya Gennadievna Akhremko

ABSTRACT

This work is devoted to pig spleen proteome study. Spleens were taken from Duroc pigs (females, 145 – 160 days old) and typical two-dimensional electrophoregrams were obtained. On proteomic maps after visualization and image analysis there were detected 600 fractions, including organ-specific proteins – 3 62 fractions. Among the identified constitutive fractions, the highest expression was observed (Vol spots more than 3.0E + 07) four protein spots S1, S9, S12 and S21, which are supposedly Annexin A1 (MW 38.76 kDa), Ectonucleoside triphosphate diphosphohydrolase 1 (MW 57.75 kDa) Pro-cathepsin H CD59 (MW 37.45 kDa) and glycoprotein (MW 13.79 kDa), respectively. Obtained electrophoregrams analysis using information resources made it possible to identify different active compounds in spleen with various functions, mainly immunoregulatory – glycoprotein CD59 (Mm 13.79 kDa) and ATP-dependent RNA helicase (Mm 107.58 kDa); the intensely expressed LIM-domain of the actin-binding protein (Mm 83.99 kDa). The results obtained are a prospect for immunomodulating biologic development based on animal raw materials for farm animals.

Keywords: spleen; two-dimensional electrophoresis; pork; proteomic

INTRODUCTION

Farm animals' organs and tissues are an inexhaustible resource of compounds involved in various regulatory and compensatory oraganism reactions **(Chernukha et al., 2016; Kotenkova, Lukinova and Fedulova, 2017)**. Modern researches are aimed to identifying, studying and isolating proteins that are potentially capable of exhibiting biological activity.

Today, one of the most effective ways to study active compounds derived from animal raw materials is complex tissue-specific proteins analysis at molecular level, which is commonly called the "proteomic approach". The main proteomics method, still relevant today, is two-dimensional electrophoresis, used to study protein changes and identify functional species and tissue-specific compounds **(Chernukha et al., 2017)**. Two-dimensional electrophoresis technology allows us to separate thousands of proteins with high resolution, and characterize the isolated protein fractions using mass spectrometric methods. Obvious advantage proteomic approach over others lies in its ability to detect alternative proteins forms that are the result of co-and/ or post-translational modifications.

Currently, animal tissue researches are aimed at studying biological processes in order to identify product quality and safety markers (for example, species, autolysis, quality defects) **(Mora, Gallego and Toldrá, 2018)**. However, scientific projects devoted to productive animals individual organs proteomic analysis for active protein components isolation and biologics creation based on them have great potential.

This work is devoted to protein composition comparative study of pigs spleen and resulting two-dimensional electrophoregrams analysis in order to identify potential constitutive proteins to create biological preparations of immunomodulatory action.

Scientific hypothesis

Productive animals' organs and tissues are bioactive protein compounds source. Spleen as an immune organ may contain physiologically active proteins with a pronounced immune orientation.

MATERIAL AND METHODOLOGY

Duroc pigs spleen was selected as study object. In order to level the geographic population characteristics, animals were selected from healthy females of 145 – 160 days old on three farms: Lipetsk region (C1); Voronezh region (C2); Tyumen region (C3).

Two dimensional gel electrophoresis (2-DE)

The samples described above were subjected to 2-DE. Proteins were separated by IEF in the first dimension and SDS-PAGE in the second dimension essentially as described by Hirano **(Hirano, 1982)** with slight modifications **(Kimura et al., 2003)**. IEF in the first dimension was performed at 3650 $V.h^{-1}$. The anodic and cathodic electrode solutions used for IEF were 0.02 M H3PO4 and 0.02 M NaOH, respectively, in

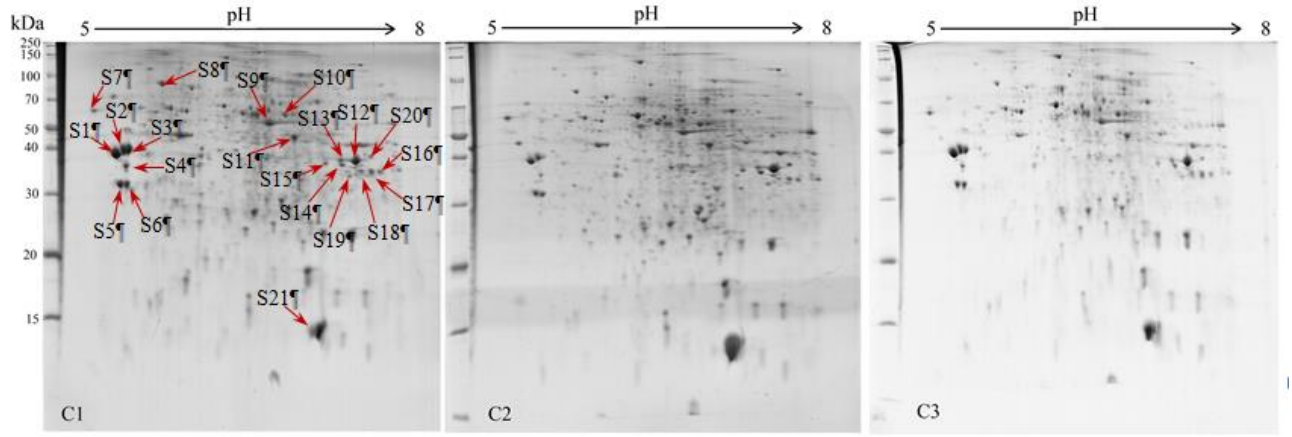

Figure 1 2D PAGE of pig's spleen.
Note: C1 – AgroEko, C2 – SGC, C3 – Tumen. Spots showing differential expression were marked excised.

2.4 mm × 160 mm tube gels. After IEF, the extruded tube gels were incubated for 10 min, in 2.5 mL of equilibration buffer I (6 M urea, 20% w/v glycerol, 2% w/v SDS and 1% w/v DTT in 50 mM Tris-HCl buffer, pH 8.8) followed by equilibration buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 375 mM Tris/HCl buffer, pH 8.8). For SDS-PAGE, the equilibrated tube gels were transferred to a 12.5% polyacrylamide gel (170 mm × 180 mm × 1.5 mm). Electrophoresis was carried out with a gel running buffer containing 25 mM Tris-HCl, 192 mM glycine, and 0.1% w/v SDS at 30 mA per gel until the bromophenol blue front had reached the bottom ofthe gel.

Protein visualization and image analysis

Protein spots were visualized by staining with Coomassie Brilliant Blue G-250. For computerized densitometry, two-dimensional electrophoregrams were used, which were in a wet state. Their full digital images and/or images of individual fragments were obtained using a Bio-5000 plus scanner (Serva, Germany). Scanned images were analyzed with ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). Spots were detected and quantified automatically. The relative optical density (OD) and relative volume were computed to correct for differences in gel staining. These measures take into account variations due to protein loading and staining, by considering the total OD or volume over all the spots in the gel. The digitized 2DE images of cortex were then compared by the matching method.

Protein spots interpretation on spleen two-dimensional electrophoregrams was carried out in accordance with the **Swiss-Prot database (2002)**.

Statisic analysis

The experimental data between three organs were analyzed using student's t-test, and data among several groups were analyzed by one-way ANOVA by ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). A p value <0.05 was considered significantly different. All results are presented as mean ±SD from at least three independent experiments.

RESULTS AND DISCUSSION

As a result of spleens 2-DE gels quantitative proteomic study images from pigs at three different farms (Figure 1) using ImageMaster ™ 2D Platinum software, it was found that, on average, there are about 597 spots on each gel. At the same time, the most pronounced protein expression was detected in samples C2 – 624 fractions, the least pronounced protein expression was observed in samples C3 – 570 fractions. Two-dimensional electrophoregrams comparative analysis in all samples revealed 362 major (constitutively present) fractions.

Among identified constitutive fractions (Figure 2), there were noted high expression protein spots S1, S9, S12, and S21, presumably Annexin A1 (MW 38.76 kDa), Ectonucleoside triphosphate diphosphohydrolase 1 (NTPDase 1, MW 57.75 kDa), Pro-cathepsin H CD59 (MW 37.45 kDa) and glycoprotein (MW 13.79 kDa), respectively. The biology of protein Annexin A1 functions, as revealed by studies **D'acquisto, Perretti and Flower (2008)** using transgenic animals, peptide mimetics and neutralizing antibodies, speaks to its role as a key modulator of both innate and adaptive immune systems. **Lemmens et al. (2000)** found that NTPDase 1 possesses both immunological identity and functional characteristics of vascular ATPDase. It is also known that Prokatepsin H regulates the signaling pathway of immune response **(Gladue et al., 2014)**, and Glycoprotein is a strong inhibitor of membrane attack complex and nonspecific immune response **(Maher et al., 1998)**.

In accordance with the information databases resources, the following functional compounds were found in spleen samples: the leptin receptor (Mm 132.52 kDa), which acts as factor regulating appetite, causing decrease in food intake and an increase in energy consumption, also regulates bone mass and secretion of hypothalamic-adrenal pituitary hormones **(Ruiz-Cortés et al., 2000)**; in small quantities, the transmembrane glycoprotein 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Mm 97.15 kDa), which limits the rate of cholesterol biosynthesis and participates in the biosynthesis of isoprenoids necessary for the normal functioning of cells **(Chen et al., 2012)**.

Fractions, involved to innate immune response mechanisms, were identified, such as glycoprotein CD59 (Mm 13.79 kDa) and ATP-dependent RNA helicase (Mm 107.58 kDa) **(Zhang et al., 2000)**; the intensely expressed LIM-domain of the actin-binding protein (Mm 83.99 kDa),

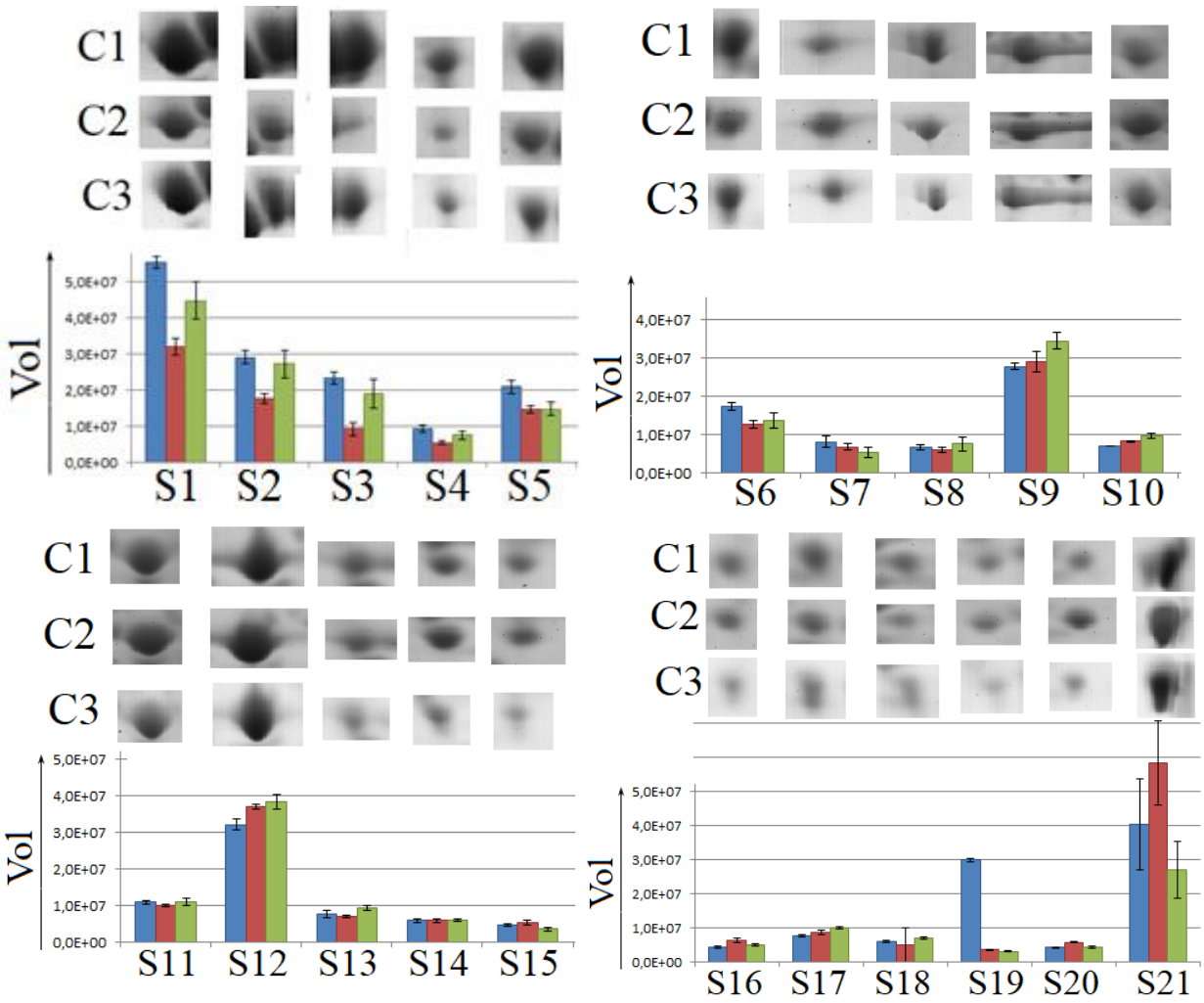

Figure 2 Relative vol change in differentially expressed proteins in spleens (blue – C1, red – C2, green – C3).
Note: Spot intensities were normalized by total valid spot intensities and mean of values from duplicate analytical gels from three replicates. Data represented are means ±SD of three independent experiments.

which is involved in the regulation of the cytoskeleton of actin, which increases the number and size of actin stress fibers, as well as inhibits the depolymerization of actin filaments **(Wang et al., 2007)**.

Also on two-dimensional electrophoregrams, a proteins group was identified that is characteristic of all analyzed spleens and plays an important role in innate immune response and inflammatory processes regulation: interferon stimulator protein (Mm 41.8 kDa), chemokine-like receptor 1 (Mm 41.38 kDa), platelet activating receptor (Mm 39.43 kDa); TYRO tyrosine kinase binding protein (Mm 11.61 kDa), activating macrophages and neutrophils, directly involved in immune response **(Xie et al., 2010; Huang et al., 2010;. Yang, Diehl and Roudebush, 2003; Yang et al., 2003; Yim et al., 2000).**

CONCLUSION

Studies of pigs spleens two-dimensional maps made it possible to establish proteins wide range presence — up to 600 fractions, of which 362 fractions are structural.

Detected fractions on the obtained maps are predominantly physiologically active, wherein their activity consists mainly in participation in various immunoregulatory reactions. Thus, in spleen samples there are found both compounds with immunoregulatory function (glycoprotein CD59, ATP-dependent RNA-helicase, LIM-domain of actin-binding protein), and factors involved in metabolism regulation (leptin receptor, transmembrane glycoprotein 3-hydroxy- 3-methylglutaryl-coenzyme A-reductase). It can be explaned by spleen biological role in organism and, as a result, a special structure – the division into red and white pulp and marginal zone, which produce compounds of various specificities.

This work is the first step to development of immunomodulating biologic based on animal raw materials for farm animals.

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FOOD ALLERGY AND FOOD INTOLERANCE KNOWLEDGE OF FOODSERVICE WORKERS IN HUNGARIAN SCHOOLS

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ABSTRACT

To provide food for children with food allergy or food intolerances represents an increasingly important role in school catering services. The number of children with food intolerances is growing continuously; therefore, it is necessary to improve the knowledge of foodservice workers, who are responsible for food provision in school catering units in relation with food intolerances, food allergies. The main goal of our research is to assess and analyze the knowledge of food service workers and food handlers on food intolerances and to determine those factors, which may influence their knowledge. Our research was conducted by using paper and pencil questionnaires. The mean of test results was 89.16% while deviation was 12.26%. There were no correlations between the test results and respondents' education level, age group and the number of years working in food catering sector, and only partial correlation was detected with the job of the respondents. Based on the answers the food handling techniques of diet foods represented the poorest results. Our findings proved that the knowledge and food handling practice of food handlers regarding food intolerances and the preparation of diet meals should be improved.

Keywords: food hypersensitivity; food allergy; food intolerances; food handler; school catering; knowledge test

INTRODUCTION

Food allergies and food intolerances are among the most frequent reasons of bad or unfavorable reactions on certain food or meals. Food intolerances are those side effects, which are resulted by any ingredients, components or additives of meals or food products, and are not derived from immune system problems, but it is a caused by any other problems when the human body is not able to digest, to absorb or to metabolize certain food or food components. The most frequent food intolerances are lactose and fructose intolerances **(Mahan and Swift, 2017)**.

Food intolerances generally produce undesirable symptoms in the digestive system, such as abdominal distension, pains, diarrhea and vomiting. The symptoms and impacts of food allergies can be more dangerous **(Caballero, 2013)**.

Food allergies are the abnormal reactions of the immune system on specific components of food (mostly proteins) which are recognized by the allergen-specific immune cells, generating specific immune responses which will result specific symptoms **(L'Hocine, Achouri and Pitre, 2018)**. The reactions on a given allergenic substance may be different in different individuals, from the less intensive to the dangerous forms, which may be even life threatening reaction. Some allergenic substances will cause abdominal discomfort, skin rashes, vomiting or diarrhea, but others may cause difficulties in breathing, drowning or anaphylaxis as well **(Bird, Jones and Burks, 2019)**.

In Europe, based on the information from parents, one from 20 children has any type of health problems because of food allergies **(Nwaru et al., 2014)**. In Hungary, in 72% of the schools there is at least one pupil or student who claimed for dietary menus. The most frequent food allergies and intolerances are caused by milk, eggs and gluten. As a consequence, in 2017, the most frequently required dietary meals were lactose-free, milk protein-free, gluten-free and egg-free dietary menus **(Bakacs et al., 2017)**.

The manifested cases of food allergies and food intolerances show an increasing tendency, so it is among the most important health and food safety questions of our time, which will bring more and more challenges for the food industry and catering industry. The only treatment for allergies and intolerances is the absence of the harmful substances, so these substances should be deleted from the everyday life, everyday food of the hypersensitive persons **(NIAID-Sponsored Expert Panel, 2010)**.

The answer for the question of how to avoid these problems, how to avoid these harmful ingredients in order to keep health, will depend not only on the knowledge of

the patients and their families, but also on the knowledge of their environment, such as school, workplace, food industry, governmental bodies and the authorities.

In public catering, special food and meals shall be provided for those children, who confirm their disease with the certification of specialists. The meals for them may be prepared on the spot, or food may be provided by special delivery service, and the prepared meals shall contain all those nutrients and food types which are given for the relevant age group of healthy children as it is given in an official decree **(No. 37/2014 decree of Hungarian Ministry of Human Capabilities)**.

To avoid the contamination with allergenic components and to keep food safety requirements a high level of knowledge is required **(Sicherer and Sampson, 2018)**. In order to meet these strict requirements, and to prepare the appropriate food and meals for hypersensitive consumers, catering services shall comply with three main requirements.

Firstly, the caterer shall know which are those food types or ingredients which shall be avoided by the hypersensitive person in the given diet, and caterers shall provide the relevant information for these customers.

Secondly, the caterers shall provide information for the consumers about the dietary food or meals **(Hattersley and King, 2014)** as well as the nutritional point of view in order to allow consumers to make healthy food choices **(Šedík et al., 2018)**. Each catering services (school kitchens, fast food or street food caterers, restaurants) shall give the appropriate information for the consumer about the food itself, the meals displayed at the menu card, the ingredients, the appearance of different allergenic components, the food preparing technology and storage matters, as not only the absence of allergenic components is necessary, but also the storage and the avoidance of cross contamination **(Abbot, Byrd-Bredbenner and Grasso, 2007)**.

This procedure is highlighted by the **decree of the European Parliament and Commission (1169/2011/EU)** which outlines the compulsory information, which shall be given by the caterers at each stages of the food supply chain. The minimum requirement is to indicate the 14 main allergenic materials, namely cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame seed, sulphur dioxide and sulphites, lupin and molluscs. Ingredients containing these allergens shall be indicated on menu cards and food labels.

Thirdly – and this requirement has a critically high importance – caterers shall provide appropriate training and education for the kitchen staff, to inform them about the importance of allergies and food intolerances. Information shall also be given about the special steps of food handling in the preparation of dietary food and meals. The knowledge of the food handlers is one of the most important influencing factors in the course of health protection of hypersensitive consumers (i.e. patients with food allergies or food intolerances).

The main objective of our research is to evaluate the knowledge of the kitchen workers in relation with food allergies or food intolerances.

Scientific hypothesis

Based on the previous research results and own experiences two scientific hypotheses were formulated.

Hypothesis H1: We assume that the knowledge level of the food handlers in school catering services in relation with food intolerances is rather low, as these workers do not have such education or training.

Hypothesis H2: We assume that the job type, the age, the time spent in catering sector (work experiences) and the education level are correlated with the knowledge about food intolerances.

MATERIAL AND METHODOLOGY

Structure of the questionnaire

The goal of the survey was to evaluate the general knowledge of food handlers on food allergies and food intolerances, by conducting a questionnaire survey. The questionnaire was prepared by the help of food experts, and the suggestions of literature sources dealing with this topic were also taken into consideration. **(Ajala et al., 2010; Dzwolak, 2017; Lee and Sozen, 2018)**. The first version of the questionnaire was filled in after preliminary discussions by 24 kitchen workers, and based on the experiences and suggestions, some modifications were conducted. The final version of the questionnaire contained 12 questions, from which the 4^{th} question included 9 sub-questions. Three questions were related to the knowledge of food and meals which may cause food intolerances, 5 questions were related to the main characteristics of food intolerances and 4 questions were related to the appropriate preparation and handling techniques of dietary meals.

Besides the questions related to the knowledge of food handlers, additional demographic questions were also given to help in the deeper analysis of the results, namely age, job/profile, work experiences in the catering sector, educational level and skills. Each proper answer represented one point and in case of question 4, partial points were also given. The maximum result was 12 points. The results are shown in the percentage of the maximum points.

Study samples

The survey was conducted in June of 2018. The questionnaires were filled in during special training days organized for the food handlers, it means that workers should not work in their shifts at that day. All food workers were employees of different school catering units (kindergartens, primary and secondary schools), some of them worked at cooking kitchens and the other part worked in institutions with serving kitchens. 215 workers filled in the questionnaire, 180 of them worked as kitchen maid, 24 as cook and 11 workers as storage manager.

The kitchen workers of the sample previously took part regularly at compulsory trainings in food hygiene topics, but they did not have specific knowledge about food allergies and food intolerances before filling in the questionnaire. They could rely on only their general knowledge and experiences while responding the questions.

Statistical analysis

Data processing and statistical analyses were performed by using the IBM SPSS Statistics 22.0 for Windows. On one hand, in order to compare means between two groups, we used the independent two-sample t tests. On the other hand, when comparing more than two groups, we used one-way variance analysis (one-way ANOVA). The equality of group variances was tested by Levene-test. Significant differences were detected by using the Duncan's multiple range test in the case of equal variances; while in case of different variances, we used the Tamhane-test.

RESULTS AND DISCUSSION

Sample

The average age of the respondents at the time of the survey was 49 years, the youngest food worker was 22 years old, and the oldest was 75 years old. 35% of the respondents were in the age group of older than 55 years, 48% of the respondents were between 40 – 54 years old and 15% was younger than 39 years old. Five respondents did not indicate their age group, so their answers were not taken into consideration in calculations related to age. The majority (92%) of the respondents was female. The general distribution of educational level showed a relatively balanced image: 28% of respondents indicated that their highest educational level is elementary school, 36% of them finished basic professional level of education (semi-skilled) and 34% of them had secondary or higher education level. Three respondents did not answer for the question related to the educational level. 118 kitchen maids worked at serving kitchens, the others worked at cooking kitchens, from this 62 worked as kitchen maids. Nearly half of the respondents (102 food handlers) indicated that they have more than 10 years of experience in the catering sector (Table 1).

Test results

The average result of the tests was 89.16%, standard deviation was 12.26%. The worst result was 22.92%, the best result was 100%.

According to the job (position), there were significant differences between kitchen maids and cooks. Although the average result the storage managers was better than the results of kitchen maids, significant differences were not detected (Table 1). According to the statistical analyses, there were no correlations between the educational levels, age of respondents, work experience, and there were no differences in the knowledge level of kitchen staff working in cooking and serving kitchens (Table 1).

Results on questionnaire questions

The distribution of the answers of the 215 respondents is shown in Table 2. The questions were separated into three groups:

- food knowledge related to meals which may have impact on hypersensitive individuals (i.e. allergenic food) were represented by questions No. 1., 2. and 4.,
- knowledge on the symptoms of food allergies and intolerances (i.e. allergenic reactions) were represented by questions No. 3., 5., 6., 7. and 8.,
- knowledge of the special handling techniques of the dietary foods and meals (diet food handling) were represented by questions No. 9., 10., 11 and 12.

Food knowledge

The knowledge of the respondents about those food components and meals, which may cause problems for hypersensitive individuals was answered successfully with 91% ±13% result, there were no differences according to the age and job of the food handlers.

Nevertheless, the result of workers with professional education showed significantly better results, compared to workers with elementary education. Other correlations were detected according to the working experience and the knowledge of food that may cause harmful reactions for hypersensitive consumers. Those workers, who had less than one year of working experience, reached worse results than those who had more than 5 years of working experience. The workers of cooking kitchens also reached better results in total, but in case of kitchen maids, their group did not show such differences according to their kitchen type (i.e. cooking or serving kitchens).

Table 1 Descriptive data of food handlers' knowledge scores.

		N	% ±StDev
Total		215	89.16 ±12.27
Position	Kitchen maid	180	87.87 ±12.74[a]
	Storage manager	11	94.88 ±7.02[a,b]
	Cook	24	97.73 ±4.11[b]
Educational level	Elementary	60	87.33 ±15.69[a]
	Semi-skilled	78	90.09 ±8.9[a]
	Skilled	74	89.84 ±11.98[a]
Age	Age range: 55 and above	74	88.82 ±12.47[a]
	Age range: 40 – 54	101	89.6 ±12.86[a]
	Age range: below 39	35	87.38 ±10.5[a]
Work experience	less than 1 year	35	86.61 ±13.59[a]
	1 – 4.99 years	43	87.84 ±13.25[a]
	5 – 9.99 years	35	87.8 ±11.6[a]
	10+ years	102	91.05 ±11.44[a]
Cooking/serving kitchen*	Cooking	62	90.29 ±11.54[a]
	Serving	118	86.6 ±13.19[a]

Note: *Kitchen maids only. [a, b] Results indicated by different letters are statistically different (p <0.05 Student's t-test and ANOVA with Duncan's multiple range test).

Table 2 Food intolerance questionnaire and correct answers in %.

Question	Question type	Kitchen maid (n = 180)	Cook (n = 24)	Storage manager (n = 11)	Total (n = 215)
1. Which of the following items contains gluten? (Apple, Pork stew, **Spaghetti,** Potato with parsley)	Allergenic food	92	100	100	93
2. Which of the following items are risky for guests who have food allergies? (**Pasta with walnut**, Fresh salad, Cooked rice, Fried leg of chicken)	Allergenic food	97	100	100	98
3. What is lactose intolerance? (Intolerance against cereals, **Intolerance against sugar of milk**, Intolerance against nuts, Intolerance against egg)	Allergic reactions	96	100	100	96
4. Check the food items that are considered as major food allergen [b,c]	Allergenic food				
a) **Milk**		91	88	100	91
b) Chocolate		63	42	27	59
c) **Egg**		79	83	91	80
d) **Peanut**		88	92	100	89
e) Strawberry		51	50	55	51
f) Carrot		97	100	100	97
g) Tomato		94	100	82	94
h) Orange		94	100	100	95
i) **Almond**		73	75	73	73
5. Individuals with food allergies can safely consume the foods that cause the allergies if only a small amount is consumed. (True, **False**)[a]	Allergic reactions	86	100	91	87
6. Can high temperature (deep-frying, cooking) destroy food allergens? (Yes, **No**)[a,c]	Allergic reactions	89	100	100	91
7. If someone has an allergic reaction, is it correct to offer water in order to ‘‘dilute” the allergen and stop the reaction. (True, **False**)[a]	Allergic reactions	97	100	100	98
8. If you remove allergenic food items (such as walnuts) from a finished dish, will it prevent the client from having an allergic reaction? (Yes, **No**)[a,c]	Allergic reactions	91	92	100	92
9. Gluten-free meal can be handled together with regular utensils. (True, **False**)	Diet food handling	80	88	100	82
10. The utensils used for handling gluten-free food can be stored with other utensils. (True, **False**)	Diet food handling	71	92	100	75
11. Gluten-free meal can be transported only in airtight container. (**True**, False)	Diet food handling	85	96	91	87
12. Gluten free meal must be handled separately. (**True**, False)	Diet food handling	87	88	100	88

Note: [a] These questions are from **Ajala et al. (2010)**; [b] Similar question has been published by **Ajala et al. (2010)**; [c] Similar question has been published by **Lee and Sozen (2018)**. Correct answers are indicated by **bold** letters.

Knowledge of food allergies and intolerances

The respondents of the survey reached the best results in these field, the average result and deviation was 92% ±15%. There were no differences according to job, age and working experience, moreover the type of kitchen also did not influence the results. The results of respondents with elementary educational level were worse than the responses of skilled and semi-skilled kitchen workers.

Handling of dietary food and meals

This part of the survey brought the worst results, the average result and deviation was 83% ±22%. The storage managers reached better results than the kitchen maids, but the cooks did not differ from the other groups. The results of kitchen maids working in cooking kitchens were better than of those who worked in serving kitchens. This part of the results was not influenced by the age, educational level and working experience.

Discussion

Nowadays, the importance of the existence of food intolerances is increasing in the school catering sector, and one of the most important players of this process is the group of food handlers, who works at the end of the food chain. Food handlers serve the consumers directly, i.e. food handlers will make the final decision about the food (meal): is it safe or not? In this situation, the responsibility of the food handlers is very high, and the knowledge of these workers will determine the final objective, i.e. to serve the appropriate meal for each customers. As the number of children with food allergies and intolerances showed a growing tendency in the past decades, so it is necessary to improve the knowledge level of food workers in this specific topic. The primary goal of our research was to explore and evaluate the knowledge of kitchen workers of the school catering sector in relation with this topic, and to determine those factors, which may influence the knowledge on food hypersensitivity issues.

Our survey was conducted by a paper-and-pencil questionnaire, which was elaborated based on the findings of different literature sources. Based on the feedbacks, we could detect some problems. For example, the 4th question of our questionnaire was not appropriate for measuring the knowledge properly. In this question, chocolate and strawberry was indicated by almost 50% of the respondents as major allergens. Of course, chocolate and strawberry allergy is existing, but they are not so frequent and dangerous, so the term “major allergen” is not clear in this aspect. This problem was also highlighted by previous researchers as well, such type of questions cannot be understood clearly, so the answers might be very diverse **(Ajala et al., 2010**; **Lee and Sozen, 2018)**.

The respondents of the survey have not passed previously any specific training in the topic of food hypersensitivity; they have finished only general training courses in food hygiene topics. Thus, according to our first hypothesis, they do not have high level of knowledge, or they have different knowledge levels related to topics in food allergy and food intolerances. In our research, the average score of 215 food handlers was 89.16% with a deviation of 12.27%, which was higher than the preliminary expectations. The scores on the different questions showed a wide range between 75 – 98%, excluding the results of question 4, which was not considered in the final evaluation. The findings of other literature sources showed lower scores for such questions: according to the findings of **Lee and Sozen (2018)**, the results of the food handlers were between 41.5 – 89.5%, and the results of **Ayala et al. (2010)** were between 69 – 84%.

Based on the abovementioned facts, we rejected our first hypotheses (H1), because the knowledge level of Hungarian food handlers, who did not have previous specific knowledge on food hypersensitivity, cannot be considered as low, even in international comparison.

According to our results, there were some correlations between the knowledge of the food handlers and their job (position). For the position of kitchen maid, no educational requirements are needed, therefore, it was assumed, that their knowledge will be lower than the knowledge of the skilled worker cooks and storage managers. This assumption was verified only in case of the comparison with cooks. The results of other previous researches on the knowledge of food hygiene issues **(Illés et al., 2018)** also showed that there is no correlation between the knowledge and working position of the food workers working in positions like kitchen maids, cooks and storage managers. There were no correlations at all between the test results and the educational level, working experiences and age, in addition, the type of the working place (i.e. cooking or serving kitchens) have not shown any correlations with the knowledge of food handlers. As cooks and storage managers work only in cooking kitchens, an additional analysis was conducted only for the evaluation of kitchen maids, who work in both kitchen types (Table 1).

Based on the abovementioned results, H2 hypotheses was rejected, because the age, the working experience in the catering sector and the educational level did not show correlations with the knowledge level, while the job profile (position) was just partly influenced by the knowledge on food hypersensitivity.

During the analysis of the different fields, it was an alarming observation that the worst results were achieved in the questions related to handling of dietary food. These questions were connected to the everyday working processes and practices of food handlers. Based on these results it is clearly concluded that there is need for specific education and trainings in this topic. Another important observation was related to the knowledge of allergens. Respondents, who have only elementary education level, reached lower scores in general knowledge on the reasons of food intolerances and allergies. To provide safe dietary food for the consumers at cooking or serving kitchens is the responsibility of the food catering service. Therefore, food caterers and food services shall ensure the traceability of allergenic components, which shall be built into their monitoring system based on the principles of HACCS and GHP, and this traceability process is to be monitored by the authorities **(Fontcuberta-Famadas et al., 2018)**. In the course of preparing dietary meals, the general principle is that preparation of dietary food should be separated in time and place. The cleanness of the devices and utensils should be ensured, because the surfaces of the equipment may be a primary source of cross-contaminations **(Ortiz et al., 2018)**. The production and supply of allergen-free food may have several hidden dangers, for which the food

producer or food provider shall take the responsibility. Besides other previous researches **(Ahuja and Sicherer, 2007)**, our survey results also highlighted that the sources of such dangers frequently arise from the non-appropriate knowledge of food handlers.

CONCLUSION

A contemporary challenge of food providers, particularly for school catering services is to prepare and serve food for children with food intolerances or food allergies. In managing this process, and in the prevention of diseases, harmful reactions and diet mistakes, the food handlers – as players who work at the end of the food chain – play an important and active role.

Our research findings highlighted that the general principles of preparing dietary food and meals are not clear enough for the food handlers. In order to manage the process properly, and to prepare and serve healthy food for hypersensitive consumers in a safe and appropriate way, the knowledge of the kitchen workers shall be improved not only in general, but also this special field.

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INFLUENCE OF TEMPERATURE, HUMIDITY, AND DILUENT TYPE ON SURVIVAL OF *SALMONELLA* SPP. ON THE SURFACE OF RAW TOMATOES

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ABSTRACT

Tomatoes are an important commodity, placing fourth among most popular vegetables in the U.S. However, fresh tomatoes lack a final pathogen elimination step and have been implicated in *Salmonella*-related outbreaks. The purpose of the study was to evaluate survival of *Salmonella* post-drying in three diluents on the surface of green mature tomatoes at 12 °C or 25 °C. Additionally, low and high air relative humidity influence was evaluated at 25 °C on pathogen survival. A five *Salmonella* rifampin-resistant strain cocktail was double-washed in buffered peptone water (BPW) and resuspended in 0.1% peptone, BPW, or fresh tomato serum. Inoculum (0.1 mL) was allowed to dry on the surface of tomatoes. For study I, tomatoes were placed in 12 °C and 25 °C incubators with no humidity control and sampled on days 0, 1, 3, and 5. For study II, tomatoes were sampled on days 0, 1 (biosafety hood storage) and on day 5 after storage in two 25 °C incubators (low and high relative humidity). *Salmonella* was recovered from tomatoes (20 mL BPW) and plated (TSA-rif80, 37 °C, 48 hours). Post-drying *Salmonella* counts (ca. 4.5 – 5.0 $\log_{10}$ CFU.mL^{-1}) remained at 4.03 and 4.40 $\log_{10}$ CFU.mL^{-1} in serum after 5 days of storage at 12 °C and 25 °C, respectively. Conversely, corresponding counts in BPW and peptone were lower at ca. 1.4 to 1.8 and 2.2 to 2.8 log units at 12 °C and 25 °C, respectively. At low humidity, post-drying *Salmonella* counts showed highest decline for peptone (final 1.98 $\log_{10}$ CFU.mL^{-1}) compared to BPW (3.79 $\log_{10}$ CFU.mL^{-1}) and tomato serum (4.75 $\log_{10}$ CFU.mL^{-1}) on day 5. Counts declined rapidly to 0.03, 0.56, and 0.44 $\log_{10}$ CFU.mL^{-1} for peptone, BPW, and tomato serum, respectively, at high humidity on day 5. To summarize, it was shown that increased solutes have protective effect on *Salmonella* in desiccated conditions, while high humidity storage causes accelerated death of stationary culture within five days storage period.

Keywords: tomatoes; *Salmonella*; humidity; refrigeration; survival

INTRODUCTION

Tomatoes are an important commodity, placing fourth among most popular vegetables in the U.S. According to **FAOSTAT (2017)**, top ten tomato producing countries in the world were China, India, Turkey, USA, Egypt, Iran, Italy, Spain, Mexico, and Brazil, with Slovakia present in the top twenty and producing as much as 21,964 tonnes in 2017 alone. Enteric pathogens, such as *Escherichia coli* O157:H7 and *Salmonella*, may be present on fresh produce as contamination from environment and may persist on the surfaces **(Sreedharan et al., 2015; Tokarskyy et al., 2018)**. *Salmonella*-associated tomato outbreaks were recorded in the United States on numerous occasions **(CDC, 2002; Croby et al., 2005)**. It is generally believed that pathogen will grow in the tomato flesh at ambient temperature if introduced through stem scars, wounds, and abrasions **(Wei, 1995; Zhuang, Beuchat and Angulo, 1995; Shi et al., 2007; Beuchat and Mann, 2008)**. As for the fate of the pathogen on the healthy tomato surface, most studies agree that *Salmonella* populations decline over time, depending on bacterial strain, humidity, and tomato storage temperature **(Yuk, Warren and Schneider, 2007; Tokarskyy et al., 2018)**. Conversely, a study by **Iturriaga, Tamplin and Escartín (2007)** showed the potential for *Salmonella* Montevideo to colonize and grow on the surface of healthy undamaged tomatoes, but those results could have been due to the presence of micro abrasions on the surface where pathogen could have been introduced, or possibility of the pathogen introduction onto the stem part during wash-off step **(Wei et al. 1995)**. Therefore, *Salmonella* will likely to die on the surface of healthy tomatoes, and its behaviour might mimic survival rate on non-biological inanimate objects.

A systematic review by **Kramer, Schwebke and Kampf (2006)** suggested that nosocomial bacterial pathogens, including *Salmonella* and *Escherichia coli*, persist on inanimate objects longer at higher inocula, higher solute concentration, higher humidity, and lower temperature. Early studies on bacterial desiccation/drying on glass have shown that solutes overall protect bacteria in desiccated

state. For example, **Hirai (1991)** showed, using Rodac plate technique, that *Salmonella* counts in sterile distilled water dried on glass surface decreased to non-detectable level after 7 hours; however, *Salmonella* was detectable for over 5 days if suspended in 2% bovine serum albumen before glass inoculation.

Conversely, humidity effects might be more complicated as **Møretrø et al. (2010)** showed that shigatoxin-producing *Escherichia coli* dried on plastic or steel had highest inactivation rate at 85% relative air humidity, while survived the best at 98%. It can be argued that microorganisms in dried inoculum survive better at low humidity (low metabolic activity) compared to high humidity, where stationary culture, still metabolically active, slowly dies off. However, at low inoculation levels and high organic matter and high humidity might stimulate growth.

It is generally believed that *Salmonella* survives desiccation very well and may persist in dry and low water activity foods. Several *Salmonella* foodborne outbreaks involving dry/low water activity foods, such as peanut butter, were recorded. **Li, Megalis and Tortorello (2010)** has shown that dried (a_W = 0.21) *Salmonella* Typhimurium LT2 culture had 5-log reduction in numbers at 97% relative air humidity, while only 2 log reduction was observed at 33% relative humidity, with similar trend observed for *Salmonella* Tennessee. However, only desiccation-injured pathogens (drying to a_W = 0.21) were impacted by high humidity, while cells dried to higher water activity (a_W = 0.55) survived high relative humidity better.

Stine et al (2005) came to an overall conclusion that lower relative humidity stimulates survival of bacterial pathogens and indicators, such as *Escherichia coli*, *Salmonella enterica*, and *Shigella sonnei* on the surface of lettuce, and bell pepper. However, controversial data exist for *Salmonella* survival on tomatoes, which might be attributed to the phenotypic strain variations. For example, **Rathinasabapathi (2004)** showed that *Salmonella* Montevideo spiked on the surface of pericarp discs cut from green mature tomato can survive with little reduction for at least 6 days at 100% relative humidity. However, **Wei at al. (1995)** have shown that *Salmonella* Montevideo introduced on the surface of unbroken skin at 4 $\log_{10}$ CFU per site survived for at least 48 hours but could not be consistently detected after 5 days. **Lang, Harris and Beuchat (2004)** showed that *Salmonella* counts in 5% horse serum on the spot-inoculated tomatoes decreased 0.8 log after 1 hour drying and 2.2 $\log_{10}$ 24 hours post-drying from initial 7.22 $\log_{10}$ CFU per tomato. However, **Guo et al. (2002)** found that a cocktail of five *Salmonella* strains diluted in sterile tap water and inoculated on the surface of green tomato at 7.72 $\log_{10}$ CFU per tomato experienced only 1 $\log_{10}$ CFU per tomato reduction on day 1 and 3 log reduction on day 7 at 20 °C and 70% relative air humidity. Similarly, **Allen et al. (2005)** showed persistence of *Salmonella* in phosphate buffered saline dried on the surface of tomatoes for at least 14 days at 30 °C/80% RH, 20 °C/60% RH, and 20 °C/90% RH.

Other studies have used deionized water, tryptic soy broth, 5% sterile horse blood, tomato serum, soil, 0.1% peptone water, buffered peptone water, phosphate buffered saline, among others, to dilute *Salmonella* culture before placing on the surface of tomatoes. As noted by **Wei et al. (1995)**, TSB as a diluent supported better bacterial survival on tomato surface and provided protection against chlorine treatment. According to the researchers, *Salmonella* Montevideo grew in TSB, but died rapidly in Butterfield's buffer or tomato serum, while death rate in deionized water was slower. **Guo et al. (2002)** showed that *Salmonella* on tomato in contact with soil was capable to grow up to day 4 and persisted thereafter up to day 10.

Many of the *Salmonella* spot-inoculation studies were done with high level of inoculum (~10^7 CFU per tomato). Low level of inoculation resulted in quick bacterial die-off. It can be argued that such high inoculum might mimic high solute diluent, where solutes from bacteria themselves might protect them in bulk. For example, **Wei et al. (1995)** showed that *Salmonella* Montevideo die-off at <5.12 $\log_{10}$ CFU.mL^{-1} was significantly faster in Butterfield's buffer, tomato serum, and deionized water, compared to 8.15 $\log_{10}$ CFU.mL^{-1} culture. When bacteria were inoculated at 2.85 – 3.86 $\log_{10}$ CFU per tomato in deionized water, the bacterial die off occurred overnight, while survival for 3 days was observed at 9.48 $\log_{10}$ CFU per tomato level. According to **Kusumaningrum et al. (2003)**, *Salmonella* Enteritidis was recovered from inoculated steel squares after drying for at least 4 days at high contamination level (10^5 CFU.cm^{-2}), while at moderate level (10^3 CFU.cm^{-2}) and low level (10 CFU.cm^{-2}) inoculation counts went below detection limit within 24 hours and 1 hour, respectively. In addition, milk residue and chicken fillet suspension improved survivability compared to 0.1% peptone +0.89% saline solution **(Kusumaningrum et al., 2003)**.

Scientific hypothesis

It is hypothesized that high organic solute concentration in inoculation diluent (buffered peptone water and natural tomato serum), as well as low humidity and low temperature, may improve survival of *Salmonella* on the surface of undamaged tomatoes. It is expected that stationary phase *Salmonella* culture on spot inoculated tomatoes stored in high humidity will experience rapid die-off as compared to tomatoes held at low humidity.

MATERIALS AND METHODOLOGY

Rifampin preparation and microbiological media supplementation

Rifampin stock solution (10,000 ppm) was prepared by dissolving 0.4 g of rifampin (Fisher Scientific, BP26795, Pittsburgh, PA) in 40 mL HPLC grade methanol (Fisher Scientific), filter-sterilized (0.2 µm nylon filter), and stored in the dark at 2 °C for no longer than 1 month. Tryptic Soy Broth (TSB) with rifampin at 100 ppm was prepared by aseptic addition of 0.1 mL of rifampin stock solution to 9.9 mL of sterile TSB (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Tryptic Soy Agar (TSA) with rifampin 80 ppm was prepared by aseptically addition of 8 mL of rifampin stock solution to 1 L of sterilized and cooled to 45 °C TSA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). TSA-rif80 was used either for pour plate method (1 mL of analyte with 15 – 18 of liquid TSA-rif80) or spiral plating on pre-poured TSA-rif80 plates (WASP2

spiral plater, Don Whitley Scientific Limited, West Yorkshire England).

Tomatoes and tomato serum preparation

Green mature unwashed and unwaxed round tomatoes (variety Florida 47) for inoculation studies were acquired from local packinghouses. Eight tomatoes were ripened, washed, trimmed, chopped, and 945 grams were homogenized into slurry (1-minute, Waring blender, Waring Products Inc., Torrington, CT, USA). Approximately 100 mL of slurry was centrifuged (10 minutes, 5,000 rpm, SG-3 rotor, Sorvall RC-5B, DuPont Instruments, Corp, Parkersburg, WV, USA) and the supernatant was filter sterilized (0.2 µm nylon filter), stored at 2 °C, and used as a "tomato serum" diluent for *Salmonella* within 24 hours of preparation. Absence of rif resistant microflora in the serum was confirmed on day 0 and day 5 (25 °C) using pour plating using TSA-rif80.

Bacterial strains and inoculation

Salmonella rif-resistant strains (derivatives of Typhimurium ATCC 13311, Braenderup ATCC BAA-664, Enteritidis ATCC 4931, Newport ATCC 6962, and Javiana ATCC BAA-1593, American Type Culture Collection, Manassas, VA, USA), grown in three consecutive TBS-rif100ppm (37 °C, for 12 hours, 1st broth; 12 hours , 2nd broth; and 18 hours, final transfer), were combined 2 mL each, double-washed in BPW (4,000 g, 10 minutes), and finally resuspended in 10 mL of 0.1% Bacto™ peptone **(**Becton, Dickinson and Company, Franklin Lakes, NJ, USA), buffered peptone water (BPW, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), or tomato serum. Total solids and pH of the uninoculated diluents were measured (Brix, refractometer; pH meter). The resulting cocktails were diluted 1:10 in corresponding diluent and 0.1 mL of inoculum was spotted as ten 10 µL drops around blossom end of each green tomato. Inoculated spots were allowed to completely dry on tomatoes for 90 minutes in a biosafety hood.

Tomato storage

For study I, inoculated green tomatoes were placed in 12 °C and 25 °C incubators for 5 days after initial inoculum pre-drying in biosafety hood for 90 minutes, with no humidity control or monitoring.

For study II, green tomatoes were placed in two 25 °C incubators after initial inoculum pre-drying in biosafety hood for 24 hours. First incubator was maintained with low air relative humidity (LH) (~20 – 30% RH), and the atmosphere of the second one was humidified with a shallow pan filled with deionized water and soaked paper towels (HH) (~70 – 90% RH). Humidity and temperature were recorded at 10-minutes intervals (Hobo U12 dataloggers, Onset Computer Corporation, Bourne, MA, USA; software Hoboware Lite v. 3.1.0) throughout the study.

Salmonella enumeration

For study I three inoculated tomatoes and one negative control tomato were randomly pulled on day 0 (90 minutes dry) and afterwards from each incubator (12 and 25 °C) on days 1, 3, and 5.

For study II same set of tomatoes was analysed immediately after inoculation, after 90 minutes pre-drying in biosafety hood, on day 1 (24 hours dry in biosafety hood), and day 5 (25 °C, LH incubator and HH incubator). Each tomato was transferred to 20 mL BPW in sterile stomacher bag and pathogen was recovered by 20 seconds shake, followed by 20 seconds rub, and 20 seconds shake. The rinsate was either plated directly or serially diluted (BPW) and spiral plated (WASP2 spiral plater) or pour plated (TSA-rif80ppm, 37 °C, 24 to 48 hours).

Data and statistical analysis

Each experiment (Study I and Study II) was repeated three times on three different days. Statistical analysis was performed using commercially available software Statistica ver. 10.0 (StatSoft, Inc., Tulsa, OK, USA).

Study I. A multi-factorial design was utilized to determine the influence of diluent type (BPW, 0.1% peptone, and tomato serum), storage temperature (12 and 25 °C), and storage day (day 0, 1, 3, and 5), as well as their interactions, on *Salmonella* counts on tomato surface. Fisher's Least Significant Difference (LSD) test was utilized to separate treatment means when differences (p <0.05) occurred among factors.

Study II. A two-factorial design was used to test the effects of treatment-storage factor (immediately, 90 minutes dry, 24 hours dry, day 5 LH, day 5 HH) and diluent type (BPW, 0.1% peptone, and tomato serum), as well as their interaction, on *Salmonella* counts on tomato surface. Fisher's Least Significant Difference (LSD) test was utilized to separate treatment means when differences (p <0.05) occurred among factors.

Total solids of the uninoculated diluents (BPW, 0.1% peptone, and tomato serum) were evaluated three times, once for each replication, indirectly using handheld refractometer and expressed as average value of degree Brix with standard deviation among three measurements.

Similarly, pH value of the uninoculated diluents (BPW, 0.1% peptone, and tomato serum) were evaluated three times, once for each replication, and expressed as average pH value with standard deviation among three measurements.

Temperature and humidity measurements for each incubator for each replication (Study II) were averaged for all 10-minutes intervals and expressed as average value ±standard deviation.

RESULTS AND DISCUSSION

The measured solids in the diluents, indirectly expressed as ^{o}Brix, were 0.20 ±0.00, 2.50 ±0.00, and 4.93 ±0.15, for 0.1% peptone, buffered peptone water, and tomato serum, respectively. The corresponding pH values were 6.99 ±0.23 (0.1% peptone), 7.16 ±0.04 (BPW), and 4.29 ±0.10 (tomato serum).

Peptone diluent used at concentration 1 g.L^{-1} contained enzymatic digest of protein with no salt. Conversely, BPW used at recommended 20 g.L^{-1} concentration contained enzymatic digest of protein (peptone) 10 g.L^{-1}, sodium chloride 5 g.L^{-1}, disodium phosphate 3.5 g.L^{-1}, monopotassium phosphate 1.5 g.L^{-1} with claimed pH value as 7.2 ±0.2 by manufacturer. The composition of tomato serum remained unknown.

Table 1 Temperature and humidity variations in LH and HH incubators at 25 °C during 5 days tomato storage.

	LH incubator		HH incubator	
	Temp (ºC ±*SD*)	Humidity (% ±*SD*)	Temperature (ºC ±*SD*)	Humidity (% ±*SD*)
Replication 1	25.1 ±0.2	19.3 ±3.7	24.6 ±0.7	71.8 ±2.2
Replication 2	24.8 ±0.3	30.0 ±12.3	25.9 ±0.4	88.1 ±5.9
Replication 3	24.9 ±0.0	19.5 ±3.0	24.7 ±0.3	92.9 ±3.0

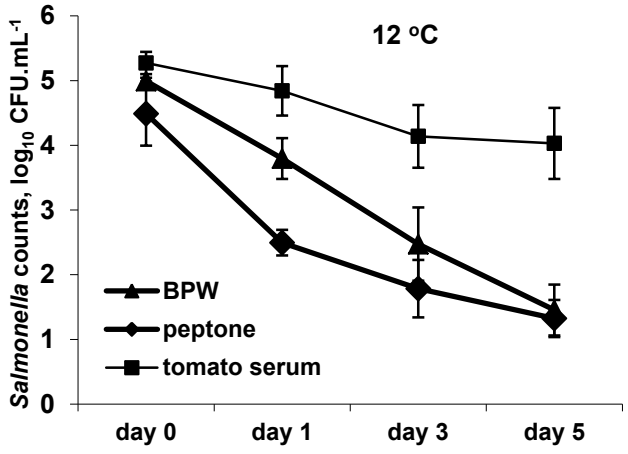

Figure 1 Survival of *Salmonella* on the surface of raw green tomatoes in BPW, 0.1% peptone and tomato serum for 5 days at 12 °C. Counts expressed as $\log_{10}$ CFU.mL^{-1} in 20 mL BPW rinsate. Error bars reflect standard deviation.

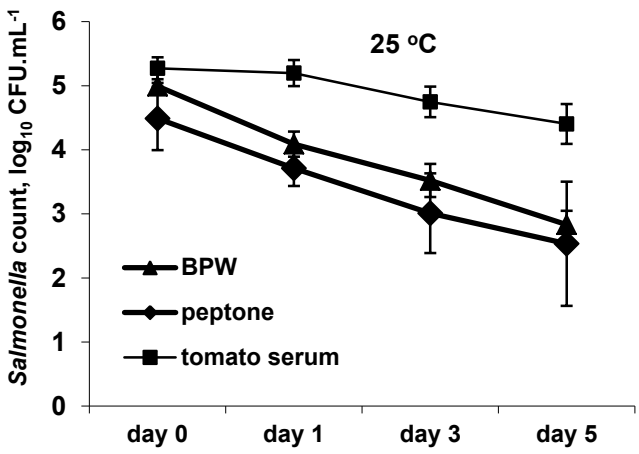

Figure 2 Survival of *Salmonella* on the surface of raw green tomatoes in BPW, 0.1 % peptone and tomato serum for 5 days at 25 °C. Counts expressed as log10 CFU.mL-1 in 20 mL BPW rinsate. Error bars reflect standard deviation.

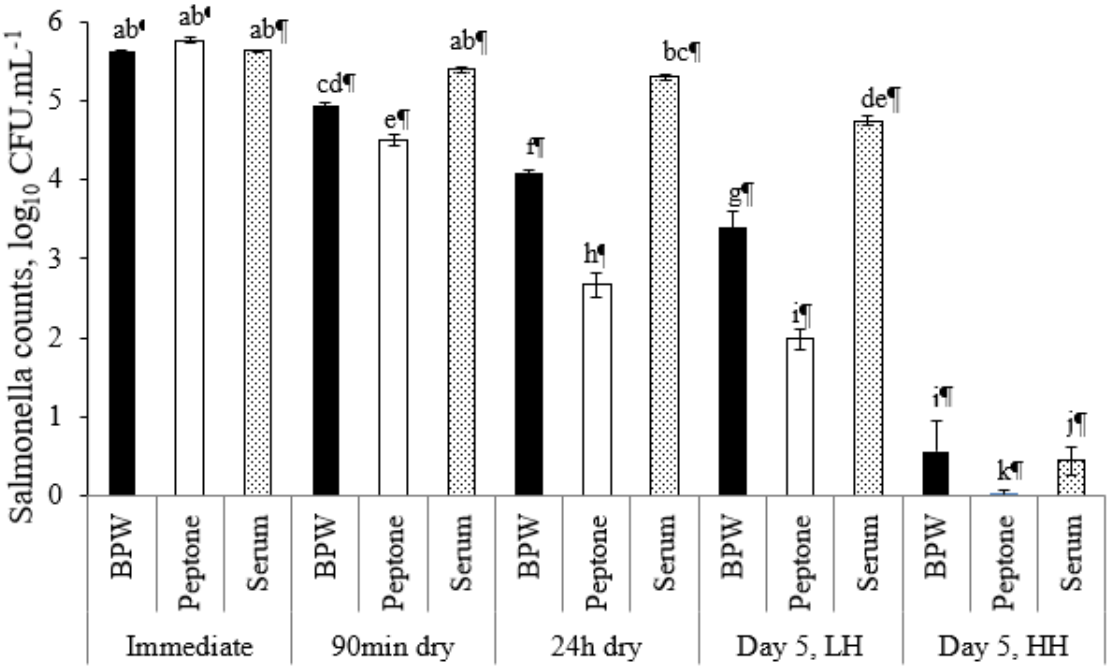

Figure 3 Survival of *Salmonella* on the surface of raw green tomatoes immediately after inoculation, 90 minutes postdrying and 24 hours postdrying in biosafety hood, and after 5 days in low humidity incubator and high humidity incubator at 25 ºC. *Salmonella* counts are expressed as mean values of $\log_{10}$ CFU.mL^{-1} in 20 mL BPW rinsate of three replications. Error bars reflect standard error of mean. Means with the same letters are not significantly different (p >0.05)

Study I. All three factors (diluent type, storage temperature, and storage day) had significant influence on *Salmonella* recovery in rinsate diluent (p <0.05).

Estimated inoculation level was ca. 5.6 $\log_{10}$ CFU.mL^{-1} per tomato as expressed by counts in BPW rinsate. *Salmonella* counts upon storage at two different temperatures is shown in Figures 1 and 2. Upon 90 minutes drying, *Salmonella* counts declined significantly the most in low-solute 0.1% peptone (4.49 $\log_{10}$ CFU.mL^{-1}, p <0.05), comparing to BPW (4.99 $\log_{10}$ CFU.mL^{-1}) and tomato serum (5.27 $\log_{10}$ CFU.mL^{-1}), which were not significantly different between each other (p >0.05). *Salmonella* counts remained at 4.03 $\log_{10}$ and 4.40 $\log_{10}$ CFU.mL^{-1} in tomato serum after 5 days of storage at 12 °C and 25 °C, respectively, with no significant difference between two values (p <0.05). *Salmonella* counts in BPW were significantly lower (p >0.05) comparing to tomato serum on day 5 at both storage temperatures, 1.45 $\log_{10}$ and 2.83 $\log_{10}$ for 12 °C and 25 °C, respectively. Moreover, there was a significant difference between two storage temperatures for BPW diluent on day 5 (p >0.05). Similarly, *Salmonella* counts in peptone were significantly lower (p >0.05) comparing to tomato serum, but not to BPW, on day 5 at both storage temperatures, 1.32 $\log_{10}$ and 2.53 $\log_{10}$ for 12 °C and 25 °C, respectively. The difference for *Salmonella* counts between 12 °C and 25 °C for peptone diluent on day 5 was significant as well (p >0.05).

Similarly, **Wei et al. (1995)** showed that *Salmonella* Montevideo dried on tomato surface in water at ca. 5.8 $\log_{10}$ CFU per area died after four days, while pathogen dried in TSB persisted and increased in numbers at 25 °C and 72% RH. Conversely, *Salmonella* died in tomato juice upon storage, which might be attributed to autoclaving **(Wei et al., 1995)**, as tomato pulp is suitable for *Salmonella* growth. **Guo et al. (2002)** showed *Salmonella* growth on tomato surface in contact with soil up to four days and persistence until day 10 thereafter, while *Salmonella* in water diluent declined by 3 $\log_{10}$ units on day 7. Similarly, **Hirai (1991)** showed that solutes overall protect *Salmonella* in desiccated state on inert glass surface. As expected, death in the presence of high solutes was slower. However, the lack of preservation effect of low temperature on *Salmonella* can be explained by rapid humidification of air in the incubator during sampling and inoculated spots temporary liquefaction, causing stress to bacteria.

Study II visual observations of inoculated tomatoes stored at high humidity suggested hygroscopic nature of diluent solids, as BPW and tomato serum inoculated spots, but not peptone water spots, liquefied. Average temperature and humidity values, both in LH and HH incubator, are shown in Table 1. *Salmonella* counts obtained during Study II experiments are shown in Figure 3. Both factors, namely, treatment-storage and diluent type, as well as their interaction, had significant influence on *Salmonella* counts (Figure 3, p >0.05). *Salmonella* counts post-drying in biosafety hood (24 hours) declined to 5.3 $\log_{10}$ CFU.mL^{-1} in tomato serum, comparing to 4.08 and 2.67 $\log_{10}$ in BPW and peptone, respectively. At this time frame, only *Salmonella* counts in tomato serum were not significantly different comparing to initial counts upon inoculation and 90 minutes post drying (Figure 3, p <0.05). At 5 days low humidity storage at 25 °C, *Salmonella* counts in tomatoes remained as high as 4.75 $\log_{10}$ comparing to decline in BPW (final 3.40 $\log_{10}$ CFU.mL^{-1}) and peptone (1.98 $\log_{10}$ CFU.mL^{-1}). Interestingly, 5 days high humidity storage at 25 °C accelerated *Salmonella* populations decline to close to below detection limit at 0.44, 0.56, and 0.03 $\log_{10}$ CFU.mL^{-1} for tomato serum, BPW, and peptone diluent, respectively. Those data further solidified the concept of solute protective effect in desiccated state. **Li, Megalis and Tortorello (2010)** has shown that day one dried (a_W = 0.21) *Salmonella* Typhimurium LT2 culture had 5-log reduction in numbers at 97% RH, while only 2-log reduction was observed at 33% RH. It has been shown that microorganisms in dried inoculum survive better at low humidity compared to high humidity. However, at a low inoculation level along with high organic matter, high humidity may stimulate growth **(Wei et al., 1995; Guo et al., 2002)**. As noted by **Wei at al. (1995)**, TSB as a diluent not only supported better bacterial survival on tomato surface, but also caused protection against chlorine treatment. According to the researchers, *Salmonella* Montevideo grew in TSB, but died rapidly in Butterfield's buffer or tomato serum.

These data can be compared to the temperature/humidity fluctuations during tomato growing season in major US tomato growing states, such as Florida and California, as well in European countries. According to the results, lower temperature storage was not more beneficial regarding rate of *Salmonella* die-off. A recommendation of high average relative humidity (>72%) within first three days of storage, might result in higher bacterial die-offs. Potentially more significant in terms of public health is that tomato surface cleanness may play an important role regarding *Salmonella* survival.

CONCLUSION

It has been shown that high solute diluent for inoculum preparation, mimicking naturally soiled tomato surface, improves survival of *Salmonella* on the surface of undamaged tomatoes at low humidity storage. Additionally, stationary phase culture on spot inoculated tomatoes stored in high humidity experiences rapid die-off. Cleanness is one of the key factors to keep tomato surface unsuitable for pathogen survival, as solutes can contribute to survival and growth.

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INFLUENCE OF TECHNOLOGICAL PROCESSING ON LIPID-LOWERING ACTIVITY OF SUBSTANCES CONTAINING IN PORCINE HEARTS AND AORTAS

Elena Kotenkova, Irina Chernukha

ABSTRACT
Edible by-products are a good source of nutrients and bioactive substances and could be used as functional ingredients or for biopeptides production natively contained in raw materials. A wide range of peptides are also formed during the enzymatic hydrolysis or food processing. The comparative results of the effectiveness of isolated certain protein and peptide fractions by ultrafiltration with the same natively presented in raw tissues, as well as the influence of heat treatment on biological activity of origin active substances are presented. The model of rat alimentary hyperlipidemia was developed by adding cholesterol and fat to the standard diet and vitamin D_2 injection *per os*. Serum lipid profile was determined on automatic analyzer BioChem FC-360. Dynamic of changes in serum lipid profile was assessed as corresponding control group medium results in ratio to certain rat data. Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE) with following identification by MALDI-TOF MS and MS/MS mass spectrometry. Consumption of native pig aorta and pig heart during 14th days led to normalization of lipid profile in serum of hyperlipidemic rats, while low molecular weight (LMUF, MW <5 kDa) and medium molecular weight (MMUF, MW = 5 – 30 kDa) ultrafiltrates of pig aorta extract did not strongly influenced on level of triglicerides and, on contrary, elevated high density cholesterol. Consumption of developed product by hyperlipidemic rats during 28th days did not lead to significant changes in serum lipid profile, while on 42nd day all ratios reached ones in group, which were treated with native raw material or isolated active fractions. The stability of developed product was confirmed by proteomic studies. Obtained results open prospects to modernization the technology, presumably use as a matrix dietary meat (e.g. poultry) with incorporated active identified components.

Keywords: by-products; heart; aorta; lipid-lowering activity; functional additives and product

INTRODUCTION
A large number of by-products are left after farm animal's slaughter; edible ones such as internal organs are a good source of nutrients and bioactive substances and could be used as functional ingredients or for biopeptides production **(Toldrá, Mora and Reig, 2016; Alao et al., 2017)**.

Natively contained in raw materials peptides or formed during the enzymatic hydrolysis or food processing demonstrated hypotensive, antioxidant, opioid, immunomodulatory, prebiotic, mineral-binding, cholesterol-lowering and antimicrobial activity **(Bauchart et al., 2006; Mine and Shahidi, 2006; Ahhmed and Muguruma, 2010; Toldrá et al., 2012; Udenigwe and Howard, 2013; Lafarga and Hayes, 2014)** and could be used as a functional ingredient in food processing for specialized purposes. Collagen, hemoglobin and casein are mostly studied, a lot of peptides were isolated, identified and their functions were determined **(Toldrá et al., 2011; Wang et al., 2013; Lafarga et al., 2016; Mohanty et al., 2016; Arrutia et al., 2017; Bamdad et al., 2017; Hongdong and Bo, 2017)**.

Technological processing, especially enzymatic treatment is a good tool for bioactive peptide generation. Nevertheless, it's known that every tissue is characterized uniqe proteome and peptidome, which are involved in maitance of its own normal physiological condition **(Fagerberg et al., 2014)**. Moreover, it was found that besides peptides with defined functions, tissues of living organisms contain powerful peptide background, which mainly consists of fragments of larger molecules or functional proteins **(Chugunov, 2010)**.

In previous studies we confirm lipid-lowering action of raw material (porcine heart and aorta), different protein fractions isolated from aorta, and functional meat product produced from porcine heart and aorta in certain ratio on hyperlipidemic rats **(Chernukha, Fedulova and Kotenkova, 2015; Chernukha, Fedulova and Kotenkova, 2018; Chernukha et al., 2018a, Chernukha et al., 2018b)**.

This paper reports the analytical results of influence of the type of technological treatment on hypolipidemic activity substances naturally presenting in porcine heart and aorta.

Scientific hypothesis

Different technological treatment could influence on biological effectiveness of substances presenting in corresponding tissue. It's known that an enzymatic treatment lead to release different active peptides from some wastes of farm animal's slaughter. On the other hand, specific by-products are already enriched with unique substances with certain biological function and involved in maitance of its own normal physiological condition. In this case we decided to compare the effectiveness of isolated certain protein and peptide fractions by ultrafiltration with the same natively presented in raw tissues. Also we studied the influence of heat treatment on biological activity of origin active substances. All these analytical results could propose final recommendation for further raw material processing to produce functional meat product.

MATERIAL AND METHODOLOGY

Native porcine heart and aorta, low molecular weight (LMUF, MW <5 kDa) and medium molecular weight (MMUF, MW = 5 – 30 kDa) ultrafiltrates of pig aorta extract, meat product containing porcine heart and aorta in ratio 3:1 were objects of study.

Ultrafiltrates production

Aorta tissues were homogenized in a grinder KENWOOD (UK) with a stainless steel plate (3 – 5 mm hole), re-frozed and then homogenized in a cutter KG Wetter 258/1336 (Germany) with the addition of distilled water in the ratio (4:1) and knife shaft speed 2000 rpm. Then homogenate were reconstituted in 0.9% NaCl solution, and extracted during 24 hours with stirrer speed 500 rpm. Extract separation was carried out by centrifugation for 7 – 10 minutes at 3,000 – 3,500 rpm on centrifuge CM-6M (ELMI, Latvia). Supernatant was collected and ultrafiltrated on PES membrane (MWCO 5 and 30 kDa) by tangential filtrationon VivaFlow 200 system (Sartorius, Germany). Low molecular weight (LMUF, MW <5 kDa) and medium molecular weight (MMUF, MW = 5 – 30 kDa) ultrafiltrates were lyophilized in INEY-4 (IPB RAN, Russia) to protein concentration 0.9 $g.L^{-1}$.

Meat product manufacture

Meat functional product was produced on ZAO "Yoshkar-OlinskiyMyasokombinat". Porcine hearts were chopped with a particle size of 2 – 3 mm and salted for 12 h. Porcine aortas were chopped with a particle size of 2 – 3 mm and homogenized in cutter at 3000 rpm for 2 – 3 min. Minced hearts with the juice were quantitatively transferred in the cutter and mixture was then homogenized at 3000 rpm for 6 – 8 min (ratio of aorta to hearts 1:3). Obtained mince was packed in cans and sterilized at 115 °C, a pressure of 0.23 MPa for 40 min. Meat product contained 17.53 ±0.95% protein, 3.82 ±0.13% fat, 0.305 ±0.015% sodium chloride, and 2.35 ±0.25% starch.

Animal experiments

Male Wistar rats (380 ±20 g) aged approximately 12 months were kept under standard conditions (temperature 20 ±3 °C, humidity 48 ±2%, day/night (from 06.00 to 18.00 hours/from 18.00 to 06.00 hours), no more than six rats per plastic cage), and water and feed were available ad libitum. Rats were obtained from Andreevka (Moscow region, Russia), acclimatized for 5 days, and grew up to 12 months of age before use in this study. The model of alimentary hyperlipidemia was developed by adding cholesterol and fat to the standard diet (standard chow (Labkorm, Russia)) and vitamin D_2 injection *per os* **(Chernukha et al., 2018b)**.

Native porcine heart and aorta testing protocol

After modeling the rats were randomly divided into three groups: control (n = 10) animals were administered standard chow, group A (n = 10) - pig heart tissue, group B (n = 10) - pig aorta tissues. All samples were mixed with standard chow in quantity 10 g per kg body weight for 14 days. According to physic-chemical protocol of raw material testing and electrophoretic study results, porcine aorta contained 21.40% protein, including approximately 10% proteins lower 30 kDa and 30% polypeptydes in all nitrogen, therefore experimental animals consumed 10 x 0.214 x 0.4 = 0.86 g target fraction per kg body weight. Porcine heart contained 13.23% protein, including approximately 14% proteins lower 30 kDa and 51% polypeptydes in all nitrogen, therefore experimental animals consumed 10 x 0.1323 x 0.65 = 0.86 g target fraction per kg body weight.

Ultrafiltratesand meat product testing protocol

At the end of modeling animals were randomly divided into four groups: control (n = 10) rats were administered 0.9% solution of sodium chloride, group C (n=10) – LMUF, group D (n=10) – MMUF. All samples were administered *per os* in dose 0.3 mg protein per kg body weight for 14 days. The dose was determined according to the recommended dose of commercial analogue - food bioactive additive containing a mixture of peptides isolated from the vessels of farm animals (Scientific and Production center of Revitalization and Health (SPRH), Russia).

Meat product testing protocol

After modeling, rats in control group were fed with standard chow, in group E – meat product ($8g.kg^{-1}$ b.w.) in mixture with standard chow during 28 and 42 days. The dose was reduced to $8g.kg^{-1}$ b.w. because in raw material experiment animals during all study left about 30 – 40% of standart chow mixrure with testes samples. During meat product testing there was no such observation.

Biochemical analysis

After the experiment, the animals were euthanized (VETtech, UK), blood samples for biochemical studies and were taken. Biochemical investigations were carried out on automatic analyzer BioChem FC-360 (HTI, USA) according to instructions applied to measurement kits

(HTI, USA). Total cholesterol (TCL), triglyceride (TG), cholesterol low-density lipoproteins (CL LDL) and cholesterol high-density lipoproteins (CL HDL) levels were measured in rat serum. Atherogenic index (AI) = (TCL - CL HDL)/ CL HDL. Dynamic of changes in serum lipid profile was assessed as corresponding control group medium results in ratio to certain rat data. Therefore ratio <1 means that experimental group value was higher control mean, ratio >1 – lower.

Proteomic study

Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). Following reagents were used: urea, acrylamide, methylene bisacrylamide, agarose, Tris, glycine, sodium dodecyl sulfate, ammonium persulfate, Triton X-100, 2-mercaptoetanol, bull serum albumine, ampholines pH 3 – 10, 5 – 8 (Sigma, United States), amberlite IRN-150L (Amersham Biosciences, Sweden). The subsequent detection of the proteins was carried out by staining with silver nitrate (Panreac, Spain) as described previously **(Kovalyov et al., 2006)**. The resulting digital images were edited in a graphic editor and the quantitative protein content was calculated using ImageMaster 2D Platinum version 7 ("GE Healthcare", Switzerland).

Protein fractions were excised from the gel, grinded and undergone trypsinolysis (Sigma, Germany) **(Zvereva et al., 2015)**. Obtained peptides were investigated by MALDI-TOF MS and MS/MS mass spectrometry on Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive ion mode in molecular weight range of 500 – 8000 Da with calibration according to known peaks of trypsin autolysis.

Bioinformatics analysis

Analysis of obtained tryptic peptides mass spectra was performed using Peptide Fingerprint option in Mascot software (MatrixScience, USA) with MH+ mass determination accuracy of 0.01%; search was performed in databases of the National Center for Biotechnology Information, USA (NCBI).

Statistic analysis

STATISTICA 10.0 software was used in this study for the statistical analyses. The results were calculated as "middle value ± standard error" (M ± SE). Significant differences were tested by one-way ANOVA, followed by the Tukey test. Differences with *p*-values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The analytical results of dynamic of changes in serum lipid profile are presented in Table 1. Maximum elevation of total CL ratio was noticed in group B (native pig aorta) and was higher value in group A (native pig heart) by 27.5%, while TG ratio was lower by 42.4%. CL LDL ratio, on contrary, in serum of rats group B (native pig aorta) was higher group A (native pig heart) value by 19.3%, as well as CL HDL ratio was higher by 24.3%. Observed changes compensated each other; therefore the AI ratio was the same and showed no statistical difference.

There were no significant changes in total CL, CL LDL, CL HDL and TG ratios between group C (LMWU) and D (MMWU). It was also noticed, that CL HDL and TG elevated control values, because ratios did not exceed 1, while CL LDL was also higer control – corresponding ratios were higher 1. Nevertheless, the AI ratios corresponded with group A (native pig heart) and B (native pig aorta) and was lower control group approximately by 2 fold. This observation was explained previously by phenomenon of CL non-LDL and non-HDL reduction in rat serum, which is also associated with atherogenic lipoprotein fractions, as well as elevation of CL HDL **(Chernukha et al., 2018)**.

Consumption of developed product by hyperlipidemic rats during 28 days did not lead to significant changes in serum lipid profile. Total CL and CL ratio did not differ from control; corresponding ratios did not exceed 1, CL HDL ratio was lower 1. There was a slight increase in TG ratio, as well as in AI ratio. On 42nd day of developed product consumption all ratios reached ones in group A – D, which were treated with native raw material or isolated active fractions. Total CL, CL LDL, CL HDL, TG and AI ratios elevated by 50.5% (p <0.05), 39.0%, 29.8%, 11.4% and 37.0%, respectively.

Summarising, we observed that in group C (LMWU) and D (LMWU) there was not such effect as in group B (native pig aorta), the ratio of TG was lower 1 and therefore higher control value. It could be explained both significantly lower concentration of ultrafiltrates dosage as well as separation of active fraction into two-lower 5 kDa and 5 – 30 kDa. Nevertheless, the ratio of AI in both group

Table 1 Lipid profile in the serum of hyperlipidemic rat model.

Groups	Cholesterols (medium control value in ratioto certain rat data)			Triglycerides	Atherogenic index
	Total	LDL	HDL	(medium control value in ratio to certain rat data)	
Group A	1.31 ±0.10	1.09 ±0.07	1.07 ±0.11	3.09 ±0.92^{a}	1.88 ±0.31
Group B	1.67 ±0.16^{b}	1.30 ±0.05	1.33 ±0.13^{a}	1.78 ±0.33	1.91 ±0.06
Group C	1.30 ±0.09	1.13 ±0.09	0.85 ±0.06^{b}	0.92 ±0.15^{b}	1.90 ±0.09
Group D	1.46 ±0.11^{b}	1.12 ±0.09	0.95 ±0.07^{b}	0.90 ±0.13^{b}	1.98 ±0.19
Group E (28 days)	1.01 ±0.04^{a}	1.05 ±0.04	0.84 ±0.07^{b}	1.32 ±0.18^{b}	1.35 ±0.09
Group E(42 days)	1.52 ±0.15^{b}	1.46 ±0.30	1.09 ±0.03	1.47 ±0.18^{b}	1.85 ±0.25

Note: $^{a-b}$ -significant differences between the experimental groups (p <0.05).

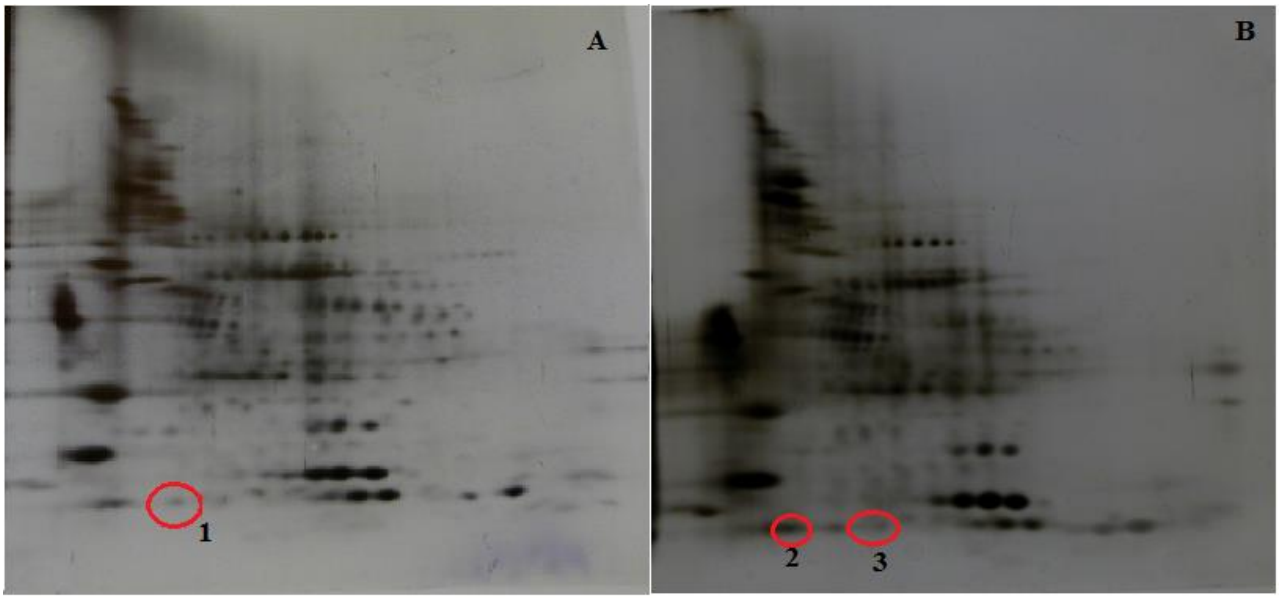

Figure 1 2DE of developed product.
Note: A – 2016 year of processing, B – 2018 year of processing, red round corresponded to fatty acid binding protein.

Table 2 The results of mass spectrometric identification (MALDI-TOF MS и MS/MS) of protein fractions.

№	Protein name; (Gene symbol)	S/M/C *	Мм/pI (exp.)**	Мм/pI (calc.)**
1	heart fatty acid-binding protein (H-FABP) (+ Acetyl (Protein N-term)*****(1)	283/13/68	14.8/5.11	14.8/6.11
2	Mixture of heart fatty acid-binding protein (*H-FABP*)***(1) + Deamidated (96Q,99N), cytochrome c oxidase subunit 5A, mitoch (*LOC100156967*)***(1) + Acetyl (Protein N-term)	122/9/56 38/17/73	15.0/5.25	14,8/6,11 16.7/6.42
3	Mixture of heart fatty acid-binding protein (*H-FABP*)***(1) + Acetyl (Protein N-term) and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 isoform X1 (*NDUFA5*)***(1)	120/4/36 113/2/36	15.0/5.60	14.8/6.11 13.3/7.79

Note: * S/M/C: Score – indicator of conformity or «scorecard»; Match peptides – the number of matched peptides; Coverage – % coverage of the entire amino acid sequence of the protein by identified peptides.
**mM/pI (experiment) – scores obtained as a result of electrophoretic mobility on the DE and mM/pI (calculation) – estimates made based on amino acid sequence data with consideration of signal peptide removal, but with no consideration of other post-synthetic modifications using the ExPASy Compute pI/Mw tool software.
***msms – indication of identification by tandem mass spectrometry, the number of sequenced tryptic peptides in parentheses.

C (LMWU) and D (LMWU) was approximately equal to group B (native pig aorta) and A (native pig heart).

In contrast to group C (LMWU) and D (LMWU) in group E there was another dynamic: on 28th day the ratio of TG was higer 1, while the ratio of AI was not so high despite of elevation CL HDL (ratio was lower 1). On 42nd day the ratio of CL HDL was higer 1, the ratio of AI was approximately equal to groups A (native pig heart), B (native pig aorta), C (LMWU) and D (LMWU).

In previous studies, several tissue-specific proteins were identified in porcine heart and aorta, as well as peptides. Mostly observed substances were decomposed during heat treatment of meat product, except fatty acid binding protein and several peptides **(Chernukha et al., 2016)**. However, it was proposed that tissue-specific proteins could be decomposed into active peptides with similar biological action or retained residual activity. In this study analytical study we revealed that meat product characterized by milder hypolipidemic action compare with native raw material or isolated active fractions; a significant effect was observed only after 42 days of consumption. Obtained results confirm our hypothesis.

There are different opinions about the relationship between the content of cholesterol or its atherogenic fractions and the risk of heart attack or stroke. While some scientists argue that there is no established relationship, others link worsening of a person after stroke or heart attack with elevated concentrations of cholesterol in blood, in particular, low-density cholesterol **(Demarin et al., 2010; Nelson, 2013; Ference et al., 2017; Hindy et al., 2018; Matthews, 2018)**. Developed product pronounced as a component of diet. Revealed long-term effect of product meets the requirements for patients receiving traditional medical treatment.

The creation of food products aimed at their constant use and positively affecting on the lipid profile can be considered as an important component of rehabilitation

therapy as accommodating diet therapy in particular for people with risk of stroke or heart attack.

The developed product was processed twice with two year interval. The 2D proteomic maps presented in Figure 1, results of fatty acid binding protein identification presented in Table 2. It was shown that there were no significant changes in major proteins, which indirectly confirm reproducibility of production technology and long shelf life. Moreover, fatty acid binding protein retained during 2-year storage.

Obtained results confirm that developed product contain peptides with residual hypolipidemic activity. On the other hand, native raw material or isolated active fractions demonstrated higher hypolipidemic effect, therefore it would be perspective to modernize the technology, presumably use as a matrix dietary meat (e.g. poultry) with incorporated active identified components.

CONCLUSION

Despite on the decomposition of target proteins and peptides after product processing, a pronounced lipid-lowering effect was noted, but less active than in case of native raw material or isolated active fractions. It was also found, that such technology treatment as ultrafiltration did not affect on activity of target compounds.

The obtained results open wide horizons for modification of the existing technology both in respect of variation of production modes and in respect of matrix changes and dosing of active proteins and peptides.

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Antioxidant effect of oregano essential oil during various storage meat time of hybrid combination ross 308

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ABSTRACT

This study was conducted to evaluate the effect of *Origanum vulgare* L. *Hirtum* essential oil on the oxidation stability of raw chicken meat. Oregano essential oil was applied in a different way, on the one hand in a feed for broiler chickens (E1) and on the other hand on a surface of chicken thighs (E2). Broiler chickens were fed during the experimental period in the all groups with commercial feed mixtures except the experimental group of E1 (with the addition of 0.05% oregano essential oil, 50 g EO per 100 g of the feed mixture). In E2 was application of oregano essential oil (0.5%) on surface of thighs 1 mL per 60 g of meat realized. The oxidative stability of the chicken meat was investigated in the same way, 8th days after vacuum-packed and stored at temperature 4 °C and 6, 9 and 12 months after vacuum-packed and storage at -18 °C. The samples of the E1 consisted of breast and thigh muscles with skin (150 g) and of the E2 thigh muscle with skin (60 g). The impact of oregano essential oil was measured by content of fat and peroxide value (PV). Fat content in both experiments was not affected by storage time and EO addition. Content of chicken meat fat in E1 in control group ranged between M = 9.64 – 12.95 g.100 g^{-1} and in experimental group contained similar amount of fat mean from M = 9.94 – 12.24 g.100 g^{-1}; E2: in control group M = 7.01 – 7.73 g.100 g^{-1} and in experimental group M = 6.15 – 8.03 g.100 g^{-1}. Measured peroxide values confirm that oregano essential oil has effect on broiler chicken meat oxidative stability, if applied to feed, manifested statistically significant differences between control and experimental group. The mean of peroxide value in control group of E1 was M = 0.58 –3.60 µmol O_2.kg^{-1} and in experimental group was M = 1.06 – 2.11 µmol O_2.kg^{-1}. We found not statistically significant difference in peroxide values, if applied oregano essential oil to raw chicken meat. The results impact of oregano essential oil on chicken meat comparable to control group, but a tendency to improve oxidative stability was indicated.

Keywords: oregano essential oil; antioxidant; chicken meat storage; oxidative stability; peroxide value

INTRODUCTION

The relationship between the consumption of meat and health is multifaceted, and it needs to be analyzed in detail, with specific attention to the relevant differences that characterize the effects of the different meat types. A variable but moderate energy content, highly digestible proteins of good nutritional quality, unsaturated lipids, B-group vitamins and minerals make poultry meat a valuable food. Epidemiological studies performed across the world, in highly diverse populations with different food preferences and nutritional habits, provide solid information on the association between poultry consumption, within a balanced diet, and good health **(Marangoni et al., 2015)**.

Meat and meat products are important sources of high-quality protein. Their acceptance of consumers depends mainly on the proportion of fat, that is responsible for its taste properties and structure **(Lorenzo a Franco, 2012)**. However, despite this benefit, fat is the cause of deterioration in the nutritional and sensory quality of the meat due to its greater susceptibility to oxidative damage **(Qi et al., 2015)**.

Angelovič et al. (2015) constate that the mechanisms of oxidative degradation can be autoxidation in presence of atmospheric oxygen.

The intake of lipids from chicken meat is variable dependent on the cut considered by consumers. Fats are mainly found in the skin and can, therefore, be easily removed during processing or consumption of meat. The lipid content varies from 1% in poor parts such as breasts to 17% in chicken wings with skin. The inclusion of skin can increase these values **(Marangoni et al., 2015)**.

Muscular lipids are highly susceptible to oxidation due to a high degree of unsaturation. Oxidation leads to a deterioration in the taste, color, structure and nutritional value of the meat. Of great importance in the oxidation of

chicken meat are some ingredients (iron content, antioxidants) as well as external factors (feeding with fodder feed, stress, killing process, temperature, processing procedures, storage conditions, etc.) **(Estévez et al., 2014)**.

The main factor influencing lipid oxidation in meat is oxygen, which reacts with unsaturated lipids to form lipid peroxides. The resulting lipid peroxides lead to the formation of various chemical compounds such as alcohols, aldehydes and ketones **(Domínguez et al., 2014)**, which are responsible for the unpleasant taste and odor and thus also for the reduction of the sensory and nutritional quality of the meat.

Lipid oxidation is possible to prevent through adding synthetic and natural antioxidants to animal feed or to processes meat and meat products. Synthetic antioxidants have been confirmed for their toxicological and carcinogenic effects. Natural antioxidants may be found in any plant part and most natural antioxidants are phenolic compounds, and the most important are the tocopherols, flavonoids, and phenolic acids **(Kumar et al., 2015)**.

The genus *Origanum* belongs to the family of *Labiatae* and includes many species that are commonly found as wild plants in the Mediterranean areas. Thirty-eight (38) *Origanum* species are recognized in the world. Due to the variability in chemical and aroma characteristics is used *Origanum* in cosmetic industries, as a culinary herb, flavoring substances of food products, alcoholic beverages and perfumery for their spicy fragrance **(Pirigharnaei et al., 2011)**.

Essential oils (EO) are volatile, natural compounds formed by aromatic plants as secondary metabolites, known for their antiseptic, bactericidal, viricidal and fungicidal, and medicinal properties and their fragrance, they are used in preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies **(Bakkali et al., 2008)**.

Dominant components of oregano EO are composed of carvacrol and thymol, followed by γ-terpinene, pcymene, linalool, terpinen-4-ol and sabinene hydrate. *O. vulgare* ssp. *hirtum*, contained a high amount of EO. The content of EO as high as 8% with carvacrol as dominant component (95%) was reported for this subspecies **(Pirigharnaei et al., 2011)**.

Freezing is one of the several methods of preserving meat and protecting its quality until it reaches the consumer **(Xia et al., 2012)**.

The main objectives of this preservation method are to inhibit microbial growth, delay metabolic activities and oxidative damages. It allows maintaining almost all product characteristics of products and to stock them for long periods **(He et al., 2013)**. Despite being one of the least aggressive preservation methods, freezing still produces modifications in foods.

Bennett et al. (2014) report that oxidation can be initiated by the formation of lipid peroxides, which can be detected by measuring the peroxide value, that quantifies the levels of peroxides and hydroperoxides formed in the initial phase of lipid oxidation. The determination of the fat peroxide value is used to determine the degree of fat loss. The low peroxide fat value usually indicates that the fat has not become rancid and will have good stability.

Fresh fats have a peroxide value of 1 – 2, whereas rancid fats have a peroxide value of 15 – 20. Rancidity is caused by oxidation and hydrolysis **(Sharma, Giriprasad and Goswani, 2013)**.

Scientific hypothesis

The aim of this paper is to study the effect of oregano essential oil supplementation on chicken meat quality and lipid oxidation stability during various storage time of meat.

MATERIAL AND METHODOLOGY

Biological material

Two experiments were carried out. The difference between the 1st (E1) and 2nd (E2) experiment was in the way of application of oregano essential oil (EO). The application of oregano essential oil was performed in the E1 in the feed and in the E2 on the surface of the chicken thighs. The oxidative stability of the chicken meat was investigated in the same way in both experiments, after vacuum-packed 8 days after vacuum-packed and storage at 4 °C and 6, 9 and 12 months after vacuum-packed and storage at -18 °C.

The E1 was conducted in poultry farm, in conditions of the welfare principles application whit deep litter breeding system. The conditions responded protection requirements for broilers chickens **Council Directive 2007/43/EC**. Microclimatic conditions (light, temperature, humidity and air exchange) were uniform for both groups in accordance with recommendations for the meat broiler chickens ROSS 308. Broiler chickens (40 one-day-old broiler chickens) were divided in the E1 into 2 groups, control (CG) and experimental – EG (n = 20). Broiler chickens were fed during the experimental period in the control group with commercial feed mixtures without oregano essential oil (EO) and in the experimental group with similar diets as in the control group but with the addition of 0.05% EO, in amount of 50 g of EO per 100 g of the feed mixture. Broiler chickens consumed of feed mixtures *ad libitum*. The experimental period lasted 38 days and tree feed mixtures were used: starter feed mixture, for chickens to 18 days of age (feed from plate feeders and water from the hat drinkers located on the floor); grower mixture, from 19 to 31 day of age (feed from the tube feeders and drank water from bucket drinkers till end of the experiment) and finisher mixture, from 32 to 38 day of age. Broiler chickens were transported to the chemical laboratory of Department of Food Hygiene and Safety, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture Nitra and killed after ending experiment. The breast and thigh muscles with skin were deboned and used for analyses (8th days after slaughter and storage at 4 °C and after 6, 9 and 12 months of storage at -18 °C of chicken meat).

In the E2 were broilers chickens killed on slaughterhouse and selected thighs randomly, after slaughtering. Chicken thighs were obtained from broiler chickens that were farmed and fed with commercial feed mixtures. Chicken thighs were deboned and used for analyses in the 8th day (measurement 30 minutes after application 0.5% of EO on the surface of thighs) and after 6, 9 and 12 months. In both measurements were thighs divided to two groups: control group (samples without treatment), experimental group (samples with application of EO on surface of thigh). Each

Table 1 Experiment scheme.

Experiment	Sample n	Storage time and temperature	Chemical analysis
E1			
Control group	20	8 days at 4 °C 6, 9 and 12 months at -18 °C	of chicken meat achieved from broiler chickens fed with commercial feed mixtures without EO
Experimental group	20		of chicken meat achieved from broiler chickens fed with 0.05% EO in the feed mixtures
E2			
Control group	6	8 days at 4 °C 6, 9 and 12 months at -18 °C	chicken thighs without application of EO
Experimental group	6		chicken thighs with application of 0.5% EO

Note: E1 – 1st experiment with EO in feed mixtures, E2 - 2nd experiment with application EO on raw thigh muscle.

group contained 6 samples which were used for oxidative stability measurements. Scheme of experiment is given in Table 1.

Laboratory testing

Sample preparation

The samples consisted of: E1: chicken muscles (breast and thigh) and skin; E2: thigh muscle and skin. Samples were deboned and collected on the day of slaughter vacuum-packed and stored at 4 °C for 8 days and at -18 °C for further analysis after 6, 9 and 12 months of storage. In the E2 were samples of experimental group with EO treated (1 ml of oregano essential oil applied on surface of thigh muscle 60 g). Time of EO treat was 30 minutes, amount 1 ml per 60 g of meat. Chemical analysis (fat content, peroxide value) of the meat samples were carried out after homogenization (according to the method AOAC 983.18), drying of the samples under the prescribed conditions at 105 °C to constant weight. The dried samples were then milled into powder using Grindomix GM 200 grinder and used to obtain fat by extraction (non-polar solvent) in a Soxhlet extractor and expressed as g per 100 g of fresh tissue.

Chemicals analyses

Oregano essential oil (*Origanum vulgare* sup. *Hirtum*) was purchased from Calendula a.s., Nová Ľubovňa, Slovensko with a total antioxidant activity of EO 93.85% and carvacrol content 57%.

Determination of fat content: was used an official method to determine fat AOAC 991.36. Crude fat content is determined by extracting the fat from sample using a chemical solvent (petroleum) under the extraction method. Lipid extraction was carried out using a device Det-gras N, model 4002842.

Fat content calculation (g.100 g^{-1}):

$$Crude\ fat = \frac{\mathrm{w2} - w0}{w1} \times 100$$

w2 – is weight of the extraction thimble with extracted fat
w0 – is weight of the empty extraction thimble
w1 - is weight of the sample

Determination of peroxide value (PV): was used an official method to determine peroxide value **IFRA (2011)** with minor modifications. Peroxide value test measures amount of iodine released from potassium iodide next estimated using a standard sodium-thiosulfate solution. Peroxide value is stated by millimoles of active oxygen per kilogram of lipids mmol $O_2.kg^{-1}$. Peroxide value was carried out using a chemical: chloroform, acetic acid, potassium iodide (freshly prepared saturated solution dark stored), sodium thiosulphate (0.01 $mol.L^{-1}$), starch.

Peroxide value calculation (mmol $O_2.kg^{-1}$):

$$PV = \frac{(V1 - V0) * \mathrm{c} * 1000 * \mathrm{T}}{w}$$

V1 – consumption of 0.01 $mol.L^{-1}$ sodium thiosulphate solution in the main test
V0 – consumption of 0.01 $mol.L^{-1}$ sodium thiosulphate solution in the blank test
c – molar concentration of the sodium thiosulphate solution
T – titre of the sodium thiosulphate solution
w – weighed portion of fat in grams

Statistic analysis

The statistical analysis was performed by the program SAS, version 9.1. The results of measurements were analysed using ANOVA for the repeated measure and Student t test for independent samples if the normality was met. If the normality wasn't met, Friedman test and Mann-Whitney test were used. Significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Fat content

The fat content of chicken meat after the application of EO to the feed and measured in raw chicken muscle homogenates depending on its storage, is reported in Table 2. In E1 mean content of chicken meat fat in control group ranged between M = 9.64 g.100 g^{-1} in 9th month and M = 12.95 g.100 g^{-1} in 12th months. The time has not a statistically significant effect on content of chicken meat fat in control group ($F(1.94, 36.864) = 2.075$, $p > 0.05$).

Table 2 Fat content of chicken meat during storage, g.100 g^{-1}.

Group	Storage period							
	8 days		6 months		9 months		12 months	
	1st experiment with EO in feed mixtures							
	M ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)
CG	11.17 ±6.97	62.39	10.95 ±2.46	22.47	9.64 ±4.40	45.64	12.95 ±2.09	16.14
EG	10.84 ±2.41	22.23	9.94 ±2.76	27.77	11.07 ±4.35	39.30	12.24 ±3.74	30.56
	2nd experiment with application EO on raw thigh muscle							
CG	7.44 ±0.98	13.17	7.01 ±1.74	24.28	7.12 ±2.11	29.63	7.73 ±2.05	26.52
EG	8.03 ±2.11	26.28	6.15 ±1.70	27.64	7.14 ±1.49	20.87	7.00 ±1.79	25.57

Note: CG – control group without oregano essential oil, EG – experimental group whit oregano essential oil, *M* - mean, *SD* – standard deviation, c_v – coefficient of variation.

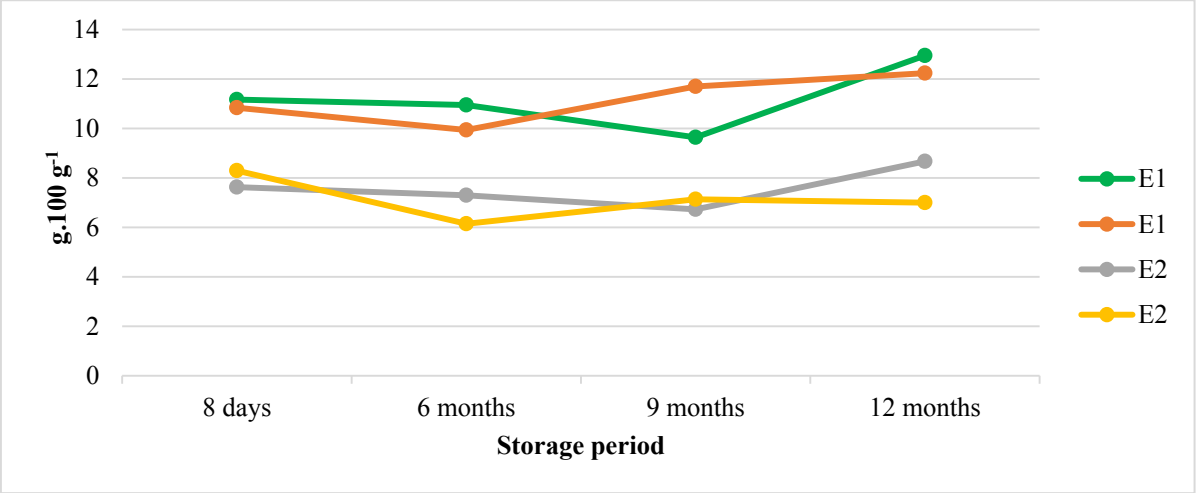

Figure 1 Fat content of chicken meat during storage, g.100 g^{-1}.

Chicken meat from experimental group contained similar amount of fat mean from $M = 9.94$ g.100 g^{-1} in 6th month to $M = 12.24$ in 12th months. We found out in experimental group too, that the time has not a statistically significant effect on content of chicken meat fat ($F(3, 57) = 1.749$, $p > 0.05$).

The difference between control group and experimental group of oregano essential oil feeding broiler chickens was not significant in measurements of fat content of chicken meat (8 days ($t(38) = 0.2$, $p > 0.05$), 6 months ($t(38) = 1.23$, $p > 0.05$), 9 months ($t(38) = -1.036$, $p > 0.05$), 12 months ($t(38) = 0.735$, $p > 0.05$)).

In E2 the fat content has been balanced during whole storage period (Figure 1). In control group was mean from $M = 7.01$ g.100 g^{-1} to mean $M = 7.73$ g.100 g^{-1}. There was not a statistically significant effect of time on fat content in control group ($F(3, 33) = 0.41$, $p > 0.05$). Fat content of chicken tight in experimental group ranged between mean $M = 6.15$ g.100 g^{-1} in 6th month and $M = 8.03$ g.100 g^{-1} at 8th day storage period. In experimental control group was not found a statistically significant effect of time on fat content ($F(3, 33) = 2.228$, $p > 0.05$).

In comparison of control group and experimental group we didn't found a statistically significant difference in individual measurements (8 days ($t(22) = -0.877$, $p > 0.05$), 6 months ($t(22) = 1.219$, $p > 0.05$), 9 months ($t(22) = -0.032$, $p > 0.05$), 12 months ($t(22) = 0.924$, $p > 0.05$)).

These results correspond to the results of **Liptaiová et al. (2010)**, which reported average fat content of chicken meat of broilers chickens fytoadditives feeding with concentration 0.1% 9.9 g.100 g^{-1}, 0.05% 9.5 g.100 g^{-1}, 0.025% 10.45 g.100 g^{-1}. The fat content of the chicken meat in the control group (without fytoadditives), was 9.8 g.100 g^{-1}. Differences in the fat content of chicken meat between the groups were not statistically significant ($p > 0.05$).

Giannenas et al. (2016) in a 42-day-long fattening experiment with 5% oregano essential oil in feed mixtures (300 g.ton^{-1}) of broiler chickens Ross 308 hybrid combination and 5% oregano essential oil and 0.5% virgin oil in feed mixtures (500 g.ton^{-1}) indicated fat content in chicken breasts without skin of 5.4 g.100 g^{-1} in both groups compared to the control group of 5.5 g.100 g^{-1}. Fat content in chicken thighs without skin 8.2 g.100 g^{-1} in both experimental groups and 8.4 g.100 g^{-1} in the control group without a statistically significant difference between the groups ($p > 0.05$).

Comparable fat content introduced in their study by broiler chickens Ross 308 hybrid combination **Čuboň et al. (2013)**, where measured in thigh muscle with skin 12.2 – 13.2 g.100 g^{-1}, **Milićević et al. (2014)** measured in thigh muscle with skin 5.19 – 9.85 g.100 g^{-1}.

Table 3 Fat peroxide value during storage, mmol $O_2.kg^{-1}$.

Group	Storage period							
	8 days		6 months		9 months		12 months	
	1st experiment with EO in feed mixtures							
	M ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)	*M* ±*SD*	c_v (%)
CG	0.58 ±0.34	58.62	1.39 ±0.54	38.85	2.40 ±1.87	77.92	3.60 ±1.70	47.22
EG	1.06 ±0.74	69.81	1.22 ±1.06	86.89	2.02 ±0.81	40.10	2.11 ±1.02	48.34
	2nd experiment with application EO on raw thigh muscle							
CG	1.76 ±0.42	23.86	1.56 ±0.53	33.97	2.22 ±2.03	91.44	3.40 ±3.84	112.94
EG	1.62 ±0.50	30.86	1.47 ±0.58	39.46	2.28 ±0.59	25.88	2.50 ±1.16	46.4

Legend: CG – control group without oregano essential oil, EG – experimental group whit oregano essential oil, *M* - mean, *SD* – standard deviation, c_v – coefficient of variation.

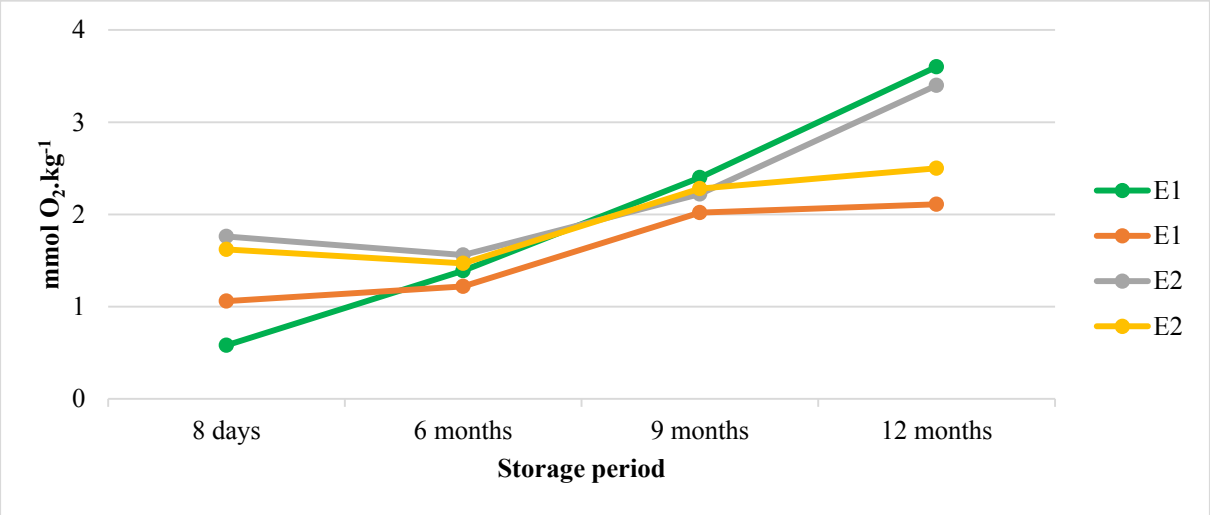

Figure 2 Fat peroxide value during storage, mmol $O_2.kg^{-1}$.

Peroxide value

The oxidative stability parameter of the peroxide value after the application of EO to the feed and measured in raw chicken muscle homogenates depending on its storage is reported in Table 3.

The mean of peroxide value in control group of E1 was M = 0.58 µmol $O_2.kg^{-1}$ at 8th day of storage time and increased during whole storage period up to M = 3.60 µmol $O_2.kg^{-1}$ in 12th months (Figure 2). There is a statistically significant effect of time on content of peroxide value in control group (F(1.823, 34.634) = 19.357, p <0.05).

Peroxide value in experimental group with EO was M = 1.06 µmol $O_2.kg^{-1}$ at 8th day of storage time and after 12th months M = 2.11 µmol $O_2.kg^{-1}$. There is a statistically significant difference between peroxide values in experimental group measured in various period (χ^2(3) = 21.211, p <0.05).

The difference between control group and experimental group with oregano essential oil feeding broiler chickens was statistically significant in first and last measurements (8 days (U = 101, p <0.05), 12 months (U = 90.5, p <0.05)). In first measurement was peroxide value significantly higher in experimental group (*Mdn* = 0.85) than in control group (*Mdn* = 0.57). In the last measurement it was inside out. Peroxide value was significantly higher in control group (*Mdn* = 3.66) than in experimental group (*Mdn* = 1.98).

Peroxide value of the samples in E2 evaluated in the control group ranged between M = 1.56 µmol $O_2.kg^{-1}$ and M = 3.40 µmol $O_2.kg^{-1}$. We didn't found a statistically significant difference between peroxide values measured in various time (χ^2(3) = 1.4, p >0.05) in control group. In experimental group we observed increased peroxide value too and there was not found a statistically significant difference between peroxide values measured in various time (χ^2(3) = 5.034, p >0.05).

In comparison between control group and experimental group with EO, we found not a statistically significant difference in individual measurements (8 days (U = 17.5, p >0.05), 6 months (U = 14.5, p >0.05), 9 months (U = 12.5, p >0.05), 12 months (U = 12.5, p >0.05)).

Sing et al. (2014) noticed significantly higher peroxide value in control group without fytoadditives during storage at 4 °C after 7 and 9 days in raw chicken meat unlike our study, where was peroxide value of E2 after 8th days similar in both groups. In our experiments, the peroxide value was comparable to 8th day and after 6th months of storage, indicated oxidative damage to chicken meat, well below the peroxide limit reported **Min and Ellefson (2010)**, which indicates, that a low peroxide value can represent either the beginning or advanced oxidation and can be distinguished based on the PV over time or by

measuring secondary oxidation products. A high quality, fresh fat has a PV of zero, on the other hand very poor-quality fats resulting in a PV ≥20 mmol.kg^{-1}. Peroxide value of chicken meat increased during the whole period of storage, but its value was not so high as to cause that meat to be damaged. In 12 months were in both experiments' numbers of peroxide value higher in control group. The increase could be caused due to the faster rate of formation of new hydroperoxides than reduction of hydroperoxides into secondary oxidation products that signify effect of supplementation oregano essential oil in experimental groups.

Results of **Dashti et al. (2015)** showed that thyme essential oil was effective in preventing oxidative spoilage. No difference of peroxide value was observed between the treated samples and control during the first month of chicken nuggets storage but differences among samples can be observed from 2nd month to the 6th month of the storage time. Control samples showed higher oxidation rate than treated samples containing different concentrations of essential oil (0.1%, 0.2%, 0.05%) throughout the storage. In 6th months, peroxide value for all three concentrations was significantly different from samples without thyme essential oil. His research demonstrated the strongest effect of 0.2% essential oil that is lower concentration like in our experiment without significant difference in peroxide value of chicken thighs.

Dzomba et al. (2014) confirmed in their study with different parts of the chosen plants improves meat oxidative stability and provides better protection as synthetic antioxidant. The profile of the untreated meat was above all other profiles. Treating meat by mixing with 50 mg of *Cleome gynandra* leaf extract gave more promising results with a peroxide value that was below 50 mmol O_2.kg^{-1} even after 26 days. It depicted the slowest rate of formation of peroxides as compared to synthetic antioxidant BHT.

Our results do not consist with authors, who reported that the peroxide value of mechanically deboned chicken meat treated with a polyphenol extract increases and thereafter decreases with storage time. They represented, that decomposition of hydroperoxides into secondary products increases at a higher rate as lipid oxidation progresses, as compared with the formation of new hydroperoxides, resulting in decreased peroxide value **(Teets and Were, 2008, Soyer et al., 2010, Hwang et al., 2013)**.

CONCLUSION

Results of our experiments indicate that the oregano essential oil and storage time not influenced the fat content in chicken meat but manifested an impact on the oxidative stability of chicken meat its application to feed. This effect was statistically significant compared with control group. We found not statistically significant difference in peroxide values, if applied oregano essential oil to raw chicken meat. The results impact of oregano essential oil on chicken meat comparable to control group, but a tendency to improve oxidative stability was indicated. We recommend the use of 0.05% oregano essential oil for broiler chickens due to its antioxidant properties in amount 50 g of oregano essential oil per 100 g of the feed mixture. Oregano essential oil appears to be alternative to synthetic additives in broiler nutrition.

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THE EFFECT OF COFFEE BEANS ROASTING ON ITS CHEMICAL COMPOSITION

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ABSTRACT

Drinking coffee has become part of our everyday culture. Coffee cultivation is devoted to over 50 countries in the world, located between latitudes 25 degrees North and 30 degrees South. Almost all of the world's coffee production is provided by two varieties, called 'Arabica' and 'Robusta' whereas the share of Arabica is 70% of the world's coffee harvest. Green (raw) coffee can not be used to prepare coffee beverages, coffee beans must first be roasted. Roasting coffee and reaching a certain degree of coffee roasting determine its flavor and aroma characteristics. In the present study the fate of sucrose, chlorogenic acid, acetic acid, formic acid, lactic acid, caffeic acid, total phenolic compounds and 5-hydroxymethylfurfural was studied in coffee (Brazil Cerrado Dulce, 100% Arabica) roasted in two ways (Medium roast and Full city roast). It has been found that almost all sucrose has been degraded (96 – 98%) in both roasting ways. During Medium roast 65% of chlorogenic acid contained in green coffee was degraded while during Full city roast it was 85%. During both Medium and Full city roasting, the formation of acetic acid but especially formic and lactic acid was recorded. The highest concentration of organic acids was recorded at Full City roasting at medium roasting times (3.3 $mg.g^{-1}$ d.w. acetic acid, 1.79 $mg.g^{-1}$ d.w. formic acid, 0.65 $mg.g^{-1}$d.w. lactic acid). The amount of phenolic substances also increased during roasting up to 16.7 $mg.g^{-1}$ d.w. of gallic acid equivalent. Highest concentrations of 5-hydroxymethylfurfural were measured at medium roasting times at both Medium (0.357 $mg.g^{-1}$ d.w.) and French city (0.597 $mg.g^{-1}$ d.w.) roasting temperatures. At the end of roasting, the 5-hydroxymethylfurfural concentration in coffee were 0.237 $mg.g^{-1}$ d.w. (Medium roast) and 0.095 $mg.g^{-1}$ d.w. (Full city roast).

Keywords: coffee; roasting; hydroxymethylfurfural; sucrose; organic acids

INTRODUCTION

Coffee is made up of modified seed of the fruit of various tropical to subtropical trees or coffee shrubs. Coffee has a large number of varieties, only a few of them have economic significance. Almost all of the world's coffee production is provided by two varieties, called 'Arabica' and 'Robusta' **(Butt and Tauseef Sultan, 2011)**. Arabica (*Coffea arabica*), is the most important botanical species, especially for the high quality of its fruits. It comes from about 70% of the world's green coffee production. Robusta (*Coffea canephora*), is the second most important variety of coffee, and its share of world production is steadily growing mainly due to its greater adaptability to habitats and disease resistance. Other reasons to increase demand of Robusta coffee in the market are the growing demand for instant coffee, which is preferentially made from Robusta coffee and last but not least, lower price of Robusta coffee compared to Arabica coffee **(Kemsley et al., 1995)**. World coffee production in 2017 was about 9.5 million tonnes, which makes coffee the second most important commodity of world trade **(FAOSTAT, 2018)**.

After processing the coffee beans by wet or dry drying technologies **(Arya and Jagan Mohan Rao, 2007; Guimar, Berbert and Silva, 1998),** coffee beans are roasted. During coffee roasting, coffee beans get brown colour and their characteristic flavour and aroma **(Yeretzian et al., 2002)**. In different countries, different roasting styles have been created according to population preferences. These roasting styles differ from each other at the roasting temperature used and the total coffee roasting time **(Moon, Yoo and Shibamoto, 2009; Dórea and DaCosta, 2005)**.

Due to the popularity of coffee in the world many researchers were involved in coffee research in the last quarter of a century. Most studies on coffee are health-related studies **(Ciaramelli, Palmioli and Airoldi, 2019; Poole et al., 2017; Ludwig et al., 2014; Butt and Tauseef Sultan, 2011; Dórea et al., 2005)**. Other studies are focused on the role of roasting conditions of the coffee in the level of selected compounds. Information on what is happening in roasted coffee beans is quite sufficient in the available literature, but there are only few studies that deal with the complex monitoring of changes in the chemical composition of coffee beans during roasting **(Wei et al., 2012)**.

In the present study the fate of sucrose, chlorogenic acid, acetic acid, formic acid, lactic acid, caffeic acid, total phenolic compounds and 5-hydroxymethylfurfural was studied in coffee roasted in two ways (Medium roast and Full city roast).

Scientific hypothesis

Higher temperature and higher time of coffee beans roasting cause higher amounts of 5-hydroxymethylfurfural, organic acids and phenolic compounds while reducing the carbohydrate content in the coffee beans.

MATERIAL AND METHODOLOGY

Chemicals and reagents

All water used in this study was ultrapure water (Elga pure lab classic, Veolia water systems, UK). All chemicals used in this study were analytical grade chemicals purchased from Sigma-Aldrich (Germany) company except of Karl-Fisher titration reagent and water standard which have been purchased from Labicom (Czech Republic).

Sample preparation

The 100% Arabica coffee (Brazil Cerrado Dulce, 4coffee, Czech Republic) was used in this study. Total amount of 25g of green coffee beans was roasted in a home coffee roaster (Gene café CBR101, 4coffee, Czech Republic). Coffee was roasted with two roasting degrees as Medium roast and Full city roast. Roasting on Medium roast degree was done at 210 °C and the total roasting time was 14 min. Roasting on Full city roast degree was done at 225 °C and the total roasting time was 19 min. Extraction of organic acids, sucrose, 5-hydroxymethylfurfural and total phenolic compounds was performed in 25 mL Erlenmeyer flasks. One gram of sample weighted on analytical balancer and 10 mL of solvent (80 °C water mixed with ethanol in 60:40 volume ratio) were used for extraction. Extraction was carried out on a magnetic stirrer for 30 minutes. After the extraction, the samples were centrifuged at 5000 rpm in centrifuge and the supernatant was filtered using nylon syringe filters (0.45 μm, Labicom, Czech Republic) and used for analysis. All samples were prepared in two replicates.

Chemical analysis

Acetic, formic and lactic acid were determined using ion chromatography (Metroohm 850 professional IC, Metroohm, Switzerland) with conductivity detector. An Agilent Infinity 1260 liquid chromatograph (Agilent Technologies, USA) equipped with ELSD detector was used for determination of sucrose. Both methods are described in detail at work published by **Diviš et al. (2018)**. Total phenolic compounds were determined using Helios gamma spectrophotometer (Spectronic Unicam, Great Britain) through the Folin-Ciocalteus method **(Singleton et al. 1999)** and expressed as gallic acid equivalent. Concentration of 5-hydroxymethylfurfural was determined on Agilent Infinity 1260 liquid chromatograph with DAD detector using Kinetex EVO-C18 column and acetonitrile mixed with water in 15:85 volume ratio. Chlorogenic and caffeic acid were determined on Agilent Infinity 1260 liquid chromatograph with DAD detector using Kinetex EVO-C18 column and mixture of 2.5% formic acid and acetonitrile in 90:10 volume ratio as mobile phase. Water content in all samples was determined by Karl-Fisher titration **(Verhoef and Barendrecht, 1977)** using KF Titrino 701 titrator (Metroohm, Switzerland)**.** The pH value was measured using pH meter with combined electrodes (WTW, Germany). All parameters for a single sample were measured in three replicates. All measured concentrations were recalculated to dry weight of coffee.

Statistic analysis

All experimental data were statistically processed using software XLstat (Addinsoft, USA). Obtained data were pre-treated by using Analysis of Variance (ANOVA) to find statistical significant differences between groups. Tukey's comparative test on the significance level 0.05 has been performed for individual parameters observed during coffee roasting. The pre-treated data were used as input parameters in Principal Component Analysis (PCA) to find correlation between the chemical composition changes during the roasting process.

RESULTS AND DISCUSSION

Coffee beans contain, in addition to water and minerals, a large number of organic substances. The main component of coffee beans are carbohydrates. The coffee beans contain various hemicelluloses, starch, oligosaccharides and mainly sucrose. The amount of monosaccharides is relatively small. Other substances contained in coffee are proteins, non-protein nitrogenous substances, phenolic substances, non-volatile organic acids, volatile substances and oils **(Arya and Jagan Mohan Rao, 2007; Farah and Marino Donagelo, 2006; Redgwell and Fisher, 2006)**.

Concentration of sucrose in green coffee beans and in roasted coffee beans is presented in Table 1 and Table 2. The results show that sucrose degradation occurs during the roasting process. Sucrose degradation is explained by sucrose hydrolysis to glucose and fructose, which may be further fragmented to form aliphatic acids, or which may participate in Maillard reactions with proteins or amino acids **(Ginz et al. 2000)**. The sucrose concentration decreased in the middle of the roasting process by 47% in the case of Medium roast and by 59% in the case of Full city roast. At the end of the roasting process, almost all sucrose has already been degraded (96 – 98%).

Another substance that has been observed to reduce the concentration during the roasting process was chlorogenic acid. Concentration of chlorogenic acid in green coffee beans and in roasted coffee beans is presented in Table 3 and Table 4. Chlorogenic acid concentration decreased in the middle of the roasting process by 45% in the case of Medium roast and by 42% in the case of Full city roast. At the end of the roasting process, 67% of chlorogenic acid contained in green coffee was degraded during Medium roast and 85% during Full city roast. Chlorogenic acid is involved in colour, flavour and aroma formation of coffee **Farah and Marino Donagelo, 2006; Farah et al., 2005)**. Major degradation products of chlorogenic acid are melanoids and low molecular weight compounds.

Strong significant correlation was found between sucrose concentration and concentration of organic acid during coffee roasting (r >0.9).

Concentration of organic acid in coffee during the roasting process is shown in Table 3 and Table 4. While the sucrose concentration in coffee decreases during roasting, the concentration of organic acids significantly increases. The most significant change was found in the lactic and formic acid content. The content of these acids in coffee beans rose almost 100 times after coffee roasting. Trend of changes in the concentration of organic acids was similar for Medium roasting and French roasting, however in the case of French roasting decrease in organic acid content was observed in the later stage of roasting. **Ginz et al. (2000)** lists the content of organic acids in Robusta coffee after roasting to be approximately 2 $mg.g^{-1}$ in the case of formic acid and acetic acid and 0.2 $mg.g^{-1}$ in the case of lactic acid. Formation of organic acids in coffee is described by Lobry-deBruyn-vanEckenstein rearrangement reaction in which fructose or glucose produced by sucrose hydrolysis is involved, and by formation of 1,2-endiole or 2,3-endiole as acid precursors **(Ginz et al., 2000)**. Formation of organic acids in coffee during roasting process did not significantly affect the pH of the coffee (Table 1 and Table 2.). This finding can be caused due to highly complex buffering effects and the wide distributions of salts and acids present in coffee. **Jeszka- Jeszka-Skowron et al. (2016)** measured pH value

Table 1 Content of sucrose, 5-hydroxymethylfurfural, total phenolic compounds and pH value of coffee roasted to Medium roast degree.

Time	pH ($mg.g^{-1}$ ±SD)	sucrose ($mg.g^{-1}$ ±SD)	HMF ($mg.g^{-1}$ ±SD)	TPC ($mg.g^{-1}$ ±SD)
0	6.09 ±0.05^{a}	70.1 ±4.9^{a}	<0.010	8.5 ±0.8^{e}
4	5.93 ±0.05^{ab}	58.5 ±2.1^{b}	<0.010	8.9 ±0.8^{e}
5	5.89 ±0.05^{ab}	57.0 ±6.4^{bc}	<0.010	9.6 ±0.9^{de}
6	5.78 ±0.05^{ab}	50.1 ±3.5^{c}	0.013 ±0.004^{d}	10.8 ±0.6^{cd}
7	5.89 ±0.05^{ab}	38.8 ±3.8^{d}	0.044±0.016^{d}	12.8 ±0.6^{ab}
8	5.68 ±0.05^{ab}	23.8 ±2.5^{e}	0.136 ±0.013^{c}	12.0 ±0.7^{bc}
9	5.73 ±0.05^{ab}	14.3 ±2.5^{f}	0.281 ±0.031^{ab}	12.7 ±0.3^{ab}
10	5.68 ±0.05^{ab}	12.2 ±1.9^{f}	0.232±0.014^{b}	13.0 ±0.2^{ab}
11	5.72 ±0.05^{ab}	6.3 ±1.8^{gh}	0.139 ±0.021^{c}	13.7 ±0.3^{a}
12	5.65 ±0.05^{ab}	4.4 ±0.5^{gh}	0.264 ±0.046^{b}	14.4 ±0.4^{a}
13	5.68 ±0.05^{ab}	4.1 ±0.7^{gh}	0.346 ±0.016^{a}	14.0 ±0.5^{a}
14	5.65 ±0.05^{ab}	3.0 ±0.4^{h}	0.224 ±0.018^{b}	13.4 ±0.4^{ab}

Note: Values in the same column with different letters are significantly different at p <0.05.

Table 2 Content of sucrose, 5-hydroxymethylfurfural, total phenolic compounds and pH value of coffee roasted to Full city roast degree.

Time	pH ($mg.g^{-1}$ ±SD)	sucrose ($mg.g^{-1}$ ±SD)	HMF ($mg.g^{-1}$ ±SD)	TPC ($mg.g^{-1}$ ±SD)
0	6.09 ±0.05^{a}	70.1 ±4.9^{a}	<0.010	8.5 ±0.8^{e}
4	5.95 ±0.05^{a}	62.8 ±3.9^{ab}	<0.010	8.9 ±0.3^{e}
5	5.95 ±0.05^{a}	59.3 ±3.4^{b}	<0.010	11.5 ±0.7^{d}
6	5.76 ±0.05^{ab}	59.5 ±5.1^{ab}	0.017 ±0.003^{h}	13.7 ±0.9^{c}
7	5.73 ±0.05^{ab}	41.6 ±4.2^{c}	0.091 ±0.006^{gh}	13.9 ±0.5^{c}
8	5.69 ±0.05^{ab}	30.6 ±2.8^{d}	0.326 ±0.018^{bcd}	13.1 ±0.7^{c}
9	5.78 ±0.05^{ab}	16.3 ±2.5^{e}	0.341 ±0.025^{bc}	13.2 ±0.9^{c}
10	5.77 ±0.05^{ab}	12.1 ±2.2^{f}	0.259 ±0.021^{de}	13.8 ±0.8^{c}
11	5.75 ±0.05^{ab}	5.7 ±1.3^{g}	0.207 ±0.013^{ef}	14.9 ±0.9^{bc}
12	5.69 ±0.05^{ab}	3.4 ±0.4^{gh}	0.549 ±0.029^{a}	15.8 ±1.2^{ab}
13	5.62 ±0.05^{ab}	2.5 ±0.3^{h}	0.510 ±0.017^{a}	14.0 ±0.3^{bc}
14	5.59 ±0.05^{ab}	2.3 ±0.2^{h}	0.408 ±0.022^{b}	14.1 ±0.5^{bc}
15	5.55 ±0.05^{b}	2.0 ±0.2^{hi}	0.406 ±0.019^{b}	14.8 ±0.3^{bc}
16	5.62 ±0.05^{ab}	1.9 ±0.3^{hi}	0.303 ±0.023^{cd}	14.6 ±0.7^{bc}
17	5.72 ±0.05^{ab}	1.7 ±0.3^{hi}	0.236 ±0.015^{de}	16.7 ±0.8^{a}
18	5.66 ±0.05^{ab}	1.5 ±0.2^{i}	0.121 ±0.014^{fg}	15.6 ±0.6^{ab}
19	5.70 ±0.05^{ab}	<1.0	0.108 ±0.017^{g}	14.4±0.9^{bc}

Note: Values in the same column with different letters are significantly different at p <0.05.

Table 3 Content of organic acids in coffee roasted to Medium roast degree.

Time	Organic acids				
	Acetic ($mg.g^{-1}$ ±SD)	Formic ($mg.g^{-1}$ ±SD)	Lactic ($mg.g^{-1}$ ±SD)	Chlorogenic ($mg.g^{-1}$ ±SD)	Caffeic ($mg.g^{-1}$ ±SD)
0	0.345 ± 0.036^{gh}	<0.005	<0.005	22.7 ± 1.8^{a}	0.741 ± 0.082^{c}
4	0.262 ± 0.055^{h}	0.032 ± 0.005^{f}	0.019 ± 0.009^{e}	17.8 ± 0.5^{b}	0.873 ± 0.033^{c}
5	0.345 ± 0.013^{gh}	0.055 ± 0.016^{f}	0.037 ± 0.006^{e}	15.7 ± 0.9^{bc}	0.971 ± 0.021^{bc}
6	0.378 ± 0.033^{g}	0.069 ± 0.004^{f}	0.036 ± 0.004^{e}	14.5 ± 0.6^{cd}	0.932 ± 0.029^{bc}
7	0.726 ± 0.067^{f}	0.133 ± 0.027^{ef}	0.039 ± 0.007^{e}	13.6 ± 0.4^{de}	1.03 ± 0.08^{abc}
8	1.07 ± 0.08^{e}	0.291 ± 0.028^{de}	0.114 ± 0.011^{d}	13.7 ± 0.3^{de}	1.05 ± 0.11^{abc}
9	1.16 ± 0.06^{e}	0.383 ± 0.014^{d}	0.099 ± 0.021^{d}	12.2 ± 0.2^{def}	1.17 ± 0.12^{ab}
10	1.21 ± 0.03^{de}	0.432 ± 0.029^{cd}	0.144 ± 0.013^{d}	11.7 ± 0.3^{ef}	1.03 ± 0.07^{abc}
11	1.39 ± 0.04^{cd}	0.571 ± 0.025^{bc}	0.198 ± 0.009^{c}	10.4 ± 1.1^{fg}	1.02 ± 0.14^{abc}
12	1.58 ± 0.07^{c}	0.633 ± 0.063^{b}	0.239 ± 0.017^{c}	8.9 ± 0.9^{gh}	1.18 ± 0.05^{ab}
13	1.81 ± 0.04^{b}	0.717 ± 0.113^{ab}	0.317 ± 0.018^{b}	7.9 ± 0.8^{gh}	1.17 ± 0.08^{a}
14	2.17 ± 0.07^{a}	0.875 ± 0.057^{a}	0.392 ± 0.022^{a}	7.2 ± 0.4^{h}	1.05 ± 0.13^{abc}

Note: Values in the same column with different letters are significantly different at $p < 0.05$.

Table 4 Content of organic acids in coffee roasted to Full city roast degree.

Time	Organic acids				
	Acetic ($mg.g^{-1}$±SD)	Formic ($mg.g^{-1}$±SD)	Lactic ($mg.g^{-1}$±SD)	Chlorogenic ($mg.g^{-1}$±SD)	Caffeic ($mg.g^{-1}$±SD)
0	0.345 ± 0.036^{j}	<0.005	<0.005	22.7 ± 1.8^{a}	0.741 ± 0.082^{abc}
4	0.214 ± 0.015^{k}	0.011 ± 0.005^{i}	0.013 ± 0.004^{i}	17.1 ± 0.3^{b}	0.852 ± 0.130^{abc}
5	0.484 ± 0.016^{i}	0.061 ± 0.008^{h}	0.046 ± 0.006^{h}	16.2 ± 0.7^{bc}	0.97 ± 0.112^{abc}
6	0.492 ± 0.012^{i}	0.104 ± 0.011^{gh}	0.053 ± 0.006^{h}	15.3 ± 0.6^{bcd}	0.932 ± 0.091^{abc}
7	0.856 ± 0.025^{h}	0.293 ± 0.041^{fg}	0.051 ± 0.004^{h}	14.1 ± 0.5^{cde}	0.974 ± 0.133^{abc}
8	1.26 ± 0.08^{g}	0.421 ± 0.013^{ef}	0.180 ± 0.025^{g}	12.9 ± 0.8^{def}	1.25 ± 0.18^{ab}
9	1.46 ± 0.09^{fg}	0.519 ± 0.038^{e}	0.208 ± 0.028^{g}	12.5 ± 0.9^{def}	1.42 ± 0.19^{a}
10	1.41 ± 0.03^{fg}	0.559 ± 0.029^{de}	0.217 ± 0.025^{fg}	12.2 ± 0.6^{efg}	1.03 ± 0.09^{abc}
11	1.72 ± 0.08^{ef}	0.732 ± 0.047^{cd}	0.315 ± 0.023^{ef}	11.1 ± 0.5^{fgh}	0.95 ± 0.07^{abc}
12	1.85 ± 0.09^{de}	0.786 ± 0.040^{bc}	0.343 ± 0.013^{de}	9.9 ± 0.6^{ghi}	1.45 ± 0.15^{a}
13	2.17 ± 0.17^{cd}	0.906 ± 0.045^{abc}	0.394 ± 0.029^{cde}	9.5 ± 0.4^{ghi}	1.22 ± 0.13^{ab}
14	3.22 ± 0.11^{a}	1.13 ± 0.21^{a}	0.628 ± 0.043^{a}	9.0 ± 0.7^{hij}	1.05 ± 0.09^{abc}
15	2.61 ± 0.12^{b}	1.07 ± 0.09^{a}	0.442 ± 0.041^{bcd}	8.6 ± 0.5^{ij}	0.951 ± 0.141^{abc}
16	2.51 ± 0.14^{b}	0.917 ± 0.048^{abc}	0.497 ± 0.021^{bc}	7.5 ± 0.4^{jk}	0.873 ± 0.062^{bc}
17	2.43 ± 0.05^{bc}	0.921 ± 0.050^{abc}	0.410 ± 0.016^{bcde}	6.8 ± 0.8^{jk}	0.852 ± 0.015^{bc}
18	2.61 ± 0.06^{b}	0.984 ± 0.033^{ab}	0.454 ± 0.061^{bc}	5.8 ± 0.5^{k}	0.755 ± 0.073^{bc}
19	2.51 ± 0.05^{b}	1.02 ± 0.05^{a}	0.513 ± 0.032^{b}	3.2 ± 0.9^{l}	0.613 ± 0.082^{c}

Note: Values in the same column with different letters are significantly different at $p < 0.05$.

of water treated Brazil Arabica green coffee to be 4.92. **Ginz et al. 2000** monitored pH changes in Robusta coffee during the roasting process and recorded pH change from 6.1 to 5.7 similar to this study (Table 1 and Table 2).

Another strong correlation was found between chlorogenic acid concentration and caffeic acid concentration in the case of Medium roast (r = 0.8025). However, in the case of Full city roast, this correlation was not significant and week (r = 0.2403). Chlorogenic acid is an ester of quinic acid and phenolic acid, mostly caffeic, ferulic or 3-hydroxycinnamic acid **(Farah and Marino Donagelo, 2006)**. During shorter roasting at lower temperatures chlorogenic acid can be hydrolysed and concentration of caffeic acid in coffee beans may temporarily increase. On the other side, with longer roasting times and higher temperatures, caffeic acid released from chlorogenic acid can be further degraded. From the results summarized in Table 4 it can be seen a significant increase of caffeic acid concentration in roasted coffee beans and subsequent reduction of caffeic acid concentration over longer periods of roasting. The formation of phenolic substances during roasting of coffee is also evident from the total concentration of phenolic compounds presented in Table 1 and Table 2. Measured concentrations of chlorogenic acid, caffeic acid or total phenolic compounds in this study are comparable with data published in literature. Chlorogenic acid concentration in green coffee beans is reported within the

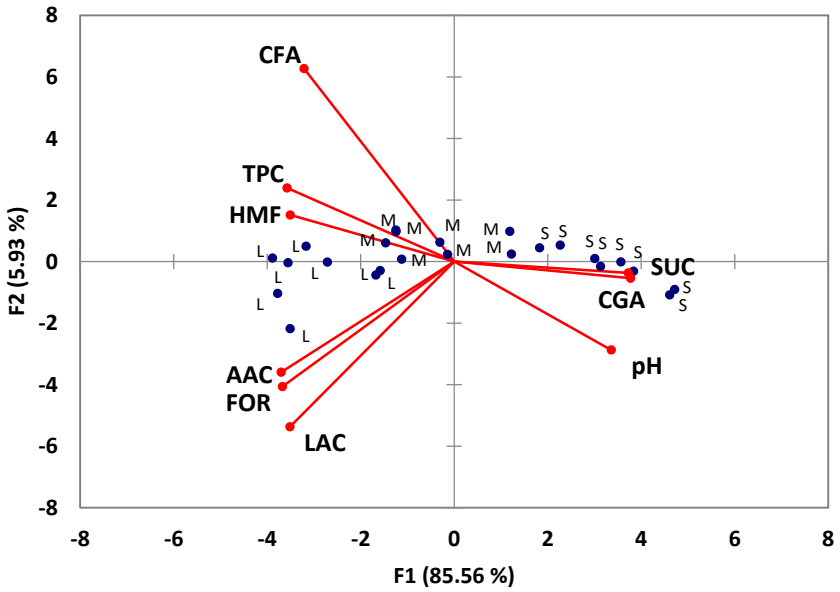

Figure 1 PCA score of the monitored analytes in coffee beans roasted on Medium roast degree. Note: S = short time of roasting (0 – 6 min), M = medium time of roasting (7 – 10 min), L=long time of roasting (8 – 14min). CGA = chlorogenic acid, SUC = sucrose, LAC = lactic acid, FOR = formic acid, AAC = acetic acid, HMF = hydroxymethylfurfural,
TPC = total phenolic compounds, CFA = caffeic acid.

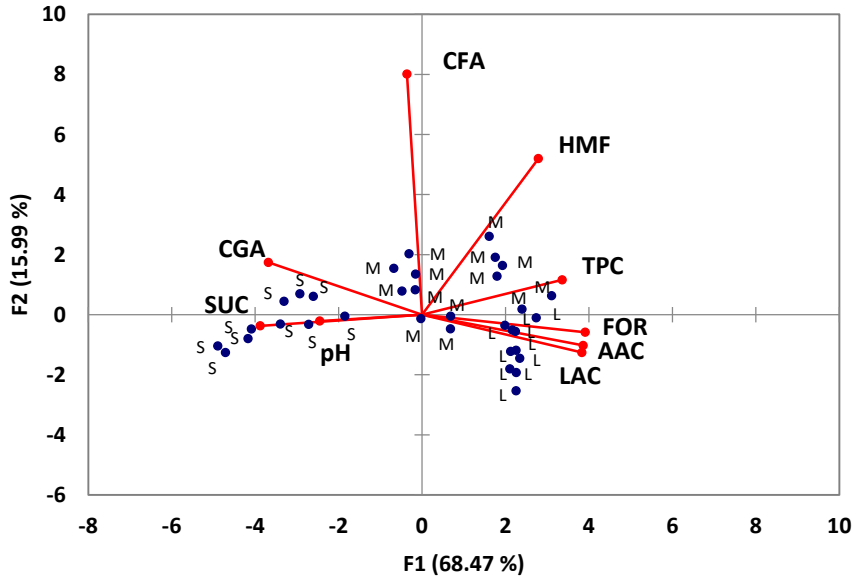

Figure 2 PCA score of the monitored analytes in coffee beans roasted on Full city roast degree. Note: S = short time of roasting (0 – 7 min), M = medium time of roasting (8 – 14 min), L = long time of roasting (15 – 19 min). CGA = chlorogenic acid, SUC = sucrose, LAC = lactic acid, FOR = formic acid, AAC = acetic acid, HMF = hydroxymethylfurfural, TPC = total phenolic compounds, CFA = caffeic acid.

range of 34 – 57 mg.g^{-1} while in roasted coffee beans in the range of 2 – 19 mg.g^{-1} **(Ludwig et al., 2014; Narita and Inouye, 2015; Farah and Marino Donagelo, 2006; Moon, Yoo and Shibamoto, 2009)**.
Total phenolic compounds content in coffee is reported to be 14 – 30 mg.g^{-1} (gallic acid equivalent) while caffeic acid content in coffee is reported to be 1.4 – 3 mg.g^{-1} **(Bauer et al., 2018; Hall, Yuen and Grant, 2018)**.

Almost all foods that are heat-treated are monitored for content of 5-hydroxymethylfurfural. This compound is generated in food by the Maillard reaction **(Antal et al., 1990)**. Increased interest in 5-hydroxymethylfurfural stems from a partially verified suspicion that this compound is a health hazard compound that may be mutagenic, carcinogenic and cytotoxic **(Abraham et al., 2011)**. The content of 5-hydroxymethylfurfural in roasted coffee is reported to be 0.3 – 1.9 mg.g^{-1} **(Murkovic and Pichler, 2006)**. In this study, maximum concentration of 5-hydroxymethylfurfural was measured to be 0.549 mg.g^{-1} in coffee during Full city roast. During Medium roast

maximum content of 5-hydroxymethylfurfural was found to be 0.346 mg.g^{-1}. Relatively interesting is the course of 5-hydroxymethylfurfural concentration during roasting. During both Medium and Full city roast two sharp maxima in 5-hydroxymethylfurfural concentration were recorded, which could correspond to the first and second crack in coffee beans. After reaching the second maximum, concentration of 5-hydroxymethylfurfural in coffee decreases, because of its degradation to organic acids **(Murkovic and Bornik, 2007)**.

To investigate the overall composition changes during the roasting process, PCA was performed on the data for all coffee bean extracts at different time of roast. The roasting process was divided into three categories according to the total roasting time (short, medium and long time). The results are shown in Figure 1 and Figure 2. The PCA plots confirmed that sucrose and chlorogenic acid degraded during the roasting process and also that with longer roasting times the content of organic acids in coffee increases. Both Figures 1 and Figures 2 also show that content of 5-hydroxymethylfurfural is the highest in medium roasting times.

CONCLUSION

This study proved that coffee roasting is a complex chemical process. The basic processes detectable during coffee roasting are the decomposition of sucrose and chlorogenic acid. Almost all sucrose is degraded during roasting independently of the roasting method. Degradation of chlorogenic acid is higher with longer roasting at higher temperatures. During the Full city roast (225 °C, 19 min) up to 85% of chlorogenic acid was degraded. Degradation products of sucrose and chlorogenic acid are low molecular organic acids and phenolic acids. Formic or lactic acid concentrations in coffee beans increased up to 100-fold during roasting. The increase in the concentration of phenolic compounds was not so steep, but it was observable. From the measured results, it cannot be clearly stated that with higher temperature and with higher roasting time concentration of 5-hydroxymethylfurfural is increasing. Highest concentrations of 5-hydroxymethylfurfural were measured at medium roasting times at both Medium and French city roasting temperatures. Conversely, at shorter roasting time and lower temperature higher concentrations of 5-hydroxymethylfurfural were found at the end of roasting.

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INCREASING OF SELENIUM CONTENT AND QUALITATIVE PARAMETERS IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) AFTER ITS FOLIAR APPLICATION

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ABSTRACT

The effect of genotype and selenium foliar biofortification in the form of an aqueous solution of sodium selenate on the content of total carotenoids, vitamin C, total polyphenols and selenium content in the tomato fruits was studied. Field experiment was held in the Botanical garden of the Slovak University of Agriculture in 2016. Seven determinant varieties of tomato in the two variants were observed. The results of experiments show that treatment of plants with the dose of Se concentration (150 g Se.ha^{-1}) at the flowering stage significantly increased the total Se content in the in tomato fruits. Foliar application of selenium had a positive effect on the increase of total polyphenol. The influence of Se biofortification on the content of vitamin C and carotenoids was not detected. Selenium foliar fertilization in dosage 150 g.ha^{-1} is suitable way of tomato fruits enriching in polyphenols, without negative effect on other antioxidants content.

Keywords: tomato; selenium; biologically active substances; biofortification

INTRODUCTION

The tomato (*Lycopersicon esculentum* Mill.) is ranked first in terms of world vegetable production. Over the last 20-year horizon, its production (more than 170 million tons per year) has doubled, while in Asian countries as China and India was recorded the most significant increase in production. The states of European Union together produce 16.9 million tonnes, while the greatest producers are Italy and Spain **(FAOSTAT, 2014)**. Within Slovakia, in 2017 were tomatoes cultivated on production area of 597 ha with a total production of 21,963 t **(Meravá, 2017)**.

Latest research highlights the relationship between consuming tomato and its products with reduced risk of various maladies like obesity, hyperglycemic and hypercholesterolemic attributes, cardiovascular disorders, and cancer insurgences. Moreover, tomato and its bioactive components hold potential to become effective modules in diet-based regimens **(Perveen et al., 2015)**. They are consumed like a functional food all over the world because of health promoting compounds in its fruit. The main reason is due to the presence of different antioxidant molecules such as carotenoids, ascorbic acid, vitamin E and polyphenol compounds such as flavanones, flavonols **(Vallverdú-Queralt, 2012)**, flavonoids **(Frusciante et al., 2007)** and other.

According to various authors, the total carotenoids content as well as lycopene in fresh tomato fruits depends mainly on genotypes, as well as on the maturity degree, growing conditions and agricultural technologies **(Carli et al., 2011; Mendelová, Fikselová and Mendel, 2013; Mendelová et al., 2015; Andrejiová et al., 2015)**. The content of carotenoids and in particular lycopene in the fruits of red tomatoes varieties is increased 10 to 14 times during vegetation **(Thompson, 2015)**.

Ascorbic acid (AA) also named vitamin C is an essential micronutrient soluble in water. It is occurring in almost all living organisms. The people, as well as a number of other animals cannot synthesize the vitamin C in their bodies and therefore it can be taken only through the diet. Following **Pinela et al. (2012)** ascorbic acid was the most abundant antioxidant in all tomato samples (following vitamins, carotenoids and phenolics).

Polyphenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens. In the last decade, there has been much interest in the potential health benefits of dietary plant polyphenols as antioxidant **(Pandey and Rizvi, 2009)**. The free radical scavenging, in which the polyphenols can break the free radical chain reaction, as well as suppression of the free radical formation by regulation of enzyme activity or chelating metal ions involved in free radical production are reported to be the most important mechanisms of their antioxidant activity **(Vladimir-Knežević et al., 2012)**. The content of polyphenols was more dependent on year and cultivar than on cultivation conditions **(Anton et al., 2014)**.

Table 1 Evaluation of the mean monthly air temperature in 2 m in the selected months in 2016.

	t, ºC	Normal 1961 – 1990	Δt, ºC	Characteristic
V.	15.0	15.1	-0.1	Normal
VI.	20.3	18.0	2.3	Extra Warm
VII.	21.4	19.8	1.6	Warm
VIII.	19.5	19.3	0.2	Normal
IX.	17.5	15.6	1.9	Warm

Note: According to climatology normal 1961 – 1990.

Table 2 Evaluation of monthly total rainfalls in selected months in 2016.

	Z, mm	Normal 1961 – 1990	% of normal	Characteristic
V.	91	58	157	Extra Wet
VI.	14	66	22	Extremely Dry
VII.	135	52	259	Extremely Dry
VIII.	35	61	57	Dry
IX.	37	40	92	Normal

Note: According to climatology normal 1961 – 1990.

Table 3 Assortment of evaluated vaarieties of tomato.

Variety	Supplier	Origin
Brixol F1	Unigen Seeds	USA
Durpeel F1	Unigen Seeds	USA
Torquay F1	Bejo Zaden	Holandsko
Townsville F1	Bejo Zaden	Holandsko
Triple Red F1	Unigen Seeds	USA
UG RED F1	Unigen Seeds	USA
Uno Rosso F1	Unigen Seeds	USA

Table 4 Agrochemical characteristics of the soil before the foundation of the experiment in 2016.

Humus, %	pH/KCl	Nutrients content in $mg.kg^{-1}$ of the soil					
		Nan	P	K	S	Ca	Mg
4.17	7.14	13.0 M	198.8 VH	487.5 VH	26.25 M	610 H	816 VH

Note: Nutrient content: M – medium content. H – high content. VH – very high content.

Tomatoes contain minerals such as calcium, magnesium, iron and potassium, as well as microelements such a copper, zinc and manganese. Moreover, the tomatoes are ranged in to the vegetables with the highest concentration of selenium (0.034 $mg.kg^{-1}$). Selenium (Se) is ultramikroelement, essential trace mineral and antioxidant. It is essential for the growth of animals and humans. That's the reason of its repeated using in biofortification programs. In the soil Se is rapidly reduced to insoluble forms and usually less than 10% of the applied Se was taken up by the crop. Another method of transferring Se to plants is foliar application of Se, either as sodium selenate or sodium selenite **(Haug et al., 2007)**. In recent years, selenium research attracts great interest because its activity plays an important role in protection against oxidative stress, initiated by redundant reactive oxygen forms (ROS) and reactive nitrogen forms (NOS). The increased risk of diseases such as cancer and heart disease, and also civilization diseases are associated with low income of selenium **(Tinggi, 2008)**.

Plants receive selenium from the growing medium in the form of inorganic compounds and they subsequently incorporate it thanks to selenocysteine in to its proteins **(Hegedűs, 2005)**. Foliar application was significantly several times more effective than the application of fertilizers **(Aspila, 2005)**. At foliar application the selenium ions diffuse from the surface of leaves to epidermal cells. There is strong correlation between solution concentration on the leaf surface and ions absorption rate, anyway, too high concentration can damage the leaf surface **(Wójcik, 2004)**. Absorption rate is limited with damage of ectodesmata **(Ježek et al., 2012)**.

The aim of the work was to monitor the effect of genotype and selenium foliar biofortification in the form of an aqueous solution of sodium selenate on the content of total carotenoids, vitamin C, total polyphenols and selenium in the tomato fruits.

Scientific hypothesis

Foliar application of selenium, in the form of sodium selenate, had a significant effect on the increase of the Se content in tomato fruits. Other antioxidants such a vitamin C and carotenoids will not be negatively affected. The concentration of total carotenoids and polyphenols is variety dependant.

MATERIAL AND METHODOLOGY

The trial establishment

An experiment was founded in 2016 in Botanical Garden of Slovak University of Agriculture (below BG SUA) in field conditions. Area is situated in very warm agro-climatic region, very dry sub-region. The mean annual temperature was 10 °C. Meteorological measurements were carried out by the help of meteorological station in the area of botanical garden, SUA in Nitra. The mean monthly air temperature and average rainfall for the year 2016 were evaluated by the climate normal 1961 – 1990 (Table 1 and Table 2). Sowing was carried out on March 14th, 2016 in a heated greenhouse BZ SUA. There were included 7 tomato determinate varieties (Table 3). According to the techniques of tomato cultivation it was done preparing of the land. According to agrochemical analysis of the soil (Table 4) in case of all three variants there was applied nitrogen fertilizer DASA®26/13 – Ammonium nitrate with sulfur (26% N and 13% of S) in dosage 254 kg.ha^{-1} (60% of recommended normative).

The field experiment was established by block method in three replications, plants were planted in uniform plant spacing 0.7 x 0.3 m. Planting of pre-growing planting was carried out on May 15th, 2016. According to necessity the supplementary irrigation was applied, as well as loosening and weeding of the crop. The application of selenium was done on June 26th, 2016 at the growth stage of fruits creation on the first inflorescences and second inflorescence establishment. Selenium was foliar applied in solution Na_2SeO_4. During the growing season the crop is in the process of setting up third-fourth inflorescence fertilization with nitrogen fertilizers DASA®26/13 (169 kg.ha^{-1}, it means 40% of the recommended normative). In the second half of vegetation protection of the plants against fungal diseases was carried out. Collection was realized in August 17th, 26th and in September 9h, 14th 2016 in full botanical ripeness of the fruits. In the small plot experiment there were observed the following options:

1st variant (K) – without applying of selenium (control),

2nd variant (Se) – selenium was applied in dosage of 150 g.ha^{-1}.

Laboratory analysis

For laboratory analysis were used only fresh fruits in full botanical maturity of all observed varieties of tomato after first harvesting of the fruits. Both control as well as the variant fortified with sodium selenite were used. An average sample of 2000 g was prepared from the harvested fruit yield. We homogenized the opposite two quarters of each sliced fruit within average sample and used it for the determination of total carotenoids and vitamin C content. Fresh fruit analysis was performed within 24 hours after harvest.

Qualitative characteristics

The qualitative characteristics were estimated in laboratory of Department of vegetable growing, SUA, in Nitra involving:

Total carotenoids estimation – Carotenoids were estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 100 Spectroquant® at 450 nm wavelengths.

Ascorbic acid estimation (AA) – HPLC method of vitamin C content estimation **Stan, Soran and Marutoiu (2014**) was used by the help of liquid chromatograph with UV detector, for separation was used RP C18 column, mobile phase was methanol:water (5:95, v/v), UV detection was adjusted to 258 nm (HPLC fy. VARIAN).

Total polyphenols content estimation (TPC) – total polyphenols were determined by the method of **Lachman (2011)** and expressed as mg of gallic acid equivalent per kg fresh mater. Gallic acid is usually used as a standard unit for phenolic content determination because a wide spectrum of phenolic compounds. The total polyphenol content was estimated using Folin-Ciocalteau assay.

Selenium content – mineralization of the plant material took place in the microwave mineralizer type CEM Mars X–press (microwave digestion oven). Quantitative determination of Se was done by using of ET-AAS method with Zeeman background correction. Atomic absorption spectrometer SpectrAA240FS (Varian, Mulgrave Virginia, Australia) was used to measure the total selenium content **(Hegedűs et al., 2008)**. Conditions for selenium measurement were set in the equipment according to the recommendations of the manufacturer **(Rothery, 1988)** for ET-AAS technique. In the research, chemicals with analytical purity were used.

Statistic analysis

The analysis of variance (ANOVA), the multifactor analysis of variance and the multiple Range test were done using the Statgraphic Centurion XVII (StatPoint, USA).

RESULTS AND DISCUSSION

Total carotenoids content

The total carotenoid content in the fresh fruits of our observed hybridized tomato varieties ranged from 8.17 to 10.28 mg.100g^{-1} of fresh matter for control variant and from 8.03 to 11.45 mg.100g^{-1} after selenium application (Table 5). The highest carotenoid content obtained UG-Red F1 variety. The average values in our tested varieties were comparable to those of **Mendelová et al. (2012)** showing an average content of total carotenoids in tomato varieties (Báb, Žiara PK, Champion, Roti) ranging from 4.8 to 7.0 mg.100g^{-1}.

Due to the foliar application of selenium, we observed a slight decrease in total carotenoid content in three hybrid varieties: Townsville F1, Triple Red F1 and Brixol F1. For other varieties of tomatoes, foliar application of selenium has resulted in increased carotenoid content.

Based on statistical evaluation of the data obtained by the multifactor analysis of variance, we can state that the variety had a proven impact on the total carotenoids content in fresh fruits and tomato juice. The effect of the variant on the total carotenoid content in fruits wasn´t statistically significant (Figure 1).

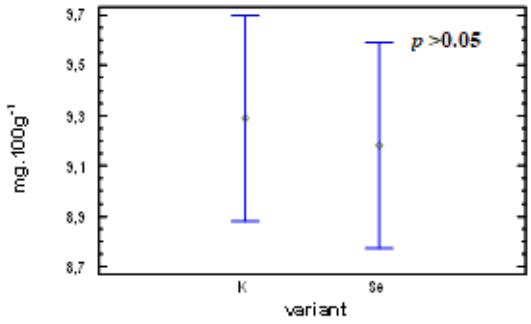

Figure 1 Graphical representation of 95% confidence intervals for the tested averages of carotenoids content in fresh tomato fruits and its variants (LSD test).

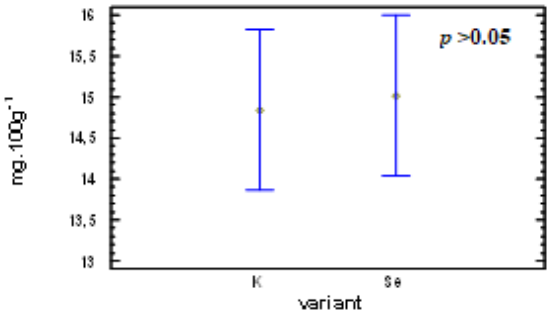

Figure 2 Graphical representation of 95% confidence intervals for the tested averages of vitamin C content in fresh tomato fruits and its variants (LSD test).

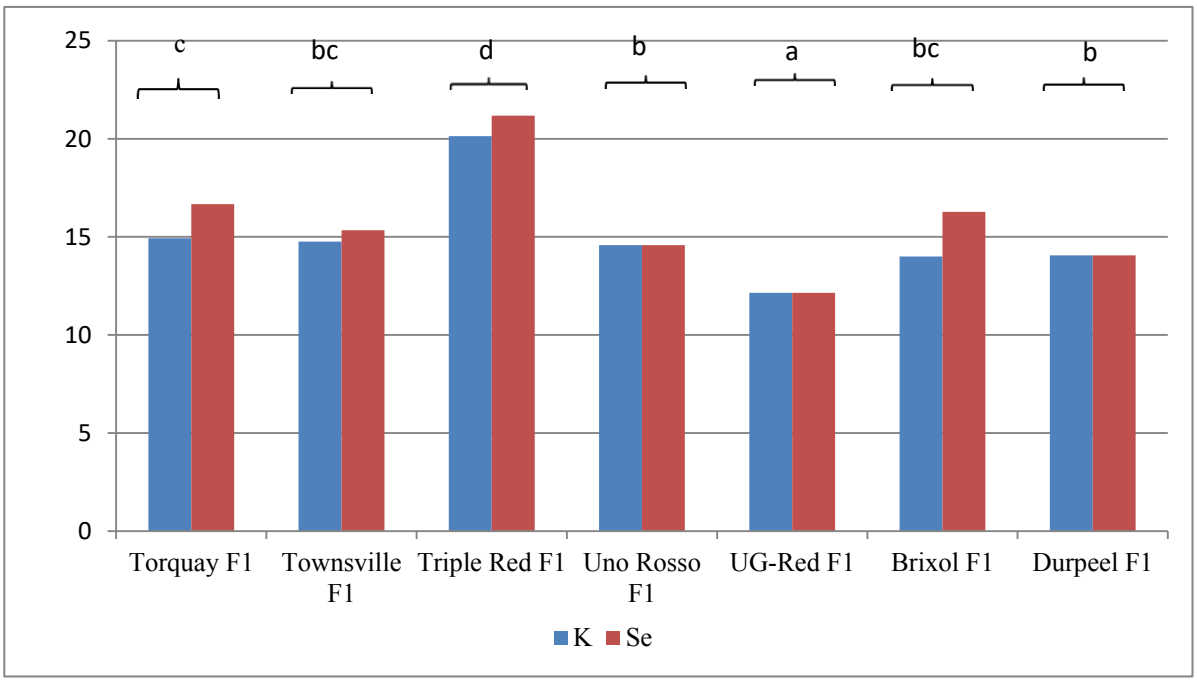

Figure 3 Vitamin C content (mg.100g^{-1} of fresh matter) in fruits depending on the observed tomato varieties and variants (K – 0 g.ha^{-1} Se; Se – 150 g.ha^{-1} Se). Note: Statistically insignificant differences between varieties are indicated by the same letters (p >0.05).

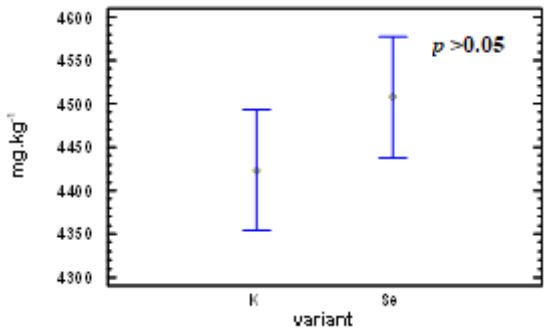

Figure 4 Graphical representation of 95% confidence intervals for the tested averages of polyphenols content in fresh tomato fruits and its variants (LSD test).

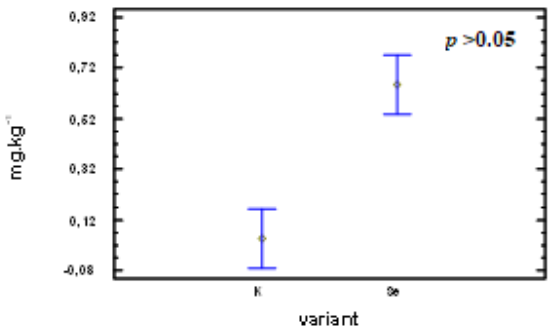

Figure 5: Graphical representation of 95% confidence intervals for the tested averages of Se content in fresh tomato fruit and its variants (LSD test).

Table 5 Carotenoids content in estimated varieties (mg.100g^{-1} FM). Nitra 2016.

Variety/variant	K	Se
Townsville F1	9.23 ±0.20ab	8.03 ±0.20a
Uno Rosso F1	8.17 ±0.14ab	8.68 ±0.40a
Torquay F1	9.82 ±0.44bc	9.80 ±1.27ab
Durpeel F1	9.84 ±0.69bc	10.05 ±1.01ab
Brixol F1	9.91 ±1.27bc	8.06 ±0.52a
Triple Red F1	10.15 ±0.14c	8.21 ±0.37a
UG-Red F1	10.28 ±0.18c	11.45 ±0.51b

Note: Average ±standard deviation. The different letters listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.05).

Table 6 Total polyphenols content (TPC) in lyophilized tomato fruits (mg GAE.kg^{-1}) depending on the observed tomato varieties and variants.

Variety/variant	K	Se
Durpeel F1	3588.30 ±73.86a	3603.40 ±45.47a
UG-Red F1	3975.00 ±73.32b	3989.00 ±51.21b
Triple Red F1	4233.55 ±56.36c	4605.28 ±49.30c
Brixol F1	4600.20 ±47.83d	4842.15 ±65.55d
Townsville F1	4752.07 ±32.17e	5223.75 ±74.36e
Uno Rosso F1	4870.83 ±43.13f	3938.53 ±75.81b
Torquay F1	4944.78 ±73.77f	5350.70 ±65.75f

Note: Average ±standard deviation, K – control (0 g.ha^{-1} Se), Se – selenium (150 g.ha^{-1} Se). The different letters listed with the mean values in the columns represent statistically significant differences between the observed varieties (p <0.001).

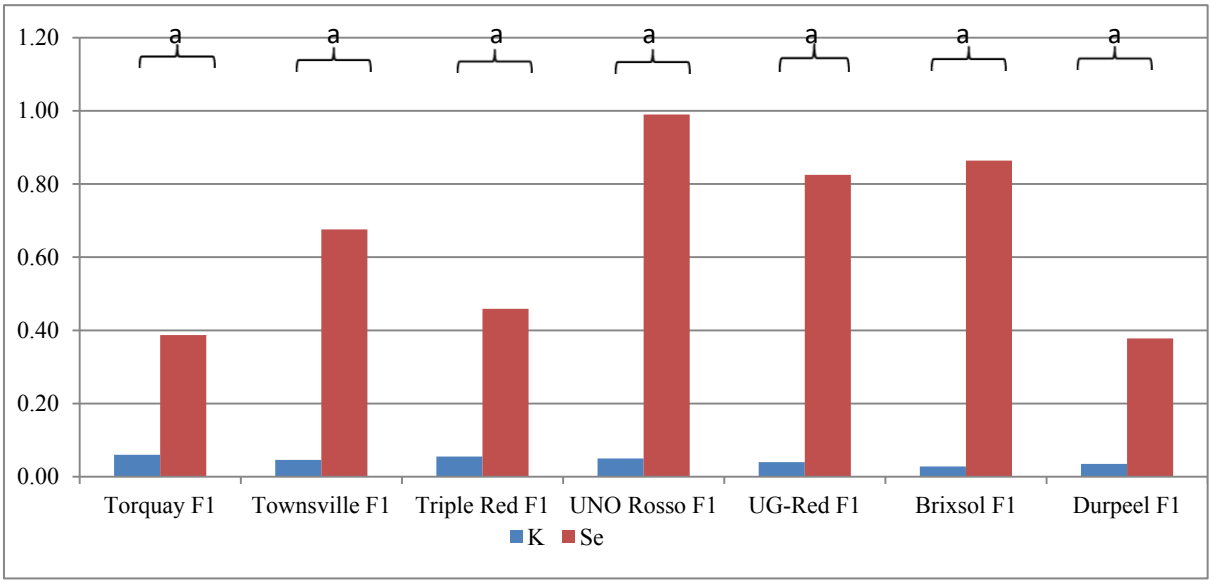

Figure 6 Selenium content (mg.kg^{-1} of dry matter) in fruits depending on the observed tomato varieties and variants (K – 0 g.ha^{-1} Se; Se – 150 g.ha^{-1} Se) Note: Statistically insignificant differences between varieties are indicated by the same letters (p >0.05).

Vitamin C content

Vitamin C (ascorbic acid) is one of the most important antioxidants in tomato. The content of antioxidants in tomato fruits depends on a number of factors such as ripeness, cultivation conditions and the variety itself **(Hart and Scott, 1995; Kotíková et al., 2011),** while the skin of fruits contains more vitamin C than the flesh **(George et al., 2004).**

Carli et al. (2011) tested 7 parental lines and 8 tomato hybrids and found that the vitamin C content in the fruits ranged from 9.1 to 16.1 mg.100g^{-1} of fresh matter. Our results show that similar levels of vitamin C content have also been found in our experiment. The vitamin C content

measured in fresh fruits of 7 hybrid tomato varieties was ranging from 11.90 to 19.44 mg.100 g^{-1} for control variant and from 11.46 to 20.14 mg.100g^{-1} of fresh mass for variant with foliar application of selenium (Figure 3).
The highest vitamin C content was in the Triple Red F1 variety in both experimental variants. Foliar application of selenium has a positive effect on the vitamin C increase only in Troquay F1, Townsville F1 and Triple Red F1 and Brixol F1 varieties.

By statistical analysis of the obtained data based on vitamin C content we can conclude, that the effect of the variety on the vitamin C content in the fruits of evaluated *Lycopersicon esculentum* varieties has been proven (Figure 3). Among the observed variants, we didn´t find any significant differences in the vitamin C content in fresh tomato fruits (Figure 2).

Total polyphenols content

During the evaluation of total polyphenol content in tomato fruit, we found that within the selenium control variant, polyphenols content ranged from 3588.30 mg GAE.kg^{-1} of dry matter in Durpeel F1 variety to 4944.78 mg GAE.kg^{-1} dry matter in Torquay F1 variety. Similar results are also reported by **Luthria, Mukhopadhyay and Krizek (2006)**, who determined the total polyphenols in tomatoes ranging from 3400 to 4400 mg GAE.kg^{-1}.

By foliar application of selenium at a dose of 150 g.ha^{-1}, all of the tomato varieties, except the Brixol F1 variety, increased the content of polyphenols in its fruits. For this variant, the content of total polyphenols ranged from 3603.40 mg GAE.kg^{-1} to 5350.70 mg GAE.kg^{-1} of dry matter (Table 6).

Kavalcová et al. (2014), based on the onion field experiments reported that the sodium selenate applied in different doses didn´t have a significant effect on the total polyphenols content in the consuming parts of evaluated onion varieties.

On the contrary, authors **Hegedűsová et al. (2017)**, on the basis of two-year field experiment, confirmed the significant influence of foliar application of selenium on increasing the polyphenols content in peas within two varieties of *Pisum sativum*.

By statistical evaluation of our results, we can conclude that the effect of the variety on the total polyphenols in the fruits was statistically significant. Although the effect of foliar application of selenium had a slight increase in the total polyphenols in the fruits, the effect of the variant on the total polyphenols content wasn´t confirmed (Figure 4).

Selenium content

To ensure the necessary income of selenium by human population, different ways of supplementing are used in the world. One of them is the addition of selenium into fertilizer preparations, which according to the authors **Ducsay, Ložek and Varga (2007)** can be considered as the most effective method of selenium intake, whereby selenium gets into individual food chain links. During the evaluation of the effect of selenium foliar application on selenium content in tomato fruit we found, that in experimental year 2016 selenium content in the fruits of studied varieties ranged in the control variant from 0.028 mg.kg^{-1} to 0.060 mg.kg^{-1} of dry matter. Foliar application of selenium caused an increase in selenium content in the fruits of observed tomato varieties and its content was in the range of 0.378 mg.kg^{-1} in a variety Durpeel F1 to 0.990 mg.kg^{-1} dry matter in a variety Uno Rosso F1 (Figure 6). **Hegedűsová et al. (2017)** also wrote about the positive effect of foliar application of selenium in the form of sodium selenate in the blooming phase of the pea,on the increase of selenium content in immature seeds (varieties: Ambrosador and Premium). They point to the fact that the total content of selenium in the consuming part is affected not only by the dose of applied selenium but also by the climatic conditions of the given growing year.

Using the statistical analysis of variance, we didn´t detect the difference in selenium content between the evaluated varieties. We recorded a statistically significant effect of the variant on the selenium content in tomato fruits (Figure 5).

CONCLUSION

Foliar application of selenium, in the form of sodium selenate, had a significant effect on the increase of the Se content, but did not affect the content of total carotenoids, and vitamin C in the fruits of tomato. The 150 g.ha^{-1} selenium dose slightly increased the total polyphenols content in the fruits. This way it is possible to enrich the tomatoes in selenium and polyphenols, which are antioxidants and to enhance healthy effects of the fruits. Based on our results, we can conclude that a shortage of daily selenium could be supplemented by consuming 250 g of fresh tomatoes foliar selenized with an average selenium content of 42 µg.kg^{-1} of fresh matter.

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HYGIENIC QUALITY AND COMPOSITION OF RAW SHEEP'S BULK MILK SAMPLES ON SELECTED SLOVAK FARMS DURING YEAR 2018

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ABSTRACT

At the control of raw ewe's milk (REM) quality is a major microbiological criterion to the total bacterial count (TBC). The aim of our work was to determine the incidence of technologically important species of microorganisms in REM in Slovak Republic. At the monitored 28 ewe´s farms, we took bulk milk samples from evening or morning milking in spring, summer and autumn during year 2018. We analyzed nutrients (fat, protein, lactose and urea) and somatic cell count (SCC). We established technologically important microorganisms (MO) of psychotrophic MO, coliform MO, thermoresistant MO, spore-forming anaerobic MO. We have found a gradual increase in milk components, except for lactose, which is apparently related to the increasing cont of somatic cells during the milking period. We found that the TBC in raw sheep's milk complied an average of 132 x 10^3 CFU.mL^{-1} per spring (min 34 x 10^3 CFU.mL^{-1}, max 501 x 10^3 CFU.mL^{-1}), 300 x 10^3 CFU.mL^{-1} in summer (min. 31 x 10^3 CFU.mL-1, max 640 x 10^3 CFU.mL^{-1}) and in autumn with an average value of 147 x 10^3 CFU.mL^{-1} (min 52 x 10^3 CFU.mL^{-1}, max 276 x 10^3 CFU.mL$^{-1)}$. The enormous occurrence of psychrotrophic bacteria was found in one farm in northern Slovakia during spring and summer, in the summer we increased our number to 3 farms, in the autumn of 2 farms. At the other farms we evaluated the average value of 12 x 10^3 CFU.mL^{-1} per spring and 28 x 10^3 CFU.mL^{-1} in summer, 130.5 x 10^3 CFU.mL^{-1} in the autumn. The count of thermoresistent MO achieved 57 CFU.mL^{-1} per spring, 15 CFU.mL^{-1} in summer and 33 CFU.mL^{-1} in the autumn. The presence of spore-forming anaerobic MO in raw ewe´s milk was found during spring at six farms out of 15, but in the summer at just one in 9, in the autumn two farms.

Keywords: SCC; TBC; microbiological quality raw ewe´s milk; milk composition

INTRODUCTION

In Slovakia sheep farming is focused on milk production. The increase in dairy yield was ensured by imports of specialized milk breeds, lacaune or East Friesian sheep and their subsequent crossing with our sheep breeds (Tsigai, Improved Valachian) **(Tančin et al., 2013)**. Ewe's milk was much more concentrated with about twice as much fat and 40% more protein that cow and goat milk. That also found that sheep milk responded differently in the cheese make procedure. It was more sensitive to rennet, coagulated faster, produced a firmer curd and yielded more cheese per unit of milk than cow milk **(Wendorff and Haenlein, 2017)**.

The quality of milk includes, in broad terms, the chemical composition, physical and technological properties, biochemical, microbiological and health indicators. In the narrower sense, we can only talk about hygienic (microbiological) aspects. Each of these features includes a number of quality features that determine the resulting quality of milk but also the quality of the dairy products. The quality of raw milk is regularly checked, because milk is the ideal environment for developing microorganisms because of its high water and nutrient content. All unwanted bacteria may not be pathogenic to humans. There are species that cause technological problems by producing thermostable lipolytic and proteolytic extracellular enzymes that pass through pasteurization in the active form. In order to avoid risks, and to ensure hygiene-sanitary quality and raw cows', sheep's and goats' milk safety, in its **Regulations (EC) Nos. 852/2004** and **853/2004 European legislation** lays down general food hygiene rules and specific ones for food of animal origin. It also sets out aspects relating to mandatory controls (EC) No. 853/2004 on raw milk production on farms, and in dairy centres and laboratories. Raw milk has to be tested for not only its physico-chemical composition, but also for its hygienic characteristics, such as microbiology, somatic cell count (SCC) **(Martínez et al., 2018)**. According to which the total bacterial count (TBC) in 1 mL of raw sheep's milk (at 30 °C) must not exceed 1 500 000 CFU and for raw milk for further processing not subjected to heat treatment, this number is reduced to 500 000 CFU. The TBC in the raw sheep's milk delivered indicates the overall level of breeding hygiene and technology of harvesting (machine

and hand milking) and milk storage. TBC reflects the hygiene of breeding conditions in milk production and is in the hands of the breeder itself. Bacterial contamination comes from a variety of sources, such as flora and pathogens present in hives, milking facilities, during storage and transport, feeding, rinsing water, udder or mastitis milk. Some of these bacteria are resistant to pasteurization or are able to grow at refrigeration temperature or to indicate fecal contamination, mastitis, or they can ferment lactic acid to butter, CO_2 and H_2, which cause late flushing of the cheese **(Gonzalo, 2017)**.

Scientific hypothesis

The occurrence of individual species of technologically significant microorganisms is influenced by the hygiene of obtaining milk in the milking process.

MATERIAL AND METHODOLOGY

At the monitored 28 ewe´s farms, we took bulk milk samples from evening or morning milking in March, April and May (spring), in June, July and August (summer) and September, October, November (autumn) during year 2018. Nutrients (fat, protein and lactose) were analyzed using the MilkoScan FT 120 (Foss Electric, Hillerød, Denmark). Somatic cell count (SCC) were set on the Somacount 150 (Bentley Instruments, Chaska, MN, USA). Urea was determined by polarimetry method. We analyzed the total bacterial count (TBC, mandatory indicator according to EC Regulation No. 1662/2006) according to **STN ISO 4833:1997**. We established technologically important microorganisms (MO) of psychotrophic MO according to **STN ISO 6730:2000** and coliform MO according to **STN ISO 4832:1997**. The presence of thermosensitive MO was detected in the Plate-Count-Agar and the presence of spore-forming anaerobic MO by liquid paraffin irrigation.

Statistic analysis

The values were evaluated through mean and standard deviation by Microsoft Excel 2013.

RESULTS AND DISCUSSION

It is known that the fat and protein content of milk is dependent on nutrition, and indirectly, nutrition will also affect the solids-non-fat (SNF) of milk. In Table 1 are presented the basic composition of milk and non-fat dry matter during the milking period. We have found a gradual increase in milk components, except for lactose, which is apparently related to the increasing number of somatic cells during the milking period and consequently the health of the milk udders.

Both fat and protein tend to increase throughout the lactation as well as **Kuchtík et al. (2017)**. This would typically result in higher cheese yields in late lactation milk **(Wendorff and Haenlein, 2017)**. As the SCC increases in the milk supply, the composition of milk also changes. As SCC increased, milkfat and the Casein/Total Protein ratio decreased. Protein recovery rate was lower in the high SCC milk while cheese yield was not significantly different.

Bocquier and Caja (2004) are reported that a high level of nutrition will reduce the level of milkfat but increase milk protein and casein. Conversely, a negative energy balance will decrease milk protein and increase milkfat. Milk protein will increase with an increased level of dietary protein. When feeding higher levels of concentrate in the diet, milkfat will be decreased and milk protein will be increased. The degree of impact from nutrition of the ewe will obviously be limited by the potential milk production capacity of the animal dictated by genetics. These trends are consistent with our results. Urea content depended on feed intensity, feeding system and pasture quality.

In Table 2, we presented the species of the most important technological types of bacteria. We found that the TBC in raw sheep's milk complied with the requirements of **Commission Regulation No. 1662/2006** with an average of 132 x 10^3 CFU.mL^{-1} per spring (min 34 x 10^3 CFU.mL^{-1} and max 501 x 10^3 CFU.mL^{-1}),

Table 1 Milk composition during the milking period.

Season	SNF		Milk composition (%)						Urea	
			fat		protein		lactose		%	
	mean	St.deviation	mean	St.deviation	mean	St.deviation	mean	St.deviation	mean	St.deviation
spring	11.26	0.59	7.50	1.61	5.56	0.58	4.84	0.45	42.39	12.44
summer	11.65	0.32	7.91	0.85	5.95	0.32	4.81	0.15	61.82	7.75
autumn	11.74	0.53	8.69	0.29	6.52	0.30	4.29	0.54	55.87	7.62

Note: SNF – solids non-fat.

Table 2 Hygienic quality of raw sheep's milk.

Microbiological characteristics (x 10^3 CFU.mL^{-1})	spring (n = 15)		summer (n = 9)		autumn (n = 4)	
	mean	St.deviation	mean	St.deviation	mean	St.deviation
TBC	132.13	87.71	300.00	217.01	147.00	124.78
Psychrotrophs MO	12.33	40.87	33.86	3.87	13.05	4.50
Coliforms MO	0.40	-	3.61	-	0.30	-
Termorezistant MO v 1 mL	57.69	29.31	13.33	11.15	40.50	33.27
SCC (x 10^3.mL^{-1})	1229.93	818.40	1411.08	770.25	2468.25	1147.14

Note: TBC – total bacteria count, SCC – somatic cell count.

300 x 10^3 CFU.mL^{-1} in summer (min. 31 x 10^3 CFU.mL^{-1} and max 640 x 10^3 CFU.mL^{-1}) and in autumn with an average value of 147 x 10^3 CFU.mL^{-1} (min 52 x 10^3 CFU.mL^{-1} and max 276 x 10^3 CFU.mL^{-1}). **Gonzalo (2017)** found similar TBC to our spring, **Martínez et al. (2018)** significantly lower values (49 x 10^3 CFU.mL^{-1}). **Vršková et al. (2017)** reported in the summer 2016 TBC range of 187 to 964 x 10^3 CFU.mL^{-1}. **Skapetas et al. (2017)** found a higher TBC of 494 x 10^3 CFU.mL^{-1} by SCC 313 x 10^3 cells in 1 mL. **Kondyli et al. (2012)** found lower TBC values in summer of 170 x 10^3 CFU.mL^{-1} than in the spring of 600 x 10^3 CFU.mL^{-1}. The microbiological quality of sheep's milk according to **Gamčíková and Hanzelyová (2009)** in the primary production is mainly affected by unmasked mastitis of ewes. **Carloni et al. (2016)** found a range between the farms at TBC of 2 to 865 x 10^3 CFU.mL^{-1} and SCC from 151 to 3384 x 10^3 cells in 1 mL. **Kološta and Dončovský (2006)** found an arithmetic mean TBC of 21.921 x 10^3 CFU.mL^{-1} of raw sheep's milk. **Ducková and Čanigová (2004)** determined the TBC from 57 x 103 to 3,400 x 103 CFU.mL^{-1} at an average of 580 x 103 CFU.mL^{-1}.

The somatic cells count (SCC) is not yet a mandatory indicator as it is for dairy cows. In the spring, 7 farms of 15 had SCC above 1000 x 10^3 cells in 1 mL. The remaining farms ranged from 131 to 825 x 10^3 cells in 1 mL. In the summer, SCC was in 5 farms over 1000 x 10^3 cells in 1 mL. The remaining four farms ranged from 60 to 965 x 10^3 cells in 1 mL. In the autumn there were 2 farms out of 4. The remaining two farms reached SCC 958 x 10^3 cells per 1 mL. **Martínez et al. (2018), Kuchtík et al. (2017)** and **Gonzalo (2017)** reported lower SCC than our results.

Season was an important effect associated with the variation of bulk tank milk prevalence for specific bacterial groups and pathogens. Psychrotrophic and coliform bacterial groups were highest in connection with more dirty beds and udders due to the wetter weather (ambient combinantion) and with beginning of milking season **(Gonzalo, 2017)**. Raw milk is stored in the primary production at 8 °C and can result in the growth of psychrotrophic microflora. Its proven relationship with a high incidence of lipolytic and proteolytic activities on milk and cheese components.

The enormous occurrence of psychrotrophic bacteria was found in one farm in northern Slovakia during spring and summer, in the summer we increased our number to 3 farms, in the autumn of 2 farms. We did not, therefore, enter statistical evaluation. We explain this by contaminating the milk in insufficiently disinfected and cooled collecting containers in accordance with statement **Ducková and Čanigová (2004)**. The remaining farms have a milking parlor and beside the dairy tank with cooling. At the other farms we are evaluated the average value of 12 x 10^3 CFU.mL^{-1} per spring and 28 x 10^3 CFU.mL^{-1} in summer, 130.5 x 10^3 CFU.mL^{-1} in the autumn. **Ducková and Čanigová (2004)** found up to 240 x 10^3 CFU.mL^{-1} psychrotrophic MO.

Thermodurics are a contaminant group of milk that contains thermophilic spore-forming bacteria which can survive pasteurization during dairy-product processing causing dairy-product spoilage in the post-processing **(Gonzalo, 2017)**. The count of thermoresistent MO achieved 57 CFU.mL^{-1} per spring, 15 CFU.mL^{-1} in summer and 33 CFU.mL^{-1} in the autumn. **Gonzalo (2017)** found a high incidence of thermoresistent MO (930 CFU in 1 mL) by the ewes.

Several studies in ewe bulk tank milk showed that the main on-farm management risk factors associated to an increase of spore counts were farm-made total mixed ration, the silages and wet brewer's grains used for feeding, and the presence of dust in the milking parlour **(Arias et al., 2013)**. The presence of spore-forming anaerobic MO in raw ewe´s milk was found during spring at six farms out of 15, but in the summer at just one in 9, in the autumn the count rose to two farms.

CONCLUSION

The amount of microorganisms in milk gives us an overall picture of the level of hygiene in the primary production. The degree of contamination of raw cows' milk with mesophilic and psychrotrophic microorganisms affects the dairy health and hygiene of dairy ewes, the hygiene of the milkers and the environment in which the ewes are farmed and milked, the methods used for the preparation of the udder and the milking technique, the methods used for cleaning and sanitizing milking equipment and bulk tank milk. Depending on the species of microorganisms found in milk, we can identify the source of contamination and then use the correct methods to eliminate them. For small ruminants, milk hygiene is important for serious economic and sanitary consequences for farmers, the processing industry and consumers due to the interrelationship between loss of production, yield in cheese production, excreted milk (and its safe disposal) and consequently the safety of dairy foods for the consumer. Consumers' demands on "natural" food, heat untreated, added preservatives or increased salt concentration are increasing. Such foods also include raw milk.

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ANTIMICROBIAL ACTIVITY OF RESVERATROL AND GRAPE POMACE EXTRACT

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ABSTRACT

Resveratrol is commonly found in food and drinks, including red wine and grapes. Grape extracts have a potent antimicrobial activity *in vitro*. The antimicrobial activity of plant extracts is the base of their potential application in food preservation agents, pharmaceuticals, cosmetics, alternative drugs and natural therapies. The aim of our study was to evaluate the antimicrobial activity of resveratrol and Blue Frankish pomace extract against Grampositive and Gramnegative bacteria as well as yeasts from the genus *Candida*. Six bacterial strains (three Grampositive bacteria *Staphylococcus aureus* CCM 2461, *Enterococcus faecalis* CCM 4224 and *Listeria monocytogenes* CCM 4699; three Gramnegative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa CCM* 1959 and *Salmonella enteritidis subsp. enteritidis* CCM 4420) and three yeast strains (*Candida albicans* CCM 8186, *Candida krusei* CCM 8271 and *Candida tropicalis* CCM 8223) were evaluated using the antimicrobial assay. Pure resveratrol and grape pomace extracts of red variety Blue Frankish were used. Our results show that resveratrol and red grape pomace extract have a very good antimicrobial activity against Grampositive bacteria when compared with Gramnegative bacteria and yeasts.

Keywords: grape pomace extract; resveratrol; pathogenic bacteria and yeasts; antimicrobial activity

INTRODUCTION

Winemaking is currently one of the most relevant agro-industrial activities in the world. Undoubtedly, grapes are an abundant fruit crop worldwide, with *Vitis vinifera* being the species most frequently cultivated for wine production **(Pareja et al., 2015; Barba et al., 2016)**.

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring stilbenoid which has been gaining considerable attention in the medical field due to its diverse biological activities – it has been reported to exhibit antioxidant, cardioprotective, anti-diabetic, anticancer, and antiaging properties. Given that resveratrol is a phytoalexin, exhibiting an increased synthesis in response to infection by phytopathogens, there has been interest in exploring its antimicrobial activity **(Chan, 2002)**.

Although there is still very limited work on the antibacterial activity of resveratrol, it has been shown that resveratrol exhibits antibacterial activity against several Gram-positive and Gram-negative foodborne bacteria **(Chan, 2002; Tegos et al., 2002; Paulo et al., 2010; Paolillo, Carratelli and Rizzo, 2011; Alvarez, Moreira and Ponce, 2012; Alvarez, Ponce and Moreira, 2013; Kumar et al., 2012; Plumed-Ferrer et al., 2013; Augustine et al., 2014; Ferreira et al., 2014; Morán et al., 2014; Promgool, Pancharoen and Deachathai, 2014; Subramanian, Soundar and Mangoli, 2016; Duarte et al., 2015; Kim et al., 2014; Makwana et al., 2015; Ferreira and Domingues, 2016; Liu et al., 2016; Seukep et al., 2016; Silva et al., 2016; Surendran Nair et al., 2016; Klancnik et al., 2016; Lai, Chiu and Chiou, 2017; Lee and Lee, 2017; Oliveira, Domingues and Ferreira, 2017)**.

Based on the available literature, resveratrol has been demonstrated to exhibit different antibacterial activities against numerous strains of foodborne pathogens including *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, and Listeria monocytogenes, E. coli* O157:H7, *Salmonella* Typhimurium, *Vibrio cholera, Campylobacter jejuni, Campylobacter coli, Arcobacter butzleri*, and *Arcobacter cryaerophilus* **(Ma et al., 2018)**.

Grape pomace is a potential source of natural antioxidant and antimicrobial agents. The phenolic compounds in grape pomace extracts exhibit antioxidant, anticancer, and antidiabetic properties **(Ruberto et al., 2007; Hogan et al., 2009; Parry et al., 2011; Zhou and Raffoul, 2012; González-Centeno et al., 2013; Snopek et al., 2018)**, as well as antibacterial activity against *E. coli, L. monocytogenes,* and *S. aureus* **(Ozkan et al., 2004; Darra et al., 2012)**.

More recently, the impact of the gastrointestinal digestion step on the phytochemical content in food, and on their bioactivities (mainly antioxidant capacity), have attracted special attention, which is evidenced by a great number of publications dedicated to this theme

(Gumienna, Lasik and Czarnecki, 2011; Tavares et al., 2012; Correa-Betanzo et al., 2014).

The aim of this work was to demonstrate the antimicrobial properties of resveratrol and red grape pomace extract towards both Grampositive and Gramnegative bacteria as well as yeasts. The microorganisms' sensitivity to resveratrol and red grape pomace extract were determined using the disk diffusion method, while the broth microdilution method was selected to determine the minimal inhibitory concentration (MIC).

Scientific hypothesis

Antimicrobial activity of the resveratrol against bacteria and yeasts.

Antimicrobial activity of red grape pomace extracts against bacteria and yeasts.

To find the lowest minimal inhibition concentration of resveratrol and pomace extract.

To find the most sensitive and most resistant microorganisms to resveratrol and pomace extract.

MATERIAL AND METHODOLOGY

Grape

Ripe grapes from wine cultivars grown in Vrbové (48° 37′ 12″ N, 17° 43′ 25″ E) Slovakia were collected. For the antimicrobial activity, the red variety Blue Frankish grape pomace extracts were used (Figure 1).

Resveratrol

Pure reveratrol was obtained from Sigma Aldrich.

Pomace extract preparation

Pomace extracts were prepared from a single production lot. A portion of the pomace samples (50 g) was immediately freeze-dried after receiving. The samples were extracted with 96% ethanol at a 1:10 ratio (m/v) using overnight shaking. The extracts were filtered through Whatman No. 2 filter paper to remove unwanted residues. After evaporating the organic solvent, the filtrates were dissolved in dimethyl sulfoxide (DMSO) at 20 $mg.mL^{-1}$ as the stock solution and stored at -20 °C for further investigation.

Figure 1 Grape Blue Frankish.

Microorganisms

Nine strains of microorganisms were tested in this study, including three Grampositive bacteria *Staphylococcus aureus* CCM 2461, *Enterococcus faecalis* CCM 4224 and *Listeria monocytogenes* CCM 4699; three Gramnegative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa* CCM 1959 *and Salmonella enteritidis subsp. enteritidis* and three yeast strains: *Candida albicans* CCM 8186, *Candida glabrata* CCM 8270, *Candida krusei* CCM 8271 and *Candida tropicalis* CCM 8223. All tested strains were collected from the Czech Collection of microorganisms (Brno, Czech republic). The bacterial suspensions were cultured in the Muller Hinton broth (MHB, Oxoid, Basingstoke, United Kingdom) at 37 °C for 24 h and yeasts were cultured in the Sabouraud dextrose broth (SDB, Oxoid, Basingstoke, United Kingdom) at 25 °C for 24 h.

Some aspects were considered for the selection of the microorganisms in this study: *S. aureus*, *E. faecalis, L. monocytogenes, S. enteritidis* and *E. coli* are known to cause foodborne diseases, *P. aeruginosa* is commonly resistant to multiple antibiotics **(Stover et al., 2000)** and *C. albicans*, *C. tropicalis* and *C. krusei* are the main fungi responsible for invasive bloodstream fungal infections, a significant cause of mortality in immunocompromised patients **(Selvarangan et al., 2003)**.

Disc diffusion method

The agar disc diffusion method was used for the determination of antimicrobial activity of the pomace extracts. Briefly, a suspension of the tested microorganism (0.1 ml of 10^5 cells mL^{-1}) was spread onto Mueller Hinton Agar (MHA, Oxoid, Basingstoke, United Kingdom) and Sabouraud dextrose agar (Oxoid, Basingstoke, United Kingdom) at 25 °C. Filter paper discs (6 mm in diameter) were impregnated with 15 µL of the pomace extract and placed on the inoculated plates. Tetracycline was used as a positive control to determine the sensitivity of the studied microorganisms. The plates were kept at 4 °C for 2 h and subsequently incubated aerobically at 37 °C for 24 h and 25 °C for 48 h for bacteria and yeasts, respectively. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of the sample that will inhibit the visible growth of microorganisms. Pomace grape extracts were dissolved in DMSO (conc. 20 $mg.mL^{-1}$). MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (**CLSI, 2019**) in Mueller Hinton broth (Oxoid) for bacteria and Sabouraud dextrose broth (Oxoid) for yeasts. Briefly, the DMSO solutions were prepared as serial two-fold dilutions to obtain a final concentration ranging from 3.9 to 2000 $µg.mL^{-1}$. The range of resveratrol concentrations tested was 2 – 512 $µg.mL^{-1}$, before the addition of the cells. Each well was then inoculated with the microbial suspension at the final density of 0.5 McFarland. After a 24 h incubation at 37 °C for

bacteria and 25 °C for yeasts, the inhibition of microbial growth was evaluated by measuring the well absorbance at 570 nm using an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after the experiment. Wells without resveratrol and pomace extract were used as positive controls of growth. Pure DMSO was used as a negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used pomace grape extracts. The results were expressed in $\mu g.mL^{-1}$.

Statistic analysis

All experiments were carried out in triplicate and the results are reported as means with standard deviations. The experimental data were subjected to analysis of variance (Duncan's test), at the confidence level of 0.05 using the XL STAT 2019 software.

RESULTS AND DISCUSSION

The disk diffusion method shoved that resveratrol exhibited a better antibacterial activity against all tested Grampositive bacteria (Table 1), when compared to Gramnegative bacteria. The lowest antimicrobial activity was found against yeasts which is consistent with previous studies using similar strains **(Paulo et al., 2010)**. The antimicrobial activity using the disc diffusion method ranged from 10.00 ±2.00 (*C. albicans*) to 22.67 ±2.08 (*E. faecalis*).

Similar results were found in case of the pomace extract. The best antimicrobial activity was found against *E. faecalis* while the lowest antibacterial activty was detected against *C. krusei*.

The high antimicrobial effect was observed in the variants with grapevine seeds against *E. coli* **(Jakubcová et al., 2015)**.

In study of **Chan (2002)** established that resveratrol conferred the antibacterial effect. DMSO did not reduce the growth of the tested bacteria, except for a slight decrease in the case of *E. faecalis* at 3.3%. When present at 171 $\mu g.mL^{-1}$ of resveratrol in 1.7% DMSO, the growth of *S. aureus* was inhibited by 80 – 90%. A similar degree of inhibition was observed in case of *E. faecalis* and *P. aeruginosa* at 342 $\mu g.mL^{-1}$ of resveratrol in 3.3% DMSO.

In our work, resveratrol concentrations ranging from 2 to 512 $\mu g.mL^{-1}$ have been tested, and its MIC for all Grampositive bacteria (S*taphylococcus aureus, Enterococcus faecalis and Listeria monocytogenes*), Gramnegative bacteria (*Escharichia coli, Pseudomonas aeroginosa* and *Salmonella enteritidis*) and yeasts (*Candida albicans, Candida krusei* and *Candida tropicalis*) were determined. The microorganism that presented the highest sensitivity towards resveratrol was *Enterococcus faecalis* (MIC 64 $\mu g.mL^{-1}$), followed by *Staphylococcus aureus* and *Listeria monocytogenes*, which have presented with a MIC of 128 $\mu g.mL^{-1}$ (Table 2). Similar results with similar bacteria were obtained in the study by **Paulo et al. (2010)**. In this work, resveratrol concentrations ranging from 3.125 to 400 $\mu g.mL^{-1}$ have been tested, and its MIC for all Grampositive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*) was determined. The microorganism that presented the highest sensitivity towards resveratrol was *Bacillus cereus* ATCC 11778 (MIC 50 $\mu g.mL^{-1}$), followed by *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, with a MIC of 100 $\mu g.mL^{-1}$.

It was not possible to obtain resveratrol concentrations higher than 400 $\mu g.mL^{-1}$ due to its poor solubility **(Jeandet et al., 2002)**, and therefore its MIC on Gram-negative bacteria was not determined. Consequently, the efficacy of inhibition (in terms of percentage) presented by different

Table 1 Screening of antimicrobial activity of resveratrol using the disk diffusion test in mm.

Bacterial strains	Resveratrol	Pomace extract
Staphylococcus aureus	22.33 ±1.15^{a}	16.66 ±1.53^{b}
Enterococcus faecalis	22.67 ±2.08^{a}	17.67 ±1.53^{a}
Listaria monocytogenes	19.00 ±1.00^{a}	16.67 ±1.53^{b}
Escherichia coli	14.33 ±2.08^{a}	9.00 ±1.00^{b}
Pseudomonas aeroginosa	15.67 ±1.15^{a}	13.00 ±1.73^{b}
Salmonella enteritidis	15.00 ±1.00^{a}	13.33 ±1.53^{b}
Candida albicans	10.00 ±2.00^{a}	6.00 ±1.00^{b}
Candida krusei	12.33 ±2.52^{a}	4.67 ±0.58^{b}
Candida tropicalis	11.33 ±1.53^{a}	5.33 ±0.58^{b}

Note: mean ± standard deviation; different letters in column denote mean values that statistically differ one from another.

Table 2 Screening of antimicrobial activity of resveratrol using the broth microdilution method in $\mu g.mL^{-1}$.

Bacterial strains	Resveratrol	Pomace extract
Staphylococcus aureus	128	500
Enterococcus faecalis	64	250
Listaria monocytogenes	128	250
Escherichia coli	512	1000
Pseudomonas aeroginosa	256	500
Salmonella enteritidis	256	500
Candida albicans	512	500
Candida krusei	512	1000
Candida tropicalis	256	1000

concentrations of resveratrol was used to evaluate its activity against these bacteria. At a concentration of 400 $\mu g.mL^{-1}$, the inhibition percentages observed for *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* were respectively, 81, 80 and 58%. In addition, it was not possible to determine the MBCs, since the maximum tested concentration was 400 $\mu g.mL^{-1}$.

Kačániová et al. (2018) tested four strains of bacteria (two Gram-positive bacteria *Staphylococcus aureus* CCM 2461, *Bacillus cereus* CCM 2010; two Gram-negative bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa CCM 1959)* and four yeasts strains (*Candida albicans CCM 8186, Candida glabrata CCM 8270, Candida krusei CCM 8271 and Candida tropicalis CCM 8223*). For the detection of the antimicrobial activity, the grape pomace extracts of white variety Pálava and red variety Dornfelder were used. *Pálava pomace* extracts were less efficient against the microorganisms tested and Dornfelder extracts were more active against Grampositive bacteria and yeasts. The best antimicrobial activity of pomace extract Blue Frankish grape in our study was found to be similar to resveratrol against Grampositive bacterial strains. The antibacterial activity of four grape pomace extracts was evaluated in the study of **Xu et al. (2015)**. All extracts exhibited antibacterial activity against *L. monocytogenes* and *S. aureus*, but no antibacterial activity was detected against *E. coli* O157:H7 and *S. typhimurium*. Our results partially agree with previous studies on the antimicrobial activity of whole grapes or grape pomace extracts against both Gram-positive and Gram-negative bacteria, with the most pronounced effects against Gram-positive bacteria **(Darra et al., 2012)**.

CONCLUSION

The present study showed that resveratrol and red pomace extract of Blue Frankish grape showed better antibacterial activity against Grampositive *Staphylococcus aureus, Enterococcus faecalis and Listeria monocytogenes* when compared with Gramnegative bacteria and yeasts using both methods, the disc diffusion and the minimal inhibiotn concentration.

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TIN COMPOUNDS IN FOOD – THEIR DISTRIBUTION AND DETERMINATION

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ABSTRACT

The aim of this work was optimization of the methods of trace- and ultratrace analysis, such as ICP-OES, ETA-AAS for charting the resources of individual forms of tin in foodstuffs. Increase of the sensitivity of the method of ICP-OES was achieved using the techniques of generation of hydrides, which was also optimized. Based on the information available on the occurrence of the different forms of tin, it appears that many of these organometallic compounds are contained in marine animals; attention has mainly focused on organisms such as marine fish, crustaceans, molluscs and algae. Tin compounds of predominantly inorganic origin can be found in foods and beverages which are packed in cans with a protective tin coating, too. The above mentioned methods have been applied to the analysis of selected beverages with low content of tin such as Coca Cola, Sprite, Fanta, Gambrinus 10°, PowerKing, and milk in the cans. Furthermore samples of animal origin as Sardines in oil, and Hunter's salami were examined, too. Prior to the determination of tin, samples need to be appropriately modified or analysed. Decomposition of the samples was done in the microwave system. Low pressure ion exchange chromatography with on-line detection of ICP-OES was used for separation of inorganic tin compounds. Separation of organically bound tin compounds was performed by HPLC on a column of ACE C-18, 3 µm, 15 cm × 1.0 mm with off-line detection by ETA-AAS. All of the above forms of tin compounds can be separated with this column. Due to the improvement in the detection of organically bounded tin, HPLC with identical ACE C-18 column coupled online for example with ICP-MS or spectrofluorimetry could be recommended.

Keywords: foods; tin; speciation of organotin; HG-ICP-OES; HPLC-ETA-AAS

INTRODUCTION

One of the indicators of the toxicological quality of food is the content of toxic mineral compounds. Lead, cadmium, mercury and arsenic belong among the most toxic elements. In higher concentrations trace elements such as, tin, chromium, cobalt, copper, molybdenum, nickel, selenium, vanadium, and others may show toxic effects. For these elements, the maximum allowed quantity, the permissible maximum quantity or special dose are specified in food legislation in the Czech Republic.

For humans or for other animals, tin is an essential element, but in larger quantities it appears as toxic. Its biological importance for humans has not yet been fully elucidated; it is assumed that it is indispensable for optimal growth and the formation of blood. Tin compounds are primarily located in the Earth's crust and also due to the human activities in the air and water flow.

The most dangerous form of tin are the compounds where tin is organically bounded in organometallic compounds. In spite of its potential toxicity, tin is used in large quantities in plastic and canning industry and for destruction of pests in agriculture, where it gets into agricultural products and from there into the food. Toxic effects of tin are known, but its use is increasing.

Humans can absorb tin from food, during breathing, and through the skin. Absorption of tin can enter human organism in the digestive system, thus food control has been emphasized quite intensively recently.

The average content of tin in the Earth's crust is about 3.0 $mg.kg^{-1}$. In nature, tin occurs as the mineral cassiterite (stannic oxide SnO_2,) and as an ingredient in some sulphides.

Metal tin is an important component of common alloys (bronze). The tinplate cans are made of steel sheets used in the food industry. Consumption of tinplate in food packaging is still growing and the largest share goes to the production of cans for the distribution of beer and other beverages **(Greenwood and Earnshaw, 1993)**.

A large quantity of tin is consumed for the production of organometallic compounds. Tin is used as a component of special paints for ships and other bodies subjected to the long-term effects of the sea water. Tributyltin compounds are used for the preservation of wood. Tributyltin oxide

$O(SnBu_3)_2$ is an excellent agent for protection of the wood. R_3SnX is also used as an antimicrobial agent to exterminate of slime mould in the paper and pulp.

Triphenyltinacetate and triphenyltinhydroxide are applied in agriculture as fungicides. For example, Bu_2SnOH and Ph_3SnOAc inhibit the growth of fungi, such as potato blight and similar infections, in sugar beet, peanuts and rice. Tin also serves as a mite-killer on apple and pear trees. Other R3SnX compounds are effective at killing insects as chemosterilant or by killing of larves **(Velíšek, 1999)**.

Dibutyldilaurate and analogue octyltin compounds are used as stabilizers for plastics (PVC). The most effective stabilizer compounds (R_2SnX_2 – R = octyl-, X = laurate, maleate). For the packaging of food products, the polymeric cis-butenedioic and ($Oct_2SnOCOCH$ = CHCOO)n and $Oct_2Sn(SCH_2COO\ Oct)_2$, with S,S´-bis (isooctyl-mercaptoethanolate), which is used in cases where a colorless, non-toxic, highly transparent material is essential, were approved.

Another no less important application of organotin compounds is their use as curing agents in vulcanization of silicone under normal temperature **(Greenwood and Earnshaw, 1993)**.

On other hand, the inorganic forms of tin are less toxic for human organism. Inorganic tin is just difficult to be absorbed by the organism and is usually excreted in the urine. According to the study from 1997, tin (II) is more toxic than tin (IV). Due to the presence of humic acids in the ground waters that reduce tin (IV) on tin (II) its concentration is increasing **(Pawlik-Skowronska, Kaczorowska and Skowronski, 1997; Rüdel, 2003)**. Inorganic tin is not carcinogenic or teratogenic.

Tin in all organic industrial compounds is tetravalent. Organic alkyl or aryl groups are groups that are covalently bonded with the central atom of tin. Mono-, di- and trisubstituted butyltin, phenyltin, and their derivatives are considered its most important compounds. The solubility of organically bounded tin compounds depends on pH, ionic strength and temperature of environment.

Degradation of organotin compounds occurs in biotic or abiotic processes. The transformation is similar for both cases **(Rüdel, 2003)** it leads through the dealkylation or dearylation on inorganic tin compounds **(Hoch, 2001)**.

The European Union (EU) limits maximum levels for certain contaminants with a view to reduce the content of these substances in foods at such a low level, which is yet to be achieved in compliance with the good manufacturing or agricultural practices. The aim is to achieve a high level of public health protection, especially for vulnerable groups of the population: children, allergies, etc. **(Commission Regulation (EC), 2006)**.

Tin gets into tinned food via further decomposition of the inner walls of tinned food cans. The decomposition of tinplate is dependent on the food matrix, pH, the presence of oxidizing substances (anthocyanines, nitrates, ions of iron and copper), and the presence of air (oxygen) in the area of food, time and storage temperature.

Nowadays, corrosion and dissolution of tin cans will be suppressed by a varnish, which greatly reduces the penetration of tin in food products.

Tin cans contain mainly canned vegetables, fruits, juices and other beverages, fruits in sweet pickle, mixed fruits in brine, canned milk and pickled mushrooms (Figure1).

For the determination of trace elements as well as for detection of tin in foodstuffs, it is necessary to choose the analytical methods, which are able to detect very low, trace amounts of analyte. Atomic Spectrometry methods – atomic absorption, emission and mass spectrometry are suggested for analyses of tin in food.

The most common method is atomic absorption spectrometry (AAS). When you use flame atomization the limit of detection is on the level of 0.1 $mg.L^{-1}$; using the electrothermal atomization even decreases this limit. Additional options for the determination are represented by different extractants (e.g. with hexane, toluene, chloroform r methanol), conversion of the compounds into volatile derivatives (by reaction with Grignard´s reagents or reaction with $NaBH_4$ result in volatile hydrides usable for atomic absorption spectrometry determination) and separation by gas chromatography. It is also possible to use liquid chromatography with mass spectrometer with ICP (inductively coupled plasma).

These methods offer for many elements excellent limit of detection, so they are suitable for the determination of trace amounts of elements in biological materials. Comparable results are achieved using sensitive electroanalytical methods, mainly a modification of polarography and voltamperometry, electrogravimetry or fluorescence.

For the determination of organically bounded forms of tin, in particular of tributyltin compounds (including degradation products – di- and monobutyl derivatives) are also used the above methods (AAS, ICP-OES and ICP-MS), in connection with sepraing in on-line or off-line arrangement (Figure 4).

To determine total tin detection of different forms (separation of inorganic and organically bound forms of tin) is performed both via complete mineralization of samples and in dry (combustion and subsequent dissolution in acid) or wet way (oxidation reactions in the strongly acidic environment). Mineralization by the wet way can be further performed under the hood in an open or closed system. Mineralization in a closed system can be done under high or low pressure and either common or

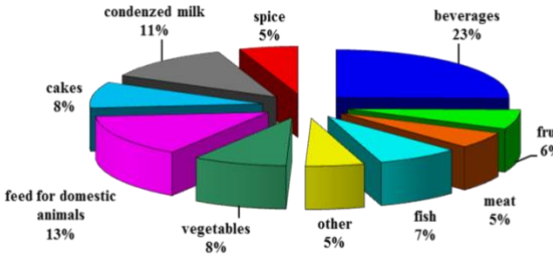

Figure 1 The most frequently observed types of canned food on the tin content **(Perring and Basic-Dvorzak, 2002)**.

microwave heating can be employed **(Mader and Čurdová, 1997)**.

If the determination of the different forms of tin is required, treatment of samples keeping tin compounds in their original form is essential and also maintaining their initial relationship in the sample is crucial. For these purposes very gentle procedures must be employed. They are based on the extraction with helping of ultrasonic wave or with the support of many microwaves in pH buffered environments. If the separation or isolation of the individual forms in one step is impossible, it is necessary to use a multi-step extraction or specific extraction or the conversion of other forms, and then multi-step analysis must be carried out **(Simon et al., 2002)**.

Scientific hypothesis

Since the determination of the tin compounds in foods is of great importance, particularly in terms of the occurrence of organotin compounds that accumulate in human adipose tissue, many techniques have been used for this purpose. Studies using different techniques, such as graphite furnace atomic absorption spectrometry (GF AAS) and cold vapour atomic absorption spectrometry (CV AAS), have been reported.

The inductively coupled plasma optical emission spectrometry (ICP-OES), which makes it possible simultaneous determinations on more different wavelength and allows rapid an effective analysis, is also reported. The design of experiments is an important approach and has been successfully employed in sample preparation procedures to identify the optimum conditions and select the proportions between the reactants, allowing a faster acquiring of results, minimizing costs and time involved. The use of diluted reagents in decomposition or extraction procedures, which leads to media with reduced acidity and also decreases the amount of corrosive substances, is an example of the experimental design application. Chemometric tools were used to establish the appropriate experimental conditions for determination of total tin content by CV AAS or by hydride generation atomic emission spectrometry with inductive coupled plasma (HG ICP-OES) and combination of separation step (EC or HPLC) with the same methods of detection for determination of tin (II) and tin (IV) and organotin compounds in biological samples.

Thus, this paper purpose is a multivariate optimization of an analytical method through all parameters outside the device parameters conclude selection the most suitable wavelength, concentrations of reagent solutions, correction of interferences and inlet flows of gases and solutions to determine the individual species of tin employing ICP-OES.

MATERIAL AND METHODOLOGY

Material and reagents

All reagents used were analytical grade, and solutions were prepared with ultrapure deionized water obtained from a reverse osmosis water purification system and ultrapure system (Aqua Osmotic 02 Tisnov, CR and Purelab ULTRA, Elga, UK). The nitric acid (68%), hydrochloric acid (37%) and hydrogen peroxide (31%) used were of Analpure Ultra (Analytika, s.r.o., CR). External calibration was prepared from stock solution of tin of 1000 $mg.L^{-1}$ (Astasol®). Also, 1000 $mg.L^{-1}$ stock solutions organotin compounds were prepared from dibutyltin dichloride (97%), triphenyltin chloride (95%) and tributyltin chloride (95%), Sigma – Aldrich s.r.o. CR and diluted according to the working range required. The calibration curves were prepared in a suitable range of concentrations in accordance with the purpose.

Other reagents such as sodium tetrahydridoborate NaBH4, NaOH, KOH for hydride generation used and organic solvents and acids (methanol, acetonitrile, acetic acid and triethanolamine) were HPLC grade from Sigma – Aldrich s.r.o. CR.

The glassware used in the experiments was previously decontaminated with a nitric acid solution (10% v/v) for 24 h **(Dantas et al., 2013)**, subsequently washed with ultrapure water and dried at room temperature.

Instrumentation

For the analysis of the food samples an inductively coupled plasma optical emission spectrometer with axial view (ICP-OES, Thermo Jarrell Ash, IRIS/AP,USA) and an atomic absorption spectrometer with electrothermal atomisation (Thermo Elemental, Solaar M6, UK) were used, and the operating conditions are detailed in Table 1 and Table 2. For preparation of samples for total tin content analyses a microwave digestion system MLS 1200, Milestone it was used and for separation of organotin forms an HPLC system (Dionex RS 3000 Ultimate, USA) was used.

Optimization strategy and analysis of the data

In order to achieve the best working conditions providing the lowest detection limit were performed an optimization, which includes the choice of the best wavelength for tin and appropriate instrument settings. Sensitive wavelengths for tin have been selected from a database of TEVA™ (Thermo Elemental Validated Analysis, ICP-OES software, v. 1.4.0. for controls the optical emission spectrometer with inductively coupled plasma). It was tested a total of six wavelengths, which is able for the tin determination. Of these six wavelengths were chosen two (Table 2), which was the signal for tin the best response (SBR).

Other parameters were setting the input of the plasma on 1150 W, auxiliary gas flow 1 $L.min^{-1}$, the uptake of the sample to the nebulizer 1.85 $mL.min^{-1}$.

Statistic analysis

Validation of analytical method

The precision of the method was evaluated via the repeatability and can be expressed as the relative standard deviation (RSD) of a set of measurements. Accuracy expresses the difference between the value found experimentally and a reference value. In this study, Trace elements in CRM material fish tissue (NIES-11) was used to establish the accuracy through the values calculated using the Eq. (1). This approach is directly related to international standards.

Recovery (%) = [found value/certified value] x 100 (1)

Where found value is the analyte concentration determined by the proposed method and the certified value is the concentration value of the analyte reported in the SRM certification document. The precision and accuracy expressed as RSD (%) and recovery (%), respectively, obtained for the optimized analytical method calculated on the certified values and the found values for CRM. The recovery between the certified values and the found values ranged from (87.0 ±1.0) % (for tributyl tin) and about (92.0 ±5.0) % for total tin content, and the RSD values obtained were better than 5% (n = 3).

The tin content in different samples of foods was measured by HG-ICP-OES. Samples before the determination were decomposed in the microwave device by mineralization step. As samples were selected common beverages: Coca Cola, Sprite, Fanta, Gambrinus 10°, PowerKing and picnic in the cans. It was also determined a total content of the tin in caned Sardines in vegetable oil and in the hunter salami as a potential possible source of organotin compounds.

Samples of drinks before mineralisation step in the ultrasonic bath were degassed. For the decomposition of samples of beverages and foodstuffs was take up 10 mL or 1.0 g of samples and added 8 ml of HNO_3 and 2 mL of HCl. After this operation the sample was evaporated to near dryness, almost added to it 1 mL of HCl and volume was fill up on 10 mL. Values measured during both the wavelengths used are listed in Table 8.

The analysis of the data obtained from the experimental design was performed using Statistica® 12.0 software (StatSoft, USA). The measurements were performed in triplicate and the data are expressed as mean ±95% confidence interval (CI).

RESULTS AND DISCUSSION

Optimization of the hydride technique

The hydride generation technique was used for the reduction of the limit of detection. This technique based on reaction of $NaBH_4$ with acid to produce hydrogen, which generate volatile hydride with suitable ions of metal. These ions are then carried into the carrier gas stream into discharge of the ICP. If we compare the direct method of measuring tin and the hydride generation technique we can change measurement extent from the range ppm ($mg.L^{-1}$) to ppb ($\mu g.L^{-1}$) levels. The optimization of the concentration of sodium tetrahydroborate for the hydride generation for the correct determination was performed.

Optimization of the concentration range $NaBH_4$ was selected on the literature **(Hosick, Ingamells and Machemer, 2002)** where the optimum of concentration was about 2.4% of $NaBH_4$. Therefore, the optimization of reducing reagents at concentrations of 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 5% of the $NaBH_4$ dissolved in 0.1% KOH was performed.

Similarly for the acidification of the samples has been used 0.10; 0.25; 0.50; 0.75; 1.0 M hydrochloric acid.

As the most sensitive appeared wavelength of tin 189.989 nm (with the highest ratio of intensities (IR), which is the ratio of full intensity to the intensity of background or blank on the same selected wavelength), therefore the final optimization was carried out and the final results of processing of the content of tin was expressed only at this wavelength. The results of the optimization steps with a concentration of 100.0 ppb tin are presented in Table 3 and Table 4.

Calibration of hydride technique for the determination of different form of tin

The calibration solutions of standards of different forms of tin were measured at wavelengths 189.989 and 242.949 nm. The concentration of the calibration solutions was selected 50; 100; 150 $\mu g.L^{-1}$ and as blank was used a 0.25 M solution of HCl. The calibration solutions of Sn (IV) species were prepared from standard calibration solution 1.0 $g.L^{-1}$ in 0.25 M of HCl solution into the 100 mL flasks. Calibration solutions of Sn (II) were prepared by loading the appropriate amount of $SnCl_2.2H_2O$. This amount was transferred to 100 mL volumetric flask and fill up with deionised water. From this stock solutions have been further prepared calibration solutions of the above concentration.

Evaluation of the operating conditions obtained for the ICP-OES

This procedure made it possible to select the more sensitive lines, free of interference (Table 1), that were used to calculate the limits of detection (LOD) and the limits of quantification (LOQ) through the background equivalent concentration (BEC) and the signal-to-background ratio (SBR). The LOD and LOQ values were calculated using the BEC and the SBR, according to International Union of Pure and Applied Chemistry (IUPAC); $BEC = C_{standard} / SBR$, where $SBR = (I_{standard} - I_{blank}) / I_{blank}$; Cstandard is the reference element concentration in the solution; and Istandard and Iblank are the emission intensities for the reference element and blank solutions, respectively at the selected wavelength **(Da Costa et al., 2013, Schiavo et al., 2009)**.

The LOD was then calculated as ($3 \times RSD_{blank} \times BEC / 100$) and the LOQ as ($3.3 \times LOD$), where RSDblank is the relative standard deviation of ten measurements of the emission intensity of the blank solution.

Separation of inorganic forms of tin by ICP-OES method

Ion-exchange chromatography

Forms of tin were separated by ion-exchange chromatography with on-line detection of ICP-OES. The measurement was done by using the time scan module in the TEVA software. Before choosing a suitable sorbet is necessary to find out as much as possible information about samples, which considered the properties of the matrix and the analyte, and based on this knowledge, then choose the type of phase and size of columns. Offer of sorbents is similar as for filling for liquid chromatography. All sorbents, which were used, had to be activated at first. Activated sorbents under reduced pressure using the sorption apparatus were implemented in polypropylene columns. After filling the columns were washed up with deionised water.

Tin in the compounds occurs in the form cations Sn (II) and Sn (IV). Therefore ion changers were selected as cation exchangers. The most appropriate cation exchanger is like this the tin ions are relatively closely retained and they are not washed up with water. Another important parameter is the selection of appropriate eluent reagents.

Due to the stability of the plasma is not appropriate use of elution agents containing higher amount of organic compounds or high concentrations of salts.

In work were tested exchangers Amberlite IRC 50, Cellulose CM 23, Servacel CM 3, Dowex 50WX, Trisacryl M CM and Sephadex CM 50 with elution reagents 1 M HCl and 0,5 M NaOH. The most of them - Amberlite IRC 50, Cellulose CM 23, Trisacryl M CM and Sephadex CM 50 – were suitable for separation of Sn (IV). Perfect separation of Sn (II) and Sn (IV) ions is complicated how is describe in literature **(White et al., 1998)** that the solutions of salts of Sn (II) ions are very unstable, easily oxidized by air and especially on light. Therefore, the study was focused on time dependency of Sn (IV) conversion on Sn (II) species on the light. In the previous chapter, it was found that when using a column filled with exchanger Amberlite IRC 50, tin in the form Sn (II) is washed out at the sampling and Sn (IV) is retained on the exchanger and washed out with 1 M HCl. This phenomenon was used for determining the time conversion dependency Sn (II) on Sn (IV) species. The functional range of the pH for ion exchanger which was used Amberlite IRC 50 is 5-14, in order to the pH of the sample is not need regulate. Results of measuring dependences of peak areas on time in derivation and logarithmical modes are shown in Table 5 and Figures 2 and 3.

Determination of tin compounds by ETA–AAS method

Optimization of method

For the determination of tin in samples by electrothermal atomisation in graphite furnace an ELC (Extended Life Cuvette) and Sn hollow cathode lamp has been used. According to literature **(Chen et al., 1996)**, where it was performed by optimizing the working conditions for the determination of Sn by ETA AAS method. For background correction a Zeeman correction method has been selected and as matrix modifier a combination of Palladium and Ascorbic acid was used. The unit was setting to the basic working conditions recommended by the manufacturer (Table 6 and Table 7).

Application of optimized method to food samples

After settinμ the optimal conditions a calibration method for different forms of tin was performed. Calibration solutions were measured at a wavelength of 224.6 nm with calibration functions y = 0.0073x + 0.0502 and R^2 = 0.9977 for tributyltin chloride; y = 0.006x + 0.0524 and R^2 = 0.9975 for dibutyltin dichloride and y = 0.004x + 0.0457, R^2 = 0.9962 for triphenyltin chloride. The concentration of the calibration solutions 10, 30, 50 $\mu g.L^{-1}$ has been prepared. A solution of 50 $\mu g.L^{-1}$ of different forms of tin with the addition of 0.5 ml 65% HNO_3 into a 50 ml volumetric flask and added a mixture of MeOH: H_2O (40: 10) has been prepared from standard solutions **(Schiavo et al., 2009)**. As blank and dilution solution 0.65% HNO3 has been used. As a matrix modifier a solution of $Pd(NO_3)_2$ and 1% solution of ascorbic acid were used. This mixture with sample by autosampler was sampled to the ELC of ETA AAS.

Due to the improvement of the detection of organically bound tin and streamlining of analysis would be suitable HPLC with column ACE C-18 connect in on-line arrangement, for example with ICP-MS or fluorimeter (**Gonzáles-Toledo et al., 2001**).

Table 1 Characteristics and operating conditions used for analysis by ICP-OES with axial view.

Parameter	Characteristics
Radio frequency power (W)	1150
Plasma gas flow rate ($L.min^{-1}$)	15.0
Auxiliary gas flow rate ($L.min^{-1}$)	1.5
Sample uptake rate ($mL.min^{-1}$)	1.85
Nebulizer gas flow rate ($L.min^{-1}$)	1.00
Nebulizer type	Concentric
Spray chamber	Type cyclone
Replicates	3
Injector tube diameter (mm)	2.0
Signal integration time (s)	1.0
Wavelength (nm)	Tin 189.989
	242.949

Table 2 Wavelengths suitable for determination of tin.

Wavelength λ [nm]	Spectral order	relative intensity
189.989	136	150,000
242.949	107	100,000

Table 3 Values obtained for BEC, LOD and LOQ in the analysis of liquid samples of beverages by ICP-OES[a].

Analytical parameter	Sn(II) 189.989 nm Direct method	Sn(IV) 242.949 nm Direct method	Sn(II) 189.989 nm Hydride generation	Sn(IV) 242.949 nm Hydride generation
BEC ($mg.L^{-1}$)	0.2185	0.9422	0.0298	0.0967
LOD ($mg.L^{-1}$)	0.0036	0.0232	0.0006	0.0011
LOQ ($mg.L^{-1}$)	0.0107	0.0697	0.0017	0.0033

Note: [a] For solid samples values of LOD and LQD is necessary multiplied with factor for mass of 1.00 g of samples and completed to 10.0 mL with deionised water and expressed in $mg.kg^{-1}$.

Table 4 Dependency of intensity ratios (IR) on the concentration of $NaBH_4$ in the solution.

% $NaBH_4$	IR	% RSD
0.5	13.57	0.6973
1.0	32.95	0.8726
1.5	49.96	0.8841
2.0	64.77	1.3630
2.5	80.50	0.7996
3.0	90.37	0.6808
3.5	78.21	0.7758
4.0	73.77	1.6490
5.0	18.40	5.5600

Table 5 Dependence of the peak area at the time by ICP-OES method in time scan model.

	Peak area	
t [s]	Sn (II)	Sn (IV)
20	6659.5	10211.0
420	2929.5	13678.0
780	1720.5	14204.0
1140	620.5	15217.0
1560	417.0	15450.0
1920	145.5	16006.5

Table 6 Working conditions for determination of tin by ETA-AAS method.

Parameter	Setting
Wavelength	224.6 nm
Background correction	Zeeman effect
Time of measurement	3 s
Slit	0.5 nm
HCL current	75% I_{max}

Table 7 Temperature – time programme for ETA AAS.

Phase	Temperature [°C]	Time [s]	Ramp [°C.s^{-1}]	Flow of Ar [L.min^{-1}]
1	100	30	10	0.2
2	120	10	50	0.2
3	800	20	150	0.2
4	2300	3	0	0
5	2600	3	0	0.2

Table 8 Content of tin in food samples obtained HG-ICP-OES method.

	c [mg.L^{-1}]
Sample	189.989 nm
Coca Cola	0.1766 ±0.0629
Sprite	0.0903 ±0.1159
Fanta	0.0275 ±0.0390
Gambrinus 10° − beer	0.0360 ±0.0100
PowerKing	0.0227 ±0.0015
Piknik	0.3758 ±0.0078
Sardines in vegetable oil	1.0791 ±0.0892
Hunter salami	0.7016 ±0.1250

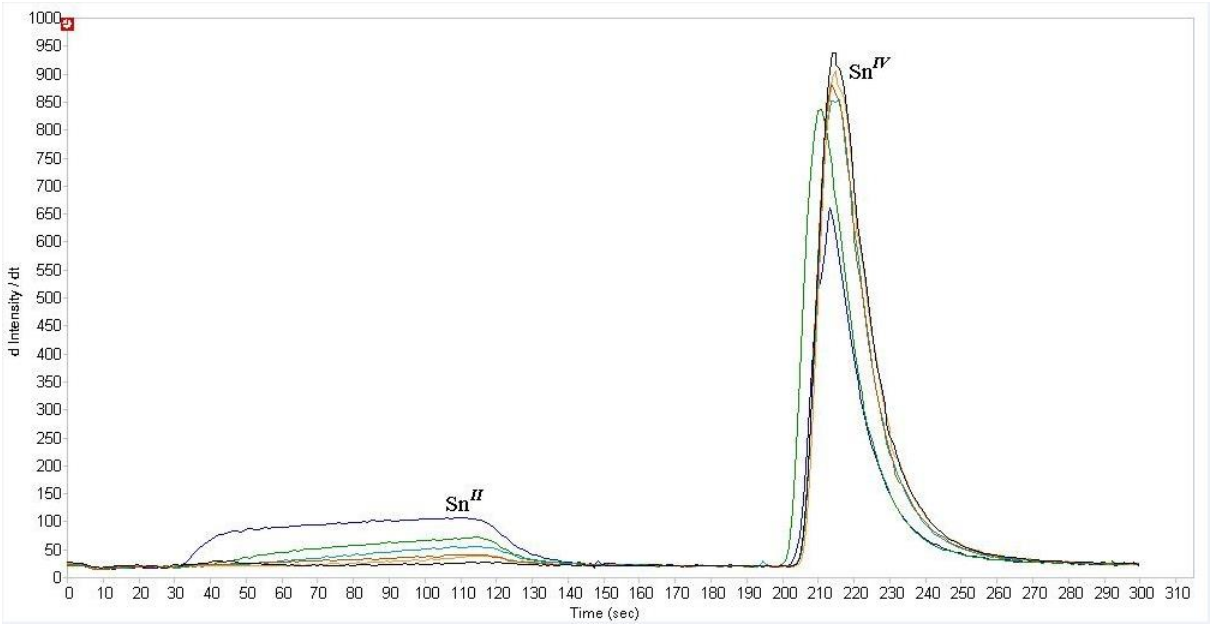

Figure 2 Time dependence of conversion Sn (II) on Sn (IV) – time scan model.

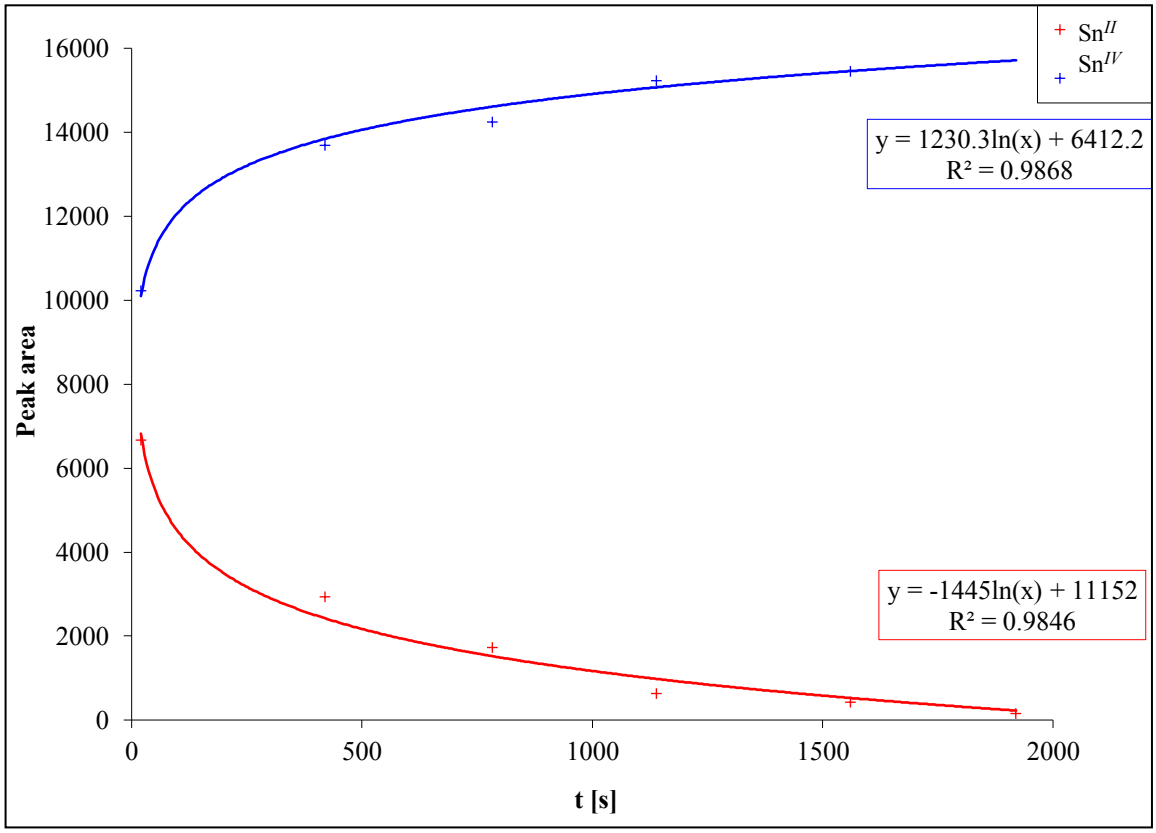

Figure 3 Logarithmical dependence of conversion of Sn (II) on Sn (IV).

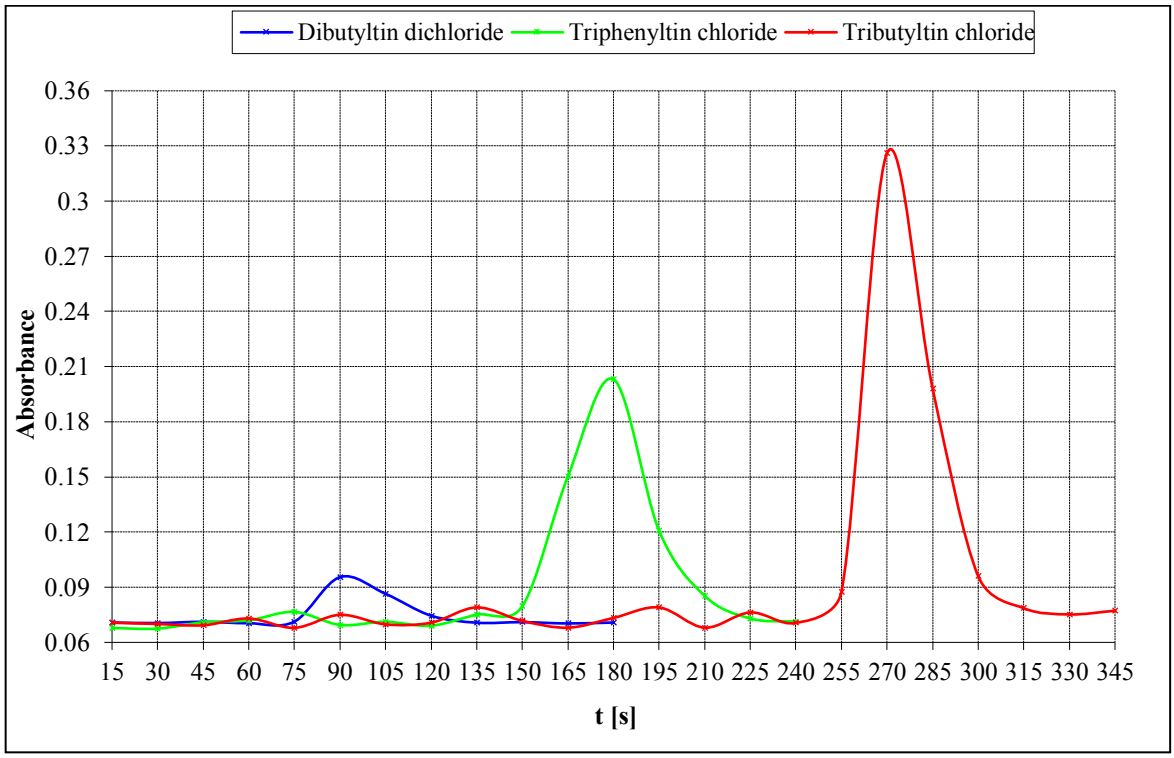

Figure 4 Dependence of absorbance on time for the separation of organotin compounds in the mixture by off-line ETA-AAS method.

CONCLUSION

In this work were simultaneously developed and tuned the three parts of the methodology on the determination of different forms of tin in food and beverages. The method for determination of total tin content in food materials by ICP-OES has been tuned. Increase the sensitivity of the method of ICP-OES was achieved using the techniques of generation of hydrides, which was also optimized. Hydride generation technique reduces the limit of detection, so it can be used for samples with low content of tin. This method has been applied to the analysis of real samples. Selected samples with low content of tin were drinks Coca Cola, Sprite, Fanta, Gambrinus 10°, PowerKing and picnic in the cans. Furthermore, Sardines in vegetable oil, and Hunter salami.

Simultaneously the method for separation of inorganic forms of tin was developed and at optimisation has been used low pressure ion exchange chromatography with on-line detection with ICP-OES. As appropriate ion exchangers shown cation exchangers Amberlite IRC 50, Cellulose CM 23, where elution reagent was 1 M solution of HCl and Trisacryl M CM, 50 CM-Sephadex, where elution reagent was 0.5 M NaOH solution. The best response was on Sephadex ion CM 50. The peak was detected during 40 seconds, and was sufficiently narrow and tall.

Similarly separation of organically bound tin was performed by HPLC on a column of ACE C-18 3 mm 15 cm × 1.0 mm with off-line detection by ETA AAS. Elution reagent was degassed mixture of acetonitrile-water-glacial acetic acid with 0.05% triethylamine (65: 23: 12) about pH 5. All of the above forms of tin can are with this column separated.

Since the performed analyses (inorganic forms of tin and organically bound tin) and information available on the occurrence of the forms of tin, it appears that many of these organometallic compounds are contained in marine animals, attention was necessary mainly focused on organisms such as marine fish, crustaceans, molluscs and algae. Based on these findings and the results of this work on the optimization of analytical procedures and the preparation of samples for analysis (homogenization, the selection of reagents and techniques for the extraction of individual forms, other modifications, such as pH adjustment, adding specific reagents, etc.) it will be possible to measure samples of the food and beverages by using this methodology that are created and optimized for the determination of the different forms of tin.

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BIOGENIC AMINES IN SMEAR RIPENED CHEESES

Olga Cwiková, Gabriela Franke

ABSTRACT

Cheeses belong to high protein foods in which enzymatic and microbial activities form amino acids, which are then converted into biogenic amines (BAs) by the activity of bacterial decarboxylases. The most important conditions for BA formation include the presence of microorganisms, the availability of substrate, temperature and storage period, water activity, salt concentration, and the hygiene of the manufacturing process. Tyramine, histamine, 2-phenylethylamine, tryptamine, cadaverine, putrescine, spermidine and spermine were detected in smear ripened cheeses stored in different temperature regimes. The highest (p <0.05) total BA content was found when storing the cheeses at the end of BBD (best before date) after 35 days in storage regime (A) or (C). During storage in regime (B), the total BA content (p <0.05) after 49 days of storage was higher than on the production date (B/0). During storage, the tyramine content in regime (B) did not change (p >0.05), while in the temperature regimes (A) and (C), the highest levels of tyramine and putrescine content were recorded in cheeses at the end of BBD after 35 days ripening. The content of polyamines in cheeses was higher (p <0.05) at the end of storage than at the beginning, in all temperature regimes.

Keywords: biogenic amines; polyamines; smear ripened cheese;storage temperature and period

INTRODUCTION

Cheeses are food that is often associated with the content of biogenic amines (Bas) **(Poveda, Molina and Gómez-Alonso, 2016)**. During ripening, substantial changes in the composition of cheeses take place **(Pinho et al., 2004)**. Protein degradation leads to accumulation of free amino acids, which are then converted into BAs by bacterial decarboxylases **(Komprda et al., 2007)**. Contaminant microorganisms such as enterococci, Enterobacteriaceae and lactic acid bacteria, such as lactobacilli **(Madejska, Michalski and Osek, 2017)**, contribute to the formation of BA.

Among the most important BAs found in foods are tyramine, histamine, cadaverine, 2-phenylethylamine, tryptamine, putrescine, spermine, and spermidine **(Önal, Tekkeli and Önal, 2013)**. Smear ripened cheeses have a higher BA content compared with other types of cheeses, which is related to the high protein content, extensive proteolysis, and highly active microbes with decarboxylase activity **(Samková, Dadáková and Pelikánová, 2013; Torracca et al., 2016)**.

Typically, these cheeses contain up to hundreds mg.kg^{-1} of histamine, tyramine, putrescine, and cadaverine, up to tens mg.kg^{-1} of 2-phenylethylamine as well as minor content of tryptamine **(Standarová et al., 2010)**. Cheeses also contain polyamines (Pas), such as agmatine, spermidine, and spermine **(Novella-Rodríguez et al., 2003)**.

The aim of this paper was to find out if the storage temperature and period could affect the BA content in smear ripened cheeses.

Scientific hypothesis

The formation of BAs in cheeses is affected by the storage temperature – the higher the temperature, the higher the BA content.

The length of storage of cheeses affects their safety in terms of high BA content.

MATERIAL AND METHODOLOGY

The cheese for testing was delivered as small rounds (diameter 45 mm, height 10 mm), where a 100 g package contained 5 pieces of these round portions. Prior to shipment, the cheeses ripened for 7 days, while the best before date (BBD) on the package was 28 days. A total of 5 batches of the product were analysed.

The samples were divided into three groups designated as (A), (B), and (C) and stored in different temperature regimes. Sampling and subsequent analyses were performed on the day of manufacture (A/0 = B/0 = C/0), at the end of BBD (A/35, B/35, C/35), two weeks after BBD (A/49, B/49), and eight weeks after BBD (C/91).

Samples in the temperature regime (A) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. These cheeses

were then stored for 14 days after the BBD expired at 6 °C, i.e. 49 days in total (6 °C/35 days → 6 °C /14 days). Samples in the temperature regime (B) were stored at 6 °C after production for 28 days (7 days before and 21 days after shipment), i.e. 28 days, then they were frozen and stored at -18 °C for 7 days. Subsequently, after BBD (35 days), the cheeses were stored at 6 °C for 14 days, i.e. 49 days in total (6 °C/28 days → -18 °C/7 days → 6 °C/14 days). Samples in the temperature regime (C) were stored at 6 °C after production for the entire BBD (7 days before and 28 days after shipment), i.e. 35 days. They were then frozen and stored for 49 days (7 weeks) at -18 °C. After 49 days of freezing, the cheeses were stored at 6 °C for 7 days, i.e. 91 days in total (6 °C/35 days → -18 °C/49 days → 6 °C/7 days).

The analyses were carried out in the laboratory of the Institute of Food Technology, Faculty of AgriSciences of Mendel University in Brno.

BA Assay: A cheese sample was grated to a particle size of about 3 mm and 10 g was weighed into an 85 mL plastic centrifuge tube. After addition of 20 mL of 0.1 M HCl and 0.5 mL solution of internal standard (1,7-diaminoheptane) with concentration of 1 $mg.mL^{-1}$, the sample was extracted with a disintegrant for 2 minutes. The suspension was centrifuged at 755 g for 10 minutes at 4 °C in order to separate the solid and fat. The supernatant was filtered and the solid was re-extracted by the same procedure. The combined extracts were added up to 50 mL with deionized water and filtered through a nylon membrane filter. The BA derivatization was carried out with dansyl chloride, where 1 mL of extract or standard was mixed with 0.5 mL of saturated Na_2CO_3 (pH adjusted to 11.2). In a 4 mL sample vial, 1 mL of derivatizing agent (5 mg of dansyl chloride in 1 mL of acetone) was added and stirred for 1 minute on an agitator. Derivatization was carried out for 1 hour at 40 °C without light access with occasional shaking at 15 minutes intervals. After derivatization, 250 µL of 10 mM ammonia was added to remove unreacted dansyl chloride and again stirred for 1 minute on the agitator. Ammonia reacted with excess dansyl chloride and the resulting reaction product was eluted before BA. Containers with dansyl chloride acetone solution and all standards and extracts were immediately after adding packaged in aluminium foil because of their photolability. After a 30-minute reaction, the hydrophobic amine derivatives were extracted with diethyl ether (3 x 1 mL) while the hydrophilic amino acid derivatives remained in the aqueous phase. The organic phase was evaporated to dry state with a stream of nitrogen and the evaporation residue was dissolved in 0.5 mL ACN or in 1 mL ACN (standard) and the solution was again filtered through a 0.45 µm nylon membrane filter and dosed on a chromatographic column. The amount of the injected real sample was modified as needed, in the case of standard it was 10 µL.

Biogenic amines were separated on a Zorbax Eclipse XDB C18 column (150 mm x 4.6 mm, particle size 5 µm) with Meta Guard ODS 2 pre-column (30 mm x 4.6 mm, particle size 5 µm) at a flow rate of 0.8 $mL.min^{-1}$ using the HP 1100 Chromatograph. Separation after derivatization with dansyl chloride was carried out by gradient elution with H_2O/ACN (time 0 – 23 minutes: H_2O 35 – 0%, ACN 65 – 100%) followed by detection with a photometric UV/VIS detector at 254 nm. To identify the separated substances in the samples, a comparison of the retention times of the standards and the substances present in the sample was used. During the analysis, the UV spectra of the eluates were captured at peak and then compared with the spectra of the standard substances, and the identity of the substances was confirmed or refuted on the basis of the so-called identity factor.

All BA standards were supplied as hydrochlorides and their concentrations after derivatization with dansyl chloride were expressed in $mg.kg^{-1}$ of the original (fresh) sample (not per kg of dry weight) to better express the conditions at consumption. The BA concentration in a sample was corrected by the internal standard method. The internal standard was prepared by dissolving 100 mg of 1,7-diaminoheptane in 100 mL deionized water (concentration 1 $mg.mL^{-1}$).

The BA/PA standards stock solution was prepared as a mixed standard for all amines to be analyses (tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) by dissolving 100 mg of each of the amines (in the form of hydrochloride) in 100 mL of deionized water (standard concentration of each amine 1 $mg.mL^{-1}$). The BA/PA standard working solution was obtained by mixing 0.5 mL BA/PA standard stock solution with 0.5 mL internal standard solution and subsequent treatment to a volume of 50 mL. The final concentration of each amine was 10 $\mu g.mL^{-1}$. The 0.1 M HCl prepared by dissolving 3.5 mL of 35% HCl in deionized water and filled to 1 litre was used as the extraction agent. The derivatizing agent was prepared by dissolving 5 mg of dansyl chloride in 1 mL of propan-2-one.

The following biogenic amines were determined: tyramine, histamine, 2-phenylethylamine, tryptamine, cadaverine, putrescine, spermidine, and spermine. The amine content of each sample was measured in duplicate.

Statistical analysis

Statistical evaluation was performed in the Statistica Statsoft programme, version 12, and Microsoft Excel 2010. Basic statistical characteristics, such as mean and standard deviation of the mean were calculated.

In order to compare the BA content during the storage period within the given temperature regime, a simple scattering analysis method (ANOVA) including the Duncan post-hoc test was used. Normality was tested by the Shapiro-Wilk test. The proportion of factors (temperature and storage period including interactions) on the total variability of BA content in cheese was calculated using the general linear model (ANOVA with interactions).

RESULTS AND DISCUSSION

At the beginning of storage (A/0, B/0, C/0) the total content of the monitored BA was 64.9 $mg.kg^{-1}$ of cheese (Figure 1). During ripening in storage in the temperature regimes (A) and (C), the total BA content first increased (p <0.05) to 259.7 $mg.kg^{-1}$. Then it decreased (p >0.05) to 190.4 $mg.kg^{-1}$ in regime (A) and to 140.7 $mg.kg^{-1}$ in regime (C).

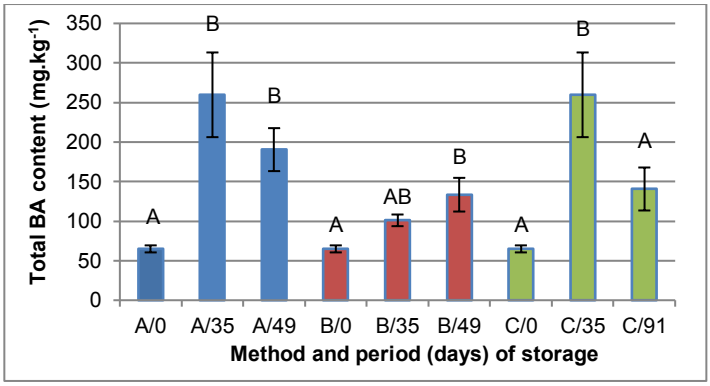

Figure 1 Comparison of the total content of biogenic amines (mg.kg^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed on the production date (0), at the end of the best before date after 35 days of ripening (A/35: storage at 6 °C/35 days, B/35: Storage at 6 °C/28 days and at -18 °C/7 days, C/35: storage at 6 °C/35 days) and at the end of storage after 49 or 91 days of ripening (A: storage at 6 °C/35 days and at 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/7 days and at 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6 °C/7 days). Averages marked with different letters are statistically different within a given factor (storage period) (p <0.05); n = 15.

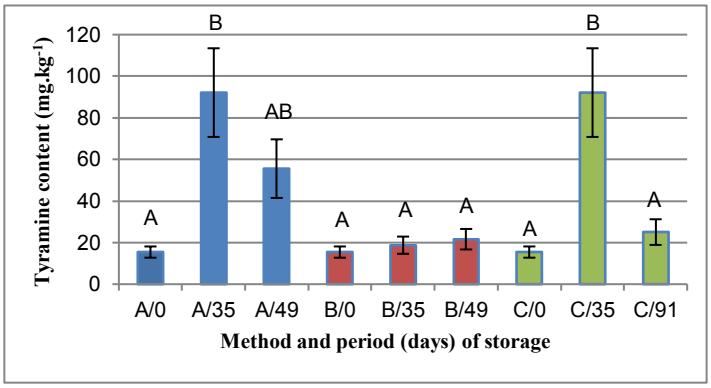

Figure 2 Comparison of tyramine content (mg.kg^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed on production day (0), at the end of the best before date after 35 days of ripening (A/35: storage at 6 °C/35 days, B/35: storage at 6 °C/28 days and at -18 °C /7 days, C/35: storage at 6 °C/35 days) and at the end of storage after 49 or 91 days of ripening (A: storage at 6 °C/35 days and 6 °C/14 days, B: storage at 6 °C/28 days and at -18 °C/7 days and 6 °C/14 days, C: storage at 6 °C/35 days and at -18 °C/49 days and at 6° C/7 days). Averages marked with different letters are statistically different within a given factor (storage period) (p <0.05); n = 15.

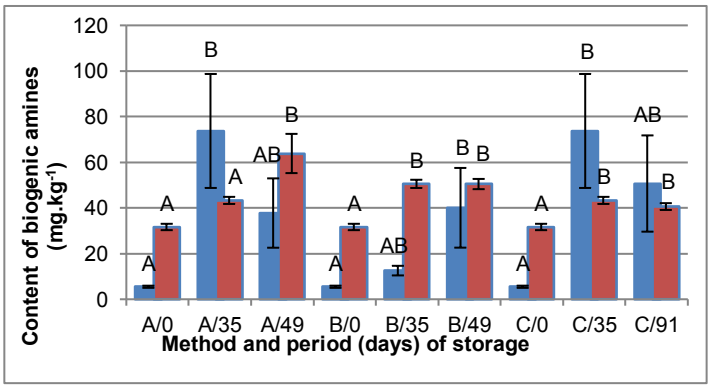

Figure 3 Comparison of putrescine and polyamine content (mg.kg^{-1}) in smear ripened cheeses stored in different temperature regimes (A, B, C) and analysed on production day (0) at the end of the minimum shelf life after 35 days of ripening (A/35: storage at 6 °C/35 days, B/35: storage at 6 °C/28 days and at -18 °C/7 days, C/35: storage at 6 °C/35 days) and at the end of storage after 49 or 91 days of ripening (A: storage at 6 °C/35 days and 6 °C /14 days, B: storage at 6 °C/28 days and at -18 °C/7 days and 6 °C/14 days, C: storage at 6 °C/35 days and at -18° C/49 days and 6 °C/7 days). Averages marked with different letters are statistically different within a given factor (storage period) (p <0.05); n = 15. Putrescine is marked blue, polyamines spermidine and spermine red.

The reduction in the total BA content of cheeses stored in temperature regime (C) was statistically significant (p <0.05); lower total BA content was recorded at the end of storage (C/91) than at the end of BBD (C/35). When stored in regime (B), the total BA content was higher (p <0.05) at the end of ripening (B/49) than on production date (B/0). The highest total BA value was recorded during storage in regime (A) after 35 days of ripening, namely 571.4 mg.kg^{-1} of cheese.

Higher total BA content compared to ours was detected in smear ripened cheeses by **Pleva et al. (2014)**, namely up to 1,000 mg.kg^{-1}. In 5 samples they show even higher values (1,000 – 6,000 mg.kg^{-1} of cheese). They ascribe the increased BA content to incorrect storage of cheese at stores. According to **Komprda et al. (2012)**, the impact of storage temperature on the variability of BA and PA content is 46% on average. The storage temperature has been reported by **Komprda et al. (2012)** for BA and PA variability has an average effect of 46%. Higher total BA content, as compared with our data, was also found by **Samková, Dadáková and Pelikánová (2013)** when cheeses were stored at 5 °C, namely 514 mg.kg^{-1}. After an additional two weeks of storage, the BA content further increased to 660 mg.kg^{-1}, with the highest recorded value of BA being 2,076 mg.kg^{-1} of cheese. **Standarová et al. (2010)** for the same type of cheese, and at the same conditions at the end of BBD, detected 1,500 mg.kg^{-1} BA; **Dičáková and Dudriková (2007)** 2,477 mg.kg^{-1}; **Rejchrtová (2015)** 593 mg.kg^{-1} and 66 days after the production 1,218 mg.kg^{-1}, which are much higher values than our results. According to **Standarová et al. (2009)**, there are also differences in BA content depending on the month of cheese production.

During storage, in temperature regimes (A) and (C) the overall BA content decreased. The reduction in the overall BA content between the 6th and 7th week of storage was reported by **Standarová et al. (2010)** for cheeses stored at 5 °C. In contrast, no significant changes in the overall BA content of long-frozen (-18 °C) cheeses were found by **Andiç et al. (2010)**, and they explain this finding by slowing down or cessation of microorganism activity. In our experiment, there was apparently for these reasons a reduction (p <0.05) in the overall content of the monitored BAs in regime (C).

Legislative limits on BA content in cheeses are currently not given. **Spanier, Bruin and Van Roode (1991)** state the content of histamine + tyramine + putrescine + cadaverine to be 900 mg.kg^{-1} of cheese on maximum. This value was not exceeded in our experiment.

During ripening, 8 biogenic amines were monitored. As for tryptamine, it was not detected in cheese at the beginning of storage at all. Its content was found in cheese only after 35 days of ripening in regimes (A) and (C), averaging 2.7 mg.kg^{-1}. Under conditions of storage regime (B), tryptamine was not detected in cheeses at all. The same results were obtained by **Standarová, Borkovcová and Vorlová (2008)** when analysing 215 samples of cheeses consumed in the Czech Republic. Tryptamine content in these cheeses was low or tryptamine was not detected at all. Low, up to 5.9 mg.kg^{-1}, or undetectable amounts of tryptamine were also found by **Standarová et al. (2009),** however in the Niva cheese taken from the Czech distribution network. **Samková, Dadáková and Pelikánová (2013)** found at the end of BBD of the same type of cheese tryptamine at 5.3 mg.kg^{-1}, after two more weeks of storage, the content of 5 mg.kg^{-1}, which is more compared to our results. **Pleva et al. (2014)** just like us did not detect any tryptamine in smear ripened cheeses.

According to **Novella-Rodrigues et al. (2003)** and **Buňková et al. (2010)** cadaverine along with tyramine and putrescine belong among the most important BAs in ripened cheeses. As in our experiment, these authors recorded the highest cadaverine values at the end of BBD after 35 days of storage. However, the values we found (26.9 mg.kg^{-1}) were more than ten times lower than those of the authors above. Higher cadaverine contents in cheese compared to our results (up to 2,400 mg.kg^{-1}) were reported by **Pleva et al. (2014)** and **Standarová et al. (2010)** after 28 days of storage at 5 °C (>400 mg.kg^{-1}). Also, **Samková, Dadáková and Pelikánová (2013)** report, compared to us, a higher cadaverine content of 176 mg.kg^{-1} at the end of BBD and 249 mg.kg^{-1} two weeks after the end of BB with the same type of cheese.

As for histamine, the highest values in our experiment were detected at the end of BBD during storage at 6 °C, with an average content of 17.5 mg.kg^{-1} of cheese and the highest recorded value of 49.5 mg.kg^{-1}. **Pleva et al. (2014)** report higher histamine content (373.8 mg.kg^{-1}) compared to ours for the same type of cheese. **Mayer, Fiechter and Fischer (2010), Standarová et al. (2010),** and **Loizzo et al. (2013)** also reported higher histamine content in smear ripened cheeses ranging between 168.3 mg.kg^{-1} and 500 mg.kg^{-1}. **Rejchrtová (2015)** also detected higher histamine levels at the end of BBD compared to our results to be 110 mg.kg^{-1} of cheese and after 66 days of manufacture to be 142 mg.kg^{-1}. **Samková, Dadáková and Pelikánová (2013)** found at the end of BBD when stored at 5°C the histamine content of 51.5 mg.kg^{-1} of cheese and after two more weeks of storage 55.3 mg.kg^{-1}, which is also higher than our results. Decrease in the histamine content (similar to our experiment) between 30th and 60th day of ripening was reported by **Martuscelli et al. (2005)** in the Pecorino Abruzzese cheese. **Dalgaard et al. (2006)** report that short-term freezing had a significant effect on the subsequent decrease in histamine content (in muscle tissue of sea pike). This corresponds to our results when, in regime (B) at the end of BBD, the average histamine content was 8.0 mg.kg^{-1} of cheese and in regime (A) 17.5 mg.kg^{-1} of cheese. **Standarov, Borkovcová and Vorlová (2008)** report that the histamine content of 100 mg.kg^{-1} of cheese may already induce intoxication in human organisms. However, this level of histamine content was not recorded in our experiment.

Cheeses typically contain up to tens of mg.kg^{-1} of 2-phenylethylamine. In our experiment, at the end of BBD in cheeses stored at 6 °C (regime A), we have detected 5.4 mg.kg^{-1} of 2-phenylethylamine and two weeks after BBD 1.4 mg.kg^{-1}. **Pleva et al. (2014)** reported higher content of 2-phenylethylamine in the same type of cheese than in our experiment, namely 11.6 mg.kg^{-1}. In contrast, **Samková, Dadáková and Pelikánová, (2013)** detected at the end of BBD in smear ripened cheeses a content of 2-phenylethylamine lower than ours**,** namely 4.3 mg.kg^{-1}. However, after two more weeks of storage compared to our data, the content of 2-phenylethylamine was higher (2.3 mg.kg^{-1}). **Standarová, Borkovcová and Vorlová**

(2008) reported that migraine can already be induced by 30 mg.kg^{-1} of 2-phenylethylamine. This value was not recorded in our experiment.

Tyramine

At the beginning of storage (A/0, B/0, C/0), the tyramine content in cheeses was 15.4 mg.kg^{-1}. During the ripening process (Figure 2), in storage in temperature regimes (A) and (C) there was first an increase (p <0.05) of the tyramine content to 92.1 mg.kg^{-1} of cheese, at the end of BBD after 35 days. At the end of storage in regime (A), lower values were recorded, namely 55.6 mg.kg^{-1}. In regime (C) the decrease (p <0.05) of tyramine content was more pronounced, namely up to 25.1 mg.kg^{-1} of cheese. During storage in regime (B), the tyramine content did not change in the course of ripening of the cheese (p >0.05). The highest value of tyramine was recorded in our experiment after 35 days of ripening in regime (A), namely 291.7 mg.kg^{-1} of cheese. The upper limit of tyramine content in food, according to **Halász et al. (1994)**, **Shalaby (1996)**, **Silla Santos (1996)** and **Eerola et al. (1997)** ranges from 100 to 800 mg.kg^{-1} of food. According to **Latorre-Moratalla et al. (2008)**, people taking MAO inhibitors tolerate 50 – 100 mg tyramine, however problems can even be caused by 6 mg.kg^{-1} **(Novella-Rodríguez et al., 2003)**. In samples stored at 6 °C (regime A), a "safe limit" of 100 mg.kg^{-1} was exceeded in 5 out of 15 analysed samples at the end of BBD. Tyramine content >100 mg.kg^{-1} was not detected during storage in temperature regime (B) for any cheese.

In the same type of cheese, **Standarová et al. (2010)** detected the highest tyramine concentration between the 5th and 6th week of storage at 5 °C, which corresponds to our results (A/35). **Rejchrtová (2015)** reported at the end of BBD in the same type of cheese at 5 °C higher tyramine content compared to ours (>800 mg.kg^{-1}). **Samková, Dadáková and Pelikánová (2013)** likewise recorded a slightly higher average tyramine values than in our experiment at 5 °C at the end of BBD, namely 140 mg.kg^{-1}. After two more weeks of storage the value was 163 mg.kg^{-1}, while the highest tyramine reading after extended storage in their experiment was 469 mg.kg^{-1} of cheese.

In our experiment, the tyramine content as well as histamine and total BA content decreased between the 5th and 7th week of storage (A/49). According to **Leuschner et al. (1998)**, one possible explanation can be the fact that some microorganisms have the ability to degrade tyramine and histamine. In testing 32 strains of *B. linens* and coryneform bacteria, it was found that 21 of them showed histamine or tyramine oxidase activity. These authors further state that *Brevibacterium linens* (LTH 456 and LTH 3686) were able to reduce the tyramine and histamine content for Munster smear ripened cheese by 55 to 70%. According to **Bergey and Holt (1994)**, **Leuschner, Heidel and Hammes (1998)** and **Dasu et al. (2006)**, also *Pseudomonas* sp., *Seratia marcescens, Kocuria varians,* and other bacteria can decompose the created BA. **Komprda et al. (2007)** found an increasing content of tyramine with time in a Dutch type cheese from two different manufacturers. **Standarová et al. (2009)** reported similar results for cheese with blue mould in dough and **Martuscelli et al. (2005)** for Pecorino Abruzzese cheese. The latter authors observed the most significant increase in tyramine content between the 14th and 30th day of ripening, which corresponds to our results.

Putrescine

At the beginning of storage (A/0, B/0, C/0), the putrescine content in the cheeses was 5.5 mg.kg^{-1}. During ripening (Figure 3), when stored in temperature regime (A) and (C), the content of putrescine in cheeses first increased to 73.7 mg.kg^{-1} (p <0.05). After expiration of BBD and further storage, putrescine content values in cheeses were lower, namely 37.8 mg.kg^{-1} in regime (A) and 50.7 mg.kg^{-1} in regime (C). In temperature regime (B), putrescine content increased (p <0.05) during storage, when after 49 days of storage (B/49) a higher putrescine content than on production date (B/0) was recorded.

Higher content of putrescine, compared to our data, was found by **Pleva et al. (2014),** when putrescine in smear ripened cheeses was detected at >2,000 mg.kg^{-1}. **Standarová et al., (2010)** also reported that the putrescine content was higher, when after 28 days of ripening at 5 °C its content was 212 mg.kg^{-1} in the same type of cheese. In our experiment, too, the highest average putrescine content was found after 35 days of ripening at 6 °C. However, the average content was lower at 73.7 mg.kg^{-1}, while the highest recorded value was 320 mg.kg^{-1} of cheese. **Samková, Dadáková and Pelikánová (2013)** detected a higher average putrescine content of 104 mg.kg^{-1} in smear ripened cheeses at the end of BBD when stored at 5 °C. After two more weeks of storage, the putrescine content increased even more to 137 mg.kg^{-1}.

In our experiment, however, the putrescin content decreased (p >0.05) after a further 2 weeks of storage at 6 °C (A/49). In contrast, in regime (B) the putrescine content of cheeses increased, when at the end of storage (B/49) a higher (p <0.05) content of putrescine than at the production date (B/0) was detected. An increase in the putrescine content during storage (of tuna) at low temperatures (-18 °C) was also reported by **Ben-Gigirey et al. (1998)**. However, during long-term freezing (regime C), there was a decrease in the putrescine content in cheeses, which is probably related to the decrease of enzymatic activity of microorganisms **(Andiç et al., 2010)**.

Polyamines

As regards PAs (spermidine and spermine), their content in cheeses was 31.7 mg.kg^{-1} at the beginning of storage (A/0, B/0, C/0). During storage, the PA (p <0.05) content has increased in all temperature regimes. At the end of the storage period, the cheeses had a higher (p <0.05) PA content than on the day of production (Figure 3).

At present, PAs and their precursor putrescine are classified differently from the BA group. PAs may originate in a different metabolic pathway and are characterized by different biological functions. **Pleva et al. (2014)** show higher PA values compared to ours (A/35) for the same type of cheese, namely 55.8 mg.kg^{-1} on average. In contrast, **Samková, Dadáková and Pelikánová (2013)** measured lower values of PAs at the end of BBD compared to ours, namely 33 mg.kg^{-1} for cheeses stored at 5 °C. After two more weeks of storage, they found a PA

content of 48.2 mg.kg^{-1} of cheese, which is also less compared to our values (A/49).

CONCLUSION

Our hypothesis regarding the effect of temperature on BA formation in cheeses was confirmed. Their content was higher at 6 °C than when stored at freezing temperatures. Freezing does not completely deactivate the enzymes, as enzyme reactions are slowly taking place even at freezing temperatures. However, it is important to keep in mind that low temperatures recommended for food storage were used in our experiment.

The highest (p <0.05) total BA content was found when storing the cheeses at the end of BBD (best before date) after 35 days (259.7 mg.kg^{-1}) in storage regime (A) or (C). During storage in regime (B), the total BA content (p <0.05) after 49 days of storage was higher than on the production date (B/0). During storage, the tyramine content in regime (B) did not change (p >0.05), while in the temperature regimes (A) and (C), the highest levels of tyramine and putrescine content were recorded in cheeses at the end of BBD after 35 days ripening (92.1 mg.kg^{-1}, resp. 73.7 mg.kg^{-1}). The content of polyamines in cheeses was higher (p <0.05) at the end of storage: 63.9 mg.kg^{-1} in storage regime (A), resp. 50.5 mg.kg^{-1} in storage regime (B) and 40.6 mg.kg^{-1} in storage regime (C), than at the beginning (31.7 mg.kg^{-1}).

Analyses performed (ANOVA with interactions) show that greater effect on the content of BAs (tyramine, putrescine) and PAs had the period of storage rather than the method of storage (temperature regime).

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PREFERENCE MAPPING OF DIFFERENT VARIETIES OF GARLIC (*ALLIUM SATIVUM*)

Zuzana Drdolová, Patrícia Martišová, Lucia Benešová

ABSTRACT

In this work we evaluated different varieties of garlic. All varieties of garlic are rated from one harvest year. Compared samples were different in shape, taste, aroma and characteristics, which were likely to impact on consumer choice and deciding on purchases garlic. Selected indicators were part of internal sensory evaluations, which were evaluated by experts in the sensory laboratory. External part of the preference mapping was conducted among consumers relying on different varieties of garlic under their consumption. Using the internal part of the preferential mapping we summarize randomly selected characteristics within the textural properties and characteristics of taste in which we consider the possible impact on consumer´s choice. In the sensory evaluation assessors used 9 point scale to evaluate 15 selected properties across the texture, taste and aroma on 10 selected varieties according to the degree of preference. Garlic odour and textural properties were evaluated by the normal procedure, though the taste because of intense lingering aftertaste of has been evaluated in a prepared mixture after cooking. For external evaluation, we designed a questionnaire in which consumers can express their preference for individual samples based on photo and variety characteristics, using a hedonic scale from 1 to 9. Obtained data from sensory evaluation and a questionnaire survey were evaluated using statistical software XLSTAT. Preferential map summarise results from internal and external evaluation. We identified characteristics affecting the degree of consumer preferences according to the visualization of our results.

Keywords: garlic; preference mapping; aroma; taste

INTRODUCTION

Preferential mapping is a way to statistically combine analytical sensory results, consumer information and product perception. Using analytical sensory data and consumer technology, it is possible to obtain a complete product image **(Risvik, McEwan and Rødbotten, 1997)**. Preferential mapping uses a set of statistical methods aimed at detecting consumer preferences of the compared products using sensory profiles. This method is used in the food industry to develop new products, especially according to consumer requirements **(Meullenet, Xiong and Findla, 2007)**. Preferential mapping was used in previous studies of fresh fruit, including raspberries **(Villamor et al., 2013)**, apples **(Jaeger et al., 1998)**, strawberries **(Lado et al., 2010)** and tomatoes **(Sinesio et al., 2010)** to confirm product attributes on the basis of which the consumer decides. These studies focused on taste intensity, texture and appearance/colour, which are important for consumer acceptance of the product **(Oltman, Yates and Drake, 2016)**. The perception of the sensory characteristics (appearance, colour, taste, smell, texture), which determine the so-called "organoleptic characteristics", is essential to create consumer perceptions about food quality. Based on the outputs of the sensory profiles, it is possible to subtract the information obtained from the analyses **(Vietoris et al., 2014)**. External factors (size, colour, appearance) of tomatoes have been shown to be important to consumers as well as taste **(Hetherington and MacDougall, 1992; Pagliarini, Monteleone and Ratti, 2001)**. The texture also plays a major role in consumer perception of quality and taste **(Causse et al., 2003; Aurand et al., 2012)**. Taste is the biggest indicator of quality for consumers **(Aurand et al., 2012)**, but texture and appearance are crucial for fresh fruits and vegetables purchased on the basis of external characteristics where taste or taste cannot be directly assessed. By texturing is mean all mechanical, geometrical and surface properties of the products, perceptible by mechanical, tactile or auditory and visual receptors **(Dimitreli and Thomareis, 2007)**. **Oltman, Jervis and Drake (2014)** have recently demonstrated with target groups and pooled research that appearance and strength were the main drivers of buying fresh tomatoes.

Garlic (*Allium sativum* L.) is one of the oldest cultural plants used both for food and for medical use. It is a rich source of several phyto-nutrients recognized as important elements of the Mediterranean diet but is also used in the treatment and prevention of many diseases. These effects are associated with thiosulfinates and volatile sulphur compounds, which are also responsible for the distinctive, pungent odour and taste of this vegetable **(Lanzotti, 2006)**. Various varieties of garlic are available on the market, which differ from each other in their properties, size, shape, colour and taste. By determining the odour and taste components more closely, differences were found between examined garlic varieties. Their chemical composition and variety also suggest various changes in the use or changing properties after adding to the product **(Calle, 2016)**.

Scientific hypothesis

The aim of the study was to determine consumer preferences to selected varieties of garlic. By selecting different varieties, we wanted to highlight the diversity of garlic offered on market and identify the properties that are decisive for the consumer.

MATERIAL AND METHODOLOGY

We evaluated ten samples (Figure 1) of winter garlic varieties obtained from one grower harvested in one harvest year stored one month under the same storage conditions and temperature regime. Groups of thirty assessors familiar with evaluation methodologies have compared the shape, size, colour, appearance to cut, hardness, odour intensity, spicy, sweet, sour and earthy odour, authentic taste, spicy, sweet, sour, and earthy taste. We used two types of scale, an intensity scale to assess the intensity of a certain property, and a hedonic scale to assess the degree of acceptability. The assessors wrote their perceptions on a pre-prepared paper form.

Apples were used as a neutralizer for odour determination. We have included the odour neutralizer in the review due to the intense sensory burden of the evaluators that we have been alerted to during the trial evaluation. Similarly, due to the extreme sensory load and the long-lasting aftertaste when using a raw garlic sample, we chose to evaluate the taste properties of the mixture. The prepared appetite blend comprised: 250 g of chicken, 15 extruded garlic, 2 g of salt, 1 g of pepper and 2 g of red pepper. The mixture was heat treated. Garlic was added to the mixture 2 minutes before the end of the heat treatment. The external part of the evaluation was done by using a questionnaire. The questionnaire included a description of the characteristics of the varieties with the attached photographs. The evaluators had the opportunity to express their preferences on a 9 point hedonic scale. 150 respondents were involved in the evaluation.

Statistic analysis

The data obtained from sensory evaluation and questionnaire survey, were evaluated using the XLSTAT statistic software (v. 2019.1.2, Addinsoft). Internal data sets from the sensory analysis were evaluated by principal component analysis (PCA). External data sets were evaluated by agglomerative hierarchical clustering (AHC). By combining these data set we have obtained preference map.

RESULTS AND DISCUSSION

From the PCA (Figure 2), we can observe that four groups of samples have been specified. Samples 1, 5, 9, 7, 3 and 8 show similarity in all of the odour, texture, and taste characteristics. Sample 10 obtained a higher rating compared to the other samples when evaluating the sour smell of odour intensity, colour, and sour taste. Samples 6 and 4 had similar ratings.

Figure 1 Samples of winter garlic varieties.

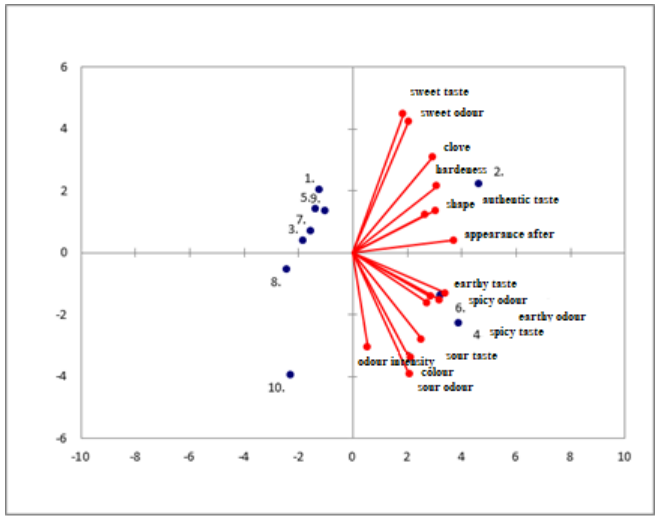

Figure 2 Evaluation using principal component analysis (PCA).

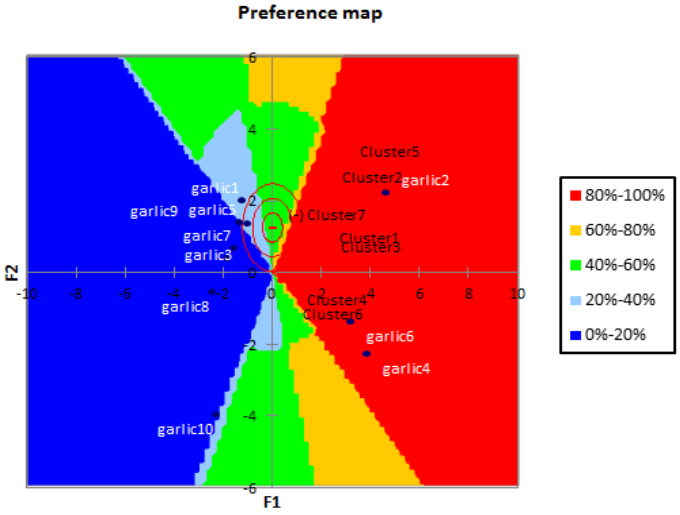

Figure 3 Preference map.

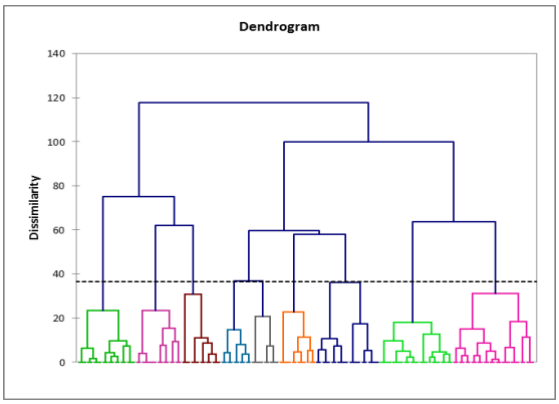

Figure 4 Agglomerative hierarchical clustering.

High values have been observed in the evaluation of the earthy taste, spicy odour, earthy odour and colour. In general, these samples, along with sample 2, had high ratings in all selected properties. Sample 2 recorded slightly lower ratings when assessing the odour intensity, spicy, sour odour and colour. **Drdolová, Golian and Vietoris (2015)** analysed the chemical composition of garlic by gas-chromatographic analysis (GC-MS) of an Agilent 6890N gas chromatograph coupled with Agilent 5973 inert mass spectrometry detector. Differences in the chemical representation of individual varieties of garlic have been noted in the intermodality. Significant differences in relative abundance have been reported for dialyl disulfide, methyl-allyl-thioacetate, and allyl-methyl-

disulfide, which are important compounds in terms of flavor formation and garlic odour. In assessing the rate of representation of these compounds, a varying proportion of the identified compounds has been demonstrated in the cross-industry comparison.

By processing the survey data for the 150 respondents (Figure 3), we reached the following results. In the highest consumer preference zone (80 – 100%), a sample of 2, 4 and 6 was placed on the basis of the specific characteristics of the products included in the survey. These samples, based on the analysis of PCA showed the following characteristics: shape, size, colour, appearance after cutting, hardness, odour intensity, spicy, sweet, sour, earthy odour, authentic taste, spicy, sweet, sour and earthy taste. The lower scoring was observed for sample 2 when evaluating colour and odour intensity. Sample 4 reported a lower rating for the sweet odour and sweet taste. Sample 6 did not report a low score in either of the endpoints, however, it received moderate evaluations in assessing hardness and spicy taste.

Samples placed in the lower consumer preference zone 5, 9 and 1 received average ratings in assessing the shape, size, appearance after cutting, hardness, sweet and earthy odour, sweet and earthy taste. Low ratings have received these samples in evaluating colour, odour intensity, spicy and sour odour, authentic taste, spicy and sour taste. Samples 3, 7, 8, and 10 were placed in the lowest consumer preference zone (0 – 20%). These samples were evaluated based on a PCA of average rating for colour, sour and earthy odour, authentic taste, spicy and sour taste. Samples 3, 7, 8, and 10 received a low rating in assessing the shape, size, appearance after cutting, hardness, sweet and earthy taste. In consumer studies on tomatoes, the consumer's emphasis on commodity appearance was more pronounced than the taste of subsequent tasting **(Sinesio et al., 2010)**.

In a similar study comparing the importance of external and internal properties of tomatoes on the market, the colour was the most significant attribute, followed by size and juiciness (evaluated after slicing), followed by strength after hand pressure, the taste itself was much less important **(Oltman et al., 2014)**. The current results from tomato evaluation confirm the importance of tomato taste and aroma, which is a key attribute for the variety modification progress. However, during the evaluation of appearance, colour, colour intensity, and size for evaluation, the assessors proceeded as in previous tomato market studies **(Oltman et al., 2014)**.

Eight clusters (Figure 4) were selected for data analysis. By a more detailed analysis of the clusters, it was found that the vast majority of the respondents, who were involved in the research, focused on garlic-based textural properties, especially shape, colour, size and hardness. The observations also show that the basic indicative characteristics of the evaluation are the sweet and spicy taste and smell as well as the degree of acidic and earthy taste and aroma in evaluating the taste and odour characteristics of garlic. **Oltman et al. (2016)** in his study distinguished consumer clusters according to the specific taste of tomatoes, which correlated with colour, taste/aroma and textural properties. Clusters consisting of assessors who consume tomatoes often submitted samples and their properties as more differentiated and concretized their evaluated properties.

Consumers focus on textural features when buying **(Causse et al., 2003)**. Another study identified a group of tomato consumers where the external strength assessed by touch was as important as the sliceability and compliance of tomatoes in the mouth. High strength was characterized in the best rated varieties in the tomato study, which was aimed at analyzing variable physico-chemical and sensory parameters and their role in the perception of tomato taste **(Piombino et al., 2012)**.

Kitchen garlic is a plant which is very often used in traditional and modern gastronomy. It is part of many foods and food products. Garlic with its distinctive taste and odour characteristics is a diversification of every meal. There are many varieties of garlic in the world that differ from those native to garlic, which are significant in addition to the taste and smell indications of their broad-spectrum positive health effects. Evaluators of garlic are very demanding, and it is necessary to select a sensitive methodology to achieve the desired result. Especially the intense smell and the long-lasting aftertaste of garlic, which prevents the ingestion of multiple samples in a row in the raw state, is a complication in sensory analysis. For this reason, we evaluated the scent neutralizer and the taste evaluation in the mixture after the heat treatment to evaluate the flavour intensity and its overwhelming after ingestion. By processing the data, we found that the evaluators in the internal evaluation section, as well as the respondents involved in the external part of the preference mapping, were key features for decision-making colour, hardness, size and appearance after cutting, and thus predominantly textural features of the evaluated plant commodity. In the highest consumer preference zone (80 – 100%), samples 2, 4 and 6 was placed on the basis of the specific characteristics of the products included in the survey. These samples, based on the analysis of the PCA of the evaluation of the characteristics examined, showed the following characteristics: shape, size, colour, appearance after cutting, hardness, odour intensity, spicy, sweet, sour and earthy odour, authentic taste, spicy, sweet, sour and earthy taste. The lower scoring was observed for sample 2 when evaluating colour and odour intensity. Sample 4 reported a lower rating for the sweet odour and sweet taste. Sample 6 did not report a low score in either of the endpoints, however, it received moderate evaluations in assessing hardness and spicy taste.

Samples placed in the lower consumer preference zone 5, 9 and 1 received average ratings in assessing the shape, size, appearance after cutting, hardness, sweet and earthy odour, sweet and earthy taste. Low ratings have received these samples in evaluating colour, odour intensity, spicy and sour odour, authentic taste, spicy and sour taste. Samples 3, 7, 8, and 10 were placed in the lowest consumer preference zone (0 – 20%). Based on the PCA, these samples received average ratings for colour, odour, sour and earthy odour, authentic taste, spicy and sour taste. Samples 3, 7, 8, and 10 received a low rating in assessing the shape, size, appearance after cutting, hardness, sweet odour, sweet and earthy taste.

CONCLUSION

Based on the results we would like to state that the samples with attractive appearance are partly overestimated by the assessors in all evaluated properties. The findings from the internal part of the evaluation were confirmed by the analysis of the clusters, which suggests that the vast majority of respondents in the research focus on garlic based on the perception of textural properties of garlic, especially shape, colour, size and hardness. The observations also show that the basic indicative characteristics of the evaluation are the sweet and spicy taste and odour as well as the degree of acidic and earthy taste and aroma in evaluating the taste and odour characteristics of garlic.

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COMPARISON OF QUALITY PARAMETERS OF THE COOKED SALAMI „GOTHAJSKÝ“ IN DEPENDENCE ON USED SALT CONTENT AND ADDITIVES

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ABSTRACT
Consumers in Czech Republic have high income of salt from food, therefore, there are efforts to reduce its content in meat products. The subject of this work was to examine differences in sensory evaluation of sliced cooked salami (Gothajský salami), manufactured according to various recipes. This type of meat product is well known primarily to the older generation of consumers, so the aim was to find out the differences in the perception of various samples between generations. The monitoring factors were salt content (1.6% or 2.0%), presence of monosodium glutamate (PG = presence or AG = absence) and group of evaluators (YC = 18 – 26 years old or OC = more than 60 years old). Older sensory panellists (OC; against YC) significantly (p <0.05) evaluated all samples more positively, especially in the taste and odour descriptors. Samples with monosodium glutamate (PG1.6 and PG2.0) were rated in the taste significantly better (p <0.05), regardless of the age of the assessors (YC and OC). Samples with reduced salt, without glutamate (AG1.6) were significantly worst evaluated (p <0.05) by both the groups (YC and OC) than PG2.0 samples.

Keywords: colour; sensory evaluation; saltiness; monosodium glutamate

INTRODUCTION
The content of salt in meat products continues to be of interest to consumer organizations and health professionals **(WHO, 2013)**. Processed meat products and bread including cereals in the group are the largest source of sodium (salt) in the European diet **(Kloss et al., 2015)**. The health consequences of excess sodium in the diet are more serious than consumers admit. with hypertension and leads to an increased risk of strokes and fatal vascular diseases **(He and MacGregor, 2010)**. There are several ways to reduce salt content. The salt content of meat products can be reduced to a level that does not affect the technological or organoleptic properties of the product. By further reducing the sodium content, it may be partly or completely replaced by other substances that do not adversely affect the sensory and technological properties. Potassium, calcium, magnesium, and sodium and potassium lactate are most commonly used **(Desmond, 2006)**. According to **Aaslyng et al. (2014)** that salt reduction from 2.2% to 1.7% did not alter the sensory properties in sausages. It is commonly assumed that sensory impairments occurring with age negatively affect older people's intake of foods in terms of both quality and quantity. Because of anatomical changes in all the senses involved in human food perception, on average seniors perceive a lower flavour intensity than younger adults, are less sensitive to changes in the flavour profile of foods and show a decreased ability to discriminate between different intensity levels of flavour and/or taste attributes. However, despite these differences in their sensory perception of foods, young adults and seniors seem to differ less in their initial hedonic appraisal of food products **(Doets and Kremer, 2016)**. Multidisciplinary approach includes evaluating psychological issues such as attitudes, beliefs, and expectations; sensory properties such as appearance, texture, flavour and odour; and marketing-related aspects such as price and brand **(Font-i-Furnols and Guerrero, 2014)**. Older consumers are more conservative in their preferences. Consumer protection and detecting of adulteration is very important and has a wide societal impact in the economic sphere **(Drdolová et al., 2017)**. Traditional consumer testing provides important information regarding acceptability but may miss important unconscious responses of consumers **(Torrico et al., 2018)**.

Unfortunately, the Czech Republic is in the income of salt content and the occurrence of diseases with this problem associated with the leading countries. However, sodium chloride and sodium nitrite have a key role in meat production. Reducing the salt content in consumers´ of known meat products is a way to rationally reduce sodium in food. Rather than developing new recipes or making a major legislation-regulated adjustment, recommendations should be made for manufacturers. Together with an assessment of such an adjustment, could be a guideline, especially for smaller producers in the regional market. This can better meet the demands of different consumer groups and will not require legislation or major

interventions in large-scale meat production **(Jůzl et al., 2018b)**. Nevertheless, the major issue when using lower salt concentrations in processed meat products is to be able to maintain the product quality characteristics without affecting the shelf-life or the economic viability of the product **(Desmond, 2006)**. Salt is predominantly used to enhance food flavour, making even unpalatable food taste better. However, taste and preservation are not the only reasons for the use of high levels of sodium in foods. The sodium level is generally kept high due to the additional functional roles it provides. The presence of salt (1.5% – 2.5% w/w) in meat products solubilizes meat proteins, activates extraction of proteins, enhancing hydration, and water holding capacity (WHC) **(Ruusunen and Puolanne, 2005)**. Colour and material of surface of packaging are important parameters for consumers **(Géci et al., 2017)**. **Tobin et al. (2013)** wrote about a problem with low-salt meat products. Main reason is that, along with saltiness, reducing sodium will also affect product texture and flavour intensity. However, lowering the salt content to 1.4% NaCl in cooked sausages has been shown to be possible while keeping an acceptable perceived saltiness, firmness, water-binding and fat retention. The meat industry is pushed to the lowest price by the retail chain, which causing meat content reduction in the products **(Fekete et al., 2016)**. One way is following the trends moving towards enhancing hygienic quality using antioxidants **(Bobko et al., 2017)** or antimicrobial agents **(Kročko et al., 2015; Kročko et al., 2017)** in recipes. Therefore, it is an attempt to reduce the use of classically used additives such as flavour enhancers (sodium glutamate), colourants (carmine) or sodium nitrite in salt mixtures **(Jůzl et al., 2018a)**.

Scientific hypothesis

We are expecting the significant effect of salami´s recipes on consumer tests by sensory evaluation. The aim of this study was to examine the importance of reduced the salt content of meat products according to the presence or absence of monosodium glutamate (MSG) for various groups of consumers.

MATERIAL AND METHODOLOGY

The cooked salamis were produced due to in three repetitions according to the quality standard of ON 57 7231 (Gothajský salami, beef H3 or H4, pork V4, pork V5 or V6 and V8 according to Czech Meat Processors Association). Samples were prepared in the pilot plant CZ 22067 (approved by the State Veterinary Administration, Czech Republic) of Mendel University in Brno. This cooked salami named Gothajský salami has name associated with city in Germany. It is delivered for retail use on shop counter where they are sliced. This salami is spiced with paprika, cumin and coriander. Due to Czech legislation, this salami is typical of pieces of pork lard, size predominantly up to 8 mm. The meat content should be min. 40% and a maximum fat content of 40%. The product must not contain mechanically separated meat or poultry mechanically separated meat. It is filled into artificial PE packaging, in our case red BETAN, calibre 75 mm, length 50 cm. For production were used typical standard machines used in industrial production (cutter, filler, smoker). Both spice mixtures from two different companies contained the E450, E451 and E452 stabilizers, spices (paprika, cumin and coriander), antioxidant E300 and E160c (pepper extract), but they differ in presence or absence of monosodium glutamate. Weights of nitrite salt mixture were weighed to produce 2.0% or 1.6% salt in the final samples. So, they were summatically produced four recipe variants PG2.0 or PG1.6 (presence of monosodium glutamate, 2.0 or 1.6% salt) and AG2.0 or AG1.6 (absence of monosodium glutamate, 2.0 or 1.6% salt). After receiving, the raw meat was kept in 2 °C and second day was coarsely ground to obtain meat emulsion in cutter (Seydelmann, Germany). Lard prepared previously to regular cubes was frozen (-18 °C) and during the production incorporated in cutter during production to the desired mosaic. Than were and filled (HTS 150, Germany) in PE casings (75/50) and treated (70 °C, 10 min in the product core) in smoker (Bastramat, Germany).

Quality evaluation of cooked salami

For chemical and sensory analysis were used commonly available methods. For the instrumental measurement of the surface colour, the spectrophotometer and CIE colour space (L*a*b*) were used. Salamis was measured and evaluated in the sixth day after production. Shelf life (in 2 – 4 °C) is set at 3 weeks. The product was sliced on a commercial rotary cutter before sensory evaluation in slices (0.5 mm).

Chemical analysis

The dry matter ($g.100g^{-1}$), the salt content ($g.100g^{-1}$) and the fat content ($g.100g^{-1}$) after homogenization of the sample (250 g) were analysed for each group (PG2.0, PG1.6, AG2.0, AG1.6) **(AOAC, 2005)**. All analysis was undertaken in duplicate.

Colour measurement

Colour space L*, a* and b* was used to determination differences in colour. The CM 3500d spectrophotometer (Konica Minolta, Japan) was used and the samples were measured (D 65, 6500 °K) on the surface in centre of the slices with SCE (Specular Component Exluded) and 30 mm slot in triplicate (3 pairs and in 2 batches). Colour variation was determined as total colour difference ΔE^*_{ab} **(Saláková, 2012)**.

Sensory analysis

Sensory analysis was evaluated by two consistently identical different groups of panellists (n = 48). Selection was based on submitted questionnaires received from trained meat products consumers. To be selected, they had to belong to a group of consumers who ever ate cooked salamis (Gothajský salami) and consumed meat products from 1 to 3 times a week. The number of women and men was not equivalent, so this factor was not evaluated. One group of young consumers (YC) was selected from students (18 – 25 years, n = 24) of course Meat Technology (bachelor study Chemistry and Food Technology, second year, MENDELU). Seniors, older consumers (OC, n = 24) were selected from the class of Institute of Lifelong Learning, members of the University of the Third Age, MENDELU. Both groups of panellists were briefly trained in the basics of sensory evaluation and the use of questionnaires. The evaluation was ongoing

Figure 1 Spice mixture with monosodium glutamate (PG).

Figure 2 Spice mixture without monosodium glutamate (AG).

Figure 3 Minced meat in cutter

Figure 4 Samples after heat treatment.

Figure 5 Samples with monosodium glutamate (PG) 1.6% (left) and 2.0% salt (right).

Figure 6 Samples without monosodium glutamate (AG) 1.6% (left) and 2.0% salt (right).

under ČSN ISO 6658 (560050) condition. Sensory analysis was undertaken at special sensory laboratory with ten chambers (Department of Food Technology).

All panellists buy and consume Czech meat products regularly. For each sample, assessors were asked to indicate their score on a 100 mm line scale. It is ranging from 0 at the left to 100 at the right. Descriptors expressed as the hedonic scores. Minimum was 0 (left) and maximum of pleasure 100 (right side of scale). Analysis were chosen as sensory panel with following descriptors: appearance, colour, texture, fat composition, consistency, odour, saltiness and taste. The samples were presented to panellists randomly and marked with a numeric code. Water and non-salted bread were used as neutralizers.

Statistical analysis

The data has been sorted and processed by analysis of variance (one-way ANOVA) and Tukey´s test to compare groups of samples according to its salt content or presence of monosodium glutamate in cooked salami´s recipes by the groups of panellists in programme STATISTICA 12. Samples were considered significant at 95% confidence level (p <0.05) and data were tested for normality by Shapiro-Wilk test.

RESULTS AND DISCUSSION

Although the recipe was free from substitutes and contained beef, it can be considered as a standard meat product of standard quality. Chemical analysis of the samples showed results that did not exceed the limit set by **Decree No. 69/2016 Collection of Laws** (40% fat) or significantly differ from the values given in the ČSN 57 7231 standard and the corresponding scheme for products of quality category (above 47 to 50% dry matter, 39 to 42% fat, 2.0 ±0.6%). Even though we were based on the norm, the fat content was lower than the standard. The reason is probably lower fat content in pork than in ČSN 57 7231. Fat content in pork has changed since the original calculations and compared to the state more than thirty years ago. There were no differences (p >0.05) in fat and protein content or in dry matter between groups of samples (Table 1). Of course, the salt content of the product varied (p <0.05) in groups with different salinity (PG2.0, AG2.0 versus PG1.6 and AG1.6). It should be noted, results could depend on the type of analysis used. State authorities

Table 1 Basic chemical analysis of cooked salami according to different salt content and presence of MSG.

Content ($g.100g^{-1}$)	Group of samples			
	PG2.0 ($\bar{x}$ ±*SD*)	PG1.6 ($\bar{x}$ ±*SD*)	AG2.0 ($\bar{x}$ ±*SD*)	AG1.6 ($\bar{x}$ ±*SD*)
Dry matter	48.52 ±0.88	48.57 ±0.74	49.09 ±0.64	49.21 ±0.89
Fat	32.52 ±1.28	33.09 ±1.11	33.04 ±1.06	33.27 ±1.15
Proteins	10.07 ±0.35	10.18 ±0.39	10.02 ±0.46	10.21 ±0.41
NaCl	2.09 ±0.09^{b}	1.63 ±0.06^{a}	2.15 ±0.08^{b}	1.68 ±0.07^{a}

Note: PG2.0 or PG1.6 = presence of monosodium glutamate, 2.0 or 1.6% salt; AG2.0 or AG1.6 = absence of monosodium glutamate, 2.0 or 1.6% salt; Means with different superscripts in the same rows show significant differences (p <0.05).

Table 2 Instrumental measurement of cooked salamis colour surface according to different salt content and presence of MSG.

Colour parameter	Group of samples			
	PG2.0 ($\bar{x}$ ±*SD*)	PG1.6 ($\bar{x}$ ±*SD*)	AG2.0 ($\bar{x}$ ±*SD*)	AG1.6 ($\bar{x}$ ±*SD*)
L* (D65)	56.10 ±0.78^{a}	59.78 ±0.98^{b}	55.70 ±0.66^{a}	58.89 ±0.73^{b}
a* (D65)	17.07 ±0.45^{b}	15.24 ±0.57^{ab}	16.39 ±0.67^{b}	14.32 ±0.62^{a}
b* (D65)	18.43 ±0.63^{b}	17.01 ±0.72^{a}	18.28 ±0.49^{b}	16.58 ±0.65^{a}

Note: PG2.0 or PG1.6 = presence of monosodium glutamate, 2.0 or 1.6% salt; AG2.0 or AG1.6 = absence of monosodium glutamate, 2.0 or 1.6% salt; Means with different superscripts in the same rows show significant differences (p <0.05).

Table 3 Sensory analysis of cooked salamis according to different salt content and presence of additives.

Descriptor	Consumer group	Group of samples			
		PG2.0 ($\bar{x}$ ±*SD*)	PG1.6 ($\bar{x}$ ±*SD*)	AG2.0 ($\bar{x}$ ±*SD*)	AG1.6 ($\bar{x}$ ±*SD*)
Appearance	YC	65.3 ±12.3	66.4 ±15.6	65.9 ±14.5	67.2 ±12.7
	OC	78.0 ±11.9	74.1 ±14.7	72.5 ±12.7	75.6 ±14.0
Colour	YC	55.3 ±18.2	50.2 ±18.1	52.6 ±16.1	49.1 ±13.8
	OC	75.0 ±14.9^{b}	59.2 ±14.5^{ab}	78.5 ±13.6^{b}	51.3 ±16.0^{a}
Fat composition	YC	54.4 ±19.1	51.5 ±16.8	55.6 ±17.3	47.1 ±13.3
	OC	70.1 ±14.1	64.2 ±15.4	68.5 ±10.7	63.2 ±12.4
Consistency	YC	59.3 ±15.8	56.2 ±17.3	59.5 ±19.0	57.2 ±14.6
	OC	75.1 ±11.3	68.2 ±12.6	74.5 ±13.3	66.2 ±15.3
Odour	YC	69.0 ±14.5	66.4 ±15.5	65.9 ±14.4	67.2 ±14.3
	OC	78.1 ±13.2	74.1 ±10.9	73.5 ±10.9	71.6 ±10.8
Saltiness	YC	58.1 ±12.9^{b}	60.4 ±13.3^{b}	60.9 ±15.2^{b}	47.2 ±13.6^{a}
	OC	80.4 ±16.8^{b}	70.1 ±14.7^{ab}	73.5 ±13.9^{b}	50.6 ±14.7^{a}
Taste	YC*	64.1 ±13.8^{b}*	52.4 ±14.3^{ab}*	50.9 ±15.0^{ab}*	43.2 ±14.2^{a}*
	OC*	80.1 ±12.2^{b}*	78.1 ±11.9^{b}*	83.5 ±13.8^{b}*	60.6 ±12.6^{a}*

Note: YC – consumers 18-26 years old, OC – consumers more 60 years old; PG2.0 or PG1.6 = presence of monosodium glutamate, 2.0 or 1.6 % salt; AG2.0 or AG1.6 = absence of monosodium glutamate, 2.0 or 1.6% salt; Means with different superscripts in the same rows show significant differences (p <0.05); Means with * designation show significant differences (p <0.05) between panellists groups YC and OC; Descriptors expressed as the hedonic scores, where 0 is the sign minimum and 100 is maximum of pleasure.

responsible for supervision of safety, quality and labelling of foodstuffs in the Czech Republic require sodium analysis, in our case we have used the determination through chlorides. However, it should not be a significant difference.

However, **Kameník et al. (2017)** states in his work that salt level determined by the two methods strongly correlated and did not differ in any meat product. After all, the results presented by **Kameník et al. (2017)** are not different from our chemical analysis.

The appearance of the food, its colour and its stability are essential for meat products to be offered to consumers at the shelves of the shops in a sliced form. This also contributes to the lighting that is in the room. In general, products with a more pronounced colour are better evaluated (higher values for red and * and yellow b *). Table 2 shows the colour values. Lightness of cooked

salamis was measured in the range of L* = 55.19 to 58.10, depending more on the salt content. Also, for parameters a * and b * for red and yellow colour (resp.) was found significantly differences (p <0.05) between groups according to its content of salt. Groups with lower saltiness had higher lightness and lower colour coordinates a* and b*. Lightness L*, it depends on the type of product. Salt, specifically sodium chloride and especially nitrite, is expressed in several ways in the meat product: it contributes to the colouring of the product, to the aroma formation, has a preservative and antioxidant effect **(Saláková et al., 2013)**. It is obvious that the lower salt content has a significant effect (p <0.05) on the colour of the products, but it did not depend on the presence of MSG (Table 2). Results showed similar colour values as at work **Jůzl et al. (2018a)**.

Table 4 shows the sensory assessment of cooked salamis and their comparison between two groups of evaluators. There were not found statistical differences (p >0.05) in appearance between groups of samples according to the content of salt in both groups of panellists. It has been confirmed that the salt content is an important aspect of the acceptability of the meat product. Older evaluators were more receptive to this, although younger evaluators would have sensible senses. It depends on the experience and popularity of the meat product. This shows the greater popularity of this product in the older generation **(Doets and Kremer, 2016)**. Those in the younger generation had the problem of distinguishing the difference in salt content.

Some strategies for innovations can be done by, for instance, not only directly lowering the amount of salt and fat in the recipe, which is the first possibility. However, some authors **(Horita et al., 2016)** describe using a salt substitute (e.g. potassium chloride or herbs), or by using animal fat replacements (e.g. starch or oil from non-animal sources), depends on consumer´s experience. Sensory evaluation has shown significant differences in taste and salinity in a group of younger panelists (YC) compared to older (OC). The presence of MSG shows that the low salt content is not so noticeable. Recent studies have even shown that sodium reduction can be beneficial for a part of the population that is defined as salt-sensitive. Senior consumers are more conservative, and have a more accurate awareness of the standard, at least in this case and cooked salamis has been confirmed. It is true that consumers are generally interested in the content of substances in food hazardous to health. Nitrites are thus negatively perceived by different consumer groups. However, nitrite replacement is a complicated intervention in the product recipe regarding its sensory and microbiological quality **(Jůzl et al., 2018b)**.

CONCLUSION

The results of the model production and analysis of four variants of cooked salamis with different spice mixtures or salt content indicate that the salt content affected the product quality parameters, however they were not considered negatively. Gothajský salami were rated more positively by older panellist group. There were also recorded several differences. From the above, it follows that the choice of seasoning mixture, the presence of other additives influences the sensory quality of the cooked salami. No significant negative result was found in the sensory evaluation, which would not reduce the salt content of the meat product's recipe.

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SOMATIC CELL COUNT DURING FIRST AND SECOND LACTATION IN EWES

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ABSTRACT

The aim of this study was to describe the frequency of distribution of ewes in SCC groups on the basis SCS (somatic cells score) per lactation and estimate changes of SCC from 1st lactation on 2nd lactation. The experiment was carried at seven farms in 1st observed period (2016 and 2017) and at eight farms in 2nd observed one (2017 and 2018). Within each of periods the same animals were sampled on their 1st and following 2nd lactation in next year of study, only. Totally 1199 milk samples from 159 ewes and 1653 milk samples from 219 ewes were collected during 1st period and 2nd period, respectively. Milk sampling were taken monthly from April to August in both periods. For evaluation only ewes with minimum three sampling per year (minimum six samples per animal) were included in the study within both periods. The ewes were divided into the five SCC groups on basis of their SCS per lactation: G1 = SCC <200 × 10^3 cells.mL^{-1}, G2 = SCC ≥200 <400 × 10^3 cells.mL^{-1}, G3 = SCC ≥400 <600 × 10^3 cells.mL^{-1}, G4 = SCC ≥600 <1000 × 10^3 cells.mL^{-1} and G5 = SCC ≥1000 × 10^3 cells.mL^{-1}. In total statistically significant impact of parity on SCC in 2nd period was detected (p <0.0001) only. From the farm point of view in 1st period only in two farms and in 2nd one in five farms significant effect of parity was found out. Thus in some farms no increase of SCC from first to second lactation was observed. When comparing the changes in SCC from the first to the second lactation in both first and second periods, 6.92% and 10.96%, respectively ewes moved from SCC group G1 to G5. The significant effect of farm management and parity on SCC was demonstrated.

Keywords: ewes; milk; somatic cell count; farm; milking

INTRODUCTION

Somatic cells in milk represent epithelial cells and leukocytes **(Paschino et al., 2019)**. Somatic cell count (SCC) is considered from many aspects as an indicator of udder health and generally is used for detection of subclinical mastitis in ewes **(Gonzáles-Rodríguez, Gonzalo and San Primitivo, 1995; Pengov, 2001; Olechnowicz and Jaskowski, 2005)**. However, there is still a big discussion among scientists about the physiological level of SCC in milk of ewes for detection of their udder health **(Persson et al., 2017)**.

Berthelot et al. (2006) reported in their study SCC <500 × 10^3 cells.mL^{-1} for healthy ewes and for infected ewes SCC >1000 × 10^3 cells.mL^{-1}, if SCC was in flock >650 × 10^3cells.mL^{-1} it showed 15% incidence of udder disease to have subclinical mastitis. The results of **Kern et al. (2013)** indicated threshold of SCC 400 × 10^3 cells.mL^{-1} in meat breeds of sheep, 300 × 10^3 cells.mL^{-1} in dairy breeds and 100 × 10^3 cells.mL^{-1} in extensive breeds as right value in detecting problems with udder health. **Hussein, El-Khabaz and Malek (2015)** determined value of SCC ≥400 × 10^3 cells.mL^{-1} in Ossimi sheep as limit for detection subclinical mastitis. The limit for the detection of subclinical sheep mastitis was determined by **Swiderek et al. (2016)** as 200 × 10^3 cells.mL^{-1}. Similar threshold of SCC for diagnosis of mastitis in Sarda sheep was considered at 265 × 10^3 cells.mL^{-1} **(Caboni et al., 2017)**. **Sutera et al. (2018)** in their study showed value SCC >500 × 10^3 cells.mL^{-1} as a possible limit in relation to milk quality.

In the study in our breeding practise **Idriss et al. (2015)** reported 78% of the samples of individual ewes <600 × 10^3 cells.mL^{-1}. **Vršková et al. (2015)** found out that 76% of Tsigai had SCC <300 × 10^3 cells.mL^{-1}. In recent study **Tančin et al. (2017)** found out that 82.03% individual milk samples were <400 × 10^3 cells.mL^{-1}, 71.79% milk samples were <200 × 10^3 cells.mL^{-1} and only 8.89% milk samples were >1000 × 10^3cells.mL^{-1}. **Oravcová, Mačuhová and Tančin (2018)** found out 60% samples with SCC ≤200 × 10^3cells.mL^{-1}.

The aim of this study was to describe the frequency of distribution of ewes in SCC groups on the basis somatic cell score (SCS) per whole lactation and estimate changes of SCS from1st lactation to SCS in 2nd lactation. The effect of farms was evaluated too.

Scientific hypothesis

The parity significantly influences the SCC in milk.

The most of the ewes have low SCC in milk. The udder heath in previous lactation affect the udder health in following lactation. The farm has impact on SCC in milk.

MATERIAL AND METHODOLOGY

The experiment was carried out during two periods in dairy practice. Seven ewes' dairy farms were involved in the study during 1st observed period in 2016 and 2017 and at eight farms during 2nd observed period in 2017 and 2018. On the farms they were kept Tsigai breed, Lacaune and on one farm Slovak dairy sheep. Tsigai (TS) breed were kept on farm 1st, 2nd, 3rd, 4th and farm 5th. Lacaune (LC) breed were kept on farm 6th, 7th, 8th and farm 9a th. On farm 9b th they were kept Slovak dairy sheep (SD) in 2nd observed period only. Within each of the period the same animals were sampled on their 1st and following 2nd lactation in next year of study. In 2 farms (1st, 3rd) hand milking was performed and remaining 7 flock were milked by machine milking. Milk sampling were taken once a month as a part of milk recording service. Milk samples were taken from April to August in 1st and 2nd observed periods. Analysis of milk samples has been performed in the certificated Central laboratory of Breeding services of the Slovak Republic (Plemenárske služby š.p. SR Bratislava).

For evaluation only ewes with minimum 3 and more sampling during each lactation within both 1st and 2nd periods were included into study. Thus minimum six observations were available per animal. A total of 1199 milk samples from 159 ewes (140 TS, 19 LC) were collected during 1st observed period. From 219 ewes (130 TS, 63 LC, 26 SD) were collected 1653 milk samples during 2nd observed period.

Statistic analysis

On the basis of SCC from milk recording the ewes were divided into the five SCC groups: G1 = SCC <200 × 10^3 cells.mL^{-1}, G2 = SCC ≥200 <400 × 10^3 cells.mL^{-1}, G3 = SCC ≥400 <600 × 10^3 cells.mL^{-1}, G4 = SCC ≥600 <1000 × 10^3 cells.mL^{-1} and G5 = SCC ≥1000 × 10^3 cells.mL^{-1} to evaluate the distribution of ewes into SCC groups in different parity and years of study. Animals were individually divided into above mentioned SCC groups on the basis of their SCS per lactation calculated as a mean from transformed individual SCC data into SCS obtained during milk recording throughout lactation. SCS was calculated according formula:

$$SCS = LOG_2(SCC/100000) + 3$$

Thus distribution of ewes on the basis of SCS into SCC groups was done by conversion of linear scores to somatic cell counts. The results were mathematically processed using the Microsoft Excel program. It was used paired t-test when comparing differences variables between first and second lactation (within observed periods). Data are presented as mean ± standard deviation. The statistical model using SAS (Mixed procedure; SAS/STAT 9.1, 2002 – 2003) can be written in the following form used for each observed period separately:

$$y_{ij} = \mu + FARM_i + YEAR_j + e_{ij}$$

y_{ij} = the measurements for SCS; μ = overall mean; $FARM_i$ = the fixed effects of farms; $YEAR_j$ = fixed effect of YEARS (two years, within each observed period), $u_l \sim N(0, \sigma c2)$; e_{ij} = random error, assuming $e_{ij} \sim N(0, I\ \sigma^2_e)$. Data are presented as LSmeans (Least squares means) ± standard error.

RESULTS AND DISCUSSION

Impact of parity on SCC was not statistically significant in 1st observed period (p <0.0868) but was significant in 2nd observed period (p <0.0001). Similar results were reported by **Romero et al. (2017)**. They found out that multiparous ewes had significant higher SCC compared with primiparous ewes (205 × 10^3 cells.mL^{-1} and 102 × 10^3 cells.mL^{-1}, resp.). Also **Takano et al. (2018)** showed in their study that multiparous Lacaune ewes had a higher incidence of intramammary infections during early lactation than primiparous ewes. SCC were higher in multiparous than in primiparous goats **(Diaz et al., 2011)**. The youngest ewes had the lowest SCC, while the oldest ewes showed in general the highest SCC **(Arias et al., 2012).** Subclinical mastitis occurred less frequently in primiparous ewes than those with two or more lactations significantly (p <0.05) and ewes on 3rd lactation had the most cases of subclinical mastitis **(Sani, Mahdavi and Moezifar, 2015)**.

Although the effect of parity on SCS in between 1st and 2nd lactation wasn´t detected in 1st observed period, we found out the effect of parity on SCC at the level of individual farms. During 1st observed period we detected the effect of parity on SCS in farm 4th and farm 9a th (Table 1). Significant effects of parity on SCS during 2nd observed period, and at farm level in farm 1st, 3rd, 5th, 8th and farm 9b th (Table 2). Breeds didn´t have impact on change of SCS in monitored farms (Table 3). Significant differences between farms with the same breed could indicate the effect of management level on farms.

Distribution of ewes in SCC groups during 1st observed period (2016 and 2017) was as followed: G1 (38.99%, 33.96% resp.), G2 (32.02%, 23.90% resp.), G3 cells.mL^{-1} (6.92%, 10.69% resp.), G4 (6.29%, 6.29% resp.) and G5 (15.72%, 25.16% resp.). During 2nd observed period (2017 and 2018) there were following distribution of ewes in SCC groups: G1 (57.99%, 35.16% resp.), G2 (21%, 20.09% resp.), G3 (6.39%, 9.13% resp.), G4 (3.2%, 8.68% resp.) and G5 (11.42%, 26.94% resp.). If compare changes from 1st to 2nd lactation in both observed periods the following changes occurred: In 1stmonitored period there were 8.81% ewes in SCC group with <200 × 10^3cells.mL^{-1} during 1st lactation which moved into SCC groups ≥600 × 10^3 cells.mL^{-1} during 2nd lactation. Even 6.92% from these mentioned ewes moved into SCC group ≥ 1000 × 10^3 cells.mL^{-1}. In 2nd observed period 15.53% of ewes were in SCC group with <200 × 10^3 cells.mL^{-1} during 1st lactation, which moved into SCC groups ≥600 × 10^3 cells.mL^{-1} in the following lactation. Even from these ewes 10.96% moved into SCC group ≥ 1000 × 10^3 cells.mL^{-1}.

Table 1 SCC during first (2016) and second lactation (2017) of the same animals.

		First observed period				
	n	**2016**		**2017**		***p* ≤0.05**
		Mean	Std.dev.	Mean	Std.dev.	
Farm 1	56	5.48	1.44	5.43	1.82	0.419
Farm 2	30	4.24	1.05	4.59	1.36	0.080
Farm 3	29	3.80	0.86	3.75	1.59	0.422
Farm 4	18	4.31	0.87	5.17	1.09	0.007
Farm 5	7	5.02	1.81	5.00	1.42	0.494
Farm 6	-	-	-	-	-	-
Farm 7	-	-	-	-	-	-
Farm 8	8	4.41	0.71	4.55	1.26	0.406
Farm 9a	11	5.05	2.17	6.43	2.66	0.047
Farm 9b	-	-	-	-	-	-

Note: n – number of observations.

Table 2 SCC during first (2017) and second lactation (2018) of the same animals.

		Second observed period				
	n	**2017**		**2018**		***p* ≤0.05**
		Mean	Std.dev.	Mean	Std.dev.	
Farm 1	37	4.65	1.45	6.00	1.59	<0.001
Farm 2	20	3.98	1.04	4.56	1.52	0.076
Farm 3	51	3.95	1.40	5.17	1.66	<0.001
Farm 4	-	-	-	-	-	-
Farm 5	22	4.23	1.16	4.39	1.34	0.024
Farm 6	17	5.06	1.61	5.37	2.06	0.303
Farm 7	30	3.93	0.80	3.85	1.52	0.384
Farm 8	10	4.13	1.38	5.42	1.80	0.007
Farm 9a	6	4.37	1.58	5.66	1.34	0.109
Farm 9b	26	3.63	1.49	5.41	2.03	0.001

Note: n – number of observations.

Table 3 Effect of farms on SCC for two observed periods of study.

		2016 – 2017			**2017 – 2018**		
		number (2n)	lsmeans	std. error	number (2n)	lsmeans	std. error
TS	**Farm 1**	112	5.46	0.14	74	5.32	0.18
	Farm 2	60	4.41	0.20	40	4.27	0.25
	Farm 3	58	3.77	0.20	102	4.56	0.15
	Farm 4	36	4.74	0.25	-	-	-
	Farm 5	14	5.01	0.41	44	4.31	0.24
LC	**Farm 6**	-	-	-	34	5.22	0.27
	Farm 7	-	-	-	60	3.89	0.20
	Farm 8	16	4.48	0.38	20	4.77	0.35
	Farm 9a	22	5.74	0.32	12	5.02	0.45
SD	**Farm 9b**	-	-	-	52	4.52	0.22

Figure 1 Frequency of distribution of ewes in SCC groups during first and second lactation in farm with machine milking.

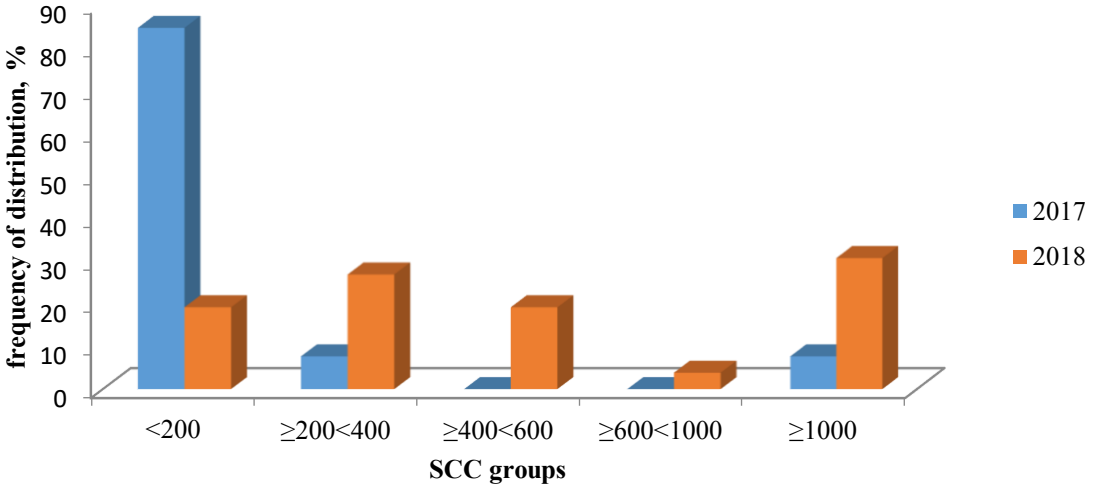
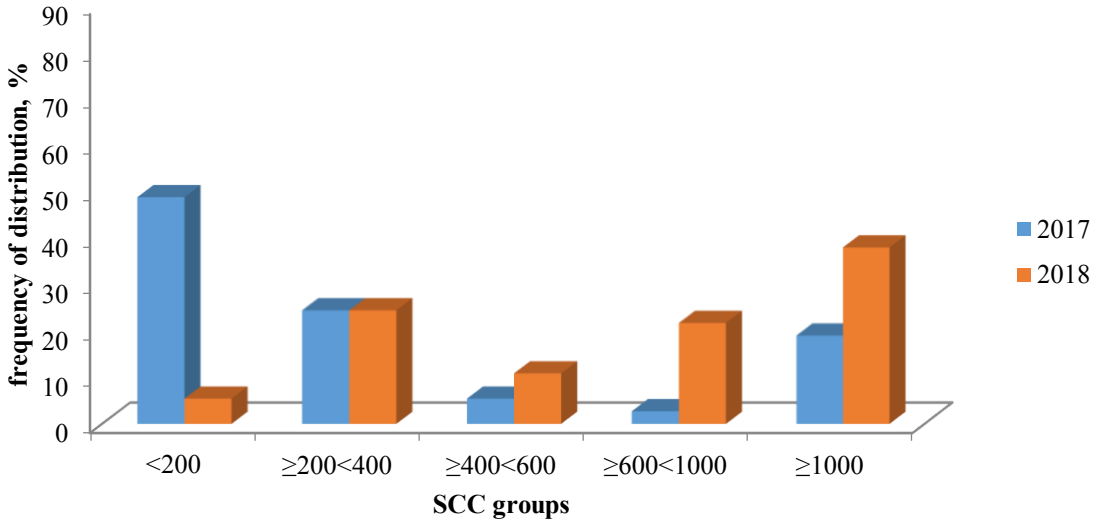


Figure 2 Frequency of distribution of ewes in SCC groups during first and second lactation in farm with hand milking.



These changes from 1st to 2nd lactation among SCC groups and clear increase of percentage of samples in SCC group ≥1000 × 10^3 cells.mL^{-1} in 2nd lactation indicate higher prevalence of subclinical mastitis. **Persson et al. (2017)** detected significant association between intramammary infection and high SCC in ewes. In contaminated samples were significantly higher SCC as compared with uncontaminated milk samples **(Ozenc et al., 2011)**. From preliminary results of **Tančin et al. (2018)** there was shown that high SCC in milk samples were associated with presence of pathogens. **Romero et al. (2017)** observed significant higher SCC in milk of primiparous and multiparous ewes with mastitis. Early diagnosis and treatment of subclinical mastitis can significantly eliminate clinical forms of mastitis **(Zigo et al., 2017)**.

Data shown in Figure 1 and Figure 2 represent examples of frequency of distribution of ewes from one farm with machine milking and another farm with hand milking during their 1st and 2nd lactation. On both figures there are presenting changes of udder heath from 1st to 2nd lactation by clear demonstration of difference between count of ewes in SCC group <200 × 10^3 cells.mL^{-1} and in SCC group ≥1000 × 10^3 cells.mL^{-1}. In both farms during the 2nd lactation there was a decrease in the distribution of ewes in the SCC group <200 × 10^3 cells.mL^{-1} regardless on the milking technique. Increase of percentage of ewes in SCC groups ≥1000 × 10^3 cells.mL^{-1} could be due to the increase prevalence of subclinical mastitis in these farms. In other

study **Marogna et al. (2010)** found out that hand milking was associated with 62% higher risk of bacterial positive samples compared to machine milking which we did not confirmed in our study. **Marogna et al. (2010**) also observed that machine milking with portable devices was associated with 40% higher risk of bacterial positive samples compared to machine milking with fixed plants. **Queiroga (2017)** detected significantly higher prevalence of subclinical mastitis in herds with machine milking than those with hand milking (p <0.0001). **Vasileiou et al. (2018)** reported increased prevalence of mastitis in farms with hand milking.

CONCLUSION

In conclusion, high percentage of ewes had SCC <200 × 10^3 cells.mL^{-1} during 1st lactation only. During 2nd but not during 1st observed period the ewes on 2nd lactation had higher SCC compared with primiparous ewes, however, clear individual farm effect was recorded in both observed periods. Also significant effect of farm management on SCC was demonstrated without connection to hand or machine milking. Thus the level of management in dairy farm has to be considered.

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COMPARISON OF CHEMICAL COMPOSITION OF EGGS FROM LAYING HENS HOUSED IN DIFFERENT PRODUCTION FACILITIES: A MARKET STUDY

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ABSTRACT

Eggs as a part of human diet dates to the prehistoric period. After the domestication of *Gallus* species, *Gallus gallus domesticus* and their eggs spread across the globe. Eggs proved to be one of the best and affordable sources of nutritionally important components in human diet, such as proteins, vitamins, lipids and some dietary significant elements. Progress in egg production methods, in European union, is recently mostly focused on the improvements in the field of welfare of laying hens, which is part of the plan set by European union council directive 1999/74/EC. Nowadays there are 4 main egg production systems divided by the way of keeping laying hens – Enriched cage, Free range, Barn and Organic. The aim of this study was to evaluate the impact of different hens housing systems on elemental (Ca, K, Mg, Na, P, Ca, K, Zn), total protein and lipid composition of whole eggs, yolk and albumen. Elemental analysis was performed by ICP-OES, total lipid content by Kjehldahl method and total lipids by Soxhlet extraction. Differences in whole egg were found in 8 from 9 tested parameters – K, Mg, Ba, Fe, P, Zn, total lipids and proteins. Assessment of the differences were done by ANOVA and Tukey`s test. Production systems were also successfully differentiated by principal component analysis. It was found that eggs from alternative production systems did not exhibit higher nutritional value than eggs from conventional cage facilities. In the case of total protein, conventional eggs contained highest average amount. It was also evident, that impact on chemical composition is difficult to assign to production system in general, which was confirmed by alternative studies from this field, which in many cases considerably differs.

Keywords: eggs; laying hens; housing; elemental analysis; proteins

INTRODUCTION

Eggs as a part of human diet dates to the prehistoric period. After the domestication of *Gallus* species, *Gallus gallus domesticus* and their eggs spread across the globe. Eggs proved to be one of the best and affordable sources of nutritionally important components in human diet, such as proteins, vitamins (A, B_5, B_7, B_9, B_{12}, K, D, E), lipids and some dietary significant elements (P, Na, K, Ca, Mg, Fe). It is also having relatively high average energy value of 547 kJ.100g^{-1} of whole raw egg **(Belitz et al., 2009; Finglas et al., 2015; Bulková, 1999)**. World egg production rises by 18 percent from 2006 to 2016 to almost 74 million metric tons, as demand for eggs is growing due to rising population, incomes and dietary acceptance. Production in the European union is projected to be quite steady with rising in next decade from 7.7 in 2016 to 8.2 million metric tons in 2026 **(WATT, 2018)**. Progress in egg production methods, in European union, is recently mostly focused on the improvements in the field of welfare of laying hens, which is part of the plan set by European union **council directive 1999/74/EC**. This regulation banned barren battery cages which were replaced by enriched cages with more space, height and nesting area. Conventional enriched cages system covered 53.2% of EU egg production in 2017 and nowadays there are 4 main egg production systems divided by the way of keeping laying hens – Enriched cage, Free range, Barn and Organic. Special group of eggs are those produces in home breeding conditions. Information about the production system must be shown as a part of code on the egg shell.

There are various motivations of modern consumers to select alternatively produced eggs - especially from organic production. This motivation is mainly driven by demand for healthy, nutritionally balanced food products and also by increased perception of the area of environmental friendliness and animal welfare. **(Kralik et al., 2008; Filipiak-Florkiewicz et al., 2017; Shafie and Rennie, 2012; Schleenbecker and Hamm, 2013)**. A growing interest of consumers for non-conventional foods prompted the research activity to assess the real impact of different egg production systems on overall quality of product. **Filipiak-Florkiewicz et al. (2017)** conducted comparison of organic, nutraceutical and conventional

eggs with focus on fatty acid profile, elemental analysis and some physical parameters like colour and rheological properties of mayonnaise made from studied eggs. Results of this study revealed significant differences ($p \leq 0.05$) in the chemical composition. Organic and nutraceutical eggs were characterized to be nutritionally more beneficial in comparison with conventional production. Similar study was proceeded by **Anderson (2011)**, focused on comparison of fatty acid, cholesterol and vitamin A and E composition of eggs from cage and rage production facilities. Significant differences ($p \leq 0.05$) were found in total fat content, which was higher in eggs from range housing. It is also worth mention a market study carried out by **Hidalgo et al. (2008)**. This paper deals with comparison of Italian eggs from barn, cage, free range and organic housing in terms of 41 physical and chemical parameters. Differences were found in whipping capacity, foam consistency, albumen quality and shell resistance to breaking. Multivariate statistical analysis differentiated cage eggs from alternative (organic, barn and free range). It was also concluded, that apart of psychological and ethical motivations, differences in quality of eggs did not justify the higher prices for alternatively produced eggs.

The aim of this study is to evaluate the impact of different hens housing systems on elemental (Ca, K, Mg, Na, P, Ca, K, Zn), total protein and lipid composition of yolk and albumen.

Scientific hypothesis

This study is based on hypothesis whether different approaches of laying hens housing can be significantly reflected in basic chemical composition of eggs. Testing of this hypothesis is an extension of previously published studies, enriching the field by actual data from Czech Republic egg market and by analysis of domestically (home) produces eggs and eggs from improved cage lined by litter material.

MATERIAL AND METHODOLOGY

Samples and sample preparation

Total of 20 batches of eggs from 5 different laying hens housing systems were obtained - Enriched cage, Enriched cage with litter bedding, Free range, Home and Organic. Every batch contained 6 eggs of M size (53 – 63 g) with similar laying period of first week in February 2018. All the batches were from different producers from Czech Republic and were acquired in standard commercial retail network. Eggs produces by home breeding and laying were obtained directly from small village farmers in Brno countryside region (Czech Republic).

Sample preparation

Every batch of 6 eggs was divided into 2 parallel parts of 3 eggs. Albumen and yolk were mechanically separated and was dried on petri dishes at 70 °C for 8 hours. Dried samples were homogenized. Total of 40 sub-samples from 5 different systems were prepared. Microwave digestion system was used for the preparation of samples for elemental analysis on ICP-OES. Amount of 200 mg of albumen and yolk samples were transferred into the teflon cartridges with 5 mL of 65% HNO_3 and 2 mL of concentrated H_2O_2 (Analytika Praha, Czech Republic). Digestion itself was performed by microwave digestion system Milestone 1200 (Milestone, USA). After the decomposition, samples were transferred into 25 mL volumetric flasks, diluted with deionized water (Elga purelab Classic, Veolia water systems, UK) and filtrated through 0.45 µm nylon syringe filters.

Determination of total proteins

Total proteins in samples of albumen and yolk were determined by standard Kjelhdahl method. Mineralization was done by mineralization unit KT-8s (C. Gerhardt & Co, Germany). Digestion of 1 g sample was proceeded at 420 °C for 90 min. Distillation of ammonia was performed by Vapodest steam distillation instrument (C. Gerhardt & Co, Germany) and collected into titration flask with 0.05 M H_2SO_4. Excess of sulfuric acid was titrated with 0.1 M NaOH. Conversion factor from nitrogen to protein content was 6.25, which is standard for eggs.

Determination of total lipids

Determination of total lipids was realized only in samples of yolk due to trace concentration level in albumen **(Finglas et al., 2015)**. Soxhlet method was selected for the extraction of lipids. Lipids from 4 g of yolk sample were extracted into 150 mL of petroleum ether by automatic extraction Soxhlet instrument Soxtherm (C. Gerhardt & Co, Germany). Extraction proceeded for 2 hours at 150 °C. Petroleum ether was distilled away from extraction flask and fats content was determined gravimetrically.

Determination of elemental composition

Elemental analysis of yolk and albumen was performed by ICP-OES Horiba Jobin Yvonne Ultima 2 (Horiba Scientific, France) with measurement conditions of 15 rpm of peristaltic pump; RF power 1300 W, argon plasma gas flow 14 $L.min^{-1}$, auxiliary gas flow 0.15 $L.min^{-1}$, sheath gas
0.7 $L.min^{-1}$ (K, Na, Mg, Ca) and 0.2 $L.min^{-1}$ (P, Zn, Fe, Mn). Instrument was calibrated by standards made from individual 1 $g.L^{-1}$ stock solutions (Analytika Praha, Czech Republic). Calibration was prepared by the standard addition method into the blank solutions used for sample digestion.

Statistical analysis

Data analysis and statistical evaluation were performed in Microsoft Excel (Microsoft, USA) and XL-stat (Addinsoft, France). Results were processed by various statistical approaches. Before the main data analysis, results were tested for outliners and data distribution. The Grubbs test for outliners did not revealed any outlined values between the 5 tested groups of eggs and data showed a normal Gaussian distribution.

Analysis of variance (ANOVA) was used to evaluate chemical parameters which exhibited statistically significant differences between the groups of eggs from different production systems. *p*-values as a result of this analysis is provided. Tukey pair test was used for evaluation of inter-group comparison.

Principal component analysis (PCA) based on Pearson correlation was used for multivariate characterization of samples and to find specific links between observations (egg samples) and original variables (chemical

composition). The main goal of this analysis was to find similarities and dissimilarities between different groups of eggs to obtain a potential sample grouping according to a corresponding egg production system. Results of PCA were visualized by the projecting the observations onto biplot of principal components and original variables.

RESULTS AND DISCUSSION

Total proteins and lipid content, combined with elemental composition, are important factors indicating the basic nutritional values of the eggs. All these chemical components are distributed differently in yolk and albumen. Summary of the analysis of eggs, produced by different production facilities are presented in Table 1. Results are divided into three parts for whole egg samples and for dried yolk and albumen.

Proteins are probably most important dietary element of eggs and has a high level of digestibility in human organism **(Geissler and Powers, 2010)**. Differences in protein content (connected to housing systems) were found to be statistically significant (p = 0.007). Deeper analysis confirmed these differences only in albumen part of tested eggs. Highest average protein content was found in eggs from free range breeding (42.6 ±8.2 g.100g^{-1}). Almost similar results were observed in eggs from conventional cage facilities (42.5 ±2.9 g.100g^{-1}). Lowest concentration of total proteins was determined in eggs from home and organic systems (31.1 ±7.1; 31.1 ±7.1 g.100g^{-1}). Comparison of these results with other studies showed inconsistency in assessment of egg production systems influence on protein composition of eggs. **Filipiak-Florkiewicz et al. (2017)** and **Minelli et al. (2016)** reported that organic eggs contained more proteins that the eggs from conventional production. On the contrary, **Matt et al. (2009)** proved that conventional eggs contained higher amounts of proteins than organic. They tested eggs

Table 1 Mean values ±SD of chemical components of yolk and albumen. ANOVA p-value significance testing and Tukey`s test.

Variable	Housing system					
	Cage (n = 4)	Litter (n = 4)	Home (n = 4)	Organic (n = 4)	Range (n = 4)	*p*
			Yolk (dry)			
Ca (mg.g^{-1})	2.55 ±0.22	2.41 ±0.23	2.45 ±0.31	2.62 ±0.37	2.41 ±0.07	n.s.
K (mg.g^{-1})	1.63**b** ±0.1	1.8**b** ±0.12	2.13**a** ±0.09	1.68**b** ±0.1	1.40**c** ±0.02	***
Mg (mg.g^{-1})	0.16 ±0.03	0.14 ±0.007	0.15 ±0.006	0.13 ±0.02	0.12 ±0.01	n.s.
Na (mg.g^{-1})	1.09**a** ±0.18	0.90**ab** ±0.01	0.82**bc** ±0.03	0.76**bc** ±0.06	0.66**c** ±0.06	**
Fe (mg.g^{-1})	0.01**ab** ±0.001	0.01**ab** ±0.003	0.02**a** ±0.003	0.01**b** ±0.001	0.01**ab** ±0.004	*
P (mg.g^{-1})	2.45**ab** ±0.05	2.80**ab** ±0.15	2.78**ab** ±0.24	2.35**b** ±0.17	3.24**a** ±0.56	*
Zn (mg.g^{-1})	0.05**ab** ±0.002	0.04**ab** ±0.001	0.06**a** ±0.02	0.04**ab** ±0.001	0.03**b** ±0.003	*
Lipids (%)	52.5**ab** ±1.23	51.8**ab** ±1.41	55.2**ab** ±0.33	43.3**b** ±3.88	50.2**a** ±0.49	***
Proteins (%)	20.9 ±1.4	18.2 ±1.2	16.4 ±2.8	18.6 ±0.3	21.2 ±4.8	n.s.
			Albumen (dry)			
Ca (mg.g^{-1})	0.436 ±0.1	0.719 ±0.001	0.456 ±0.07	0.615 ±0.11	0.50 ±0.22	n.s.
K (mg.g^{-1})	2.14 ±0.2	2.40 ±0.36	2.05 ±0.02	2.33 ±0.18	2.02 ±0.1	n.s.
Mg (mg.g^{-1})	0.177**ab** ±0.03	0.142**a** ±0.02	0.203**bc** ±0.002	0.156**bc** ±0.01	0.132c ±0.01	**
Na (mg.g^{-1})	2.79 ±0.37	2.73 ±0.19	2.35 ±0.05	2.68 ±0.05	2.48 ±0.06	n.s.
P (mg.g^{-1})	0.066**b** ±0.01	0.043**b** ±0.01	0.056**b** ±0.01	0.134**a** ±0.05	0.056**b** ±0.01	**
Proteins (%)	22**a** ±1.5	17.8**ab** ±0.9	14.6**ab** ±4.3	13.6**b** ±3.4	21.1**a** ±3.4	**
			Whole (dry)			
Ca (mg.g^{-1})	2.99 ±0.32	3.13 ±0.234	2.91 ±0.382	3.23 ±0.481	2.91 ±0.292	n.s.
K (mg.g^{-1})	3.77**ab** ±0.297	4.2**b** ±0.477	4.18**b** ±0.021	4**ab** ±0.283	3.42**a** ±0.121	**
Mg (mg.g^{-1})	0.341**bc** ±0.059	0.28**ab** ±0.017	0.355**c** ±0.005	0.291**abc** ±0.029	0.252**a** ±0.015	**
Na (mg.g^{-1})	3.88**b** ±0.552	3.63**ab** ±0.2	3.17**a** ±0.085	3.44**ab** ±0.105	3.14**a** ±0.117	*
Fe (mg.g^{-1})	0.013**ab** ±0.004	0.012**ab** ±0.0001	0.015**b** ±0.002	0.008**a** ±0.0001	0.012**ab** ±0.002	*
P (mg.g^{-1})	2.51**a** ±0.052	2.85**ab** ±0.161	2.83**ab** ±0.247	2.48**a** ±0.221	3.3**b** ±0.571	*
Zn (mg.g^{-1})	0.047**ab** ±0.005	0.039**ab** ±0.003	0.058**b** ±0.017	0.039**ab** ±0.004	0.032**a** ±0.0001	*
Lipids (%)	52.5**bc** ±1.2	51.8**bc** ±1.41	55.2**c** ±0.331	43.3**a** ±3.9	50.2**b** ±0.491	***
Proteins (%)	39.4**c** ±2.9	33**a** ±2.1	28.5**abc** ±7.1	29.6**ab** ±3.7	39.12**bc** ±8.2	**

Note: ANOVA * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; n.s.: non significant. Letters **a.b.c.d** are groups obtained by Tukey pari test. Provided when ANOVA $p \leq 0.05$.

laid in winter season (January), which is similar to our study.

It could be assumed, that conventional cage system is providing stable condition during the year, but it is not supported by the results from another cage system – with litter bed. Eggs from this system contained approximately 10% less proteins than from standard cages. It is also necessary to mention the variability of production. Highest variability of results was observed in group of eggs produces in home breeding conditions and in free range facilities. It was highly expected for eggs produces in domestic conditions due to the high variability in feed and breeding conditions. Eggs from both cage systems on the other hand showed lowest standard deviation within the observations. Overall, the correlation between the housing systems and the protein composition of eggs is not easily feasible, unless the analysis is expanded by more detailed information about feed, age, breed, climatic and other conditions. As **Lordelo et al. (2016)** concluded, those parameters are difficult to control in systems based on free movement of hens (including organic). Most problematic is observation of amount of time the hen spends in exterior where they can feed on wild plants and insects.

Another statistically significant influence (p <0.0001) of production systems was found in total lipid content in egg yolk. Highest average amount of fat was found in yolk of home produces eggs (55.2 ±0.33%). On the basis of consultation with small farmers was found, that hens are mostly fed by corn, beet, boiled potatoes, sunflower, maize and fatty pastries. It is not an exception that chicken diet is supported by rest of the meat and the offal. All these feeds are rich in lipids, which was reflected in high amount in yolk. Beside the non-standard domestic production, the lipids were most abundant in eggs from both cage systems (52.5 ±1.23 and 51.8 ±1.41%). It is probably caused by minimal possibility of animal free movement and by specific and unified diet. Fodder mixtures in cage systems are based on corn and soybean enhanced by feed fat with minimal contribution of vegetables. As in the case of proteins, eggs from organic production contained significantly lower content of fat than other tested samples (43.3 ±3.9%). The fact, that eggs from free range system contained the second least fat content (50.2 ±0.49%) offers explanation in free movement of hens. Articles published in this field again showed various results in assessment of impact of egg production systems on total lipid content. Similar results, like in presented article, was found in research of **Lordelo et al. (2016)** with lowest amount of total fatty acid in eggs labeled as organic. **Matt et al. (2009)** also proved, that conventional eggs are better source of lipids. In opposite, there is a study from **Filipiak-Florkiewicz et al. (2017**), with the finding, that organically produced eggs contained 9.2% more fat than conventional. **Anderson (2011)** found higher fat content in the eggs from the range production environment than from the cage facilities. It was explained by the contribution of wild seeds and insects on feed composition of hens. This effect will be probably weaker in winter time of year which was the case of our study. **Samman et al. (2009)** discovered only little difference between the fatty acid composition of eggs and no differences in total lipid content of conventional and organic eggs. This result is supported by the study of **Hidalgo et al. (2008)** with non-significant differences between the eggs from cage, free range, barn and organic facilities.

Among mineral compounds, statistically significant differences (p <0.05) were found within all tested elements except for calcium which exhibit stable level among all the tested samples.

The most abundant element in whole eggs was potassium. Average concentration ranges from average 3.42 to 4.2 mg.g^{-1}. Highest average concentration of K was observed in eggs from domestic farms and in eggs from cage system supported with litter bed (4.18 ±0.021 and 4.2 ±0.477 mg.g^{-1}). Laying hens can feed on straw bed in cage, which is mostly from wheat. This kind of straw contains more than 10 g.kg^{-1} of potassium **(Plazonić et al. 2016)**. Lowest average concentration was determined in eggs from free range production (3.42 ±0.12 mg.g^{-1}). In comparison with alternative studies, **Filipiak-Florkiewicz et al. (2017)** and **Matt et al. (2009)** discovered relatively higher concentration of K in organic eggs compared to conventional.

Another significant difference (p = 0.015) in microelement group was found in average concentration of **s**odium. Differences were found, similarly to potassium, only in yolk part of eggs. Highest amount of Na was observed in eggs from both cage systems (3.88 ±0.552 and 3.88 ±0.552 mg.g^{-1}). According to Tukey`s test, organic eggs were also involved in this group. The group with low concentration of potassium included eggs from home and free-range systems (3.17 ±0.085 and 3.14 ±0.117 mg.g^{-1}). It is obvious, that eggs from hens with natural free movement contains lower amount of Na, which is probably utilized in larger quantities as an electrolyte in animal organism **(Lehninger et al., 2013)**.

Content of magnesium was also significantly affected by the egg production systems. Magnesium is mostly presented in egg shell, but it can be also found in edible part of egg. If we neglect non-standard domestic production, highest average concentration of Mg was determined in eggs from conventional cage production (0.341 ±0.059 mg.g^{-1}), but according to Tukey`s test, this set of eggs belong to same group with litter and organic samples (p = 0.05). It only differs from free range production with lowest average concentration (3.14 ±0.117 mg.g^{-1}). These results can be compared with work of **Filipiak-Florkiewicz et al. (2017)**. It was confirmed that yolk of conventionally produces eggs contained higher amount of magnesium. It is likely to be caused by the stable addition of mineral additives into the feed of hens housed in cages. However, **Küçükyılmaz et al. (2012)** did not found significant differences (p >0.05) between magnesium content in edible part of conventional and organic eggs. In terms of phosphorus content, it was most abundant in eggs from free range production, but with relatively high standard deviation (3.3 ±0.571 mg.g^{-1}). Lowest average of phosphorus concentration was determined in eggs from organic and conventional cage system (2.48 ±0.221 and 2.51 ±0.052 mg.g^{-1}).

Microelements are also dietary important components of eggs, especially Fe and Zn. These elements play a role in many metabolite pathways, redox reactions and are often part of metalloproteins and its bioavailability from eggs is similar like from meat and cereals **(Geissler and Powers, 2010)** According to analysis of variance, statistically

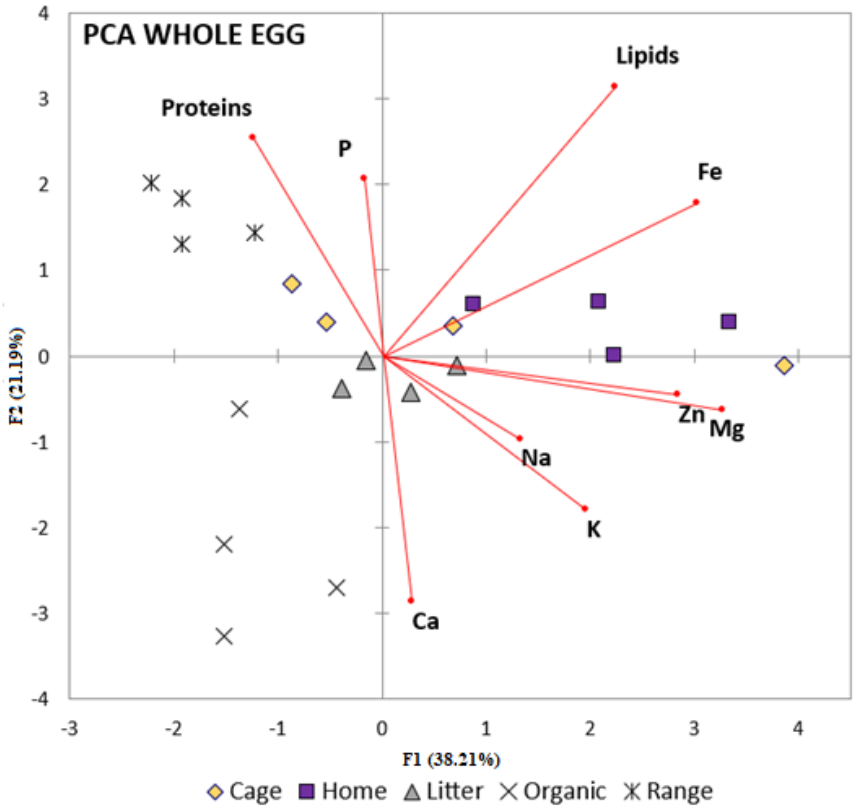

Figure 1 PCA biplot of whole egg observations with original variables projected into a 2-D factor plane of principal components F1 and F2.

significant differences were found (p = 0.019 for Fe and p = 0.014 for Zn).

Highest average concentration of iron and zinc, which are mostly presented in yolk of eggs, was found in eggs from home production (0.015 ±0.002 mg.g^{-1} Fe and 0.058 ±0.017 mg.g^{-1} Zn). This is probably caused by the fact, that domestically housed hens are often fed by animal diet based on meat and offal, which is rich source of iron and zinc. Differences between other 4 standard production systems did not exhibit significant differences (p >0.05). **Küçükyılmaz et al. (2012)** and **Filipiak-Florkiewicz et al. (2017)** determined higher content of iron in conventionally produced eggs compared to organic. Cages and its corrosion can be source of iron and zinc, which is one of probable causes of this phenomenon.

As an addition to univariate data analysis by ANOVA and Tukey`s test, multivariate classification of samples was evaluated by Pearson correlation PCA. Results of this analysis are presented in Figure 1 as a biplot of observation and variables from whole egg data (sum of yolk and albumen content) projected into 2-D plane of principal components F1 and F2. Dimension of 9 input variables was reduced to 4 principal components with eigenvalue >1 according to Kaiser`s criterion: F1 (32.75%) F2 (20.26%), F3 (17.98%) and F4 (11.86%). These components together carried 82.85% of variability. Best visual representation of PCA was obtained by 2-D factor plane of components F1 and F2. Components were correlated with original variables. Component F1 was mostly positively correlated by Mg, Fe, Zn. Component F2 positively with total lipid and proteins content, negatively with Ca. Relative clustering of observations is visible on Figure 1. There is obvious separation of egg samples from organic and free-range production, which are situated in left hemisphere of the graph with negative score for component F1. Observations from both cage systems are situated in the centre of graph and not completely differentiated which points to the fact, that overall composition of eggs from these two systems did not differentiated significantly. Another separate group was formed by observation of home-produced eggs in area of positive scores for F1. It is obvious, that even though univariate analysis by ANOVA did not provide clear picture of impact of hens housing systems on composition of eggs, it was possible to achieve classification using multivariate approach.

CONCLUSION

Results of this study confirmed the initial hypothesis and proved, that different approaches of laying hens housing can be significantly reflected in basic chemical composition of eggs. Statistical differences ($p \leq 0.05$) were defined in 8 from 9 tested parameters. It was found that eggs from alternative production systems did not generally exhibit higher nutritional value than eggs from conventional cage facilities. In the case of total protein, whole conventional eggs contained highest average amount of 39.4 ±2.9% in comparison of 29.6 ±3.7% in organic eggs. Another significant differences (p <0.0001) were found in total lipid content. Organic eggs proved to be least fatty (43.3 ±3.9%), approximately 18% less than eggs from conventional cage production (52.5 ±1.2%). This was probably closely connected with limited free movement of lying hens in cage production. This system is also characteristic with diet based mainly of corn and soybean,

which is rich on lipids. Another significant difference was found in elemental composition of eggs, specifically K, Mg, Na, Fe, P, Zn. Thanks to the combination of elemental composition and total lipid and protein content was possible to obtain clustering of observations by multivariate PCA. All tested groups of production systems were clearly separated, and it proved its important influence on chemical composition of eggs.

Important aspect of this work was comparison of presented results with other studies in this field. Common conclusion of all these studies is confirmation of significant effect of different housing systems on chemical composition and quality aspects of eggs. However, evaluation of influence of production systems, in many cases, differs considerably between studies and it was not possible to define general conclusions. For the unification of results will be necessary to expand research by more complex data about feed, climatic and other conditions and study the production in long term.

From the point of view of the consumers, these differences were in many cases negligible and the motivation to buy pricier alternative products should be still more driven by ethical and psychological aspects than desire for healthier and more nutritious product.

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IDENTIFICATION AND ANTIBIOTIC SUSCEPTIBILITY OF BACTERIAL MICROBIOTA OF FRESHWATER FISH

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ABSTRACT

The fish meat is an essential part of human diet. However, fish may be contaminated with different microorganisms, including pathogens. Antimicrobial resistance of fish microbiota may facilitate the spread of resistant microorganisms causing serious consequences for human health. The aim of the present study was to detect bacterial contamination in fish gill, gut and skin and to determine antimicrobial susceptibility of the bacterial isolates. Rainbow trout (*Oncorhynchus mykiss*) and bream (*Abramis bram*) were obtained from the market in Jelgava city. Chub (*Leuciscus cephalus*), crucian carp (*Carassius carassius*) and tench (*Tinca tinca*) were collected from fishermen. Fish samples were examined for the total bacterial count (TBC), coliforms, *Enterobacteriaceae*, *Pseudomonas* spp. and *Aeromonas* spp. Testing was done in accordance with International Organization for Standardization (ISO) standards. Identification of all bacteria was accomplished with the Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) method. The disc diffusion method was used for the detection of antibiotic susceptibility of isolated bacterial species. TBC ranged from 2.70 to 7.00 log CFU.g^{-1}, coliforms from 0 to 2.67 log CFU.g^{-1}, *Enterobacteriaceae* from 0 to 2.85 log CFU.g^{-1}. The highest contamination with *Pseudomonas* spp. and *Aeromonas* spp. was observed in chub gut samples with 1.60 log CFU.g^{-1} and 2.23 log CFU.g^{-1}, respectively. Altogether, 16 microbial genera and 31 bacterial species were identified. The dominant bacterial species belonged to *Pseudomonas* spp. (54%) and *Enterobacteriaceae*. *Pseudomonas* spp. were resistant to ticarcillin, susceptibility to ciprofloxacin showed 88% of isolates. All *Enterobacteriaceae* isolates were susceptible to imipenem. The microbial quality of the fish was acceptable, but the presence of antibiotic resistant bacteria may further cause a negative impact on public health.

Keywords: bacteria; freshwater fish; MALDI-TOF MS; antibiotic

INTRODUCTION

The bacterial contamination of freshly caught fish depends to a great extent on the quality of surrounding water. The microbiota of fish is very diverse, its composition and amount can be influenced by many different factors, such as microbial population of water and bottom mud, water source type, fish species and the conditions of their habitat. The majority of microorganisms in fish were located in gills, gut and in mucus on the fish skin, while the internal organs and muscle tissue were relatively sterile **(Austin, 2006; Cwiková, 2016)**.

Enterobacteriaceae and coliforms are indicator microorganisms that are permanently present in the intestine of humans and animals. They are not harmful to the host's organism, but under the appropriate conditions can cause a disease. The presence of indicator microorganisms in external environment indicates the contamination with human or animal faeces and could be detected with quantitative isolation of indicator microorganisms. The contamination rates are important for microbiological safety because of ability to indicate the presence of human and animals' pathogens of intestinal origin. *Enterobacteriaceae* are widespread in the nature and can be found in soil, water, fruits, vegetables, grains, flowering plants and trees **(Tosun, Üçok Alakavuk and Mol Tokay, 2016)**. *Salmonella*, *Shigella*, *Yersinia*, *Escherichia* of *Enterobactericeae* can cause intestinal foodborne infections and are transmitted by the fecal-oral or oral route with contaminated water, food or due to direct contact. Other *Enterobactriaceae* may serve as an opportunistic pathogens and *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus*, *Providencia*, *Serratia* were reported to cause infections with non-intestinal clinical signs – bacteraemia, meningitis, wound infections, genitourinary and respiratory tract injuries **(Flores-Tena et al., 2007; Dekker and Frank, 2015)**. The studies on composition of fish microbiota has been conducted in different countries but mostly the sea fish were studied **(Yagoub, 2009; Oliveira, Oliveira and Pelli, 2017)**.

Antimicrobial resistance is one of the most serious threats to public health, environmental health and food safety. Antimicrobial resistance is increasing to threateningly high levels throughout the world.

Antimicrobial resistance reduces the effectiveness of treatment of infections in humans and animals, contributes to the increase in morbidity, mortality and leads to significant economic losses **(Petersen et al., 2002; Wamala et al., 2018)**. The use of antibiotics in aquaculture for therapeutic and preventive purposes is a growing problem for world livestock and environmental health. From aquaculture, the antimicrobials may be spread in the environment in the concentration sufficient to cause microbial imbalance in animals and human with the emergence of antimicrobial resistance in the society. It has been proved that the antibiotic-resistant bacteria may be present in fish with their subsequent transfer to humans via the consumption of fish or contaminated environment **(Heuer et al., 2009)**.

The consumption of the fish in the EU is continuously increasing, however, the fish products were frequently associated with gastrointestinal infections in humans **(EFSA and ECDC, 2017)**. Nowadays, people tend to follow a healthy lifestyle by choosing fresh foods or food with optimal nutritional value like fish and fish products, which are known as the source of protein, contain omega-3 amino acids, vitamins and minerals such as phosphorus. However, the risk for consumers related to the eating of fish contaminated with non-pathogenic and pathogenic microflora with variable antimicrobial resistance rates is concerning **(Da Silva et al., 2010; Cwiková, 2016)**. Therefore, the aim of the present study was to detect bacterial contamination in fish gill, gut and skin and to determine antimicrobial susceptibility of the bacterial isolates.

Scientific hypothesis

The scientific hypothesis of this study was that the freshwater fish were contaminated with bacteria, and the bacterial isolates are resistant to antibiotics.

MATERIAL AND METHODOLOGY

Selection of samples

Altogether, 15 fish samples were collected, including 2 rainbow trout (*Oncorhynchus mykiss*) and 3 bream (*Abramis brama*) samples were obtained from the market and 3 chub (*Leuciscus cephalus*), 4 crucian carp (*Carassius carassius*) and 3 tench (*Tinca tinca*) were collected from the fishermen in June 2017 in Jelgava. Fish were caught in the river Lielupe. All fish were placed in the sterile polyethylene sampling bags, and transported n ice to the laboratory. The investigations were initiated within 2 h after deliver to the laboratory.

Sampling

Aseptically, 1 g of sample was taken from each fish skin, gills and gut. Skin samples were taken from the lateral line of the fish. Operculum was opened, and gills were dissected for preparation of gill samples. Gut samples were taken by cutting of the abdomen till the anal fin and after opening the body cavity.

Bacteriological analyses

The sample suspension of 1:10 with 0.1% peptone water (OXOID, UK) was used for bacteriological testing. An amount of 1 mL was plated onto Tryptone Soy Agar (TSA, OXOID, UK) for detection of total bacterial counts. After incubation at 30 °C for 72 h, all colonies were counted. For the detection and enumeration of coliforms, the sample suspension was plated onto Violet Red Bile Lactose Agar (VRBL, OXOID, UK). Inoculated agars were incubated at 37 °C for 24 h and all typical colonies from dark red to deep purple coloured colonies were counted **(ISO 4832:2006)**. MacConkey agar (MAC, OXOID, UK) were used for detection of *Enterobacteriaceae* and inoculated agars were incubated at 37 ºC for 24 h, and after that the typical colonies were enumerated - lactose fermenting bacteria produced red to pink and non-lactose fermenting bacteria have colourless and transparent colonies **(ISO 21528:2017)**. Pseudomonas CFC Selective Agar (OXOID, UK) were used for detection and enumeration of *Pseudomonas* spp. **(ISO 13720: 2010)**. Agar plates were incubated at 30 °C to 48 h and examined for the presence of colonies. All grown colonies were counted. Aeromonas Agar (Ryan, OXOID, UK) was used for detection of *Aeromonas* spp. The plates were incubated at 37 °C for 24 h, after that were examined for the presence of dark green, opaque colonies with dark centres.

Identification of bacterial species with MALDI-TOF Biotyper MS

MALDI-TOF Mass Spectrometry model Microflex LT/SH biotyper (Bruker Daltonics, Germany, Bremen) was used for identification of bacteria species isolated from fish. Typical bacterial colonies were selected from all agars, picked up and suspended in 300 µL of sterile distilled water and mixed. Then, a 900 µL of absolute ethanol (99%, Sigma-Aldrich, USA) was added. The solution was centrifuged at 13 000 × g for 2 min. Supernatant was removed, ethanol pipetted, and the pellet was allowed to dry at a room temperature. At first, the pellet was added and mixed with formic acid (10 µL, 70%) and then with acetonitrile (10 µL, 100%). The solution was centrifuged at maximum speed for 2 min. The supernatant was placed on a polished MALDI plate (Bruker Daltonics, Germany) and after drying a 1 µL of the matrix solution (HCCA: α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany), 50% acetonitrile with 0.025% trifluoroacetic acid (TFA) (100%, Sigma-Aldrich, USA)) was added in the spots. Sample processing was performed with MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics) using the MALDI Biotyper software package (version 3.0) and data were obtained with Realtime Classification software (RTC) (Bruker Daltonics). Data processing was carried out by Biotyper software, where sample mass spectrum was compared with the reference mass spectrum and the score values were calculated.

Antibiotic susceptibility testing of bacterial isolates

Antimicrobial resistance of *Pseudomonas* and *Enterobacteriaceae* bacteria confirmed with the MALDI TOF MS Biotyper (Brucker Daltonics) was tested with the disc diffusion method. Suspension of bacterial isolates in Mueller Hinton broth was placed onto Mueller Hinton agar (MHA, OXOID, UK). The antimicrobial discs were placed on the MHA (OXOID, UK) surface after the inoculation, agars were allowed to dry out at room temperature and

were incubated at 37 °C for 24 h. *Pseudomonas* spp. isolates were tested against ticarcillin (75 μg), cefotaxime (30 μg), ciprofloxacin (10 μg), imipenem (10 μg) and doripenem
(10 μg) (Oxoid, UK). *Enterobacteriaceae* were tested against ticarcillin (75 μg), cefepime (30 μg), ciprofloxacin (10 μg), imipenem (10 μg) and tobramycin (10 μg) (Oxoid, UK). Antimicrobial susceptibility testing was performed according to the CLSI guidelines and the results were interpreted in accordance with EUCAST breakpoint tables **(EUCAST, 2018)**.

Statistical analyses

Statistical analyses were performed by R software, version 3.4.3, for data management RStudio was used. Bacterial counts were expressed in decimal logarithms. T-test was used for calculating differences among the total bacterial count (TBC), coliforms and *Enterobacteriaceae* in fish gills, gut and skin samples. The one-way analysis of variance (ANOVA) was used to detect significant differences between the bacterial contamination rates of fish gills, gut and skin samples (p <0.05).

RESULTS AND DISCUSSION

TBC ranged from 2.70 to 7.00 log CFU.g^{-1} in all fish samples. The highest TBC was detected in gut of bream and tench, while the lowest in rainbow trout skin (Table 1). Coliforms were not detected in crucian carp gill and tench skin samples, while the highest numbers of coliforms were found in crucian carp and tench gut with 2.67 and 2.51 log CFU.g^{-1}. The highest *Enterobacteriaceae* counts of 2.85 and 2.75 log CFU.g^{-1} were identified in bream gill and crucian carp gut, while *Enterobacteriaceae* were not isolated from the chub gill, bream skin and tench gill and skin samples. *Pseudomonas* spp. and *Aeromonas* spp. were not found in rainbow trout, crucian carp and tench samples. The highest contamination with *Pseudomonas* spp. was observed in chub gill and gut samples with 1.46 and 1.60 log CFU.g^{-1}. The most contaminated fish species with *Aeromonas* spp. was the chub – 1.93 log CFU.g^{-1} in gill, 2.23 log CFU.g^{-1} in gut and 0.60 log CFU.g^{-1} in skin.

The microbiological criteria as total bacterial count (TBC) and *Enterobacteriaceae* are applied widely to ensure the microbiological quality and safety of foods. The TBC shows the general level of contamination and the shelf-life stability while the coliforms and *Enterobacteriaceae* indicate the presence of faecal contamination and possible pathogens in foods **(Tortorello, 2003)**. In the present study, the TBC were in line with **Eizenberga et al. (2015)** reported for freshwater fish from Usmas lake in Latvia with gill, skin and gut contamination rates from 1.26 to 8.08 $\log_{10}$ CFU.g^{-1}, from 1.04 to 8.61 $\log_{10}$ CFU.g^{-1} and from 1.45 to 7.36 $\log_{10}$ CFU.g^{-1}, respectively. However, our results were lower than **Terentjeva et al. (2015)** stated for bream obtained from Usmas lake in Latvia with 5.48 log CFU.g^{-1} for skin contamination with TBC and 7.24 log CFU.g^{-1} with *Enterobacteriaceae*.

Aeromonas spp. are distributed in freshwater habitats worldwide. **Stratev, Vashin and Daskalov (2015)** found the contamination with *Aeromonas* spp. in cooled rainbow trout and trout fillets at retail markets. **Abd-El-Malek (2017)** reported that the raw fish in Egypt were contaminated with *Aeromonas* spp. and majority of the isolates were identified as *A. hydrophila*. This bacterium is pathogenic not only for humans but also for fish and is commonly found in water. Inhabited in an aquatic environment, *A. hydrophila* is reported to be the cause of secondary infection of wounds, sepsis, cellulitis, pneumonitis, gastroenteritis and necrotizing fasciitis. **(Hassan and Farag, 2006)**. High prevalence psychrophilic bacteria, especially proteolytically active microorganisms

Table 1 Bacterial contamination of freshwater fish.

Fish species	Sampling site	TBC log CFU.g^{-1}	Coliforms log CFU.g^{-1}	*Enterobacteriaceae* log CFU.g^{-1}	*Pseudomonas* spp. log CFU.g^{-1}	*Aeromonas* spp. log CFU.g^{-1}
Rainbow trout (*Oncorhynchus mykiss*)	Gills	3.16[a]	1.52	2.03	0	0
	Gut	3.37[b]	1.04	1.68	0	0
	Skin	2.70[a]	1.43	2.25	0	0
Chub (*Leuciscus cephalus*)	Gills	3.58[a]	2.11	0	1.46[e]	1.93[e]
	Gut	2.97[b]	1.77	2.35[d]	1.60	2.23
	Skin	3.18[a]	0.30	0.78	0	0.60
Bream (*Abramis bram*)	Gills	3.15[a]	0.60	2.85	0.3	0
	Gut	7.00[b]	1.78[c]	2.36[d]	0	0
	Skin	3.23[a]	0.30	0	0	1.38
Crucian carp (*Carassius carassius*)	Gills	3.08[a]	0	1.18	0	0
	Gut	3.18[b]	2.67[c]	2.75[d]	0	0
	Skin	2.74[a]	0.30	1.30	0	0
Tench (*Tinca tinca*)	Gills	3.39[a]	1.04	0	0	0
	Gut	7.00[b]	2.51[c]	2.60[d]	0	0
	Skin	2.79[a]	0	0	0	0

Note: [a] no significant differences between TBC in tested fish gill and skin samples were observed (p >0.05);
[b] TBC in bream and tench gut was significantly higher than in gut of rainbow trout, chub and crucian carp (p <0.05);
[c] coliform counts in bream, crucian carp and tench gut were significantly higher than in gill and skin samples (p <0.05);
[d] *Enterobacteriaceae* counts in chub, bream, crucian carp and tench gut samples were significantly higher than in gill and skin samples (p <0.05);

– *Pseudomonas* spp. and *Aeromonas* spp. can predispose fish to a more rapid process of microbiological deterioration of fish meat quality **(Cipriano and Dove, 2011; Larsen, 2014)**.

Tested fish bacterial microbiota contained 16 microbial genera, from which the most abundant were *Pseudomonas* spp. (54%) (Figure 1). *Shewanella* spp. (5%) and *Serratia* spp. (5%) were also among the most frequently isolated. Other genera isolated were *Escherichia* spp., *Lelliottia* spp., *Leclercia* spp., *Bacillus* spp., *Citrobacter* spp. and *Rahnella* spp., *Morganella* spp., *Acinetobacter* spp., *Achromobacter* spp., *Aromatoleum* spp., *Burkholderia* spp., *Arthrobacter* spp. and *Staphylococcus* spp.

Our results agreed with **Kim, Brunt and Austin (2007)**, who found *Aeromonadaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* to be the most abundant microflora in rainbow trout intestine samples. Our study confirms the findings of **Yagoub (2009)** who isolated similar bacteria genera from fish samples.

Bacterial species isolated from freshwater fish gill, gut and skin samples are shown in Table 2. Overall, 31 bacterial species were identified with MALDI-TOF mass spectrometry. The gill microbiota was more diverse than the microbiota of gut and skin with only some *Pseudomonas* spp. were present in fish skin. *Pseudomonas proteolytica*, *P. brenneri*, *P. cedrina*, *P. veronii* and *Lelliottia amnigena* were dominant bacteria species isolated from gills and skin. Intestinal bacterial microbiota consisted of species, which were specifically found in gut – *Arthrobacter monumenti*, *P. grimondii*, *P. gessardii*, *P. synxantha*, *P. libanensis*, *P. tolaasii*, *Achromobacter xylosoxidans*, *Aromatoleum alkani*, *Burkholderia thailandensis*.

In previous studies, the skin and gill microbiota were evaluated together due to influence of water pollution on the fish microflora **(Larsen, 2014)**. **Fernandes (2009)** found that the Gram-negative bacteria were predominant. Several genera as *Bacillus* spp., *Pseudomonas* spp., *Leclercia* spp., *Acinetobacter* spp., *Citrobacter* spp., *Achromobacter* spp., *Escherichia* spp., *Serratia* spp., *Rahnella* spp. and *Staphylococcus* spp. found in our study, have been previously associated with microbiota of gill, gut and skin **(Austin, 2006)**.

Pseudomonas spp. are widespread in nature and have been isolated from aquatic environment and fish. Some species can cause a negative effect on animal and human health, eg. *P. fluorescens* is a potential pathogen that can influence the physiological processes of neurons **(Picot et al., 2001)**. Other pseudomonas can cause diseases in fish and contribute the spoilage processes of fish meat. *Pseudomonas* spp. were considered to be relatively resistant to antibiotics **(Kačániová et al., 2017)**. The members of the *Shewanella* spp., specially *S. baltica* were described in spoilage of chilled marine products **(Ge et al., 2016)**. In agreement with **Starliper (2001)** and **Aydin, Erman and Bilgin (2011)**, *Serratia liquefaciens* is a potential fish pathogen, which can lead to high mortality, causing economic losses. **Altun et al. (2013)** refers about pathogenicity of *Citrobacter braakii* isolated from rainbow trout in Bursa. *Leclercia adecarboxylata* infection was rarely reported in humans and the infection were described in patients with impaired immunity. *L. adecarboxylata* is expected to be the pathogen related to the aquatic environment **(Keren et al., 2014)**. *R. aquatilis* is prevalent in the environment and has previously been isolated from various water reservoirs, soil, clinical specimens and foodstuffs. *R. aquatilis* is an opportunistic pathogen that can cause a variety of serious gastrointestinal, urinary, respiratory and cardiovascular diseases. The consequences can be even dangerous for human life **(Alikunhi et al., 2017)**. *Bacillus cereus* is widespread in nature, it causes two types of food-borne diseases: toxicosis caused by previously produced toxin and toxicity caused by bacterial cells that produce enterotoxins in the small intestine

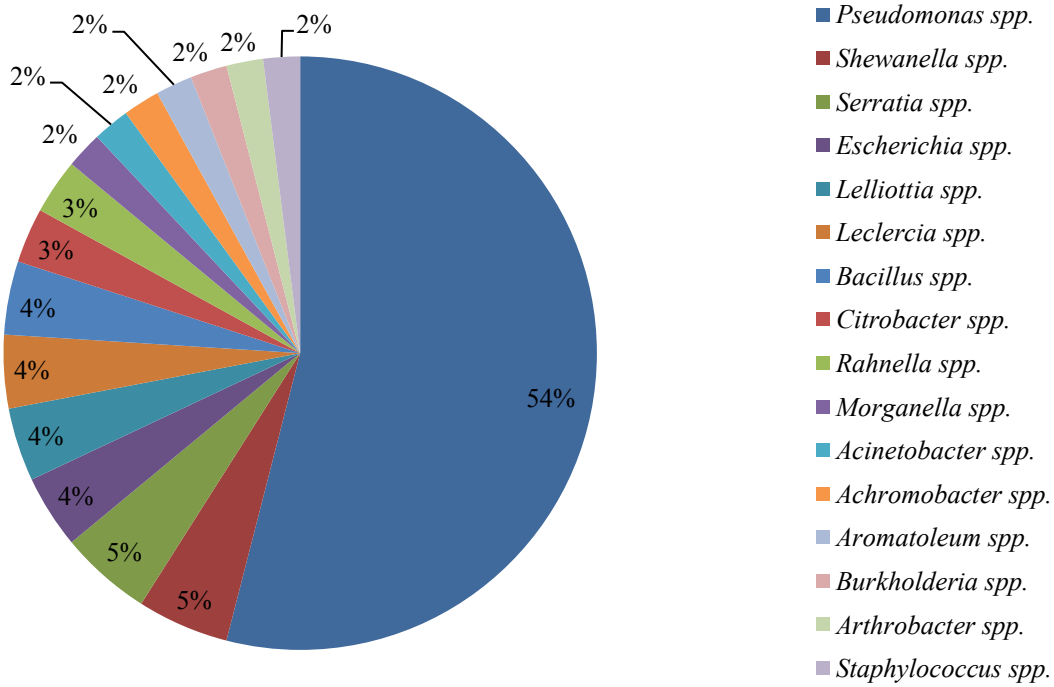

Figure 1 Microorganisms isolated from freshwater fish.

Table 2 Microorganisms wre isolated from gills, gut and skin of freshwater fish.

Bacterial species		
Gills	Gut	Skin
Lelliottia amnigena *Escherichia vulneris* *Pseudomonas proteolytica* *Pseudomonas rhodesiae* *Pseudomonas fluorescens* *Pseudomonas gessardii* *Bacillus cereus* *Pseudomonas putida Pseudomonas brenneri Pseudomonas cedrina* *Pseudomonas koreensis* *Pseudomonas veronii* *Shewanella profunda* *Shewanella baltica* *Rahnella aquatilis* *Leclercia adecarboxylata* *Citrobacter braakii*	*Arthrobacter monumenti* *Pseudomonas grimondii* *Pseudomonas fluorescens* *Pseudomonas gessardii* *Pseudomonas synxantha* *Pseudomonas libanensis* *Pseudomonas tolaasii* *Achromobacter xylosoxidans* *Aromatoleum alkani* *Burkholderia thailandensis*	*Pseudomonas brenneri* *Pseudomonas proteolytica* *Pseudomonas cedrina* *Pseudomonas orientalis* *Acinetobacter tjembergiae* *Morganella morgani* *Serratia rubidaea* *Staphylococcus equorum* *Lelliottia amnigena* *Serratia liquefaciens* *Pseudomonas veronii*

Table 3 Antibiotic susceptibility of *Pseudomonas* spp. isolates from fish samples.

Bacterial isolates	No. of isolates	TIC	FEP	CIP	IMP	DOR
		No. of resistant isolates (%)				
Pseudomonas grimondii	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas putida	1	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)
Pseudomonas fluorescens	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
Pseudomonas synxantha	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas libanensis	2	2 (100)	2 (100)	0 (0)	2 (100)	2 (100)
Pseudomonas tolaasii	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Pseudomonas taetrolens	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)

Note: TIC: ticarcillin; FEP: cefepime; CIP: ciprofloxacin; IMP: imipenem; DOR: doripenem.

Table 4 Antibiotic susceptibility of *Enterobacteriaceae* isolates from fish samples.

Bacterial isolates	No. of isolates	TIC	CTX	CIP	IMP	TOB
		No. of resistant isolates (%)				
Lelliotia amnigena	2	2 (100)	0 (0)	1 (50)	0 (0)	1 (50)
Escherichia vulneris	2	2 (100)	1 (50)	0 (0)	0 (0)	1 (50)
Staphylococcus equorum	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Rahnella aquatillis	1	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)

Note: TIC: ticarcillin; CTX: cefotaxime; CIP: ciprofloxacin; IMP: imipenem; TOB: tobramycin.

(Rasool et al., 2017). *B. cereus* was isolated from fish in India and tropical fish from Shivajinagar area of Bangalore, India **(Rasool et al., 2017; Prasad, 2014)**.

Emetic disease caused by *B. cereus* associated with tuna fish consumption was reported **(Doménech-Sánchez et al., 2011)**. *Escherichia vulneris* was originally isolated from human skin lesions, but recently the microorganism was isolated from water and fish. *E. vulneris* is present in the intestinal tract of animals, so the bacterium can cause environmental pollution **(Aydin, Celebi and Akyurt, 1997)**. Proportion of the microorganisms isolated in this study can contribute to the spoilage processes of fish and also act as infectious agents for fish and consumers.

All *Pseudomonas* species isolated from freshwater fish were resistant to ticarcillin (Table 3). *P. grimondii*, *P. fluorescens*, *P. synxantha*, *P. tolaasii*, *P. taetrolens* and *P. gessardii* were susceptible to cefepime (64%). Susceptibility to ciprofloxacin showed 91% of the isolates. Resistance to imipenem demonstrated 73% of the isolates. Most of the isolates (91%) except *P. taetrolens* were resistant to doripenem. *P. taetrolens* was sensitive to antibiotics including cefepime, ciprofloxacin, imipenem and doripenem. This study shows the high prevalence of antibiotic-resistant *Pseudomonas* spp. strains in fish. Our results agreed with **Kačániová et al., 2017** about occurrence of antibiotic-resistant *Pseudomonas* spp. in freshwater fish. Some antimicrobials were effective against *Pseudomonas* spp. in **Kholil et al. (2015)** study, who showed *Pseudomonas* spp. susceptibility to ciprofloxacin. Oxytetracycline, tetracycline and ciprofloxacin were found to be effective against *Pseudomonas* spp. also by **Mastan (2013)**.

Results on antimicrobial resistance of *Enterobacteriaceae* from fish samples are shown in Table 4. *L. amnigena* isolates were resistant to ticarcillin, however, showed susceptibility against other antibiotics. *E. vulneris* showed 100% resistance to ticarcillin and 50% to cefotaxime and tobramycin. *Staphylococcus equorum*

were resistant only to cefotaxime. *R. aquatillis* showed resistance to ticarcillin, cefotaxime, imipenem and tobramycin. Totally, 83% of isolates expressed susceptibility to ciprofloxacin with only one isolate of the *L. amnigena* was resistant. Susceptibility to imipenem was 83%, but resistance to cefotaxime was 50% among *Enterobacteriaceae* isolates. Resistant *Enterobacteriaceae* were found in water sources previously **(Guyomard-Rabenirina et al., 2017)**. **Stock and Wiedemann (1999)** reported *E. vulneris* was the species the most susceptible to ticarcillin. The antimicrobial resistance become emerging problem with international organizations and the EU institutions have recognized that the development of antimicrobial resistance raises severe consequences for human and animal health and well-being **(Singer et al., 2016)**. The present study showed the occurrence of resistant bacteria in freshwater fish.

CONCLUSION

The results of our study confirm that freshwater fish are contaminated with bacteria, but in general, the microbial quality of freshly caught fish is acceptable. Fish gill, gut and skin microflora was very diverse with *Pseudomonas* spp. and *Enterobacteriaceae* were the most abundant. Groups of microorganisms. Our results confirm that antibiotic resistant bacteria could be found in fish that represents possible public health consequences.

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ANTIOXIDANT PROFILE OF MULLED WINE

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ABSTRACT
The aim of the study was to compare chemical and nutritional profile of wine and heat-treated wine, called mulled wine. The experiment was focused on simulation of ordinary produce mulled wine by the majority of consumers. Cabernet Moravia (bottled in Velkobílovická vína s.r.o., Czech Republic) was used for the experimental production of mulled wine. Following spices were added to wine during cooking: cloves (Vitana, Czech Republic) and cinnamon (KOTÁNY, Austria). The samples of wine were heat treated in stainless steel pot for 5 minutes. The relative density, acidity, alcohol content, phenol content and antioxidant capacity were monitored in experimentally produced wine and mulled wine. The gained results showed that samples of mulled wine with added cloves had statistically significant (p <0.05) higher phenol content and higher antioxidant properties in comparison with wine before heat treatment and spices addition. The results clearly showed that mulled wine can be considered as the product with better health beneficial nutritional profile than wine from which it is produced; in addition, mulled wine sample had significantly (p <0.05) lower alcoholic content (8.27 ±0.04 vol.%).

Keywords: Cabernet Moravia; mulled wine; spice; clove; cinnamon; antioxidant

INTRODUCTION
Consumers interest in the binominal diet (diet related to health) has been constantly increasing due to clear evidences how specific dietary patterns can reduce a chronic diseases development **(Zorraquin-Peña et al., 2019)**. The most often diet connected with health benefits is the Mediterranean diet and the most common food commodity in this type of diet is wine **(Chiva-Blanch et al., 2013; Artero et al., 2015)**. Wine health benefits are mostly connected with high polyphenols content and their positive influence on human health, beside organoleptic properties **(Snopek et al., 2018; Zorraquin-Peña et al., 2019)**.

Nowdays, wine is broadly consumed and used in culinary preparation. Wine is very often thermally threated when it is added to certain meals. It is also consumed as a warm beverage with addition of spices. This popular drink is called mulled wine and its physical-chemical properties are changed due to processing **(Mudnić et al., 2011)**. It is well known that high phenol content means food with higher antioxidant activity due to phenols ability to donate hydrogen. The role of phenols in so complex system such as food is hard to predict due to the presence of other antioxidants, polyphenols, oxidative enzymes, metals, etc. This statement is crucial for food that is fortified with different polyphenols and it means that each food fortifcation should enclude a specific experimental study **(Pinelo et al., 2004)**. It was also found that thermally treated wine still posses high antibacterial properties **(Boban et al., 2010)**. Thermal treatment of wine even at lower temperatures, such as 45 ºC during 20 days, also can make changed in wine chemical and sensorial properties. It was found that this kind of treatment significantly changed floral character of the wines and increased aromas described as oak, honey and smoky **(Leino et al., 1993)**.

Cabernet Moravia is a wine variety that was made by crossing of Zweigeltrebe and Cabernet Franc. It is grown mostly in Moravian region in the Czech Republic. This wine variety contains a high content of anthocyanins, pigments and polyphenols content **(Balík and Kumšta, 2008; Bajčan et al., 2016)**.

The most often used spices for the preparation of mulled wine are cloves (*Eugenia caryophyllata*) and cinnamon (*Cinnamomum cassia*). Cloves are already known for their medical usage and antimicrobial activities. Cloves are also known to serve as preservative during shelf life of different food products **(Santin et al., 2011; Sukorini, Sangchote and Khewkhom, 2013)**. Cinnamon (*Cinnamomum cassia*) has been the one of the most commonly used spice since 2800 BCE. Cinnamon used as a spice has many beneficial effects encompassing antioxidant, antiinflamatory, antidiabetic, antibacterial and anticancer **(Ben-Arfa et al., 2019)**.

Scientific hypothesis

Mulled wine is better solution for consumers than heat not treated wine without spices addition.

The aim of the study was to evaluate heat treated wine (mulled wine) with and without spices addition and compare their antioxidant profile and chemical-physical parameters.

MATERIAL AND METHODOLOGY

The mulled wine was prepared from Moravian Country wine, dry red wine Cabernet Moravia (bottled in Velkobílovická vína s.r.o., Czech Republic). The mulled wine was made with the addition of spices: clove (*Eugenia caryophyllata*) (Vitana, Czech Republic, batch: L1104182) and cinnamon (*Cinnamomum cassia*) (KOTÁNY, Austria, batch: L327392111511). The addition of spices was done according to the Table 1.

The samples 2 to 5 were boiled in stainless steel pot during 5 minutes. In the samples 3 to 5 the spices **(**clove/cinnamon**)** were added before boiling (Table 1). The sample 1 was control sample including only wine (Cabernet Moravia).

The relative density was measured with the use of capillary tube pycnometers (capacity: 10 mL). The samples' weights were measured in analytical balance with 0.0001 g precision **(Cepeda and Villarán, 1999)**. The blank sample was distilled water and it was measured according to the following equation:

*ρv = (mpv – mpp)/Vp

*mpv: weight of pycnometer filled with wine sample; mpp: weight of empty pycnometer; Vp: volume of the pycnometer

The pH of each sample was measured by the pH meter GRYF 259 (GRYF HB, Czech Republic) with electrode PCL 124 (GRYF HB, Czech Republic).

The determination of titratable acidity was measured by the titration of sample by 0.1 M NaOH with the bromthymol blue as an indicator up to the color change to green. The concentration of titratable acid was calculated as the content of tartaric acid ($g.L^{-1}$) by formula: TA = a·0.75; where a is the volume of 0.1 M NaOH. This method is recommended by the Compendium of International Methods of Wine and Must Analysis **(OIV, 2009)**.

The determination of alcohol was measured by Ebulliometer 160450T (Laboratories DUJARDIN-SALLERON, France).

The total polyphenols content (PCA) was measured with the Folin-Ciocalteau solution diluted by water (1:10) and Na_2CO_3 (75 $g.L^{-1}$) by **Talcott, Howard and Brenes (2000)** with slightly modification. The sample of wine was diluted 100 times and then 1 mL was used for analysis. 5 mL of Folin-Ciocalteau solution and 4 mL Na_2CO_3 and then the sample was incubated in dark for 30 minutes. The absorbance was measured at 765 nm and the gallic acid was used as the standard **(Talcott, Howard and Brenes, 2000)**.

The determination of FRAP – ferric reducing/antioxidant power was measured at the absorbance of 593 nm. FRAP reagent was prepared by mixing 10 volumes of 300 $mmol.L^{-1}$ acetate buffer with 1 volume of 10 $mmol.L^{-1}$ TPTZ (2,4,6-tripyridyl-s-triazine) in 40 $mmol.L^{-1}$ hydrocloric acid and with 1 volume of 20 $mmol.L^{-1}$ ferric chloride. The dilution of sample in reaction mixture was 1:34. Absorbance readings were done after 8 minutes of incubation.

The determination of polyphenols by high pressure liquid chromatography (HPLC). A 2 g of baked cookies sample was weighed into 200 mL volumetric flask and 50 mL methanol/water (50/50, v/v) was added. The samples were left for 5 days in the dark. Extracts were then filtered by a syringe filter (Agilent Captiva Premium Syringe Filter Regenerated Cellulose, 0.45 µm, 25 mm, p/n 5190 5111) and filtrates were used directly for injection.

The HPLC mobile phase was following: A) water + 1% phosphoric acid; B) acetonitrile; with the following gradient: 10% B, from 0 to 20 min; 20% B, from 20 to 25 min; 30% B, from 25 to 35 min; 40% B, from 35 to 40 min; post time was 10 minutes. The column was Agilent ZORBAX Eclipse Plus, 4.6 × 250 mm, 5 µm. Detection was done with the use of diode array detector (DAD) at 324nm. The method was slightly modification of the method developed by **Naegele (2013)**.

Statistic analysis

Statistical significance at $p < 0.05$ was evaluated by one-way ANOVA analysis of variance, and parametric Tukey post hoc test (in the case when Levene's test showed equal variances $p > 0.05$) and nonparametric Games–Howel post hoc test (in the case when Levene's test showed unequal variances $p < 0.05$) for finding differences within individualgroups. Principal component analysis (PCA) was done (with Promax rotation) for finding overall differences among wine and experimentally produced mulled wine samples. SPSS 20 statistical software **(**IBM Corporation, Armonk, NY**)** was used.

RESULTS AND DISCUSSION

Alcohol percentage, titrated acids content and pH value of wine and developed mulled wine samples are shown in Table 2.

Titrated acids were the highest in the sample of mulled wine that was prepared with cloves addition, which can be explained by low pH of clove oil (2.81) **(Santin et al., 2011)**. Alcohol content in evaluated samples was significantly (p <0.05) reduced in mulled wine samples (samples number: 2, 3, 4, 5) due to heat treatment. In the study of **Boban et al. (2010)** during 45 minutes heat treatment of wine at 75 ºC and 125 ºC alcohol content in mulled wine was also lowered by 20% and more than 90%, respectively. The content of alcohol was almost at the same level in the both mulled wines (45 minutes treatment at 125 ºC) and dealcoholized wines (under vacuo in rotary evaporator) **(Boban et al., 2010)**. Alcohol content in wine can be up to 15% **(**vol/vol**)** in wines produced in warmer climate regions due to higher sugar content **(Contreras et al., 2014)**. At the present time there is a trend of producing wines with reduced alcohol content due to public health recommendations to lower alcohol consumption **(Grant, 2010; MacAvoy, 2010)**. The results obtained by analysis of total phenol content represented as gallic acid ($mg.L^{-1}$) and antioxidant capacity (FRAP) are shown in Table 3.

The total phenol contents in were the highest (p <0.05) in mulled wine samples with cloves addition (samples 3 and 5). The increment can be explained by cloves addition which is known as good source of phenolic compounds **(Gulcin et al., 2004)**, but also wine heat treatment leads to increase of phenolic compounds due to loss of volume **(Boban et al., 2010)**. Though, Authors **(Boban et al., 2010)** found that heat treatment and dealcoholizing of wine result in the loss of some individual phenolic compounds, but total phenol content increases. On the other side, wine and especially mulled wine represent the model of mixed polyphenolic compounds that are well protected during heating. It was also indicated that certain polyphenolic compounds serve as polyphenols' protector during heat-induced decomposition **(Yamaguchi et al., 2003)**. Although, heat-induced polyphenol interactions are very hard to predict and their paths toward degradation or increment **(Pinelo et al., 2004)**. The complexity of mulled wine phenolic content changing is also supported by the observation that even small physical-chemical properties changes of hydroalcoholic polyphenolic solutions significantly affect their solubility, perception behavior and also their interactions with other compounds, such as proteins **(Serafini, Maiani and Ferro-Luzzi, 1997; Zanchi et al., 2008)**. The differences in polyphenolic profile among wine and experimentally produced mulled wine sample can be also seen in Figure 2 that is clearly showing the increase of polyphenolic compounds, especially in samples with added cloves. Antioxidant capacity represented as ferric reducing antioxidant power (FRAP) was the highest (p <0.05) among mulled wine samples with cloves addition (samples 3 and 5). These results are corresponding with higher polyphenol contents in these samples. The finding is supported by the observation of previous studies that found higher antioxidant activity in heat treated food product in comparison to raw materials. This swift to higher antioxidant capacity is explained by two possibilities: i) the production of stronger antioxidants during heating and ii) oxidative enzymes are inactivated by thermal processing **(Boban et al., 2010)**. **Mudnić at al. (2011)** found the increase of phenol content in heat treated wine (mulled wine). The authors also stated that thermal degradation of phenolic compounds is still poorly understood and that it is hard to predict these process.

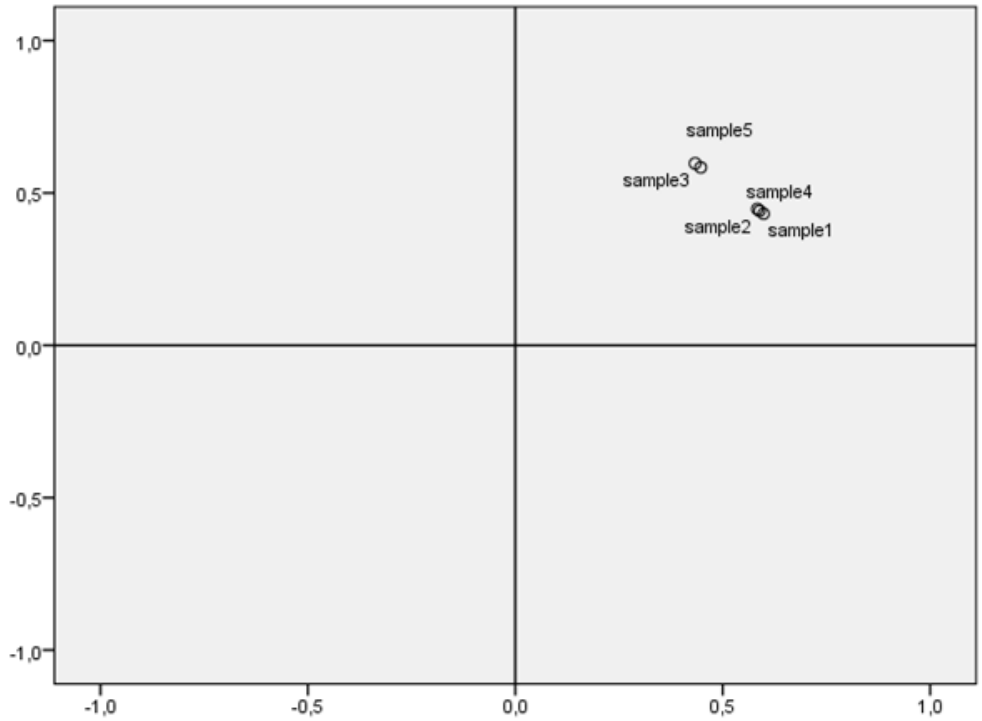

Figure 1 Principal component analysis (PCA) of wine and mulled wine samples.

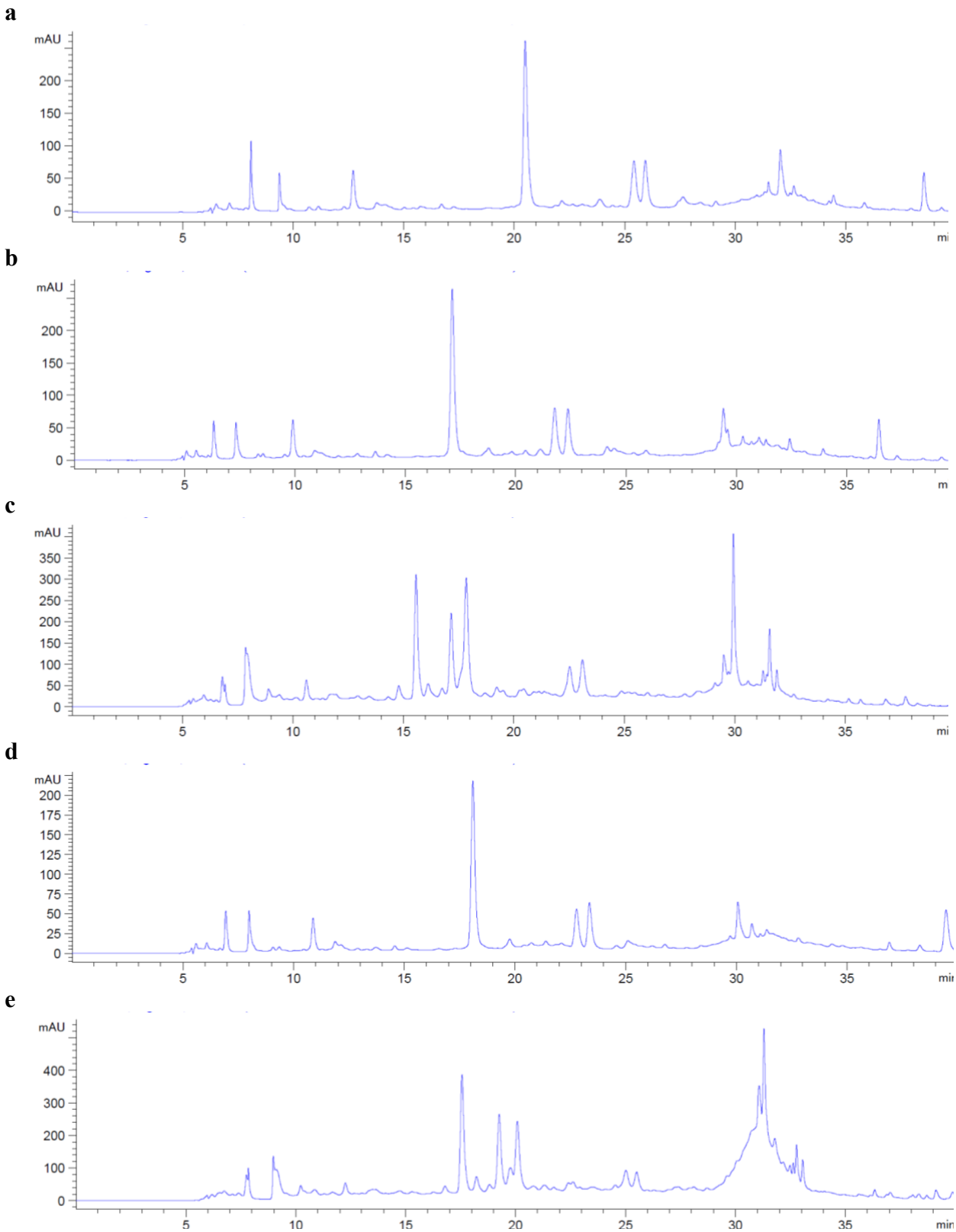

Figure 2 Polyphenolic profile of wine and mulled wine samples.
Note: *a: sample 1; b: sample 2; c: sample 3; d: sample 4; e: sample 5. According to Table 1.

Table 1 The samples used in the experiment.

Samples	Composition	Thermal treatment	Wine + spices
1	Wine	No	Wine (only wine)
2	Mulled wine without spices	Yes	Mulled wine (only wine)
3	Mulled wine + cloves	Yes	600 mL + 18 g
4	Mulled wine + cinnamon	Yes	600 mL + 17 g
5	Mulled wine + cinnamon + clove	Yes	600 mL + 17 g + 18 g

Table 2 PH, relative density, titrated acids and alcohol content in mulled wines.

	pH	Titrated acids (tartaric acid g.L^{-1})	Alcohol (vol.%)	The relative density (g.cm^{-3})
1	3.62 ±0.02	7.00 ±0.00	11.96 ±0.18^{a}	0.99 ±0.00
2	3.55 ±0.01	7.35 ±0.21	9.03 ±0.06^{c}	0.99 ±0.00
3	3.58 ±0.02	8.20 ±0.14	8.27 ±0.04^{b}	1.00 ±0.00
4	3.53 ±0.03	7.35 ±0.21	9.01 ±0.19^{bc}	0.99 ±0.00
5	3.63 ±0.02	7.65 ±0.07	9.42 ±0.18^{bc}	1.00 ±0.00

Note: *different letters (a, b, c) indicate statistically significant (p <0.05) differences.

Table 3 Total phenol content and antioxidant capacity of wine and mulled wine samples.

	Total phenol content (gallic acid mg.L^{-1})	Antioxidant capacity FRAP (μmol.L^{-1})
1	2 375.04 ±0.00^{a}	238.69 ±2.73^{a}
2	2 499.69 ±0.00^{b}	268.76 ±1.87^{c}
3	4 019.90 ±0.00^{c}	718.23 ±1.98^{d}
4	2 284.47 ±0.00^{d}	239.85 ±0,61^{acb}
5	4 165.99 ±0.00^{e}	717.61 ±1.71^{d}

Note: *different letters (a, b, c, d, e) indicate statistically significant (p <0.05) differences.

Temperature and pH affect the most degradation of phenolic compounds, though the degradation was found to be not so intense since the temperature of 111 °C degrades gallic acid during 30 minutes only by 1.3% **(Tanchev et al., 1997)**. The overall differences between wine and mulled wine samples can be seen in Figure 1. Principal component analysis found 2 seperate groups: group 1 – samples 1; 2 and 4; group 2 – samples 3 and 5 **(**Figure 1**)**.

CONCLUSION

The study clearly gives the picture about differences between wine and experimentally produced mulled wine. The health benefits of wine consumption are almost exclusively connected with high phenolic content and consequently high antioxidant properties. The sharing of this fact in many countries has raised the consumption of wine significantly. On the other hand, alcohol content of wine is the subject of constant evaluation of medical studies. The results gained by our research are indicating that during heating process of mulled wine consumption phenolic profile is not interrupted and can be even improved by the addition of regular spices the most often used to produce mulled wine, such as cinnamon and cloves. Concurrently, the heating process is significantly reducing alcohol content. Certainly, the effects of heating on wine, as representative of highly complex polyphenolic matrix, will be the subject of future studies since interection between polyphenolic compounds between them and also between other compounds present in wine has not been still explained enough.

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EFFECT OF INTRAMUSCULAR FAT CONTENT ON PHYSICAL-CHEMICAL PARAMETERS OF PORK FROM MANGALITSA AND THEIR CROSSBREEDS

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ABSTRACT

The aim of study was to evaluate the effect of intramuscular fat content on physical parameters and proximate composition in *musculus longissimus dorsi* (MLD) from Mangalitsa breed and Slovak Large White x Mangalitsa crossbreed. In the study, sixteen pigs of Mangalitsa and twenty-two pigs of Slovak Large White x Mangalitsa crossbreed were used. The pigs were reared under intensive condition and all animals were fed *ad libitum* with complete fattening feed mixture. The fattening period started from 30 kg of live weight. Then the pigs were slaughtered at 100 kg of live weight. Chemical analysis showed that MLD from Mangalitsa had lower protein content, higher moisture content (p <0.05) and higher content of intramuscular fat compared to Slovak Large White x Mangalitsa crossbreed. As regarding the cholesterol content in MLD, no significant differences were found between genotypes, but the cholesterol content was higher in MLD from Mangalitsa than in MLD from crossbreeds. The MLD from Mangalitsa exhibited lower CIE L* (p <0.01) and CIE b* (p <0.01) values 45 min *post mortem* compared to crossbreeds. Colour parameters increased after 7 days *post mortem*, which is normal due to the maturing process of the meat. Then the CIE L* value was lower in MLD from Mangalitsa (p <0.01), but CIE a* value was higher in relation to crossbreeds (p <0.05). As regards the Warner-Bratzler shear force, the meat from Mangalitsa was tenderer than in crossbreeds (p <0.05). Intramuscular fat in the meat positively correlated with colour parameter CIE a* (r = 0.324; p <0.05) as well as cholesterol content (r = 0,656; p <0.001). In contrast in the study was found negative correlations between intramuscular fat in meat with moisture content (r = -0.399; p <0.05) and protein content (r = -0.812; p <0.001). It can be concluded that the percentage of intramuscular fat significantly influenced the physical and the chemical parameters of pork. The meat from Mangalitsa is more suitable for production of special meat products (fermented and smoked).

Keywords: crossbreeds; intramuscular fat; Mangalitsa; *musculus longissimus dorsi*; pork

INTRODUCTION

Meat quality is evaluated according to quality parameters such as pH, colour or intramuscular fat content. Meat colour is one of the main quality properties which influence consumer's acceptance, but also reflects the quality of meat **(Estévez, Morcuende and Cava, 2003; Alonso et al., 2009)**. Within the qualitative parameters of meat, evaluating percentage of intramuscular fat seems to be the best way to separate indigenous pigs from commercial breeds **(Pugliese and Sirtori, 2012)**, because it is usually observed that intramuscular fat content in important cuts of pork is less than 1.5% in pig meat breeds **(Hamill et al., 2012)**. **Jeong et al. (2010)** indicated that intramuscular fat content is important factor that contribute to eating quality and it is influenced by genotype, gender, age and diet. Some reports indicate that when intramuscular fat content increases from 1% to 3%, the qualitative parameters increase at high rate. However as intramuscular fat content increase from 3% to 6%, the qualitative traits also improve, but at the lower levels.

The indigenous breeds such as Iberian and Mangalitsa are known to have desirable quality properties of meat that could be of interest to farm, giving the possibility to produce unique high-quality meat products. These indigenous breeds have a high intramuscular fat content **(Straadt, Aaslyng and Bertram, 2013)**. Mangalitsa is one of the most popular rustic pig breeds in Europe, because the meat has excellent properties, such as taste, marbling and low cholesterol content **(Pârvu et al., 2012)**. This breed is also characterized by dark colour of meat, robust constitution and slower growth rate with higher content of fat in carcass (65% – 70%) and reduced content of meat (30% – 35%) in carcass compared to commercial breeds **(Egerszegi et al., 2003; Stanišić et al., 2013)**.

In the intensive pig production, the most common crossbreeding program is to use breeds such as Large White, Duroc, Pietran, to improve some production parameters as

well as qualitative traits in meat **(Alonso et al., 2015)**. In the last several decades, pigs have been crossed for the rapid production of lean meat and to increase the slaughter yield. According to this, most recent pig breeds have less than 2% of intramuscular fat in meat with exception of Duroc breeds **(Suzuki et al., 2003; Sheard et al., 2005; Alonso et al., 2009)**.

Literature is controversial if the percentage of meat intramuscular fat in affects several quality traits. With this purpose, *the objective of this experiment* was to evaluate the effect of intramuscular fat content on physical parameters and proximate composition in *musculus longissimus dorsi* (MLD) from Mangalitsa breed and their Slovak Large White x Mangalitsa crossbreed.

Scientific hypothesis

Created crossbreeds Slovak Large White and Mangalitsa will have fattening as well as production comparable to Slovak Large White, at which the qualitative traits of meat will be a prerequisite for the production of specific products based on fermentation, smoking and drying.

MATERIAL AND METHODOLOGY

Biology material

The experiment was implemented in the Experimental centre of animal at the Slovak University of Agriculture (SUA) in Nitra. Thirty-eight pigs were studied, and they were divided into two groups with different genotype: Mangalitsa breed (n = 16) and Slovak Large White x Mangalitsa crossbreed (n = 22).

Feeding and rearing conditions

Pigs of Mangalitsa breed were reared under intensive conditions. The pens were situated outdoor. The pen consisted of concrete floor and the straw was used as bedding. The Mangalitsa pigs were housed in groups of 4. The pigs were fed by feed mixture (FM) for fatteners (Table 1), which received this feed mixture and drinking water by *ad libitum* system. The crossbreed Slovak Large White x Mangalitsa (SLW x Ma) were reared under intensive system in the indoor conditions. The size of pen was 3.10 m x 1.07 m. The pen was divided into bedding area (2.54 m^2) differenced by defecating area (0.96 m^2). The floor in the bedding area consisted of agro-pavage and the floor in defecating area was composed of grate. The crossbreeds SLW x Ma received complete feed mixture applied at the different growth phases: until 35 kg OS-03, from 35 kg to 65 kg OS-04 and above the 65 kg OS-05. The composition of complete feed mixtures and nutrient content are presented on Table 1. The pigs received drinking water and feeding with *ad libitum* system.

Sampling

Pigs were slaughtered upon reaching 100 kg of live weight, so the fattening period lasted from 30 kg to 100 kg of live weight. Pigs were slaughtered in the slaughterhouse at the Experimental centre of Animals (SUA in Nitra). Firstly, the animals were electrically stunned by electric forceps during 4 s with voltage 250 V and the amperage 1.3. The stunned animal was hooked for Achilles tendon and then was killed by bleeding. The slaughtering was realized according to **Government regulation (SR) no. 432/2012** of the coll. of Slovak Republic establishing the protection of animals during the slaughter. Samples of *musculus longissimus dorsi* (MLD) were dissected from the right-half carcasses. The MLD was taken at the level of the last *thoracic vertebra*. The dissection of carcasses was done performed according to standard practices of fattening-status and slaughter values in Slovakia.

Analysis of pH, electric conductivity and drip loss

pH values 45 min (pH_{45}) and 24 h (pH_{24}) *post mortem* in MLD were measured by pH meter Hanna HI99161 in units $log.molc^{(H+)}$. The electric conductivity was determined 45 min (EC_{45}) and 24 h (EC_{24}) *post mortem* by using an instrument Tecpro in unit $mS.cm^{-1}$. Drip loss in MLD was

Table 1 Composition of diet for pigs.

Traits	FM	OS03	OS04	OS05
Corn (%)	50	10	10	7
Barley (%)	10	22	25	28
Wheat (%)	10	44	42	49
Wheat bran (%)	0	7	8	6
Soybena meal (%)	10	13	12	7
Sunflower seed (%)	10	0	0	0
Granuled alfalfa (%)	7	0	0	0
[1]Mineral and vitamin supplement (%)	3	3	3	3

Note: [1]retinol 200 000 m.j., cholecalciferol 30 000 m.j., α-tocopherol 400 mg, riboflavin, 80 mg, pyridoxine 30 mg, cyanocobalamin 1000 mg, niacinamide 300 mg, folic acid 2 mg, pantothenic acid 300 mg, cholinchlorid 4000 mg, Cu 600 mg, Fe 3400 mg, Zn 1000 mg, Mn 1000 mg, I 30 mg, Se 8 mg.

Table 2 Nutrient composition of diets for pigs.

Traits	FM	OS03	OS04	OS05
Crude protein (g)	134	160	150	120
Metabolisable energy (MJ)	12.6	12.6	12.7	12.6
Fibre (g)	43	43	41	38
Lysine (g)	9.7	9.7	8.6	6.4

measured 24 h *post mortem* by the method according to **Honikel (1998)**.

Instrumental colour and Warner-Bratzler shear force

Meat colour was measured in MLD 24 h and 7 days *post mortem* by using spectrophotometer CM-2600d with CIE Lab space and illuminate D65 (Konica Minolta, Japan). **Commission Internationale de l'Eclairage (1975)** determined the following colour coordinates: L* (lightness, white ± black), a* (redness, red ± green) and b* (yellowness, yellow ± blue). Values were recorded from the average of three random readings across each muscle surface. After 7 day – storage at temperature 4 ±1 °C, the Warner-Bratzler shear force was analysed. The samples were heated to internal temperature of 71 ±1 °C for 30 minutes and subsequently chips in 1x1 cm sheared across fibers. Warner-Bratzler shear force of meat was determined using a Warner-Bratzler shear device Chatillon (U.S.A), in accordance with **Goodson et al. (2002)**. The shear of device was set up according to producer (capacity of tensometer was 5 kg; speed was constant 0.005 $m.s^{-1}$).

Proximate composition

The basic chemical parameters such as total water, total protein and intramuscular fat content were measured by the FT IR method (FourierTransform InfraTed) by using device Nicolet 6700 (Thermo Scientific, USA). FT IR is method of infrared spectroscopy. The homogenized sample is placed on the imagine opening of the integration sphere and absorbs infrared radiation. The final spectrum is shown molecular absorption and wavelength transmission (4000 – 10 000 nm) using an interferogram, what is made as fingerprint of sample.

Statistical analysis

The effects of genotype on studied parameters were analysed by the analysis of variance (ANOVA) using the Statistic Analysis System (SAS) package: SAS 9.2 using of application Enterprise Guide 5.1. **(SAS Institute Inc., 2012)**. Means and standard deviation (*SD*) are presented in Tables. When ANOVA was significant the means were compared using Tukey's test. The correlation between intramuscular fat content in meat and physico-chemical parameters were analysed by calculation of Pearson's coefficient. The correlation between intramuscular fat content and protein content, cholesterol content as well as Warner-Bratzler shear force was expressed by linear regression.

RESULTS AND DISCUSSION

The results of physical and chemical parameters of MLD from genotypes are presented in Table 3 and Table 4. The pH 45 min and 24 h *post mortem* was not influenced by genotypes. The values of pH decreased after 24 hours *post mortem*, what agrees with studies of **Lindahl et al. (2006)** and **Young, Bertram and Oksbjerg (2009)**. MLD of Mangalitsa pigs had slightly higher values of pH 45 min and 24 h *post mortem* than crossbreeds SLW x Ma. Similarly, **Tomović et al. (2016)** confirmed that MLD of Mangalitsa had higher values of pH compared to their crossbreed (ph_{45min} 6.37 vs. 6.26; pH_{24h} 5.72 vs. 5.53). **Sirtori et al. (2011)** indicate that indigenous breed Cinta Senese had higher value of pH in MLD compared to crossbreed Italian Large White x Cinta Senese. The indigenous pig breed Chato Murciano had also higher value of pH in MLD than their crossbreed Chato Murciano x Iberian pig, what is obtained by **Galián et al. (2007)**.

Electric conductivity (EC) 45 min *post mortem* was lower in MLD of Mangalitsa pigs compared to crossbreeds SLW x Ma. The EC increased after 24 hours *post mortem*, whereby Mangalitsa pigs had lower EC_{24} than crossbreeds SLW x Ma, Electric conductivity (EC) 45 min *post mortem* was lower in MLD of Mangalitsa pigs compared to crossbreeds SLW x Ma. The EC increased after 24 hours *post mortem*, whereby Mangalitsa pigs had lower EC_{24} than crossbreeds SLW x Ma, indicating statistical significance ($p < 0.05$). In the same way, values of drip loss were lower in MLD of Mangalitsa in relation to crossbreeds SLW x Ma. Most commonly, pH and electrical conductivity are used as indicators for pale, soft, exudative (PSE) meat **(Warriss et al., 1998; Josell, Von Seth and Tornberg, 2003; Altmann et al., 2005)**. **Mörlein et al. (2007)** applied strict levels for determination of PSE meat, when pH_{45} <6.0 – 5.8 are used as threshold levels. Then, EC_{24} values above 9 – 7 $mS.cm^{-1}$ were applied as PSE meat. Threshold parameters for drip loss were above 7 – 9% within 48 h *post mortem*. On the basis of this fact, it was found in our study PSE of meat from Mangalitsa pigs fed by LFM. According to **Lee et al. (2000)** exist linear relationship between drip loss and electric conductivity, what is in consistent with our results. In our study was not determined pale, soft and exudative (PSE) meat. As regards Warner-Bratzler shear force (W-B), the MLD from Mangalitsa showed lower values of shear force (2.63 $kg.cm^{-2}$) compared to crossbreed SLW x Ma (3.14 $kg.cm^{-2}$; $p < 0.05$). The values were notably lower than those obtained by **Stanišić et al. (2015)** in Mangalitsa breed and Landrace as well as by **Tomović et al. (2016)** in MLD of Mangalitsa and Large White. Data confirmed that Mangalitsa has more tender meat than Landrace and Large White.

As regards the instrumental colour of MLD measured 45 min *post mortem*, the value CIE L* was higher in MLD from crossbreed SLW x Ma ($p < 0.01$) compared to MLD from Mangalitsa. Similarly, the value CIE b* was higher in MLD from crossbreed SLW x Ma ($p < 0.01$) and the value CIE a* was lower compared to MLD from Mangalitsa. However, no significant differences were found in CIE a* value due to genotype after 24h. The colour parameters (CIE L*, a*, b*) in MLD increased after 7 days *post mortem*, which is common due to the maturation process of the meat. This fact agrees with studies of **Estévez, Morcuende and Cava (2003), Lindahl et al. (2006)** and **Stanišić et al. (2016)**. The CIE L* and CIE a* values were significantly influenced by genotype where Mangalitsa exhibited darker (CIE L*; $p < 0.001$) and redder (CIE a*; $p < 0.05$) MLD compared to crossbreed SLW x Ma. Although were not found significant differences for CIE b* in MLD, Mangalitsa had lower values than crossbreed SLW x Ma. It is in accordance with **Stanišić et al. (2016)**.

Table 3 Physical parameters of *musculus longissimus dorsi.*

Parameters	Mangalitsa (n = 16) Mean ±*SD*	SLW x Ma (n = 22) Mean ±*SD*	*p* <value
pH_{45min} (log.molc$^{(H+)}$)	6.09 ±0.27	6.02 ±0.19	0.425
$pH_{24hours}$ (log.molc$^{(H+)}$)	5.69 ±0.07	5.68 ±0.11	0.850
EC_{45min} (mS.cm^{-1})	3.31 ±1.27	3.92 ±0.47	0.284
$EC_{24hours}$ (mS.cm^{-1})	9.31 ±1.91	10.86 ±2.25	0.019
Drip $loss_{24hours}$ (%)	7.15 ±2.99	8.22 ±2.78	0.262
$Colour_{24hours}$ CIE L*	53.06 ±4.34	58.12 ±4.93	0.002
$Colour_{24hours}$ CIE a*	3.21 ±1.36	2.50 ±1.51	0.139
$Colour_{24hours}$ CIE b*	10.41 ±1.53	11.89 ±1.45	0.005
$Colour_{7days}$ CIE L*	54.51 ±4.67	60.46 ±4.58	0.001
$Colour_{7days}$ CIE a*	6.67 ±2.98	5.03 ±2.01	0.044
$Colour_{7days}$ CIE b*	13.14 ±2.56	14.04 ±1.92	0.228
Warner-Brazler shear force (kg.cm^{-2})	2.63 ±0.75	3.14 ±0.65	0.031

Note: SW: slaughter weight, SLW x Ma: crossbreeds Slovak Large White x Mangalitsa, SEM: Standard error of mean.

Comparing our results with those obtained by **Franci et al. (2005)**, we found that their results for CIE L* and CIE b* were lower, but CIE a* value was higher. In their study pure breed Cinta Senese exhibited darker (CIE L*) and redder (CIE a*) meat, but lower value of index b* compared to crossbreed Large White x Cinta Senese. Similarly, **Szulc et al. (2012)** obtained that pure breed Zlotnicka spotted had higher values of CIE L*, CIE a* as well as CIE b* compared to crossbreed Zlotnicka spotted x Polish Large White. On the contrary **Poto, Galián and Peinado (2007)** worked with Spanish indigenous breed Chato Murciano and they found lower values of colour parameters in MLD from crossbreed Chato Murciano x Large White than in indigenous breed Chato Murciano. On the other hand, **Renaudeau and Mourot (2007)** studied differences on meat quality between indigenous pigs Creole and Large White, where Creole pigs had lower colour parameters in relation to Large White. **Bednářová et al. (2014)** analyzed samples of pork 72 hours *post mortem* from different supliers. L* value from *m. semimenbranosus* was found from 46.13 to 48.53

Regarding the proximate parameters, water content was significantly higher in MLD from Mangalitsa than in MLD from crossbreed SLW x Ma (p <0.05). The MLD from Mangalitsa had lower protein content (p <0.05), but higher percentage of intramuscular fat than MLD from crossbreed SLW x Ma, where the difference did not reach the significance. **Franci et al. (2005)** observed that pure breed Cinta Senese had lower water content as well as protein content compared to their crossbreed Large White x Cinta Senese and the percentage of intramuscular fat was higher. Data are in accordance with our results except to water content in MLD. Our results showed higher values of water content and protein content, but lower percentage of intramuscular fat than in study of **Sirtori et al. (2011)** and **Parunović et al. (2013)**. In their study, the indigenous breeds such as Cinta Senese, compared to Mangalitsa, had lower percentage of water as well as protein content and higher percentage of intramuscular fat in meat compared to commercial breeds. According to **Serra et al. (1998)**, the pure breed Iberian had lower water and protein content, while the percentage of intramuscular fat was higher than Landrace pig meat. The results of cholesterol content were in accordance with percentage of intramuscular fat content in MLD, however no significant differences were found due to genotype. As regards the cholesterol content, **Pârvu et al. (2012)** determined, that Large White pig meat had higher cholesterol content with lower percentage of intramuscular fat (cholesterol content 41.64 mg.100g^{-1}; IMF 23.21%) compared to Mangalitsa (cholesterol content 61.24 mg.100g^{-1}; IMF 10.69%). These results are in contrast with ours. The cholesterol content of MLD was in the study of **Parunović et al. (2013)** and **Parunović et al. (2015)** higher than in our results, but they confirmed that Mangalitsa had higher value of cholesterol compared to Swedish Landrace pig meat. Similarly, **Salvatori et al. (2008)** found that indigenous pigs Casertana had higher cholesterol content in MLD than their crossbreeds Casertana x Large White. On the other hand, **Stajić et al. (2011)** indicated that Mangalitsa had lower cholesterol content in meat in relation to Landrace. Although it is saying that Mangalitsa pigs had lower cholesterol content in the meat, it can be confirmed by our study that Mangalitsa had higher cholesterol content than their crossbreeds.

Table 5 shows coefficients of Pearson's correlation between physico-chemical parameters and intramuscular fat content in *musculus longissimus dorsi*. The percentage of intramuscular fat (IMF) in MLD was positively correlated (p <0.05) with CIE a* 7 days *post mortem* (r = 0.324) and also with cholesterol content (r = 0.656; p <0.001). The relationship between percentage of intramuscular fat and cholesterol content in meat is shown by Figure 1. In contrast, the IMF in MLD was negatively correlated with percentage of water (r = 0.399; p <0.05) and with protein content in MLD (r = -0.812; p <0.001). These results are in accordance with study of **Vranic et al. (2015)** and **Tomović et al. (2016)**. Figure 2 represents the relationship between intramuscular fat content and protein content in meat by linear regression. As regarding the Warner-Bratzler shear force, no significance was found between intramuscular fat and tenderness of meat. The results are in accordance with **Jeong et al. (2010)**. On the contrary, **Barlocco et al. (2006)** found positive correlation between Warner-Bratzler shear force and intramuscular fat content in the meat. In other parameters, no significant correlations were found.

Table 4 Chemical parameters of *musculus longissimus dorsi.*

Parameters	Mangalitsa (n = 16)		SLW x Ma (n = 22)		*p* <value
	Mean	*SD*	Mean	*SD*	
Total water (%)	73.53	0.61	73.01	0.58	0.017
Total protein (%)	24.15	0.66	24.56	0.34	0.019
Intramuscular fat (%)	1.93	0.91	1.66	0.59	0.286
Cholesterol (mg.100g^{-1})	43.01	7.16	38.91	10.41	0.203

Note: SW: slaughter weight, SLW x Ma: crossbreeds Slovak Large White x Mangalitsa, *SD*: standard deviation.

Table 5 Pearson's correlation coefficient between physico-chemical parameters and intramuscular fat content in *musculus longissimus dorsi* of the pigs.

Parameters	IMF
pH_{45min}	0.071
$pH_{24hours}$	-0.186
EC_{45min}	-0.134
$EC_{24hours}$	-0.161
Drip $loss_{24hours}$	-0.221
$Colour_{24hours}$ CIE L*	-0.046
$Colour_{24hours}$ CIE a*	0.126
$Colour_{24hours}$ CIE b*	0.044
$Colour_{7days}$ CIE L*	-0.082
$Colour_{7days}$ CIE a*	0.324*
$Colour_{7days}$ CIE b*	0.134
Warner-Brazler shear force	-0.153
Total water	-0.399*
Total protein	-0.812***
Cholesterol	0.656***

Note: IMF: intramuscular fat content, ***: p <0.001,*: p <0.05

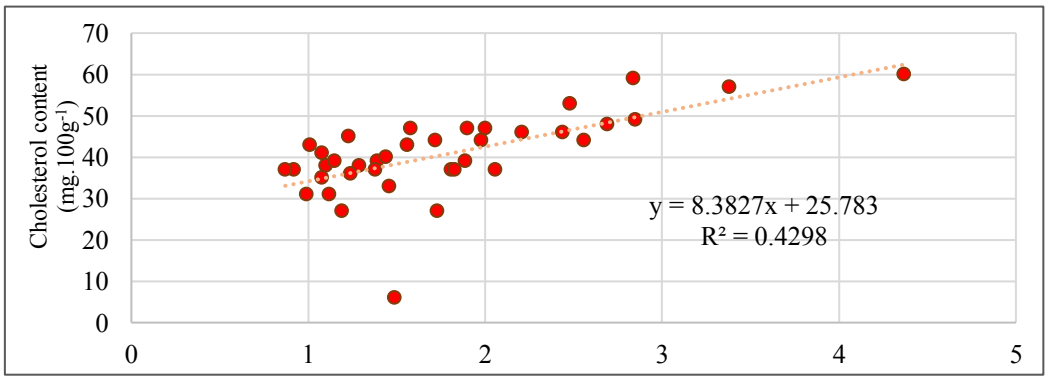

Figure 1 Intramuscular fat content in meat in relation to cholesterol content (%).

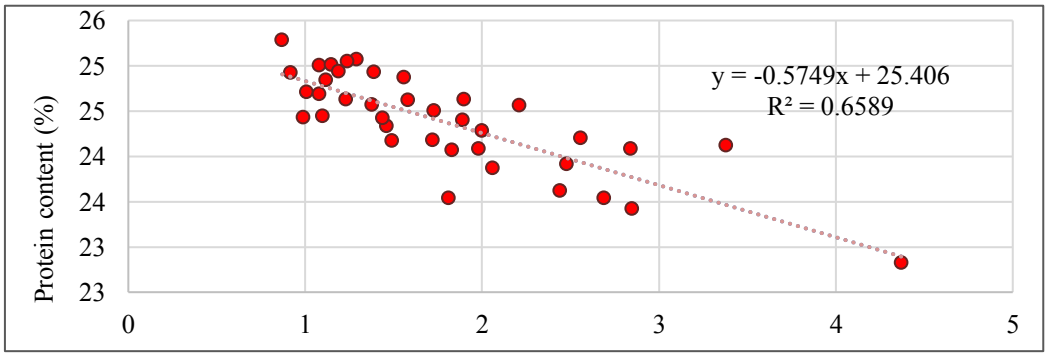

Figure 2 Intramuscular fat content in meat in relation to protein content (%).

CONCLUSION

Chemical analysis showed that MLD from Mangalitsa had lower protein content (24.15, resp. 24.56%) and higher content of intramuscular fat (1.93, resp. 1.66%) compared to Slovak Large White x Mangalitsa crossbreed. As regards the Warner-Bratzler shear force, the meat from Mangalitsa was tenderer than in crossbreeds (p <0.05). Intramuscular fat in the meat positively correlated with colour parameter CIE a* (r = 0.324; p <0.05) as well as cholesterol content (r = 0.656; p <0.001). Cholesterol content was found statistically non-significant difference (Mangalitsa 43.01 and SLW x Ma 38.91 mg.100 g^{-1}).

It can be concluded that the percentage of intramuscular fat significantly influenced the physical and the chemical parameters of pork. As regards the differences between genotypes, Mangalitsa has darker, redder and tenderer meat with lower moisture content and higher intramuscular fat content compared to crossbreeds Slovak Large White x Mangalitsa. The meat from Mangalitsa is more suitable for production of special meat products (fermented and smoked).

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ADHESION EFFECT ON ENVIRONMENT PROCESS INJECTION

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ABSTRACT

The analytical analysis of roller impact on the medium and its behavior at deformation influences are carried out, ways of choosing an optimal variant of the process for providing the maximum or minimum value of parameters (criterion) are proposed. The physical essence of the equation of energy flows of the intensity of deformation of the mass of the medium, which depends on the method of applying mechanical forces, the degree of its previous dispersion (recipe) and its physical and mechanical properties, is considered. To reveal the essence and understanding of the general research execution, a number of hypotheses for the determination of adhesion are given and a generalized approach to the definition of adhesion is given. The proposed scheme of causal relationships between the medium and roll, divided into three groups, and determine the change in the process of injection dough. The nature of the maximum increase of the forces of interaction of the dough with the high contact of the roller working body in the injection nozzle of the fuming machine is established. Violation of these mutual relations leads to the production of low-quality products and a decrease in the efficiency of the machine. These phenomena are little studied today, and the nature of adhesion requires research. It is noted that the determination of deformation processes during the passage of the process of injection of the medium by roller working bodies plays an important role in calculating the design of molding, roll-over equipment. The deformation in the knot of the injection dough by rolls and the dependence of the work performed on the influence of adhesion on the flow process in the molding machine are revealed. The contact area of the adhesive and the component forming work for overcoming the adhesion and deformation of the environment in determining the criteria that influence the process, according to each particular period of the deformation stage, are substantiated. The obtained data provide an answer to a number of questions about the possibility of regulating the process of the work of agents on the environment.

Keywords: dough; adhesion; adhesive; substrate; forming channel; deformation; stress

INTRODUCTION

Improvement of the technology of pumping, injection, mixing, separation, formation of the environment (non-Newtonian fluids) and the creation of high-performance technological equipment is an actual scientific task. Therefore, the development and calculation of new processes and equipment requires a thorough and comprehensive study of the impact of the most significant physical and mechanical properties of semi-finished baking and confectionery industries. A significant adhesive property of the environment significantly complicates the process and causes additional energy costs. This direction is given insufficient attention; therefore, the physical nature of adhesion is still not fully explored.

Adhesion refers to surface phenomena that arise when contacting heterogeneous bodies. There are different approaches to the definition of adhesion. According to the theory **(Believ, Egorenkov and Pleskachevsky, 1971)**, they can be divided into three groups:

- determination of adhesion as a process (gradual change of state);
- definition of adhesion as properties that characterize the feature of this system;
- definition of adhesion as a state (external and internal circumstances that characterize the state of the system).

Determination of adhesion as a process was given by Rebinder PA: "Adhesion (adhesion) - the appearance of a connection between the surface layers of two heterogeneous (solid or liquid) bodies (phases) that touch each other. A similar definition was given in some other sources **(Berlik and Basik, 1969; Deryagin, 1963; Li, 1979)**. That is, adhesion is interpreted as a process of convergence and removal of bodies from one another.

Often the nature of adhesion is explained by diffusion and electric theory. Adhesion is always the result of the intermolecular interaction of surfaces, different in nature.

According to this theory, with the contact of two bodies due to the Macrobroun motion, there is a rearrangement of the molecules. This occurs in such a way that large particles of molecules approach the contact surface, establish hydrogen bonds with the same molecules or with the polymer groups of the contacting body. This causes an adsorption bond in equilibrium. The influence of pressure and temperature during their increase contributes to the fairly rapid establishment of equilibrium.

In the food industry, there are many processes in which both the forces of friction and adhesion interact simultaneously. These phenomena arise when the relative displacement of the contacting surfaces of two bodies. In studies **Deryagin (1963)** established the connection between the aunt and the adhesion of the expression:

$$P = \mu N + \mu p_0 S_0$$

Where:

μ – actual friction coefficient; S_0 – contact area, m^2; N - normal load, N; p_0 – specific adhesion at the site S_0, Pa; P – external friction force, N.

The author **(Pawel et al., 2016; Stadnyk, Novak and Matenchuk, 2018)** revealed the properties of a dough for the production of bagels, which has elastic, viscous and plastic properties, that is, refers to elastic - visco-plastic bodies. Accordingly **(Gorbatov, 1979; Zimon and Yevtushenko, 1985)** noted that the adhesion of such bodies, as well as the cogenesis of particles, fluids and films, depends on the area of contact of the medium (adhesive) with the rough metal working surface of the machine.

Summarizing the above theories, we can conclude that adhesion is caused by a number of mechanisms that act on the molecular and supramolecular levels and are determined by the properties of these surfaces: physical, chemical or electromagnetic.

By the value that evaluates (determines, measures) adhesion, as a feminogenic phenomenon, a force is adopted that is defined as the force applied perpendicular to the sample, acting on a unit area and sufficient to separate it from the surface:

$$P_a = P/F_0$$

Where:

P – specific power N.m^{-2}; P_a – normal power, N; F_0 – contact area, m^2.

In this case, it is advisable to speak of adhesion strength. When separating structured masses, adhesion strength comes into competition with the cohesive strength of the mass. At tearing away the deformation and flow of the mass itself, determined by the rheological properties of the product itself. That is, it can be adhesive, cohesive and cohesive-adhesive type.

The dependence for the determination of the adhesion force was determined by M. Stefan **(Gorbatov, 1979)**:

$$P = \frac{2\eta R^2}{4\tau_0}\left(\frac{1}{h^2_1} - \frac{1}{h^2_2}\right), \quad \text{(a)}$$

Where:

R – skradius, m; $\boldsymbol{\tau_0}$ – time off (disk difference) from a distance $\mathbf{h_1}$ to $\mathbf{h_2}$, c; $\boldsymbol{\eta}$– fluid viscosity, Pa.s.

If the "adhesion" or the "force of adhesion" is evaluated by dependence (a), then such a process takes place in time, that is, adhesion is evaluated as a process that must be performed for sample discontinuation, and the distance of disc diffusion (h1 and I12) can characterize the phenomenon of cohesiveness.

Finding new ways to reduce adhesion is possible only after studying the physical nature of this phenomenon. Adhesion arises from adsorption of medium molecules (dough) on the surface of the working organ in the thinnest (up to 20 nm) surface layer. At the same time, these molecules are related to the main volume of the medium. Therefore, at all stages of the action of the roller working body on the relative displacement, the environment molecules that are adsorbed on its surface, rotate with it (Figure 1). If the forces of the intermolecular compound in the medium become weaker than the adsorption forces, then the rupture of its mass passes at a distance from the surface of the roll. Such phenomena occur in the period of tightening, compression and rolling of the medium in the formation gap formed between the rotating rolls.

Consequently, adhesion depends on the rheological properties of the medium at the time of the process, the degree of surface treatment of roller working bodies, the thickness of its layer. Investigation of the influence of physical and rheological properties of the environment and the structural features of cylindrical rolls with grooves on improving the quality of products at injection - the problem is relevant and needs to be solved in mathematical dependencies.

Figure 1 Photo of the effect of adhesion on the quality of the dough forming on the surface of an existing roll with straight grooves. Note: 1 – roll; 2 – remnants of the adhering dough after pouring; 3 – bunker with dough.

Analysis of model approximations of medium types.

Processes in the working chamber under the action of rollers occur with different effects on the environment. This process is accompanied by large deformations, which is facilitated by the profile of the roller surface and the design of the working chamber. Special attention in the study of the process of injection of rolls focuses on creating favourable conditions for tightening and compressing the environment.

For roller working valves in the injection nozzle of a fumiving machine, the maximum increase in the forces of the interaction of the dough with the high contact of the roll is characteristic. Violation of these mutual relations leads to the production of low-quality products and a decrease in the efficiency of the machine. These phenomena are little studied today, and the nature of adhesion requires research.

There are several hypotheses to determine adhesion:

- in accordance with Absorption theory Debrajina and Mc-Laren **(Berlik and Basik, 1969)**, adhesion is related to the action of intermolecular forces: physical, van der Waals or chemical, for example, covalent ion .;
- according to electrical theory B. Deryagina **(Deryagin, 1963)** - adhesion is connected with the difference in potentials on the boundary of heterogeneous bodies, that is, with the appearance in the contact area of a kind of electric molecular capacitor, caused by a double electric layer;
- according to the electromechanical theory, adhesion is associated with electromagnetic interaction, that is, with the emission and absorption of electromagnetic waves by atoms and molecules, which can be realized in condensed bodies;
- in accordance with the diffusion theory S. Vosotsky and B. Deryagin's **(Berlik and Basik, 1969)** adhesion is associated with the diffusion of ends of macromolecules through the boundary of the initial contact, resulting in the boundary (finite) case, the interface of the phases may disappear;
- according to the mechanical theory, adhesion contact is formed due to the mechanical adhesion of molecular and supramolecular formations with microniveness of the surface;
- according to the thermodynamic theory, adhesion is associated with a surface tension, which, according to the theory **(Wake, 1982)**, causes the solid-body solid-liquid surface to replace the solid-liquid interface.

In his writings **Zimon and Yevtushenko (1985)** gives preference to intermolecular interaction and partly to the Coulomb force, which determines the primary formation of the relationship between two surfaces. The forces begin to act in direct contact and a certain period after the violation of the contact.

The second group of forces, unlike the first one, manifests itself only when two bodies are contacted. These forces act as a result of primary adhesion and become one of its causes. This group includes chemical bonds, forces of electric and capillary interaction, as well as adsorption and diffusion, which are the result of contact.

With adhesion of liquid and elastic-plastic food masses, a chemical bond may occur.

To a large extent, the adhesion of the food mass depends on the presence of moisture, which can manifest itself as a capillary force.

In the gap between the contacting parts there is a meniscus of fluid. In this case, it seems to charge the particle and the surface. The value of adhesion in this case will be determined by the capillary force:

$$F_K = 4\pi\sigma_ж r$$

Where:

$\sigma_ж$ - surface tension of a liquid (water), the vapor of which is condensed between the contacting bodies.

In a liquid medium, the adhesion of the parts is less than in the air **(Zimon and Yevtushenko, 1985)**. In this case, there is a disjoint action of the thin layer of the liquid, which is on the surface of the bodies.

Among the components of the viscous medium (dough), the greatest energy of adsorption to metals is water. If it is removed from the surface of the semi-finished product, the adhesion should be reduced. At the same time, the intensity of evaporation of the surface moisture should be very large, so that the resulting thin moss on the surface of the environment prevented the removal of moisture from its volume. Such conditions can be obtained **(Stadnyk et al., 2018)** only at very high velocities - the leakage of the medium under pressure through the opening of the profile channel formed between the rotating rolls. Based on the properties of the viscous medium and the deformation of the rolls, a number of new designs of roller working valves have been proposed **(Derkach, Stadnyk and Stadnyk, 2016)**. Interestingly enough is the detection of the action of the surface of the new working body with screw grooves (Figure 2) on the adhesion properties of the medium.

In the course of the forced flow of the medium layer under the action of roller working bodies, there is a temporary slip on them at a speed. In the future, when the surface layer is squeezed and its slip on the surface of the roll, the medium layer is sealed, and the alignment of the structure takes place. This layer acquires increased gas- and form-retaining ability and penetration of water molecules (diffusion) into it inside with swelling and volume increase.

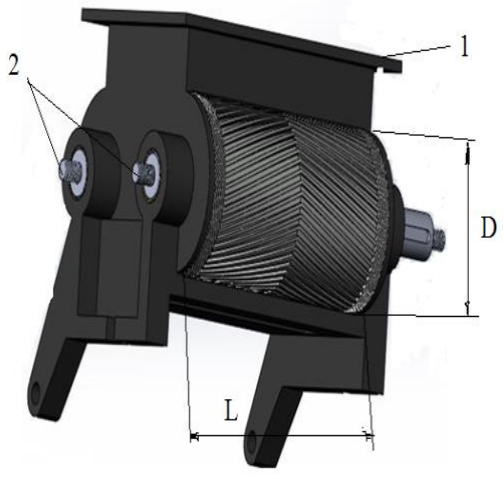

Figure 2 Nominal injection of molding machine. Note: 1 – working chamber; 2 – roller working bodies.

For even sealing and moving the mass of the medium along the surface of the roller working bodies, a gap is established between them, which regulates and smooths the resulting deformation. These deformations depend on the type of environment and requirements for finished products. Therefore, the velocity of the displacement of the medium layer on the surface of the rolls will be practically equal to its velocity. Accordingly, slipping on the surface of the rolls cannot be considered even because the surface has been loose.

Scientific hypothesis

Systems for moving the dough layer between the rollers are important components of the molding machine, which provide the role of its transportation to molding devices. The limited allowable loads of the dough require the creation of specific conditions for the synthesis of systems "roll-dough", forbearing and guiding elements and leading roller working bodies for the use of adhesion forces (measured in H) and friction in the role of forces of forces and forces of resistance. In this regard, in the interest of optimized synthesis, information is needed which relates to the phenomena and characteristics of adhesion, the possibilities of its transformation in kinematic pairs both in the direction of increasing and in the direction of reduction. Varieties of the dough with its features relate to other mechanical parameters and coefficients of friction. At the heart of interactions between rolls and the environment it is seen that they correspond to the laws of friction of Amonton-Coulomb, Euler's ratio, the concept of angle and friction cone, radius and friction wheel, reduced coefficient of friction. In turn, the wording of these concepts and definitions refers to such universally accepted assumptions and concepts of mechanics as the resultant forces of gravity, the resultant distributed forces of normal pressure, the resulting force of adhesion, the center of masses, the geometric center of contact surface, etc. In the technologies of calculations and definitions of the parameters of the systems there are regularities of statics and dynamics and the principles of independence of the forces of activity, of additivity, of Lagrange-Dahmberm.

In search of solving research tasks, simulation was carried out to assess external influences on reactions and responses of local area systems based on mathematical formalizations and with the formulation of computational experiments. The mathematical model of the forces of the adhesion factor of influence, which, unlike the coefficient of friction, acts with a stabilized value is proposed. It is important that this applies to ensure that no hitching is carried out when dragging the dough. Modified theoretical dependencies allowed to carry out the calculation of two-level experiments to create prospects for deepening the possibilities of generation of increased motive factors. Since the angle of coverage α acts as an important variational factor of influence, on the basis of analytical developments a computational experiment was carried out, in which the response function adopted the value of the specified angle. The structure of the dough leads to the need to consider the values of the coefficients of friction. Manifestations of non-isotropy with respect to the orientation of structures take place at the levels of molecular construction in the responses to the value of the coefficients of friction. The corresponding regression equations are obtained.

MATERIAL AND METHODOLOGY

A dough with a moisture content of 33%, for high quality wheat flakes on pressed yeast, was prepared in an opaque manner with a fermentation time of 60 minutes at a temperature of 32 – 33 °C. The quality of the pressed yeast corresponds to the DSTU. Characteristics of wheat flour:

- mass fraction of moisture,% – 14.5;
- the content of raw gluten,% – 28;
- resistance gluten compression on the device IDK-1, per.pril. – 54;
- gluten stretch, cm – 14.

The study of the dough injection process was carried out on the molding machine B-54 of the confectionery factory (Ternopil). It is known from work **(Zimon and Yevtushenko, 1985)** that the adhesion is determined by separating the medium from the surface, measuring the separation effort. In this effort, the adhesion resistance of the medium is calculated. Therefore, the adhesiveness of the Fvd (equal to the ratio of the fracture effort of the Fvd model to the area of the nominal contact Shk) depends on the size of the surface and adhesion, the conditions of contact and the separation of the dough. Adhesion was seen as a process that occurs in time when the surfaces of two heterogeneous bodies come into contact or are violated. For the quantity that quantitatively evaluates the adhesion, the work of the separation (strength) and the unstable process of filling the medium (dough) of the groove surface of the roll at the pressure of the previous loading Pk were used.

At the same time, determination of indicators of physical properties of the dough was carried out. The visco-plastic properties of the dough were determined using a rotating viscometer "Reotest-2" with two coaxial cylinders, using a cylinder S2 and a full range of speeds of rotation of the rotor in aI mode. Tensile displacement τ (Pa) was determined by the formula:

$$\tau = z \cdot \alpha,$$

Where

α – indicators on the scale of the device;

z – constant, the value of which depends on the cylinders used.

Dynamic viscosity η (Pa.s) was determined by the formula:

$$\eta = \frac{\tau - \tau_0}{\dot{\gamma}}$$

In order to determine the dependences of the shear rate on the shear stress and the dynamic shear stress, the law of the flow of the visco-plastic medium of Shvedov, the Branopolskaya method was used.

The force of pressure on the surface of the plasticizer was determined by registering its displacement using a computer with the integrated program "Power Graph" as

a personal recorder, which signals were fed from the built-in resistor as sensors and recorded through the input device with an illustration on the monitor. The program and a guide for its use can be downloaded at www.powergraph.ru (free working version 2.1). Minimum requirements for installing the program: operating system - Windows (98, ME, 2000, XP, Vista); operating memory - 32 MB; free hard disk space – 50 MB. To record signals in the PowerGraph program, you must pre-select the appropriate ADC driver (for example, a Joystick).

RESULTS AND DISCUSSION

Cragelskii noted that in the process of friction of two bodies at different points of contact can simultaneously be five types of fractional connection. These bonds include: cutting, plastic pressing, elastic deformation, adhesion and cohesive destruction. Relying on this theory, most inventors argue that there are two components-adhesive and deformation. Moore proposed the force of friction to determine the equation:

$$P = P_а+P_д+P_к+P_в$$

Where:

$P_а$ i $P_д$ – adhesion and deformation component of frictional forces, N; $P_к$ – cohesive component, taking into account wear on volumetric losses, N; $P_в$ – component of viscous braking in the presence of lubrication, N.

The phenomena of adhesion in the first stages of injection (delay) are explained by the theory of adsorption and diffusion, that is, the adsorption actions (van der Waals forces) on the surface of the roll. Thus, for the proper observance of the injection modes it is necessary to measure and control the adhesion forces. In this case, it is necessary to determine the strength of adhesion, modeling the conditions for the formation of adhesive forces in the working chamber of the machine, considering both the type and condition of the surface, as well as the structural and mechanical properties of the dough.

Consider the process of rolling the medium (dough) between the rollers (Figure 2). When rolling rolls of cylindrical shape, the thickness of the medium is constantly changing in the middle part of the formation, that is, in the zone of clogging. On the output of the rolls - the thickness is the same. All cycles of rolling the curved surface rolls occur without changing the gap of the middle part of the rolls from capture to capture. This allows you to smoothly effect the compression and shift of the dough with the achievement of the entire surface of the formation of the same thickness. Rolls of the same diameter are rotated with the same angular velocity ω, radius R, in length L. The working surface of the rollers is between the gaps h0 and h_1. Where: h_0 – distance between surfaces of rolls in the presence of medium; h_1 – the minimum clearance between the rollers.

When pressed, the dough is compressed, resulting in the thickness of the layer decreases, and its length and width increases. The difference between the incoming h_0 and the final one $h_1$the thickness of the dough layer will be absolute compression:

$$\Delta h = h_0 - h_1 \text{ ,mm;}$$

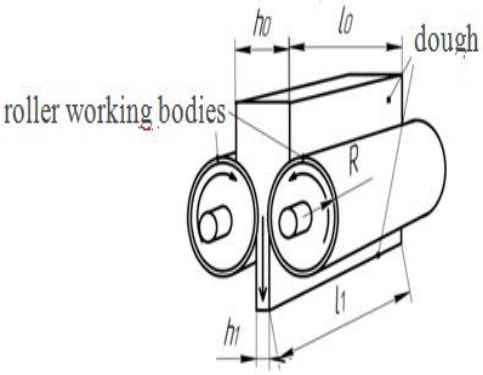

Figure 3 The scheme of rolling the dough.

The difference between the final one$l_1$and the initial one$\boldsymbol{l_0}$the width of the dough layer is the absolute width:

$$\boldsymbol{\Delta l = l_1 - l_0} \text{ , mm;}$$

The value of the deformation of the dough layer during the injection is characterized by the following indicators (coefficients):

relative compression - the ratio of absolute compression to the original thickness of the layer:

$$\varepsilon = \Delta h / h_0, \text{ and } \varepsilon = (\Delta h / h_0)\cdot 100\%$$

compression ratio - the ratio of the input thickness to the final:

$$\varepsilon_K = h_0 / h_1$$

coefficient of stretching of the dough between the rolls - the ratio of the input length of the layer –$\boldsymbol{l_0}$to the original length$\boldsymbol{l_1}$:

$$\zeta = l_1 / l_0$$

Since the volume of the dough when sprayed rolls change, then$\boldsymbol{h_0 l_0 < h_1 l_1}$, where:

$$\zeta = l_1 / l_0 = F_0 / F_1$$

Thus, the length of the layer during rolled rolls increases in proportion to the reduction of its cross-section. The coefficients of compression, stretching and expansion characterize the height, longitudinal and transverse deformation of the dough.

The volume of the dough is limited by the arcs of the capture, the lateral faces of the layer and the plane of entry of the dough between the rolls and its exit, is a deformation zone. On the basis of dependencies, one can establish regularities and calculate the rational parameters of individual operations. That is, thoroughly evaluate the influence of the design parameters of the injection nozzle. These parameters mainly include: working chamber and roller surface; diameter and angle of tightening of the mass of the test; properties of the dough after pumping and

forming; specific energy consumption; reliability and duration of the machine's operation.

So, the length of the deformation zone:

$$l = \sqrt{R\Delta h}$$

This zone is constantly in contact with the weight of the dough and, accordingly, the component of adhesion.

Obviously, in some cases, the search for geometric bindings to ensure the specified angles of roll-to-roll coverage can be significantly simplified if the initial synthesis of process equipment is resolved. However, the instability of the values of adhesion and external conditions of operation of roll systems, as well as variations of physical and mechanical parameters dough, lead to the need to search for non-standard approaches to provide the specified kinematic parameters of their displacement. The situation associated with the injection of a dough with limited strength parameters is complicated by significant initial dough masses, peculiarities of feeding and pumping mechanisms, and others like that.

The theory of frictional interaction of the dough with the supporting moving elements of the system was created on the basis of the assumptions about the limited resistance in bending deformations, compression, torsion. The transfer of the dough movement between the rotating rollers is provided by the forces of adhesion and friction that arise between them as a result of their contact. The forces of adhesion, distributed over the arc of coverage, primarily depend on the arcs of coverage, preliminary tightening (tension) and coefficients of friction.

The study of the phenomena of adhesion of the dough with rolls is conditionally divided into two cases: the dough moves along the surface of the cylindrical roll, and when there is no complete relative slip. Both of these occur in systems of transportation and use of viscous materials, the properties of which are limited to the resistance to bending, compression and torsion deformations. Let's dwell in more detail on the relations between the power parameters of the "roll-thistle" system Figure 4. This case corresponds to the system for changing directions in the trajectories of moving the dough or for creating and stabilizing the resistance during displacements and tightness (tensions) at individual sites.

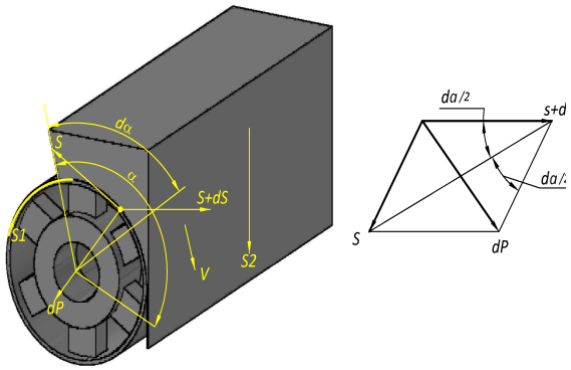

Figure 4 Scheme for extension of determining the force parameters of the roller-dough system.

We accept that a dough on a roll of a given quantity during transportation does not deform, and its speed of slipping V = const. The weight of this dough and its centrifugal force are neglected. If necessary, overcome the strength of adhesion F_{ad} we have:

$$s2 = s1 - F_{ad} \text{ and so}$$
$$F_{ad} = s2 - s1 \quad (1)$$

In the scheme dα and α, respectively, the elementary and full angles of coverage, s and s + ds-tightening (tension) of the dough. Then the elementary force of adhesion dF_{ad} will be:

$$dF_{ad} = (s+ds)-s = ds \text{ and } dFad = fdP \quad (2)$$

Where:

dP – the elemental force of the pressing, which is determined by the known forces s and s + ds.

If you ignore the values of the second order and replace the parallelograms with the diamond with sides s, then

$$dP = 2ssind\alpha/2 = 2sd\alpha/2 = sd\alpha \quad (3)$$

Then, considering the equations (2), we have:

$$ds/s = fd\alpha \quad (4)$$

By integrating the left and right parts of the condition (4) in the range from s_1 to s_2 and, respectively, from zero toα, wil have:

$$\int_{s_1}^{s_2} \frac{ds}{s} = \int_0^{\alpha} fd\alpha \ \ln\frac{s_2}{s_1} = f\alpha \quad (5)$$

$$\text{So } s_2 = s_1 e^{\alpha f} \quad (6)$$

Then, taking into account condition (1), write:

$$F_{ad} = s_1(e^{\alpha f} - 1) \quad (7)$$

Force F_{ad} is the greatest force that can be transmitted. The distributed adhesion that acts on the dough is equal to the difference s_1 and s_2, that is:

$$F_{ad} = s_1 - s_2 = s_2(e^{\alpha f} - 1) \quad (8)$$

The value of s_1 must be matched to the allowable load of the dough from the tensile strength and at the same time:

$$[s_1] \le [\sigma_0] f_0 \quad (9)$$

Where:

$[\sigma_0]$ – permissible stress, Па; f_0 – the area of its cross-section, m^2.

The condition of the transfer of motion from roll to dough is determined by the size of the angle of coverage $[\alpha]$

$$\frac{\ln\frac{s_1}{s_2}}{f} \geq [\alpha] \geq \frac{\ln\frac{[\sigma_0]f_0}{s_2}}{f} \quad (10)$$

Under the action of rollers, the force of adhesion is partially reduced due to the action of centrifugal forces. Given the last correlation between the power parameters get the form:

$$s_1 = F_{ad}\frac{e^{f\alpha}}{e^{f\alpha}-1} + \frac{qv^2}{g} \quad (11)$$

$$s_2 = \frac{1}{e^{f\alpha}-1} + \frac{qv^2}{g} \quad (12)$$

$$F_{ad} = (s_1 - \frac{qv^2}{g})\frac{e^{f\alpha}-1}{e^{f\alpha}} \quad (13)$$

Where:
v – velocity, $m.s^{-1}$;
q-is the weight of the flexible element, $kg.m^{-1}$;
g – acceleration of free fall, $m.s^{-2}$.

The given theoretical dependencies in their totality allow to carry out the calculation two-level experiments, which will create prospects for deepening the possibilities of the transfer of motion and in systems with frictional connections with quantitative estimates of the effects of various factors on the original value. The importance of the latter is related to the need to improve and optimize the systems, because even in the presence of mathematical formalization of processes or phenomena in the presence of several factors of influence the direction of finding their optimal relationships remains unknown. Object optimization refers to a set of values of control parameters that ensure the achievement of extremes of the output value and the comparison of the significance of the factors of influence.

The set of mathematical formalizations in the work allows to carry out planning of computational experiments with number of factors from two to four with reception of various functions of the response, using obtained mathematical formulas at the level of algorithms of calculations in direct application or their combinations. In the first approximation, we turn to formula (7), in which the response function is represented by the force of adhesionF_{ad}, and among the factors of influence, let's draw a tension s1 dough, angle of coverageαand the coefficient of friction $f_{т3}$. Landmark when selectin GAvalues 1 should BEAvalues 1 max, which is determined by the cross-sectional area of the test and the permissible stress$[\sigma_0]$in its stretching load and at the same time:

$$s_{1\max} \leq [\sigma]b\delta \quad (14)$$

Where:
b and δ – respectively, the width and thickness of the dough, m.

Due to the fact that there is a two-level experiment, each of the factors acquires two meanings: the upper X and the lower Xin are equal. Since the planning and processing of experiments are performed not with physical but with encoded values, then we define coded changes xi :

$$X_i = \frac{X_i - X_i^0}{h_i} \quad (15)$$

Where:
X_i^0 – the main factor level;
hi – the interval of its variation.

If the quotient space is bounded by the upper X and lower Xin, then the interval is called half the range in which the factor changes:

$$h_i = \frac{1}{2}(X_{ib} - X_{in}) \quad (16)$$

and the main factor level:

$$X_i^o = \frac{1}{2}(X_{ib} + X_{in}) \quad (17)$$

For a two-level experiment, each factor varies in two levels Xi and Xin, so the coded values of chi will have only two values: -1 and +1.

In accordance with condition (14) we will accept $S_{iв} = S1max$ and $S_{iн} = 0{,}8S_1max$. So

$$s_i^0 = \frac{1}{2}(s_{i\max} + 0.8s_{i\max}) = 0.9s_{i\max} \quad (18)$$

and the variation interval is:

$$h_i = \frac{1}{2}(s_{i\max} - 0.8s_{i\max}) = 0.1s_{i\max} \quad (19)$$

Table 1 Experiment conditions.

Size	Factors		
	S_1,H	α, rad	f
Code mark factor	X_1	X_2	X_3
The main rye, N.	0.9 $s_{1\max}$ = 2250	2.617	0.3
Variation interval, n	0.1 $s_{1\max}$ = 250	0.523	0.1
Lower level, N	$s_i^0 - h_i = 2000$	2.093	0.2
Upper level, N.	$s_i^0 + h_i = 2500$	3.13	0.4

Table 2 The matrix of planning a three-factor.

Number experience	Factors				Function feedback
	X_0	X_1	X_2	X_3	
1	+	-	-	-	3039
2	+	+	-	-	3799
3	+	--	+	-	3747
4	+	+	+	-	4684
5	+	-	-	+	4619
6	+	+	-	+	3774
7	+	-	+	+	7220
8	+	+	+	+	8777

Accordingly, we will accept:

$$\alpha_i^0 = 150^0 = 2.617 rad$$
$$\alpha_в = \alpha_i^0 + 30^0 = 180^0 = 3.14 rad$$
$$\alpha_n = \alpha_i^0 - 30^0 = 120^0 = 2.093 rad \qquad (20)$$

The basic factor of the coefficient of friction will be adopted f = 0.3, variation interval $f_\alpha^0 = 0.1$.

$$Sof_В = 0.4 \text{ and } f_Н = 0.2$$

The results of factor coding are listed in Table 1. The matrix of planning a three-factor experiment is presented in Table 2. The X_0 column is required to calculate the coefficient b_0. For three factor experiments, the regression equation has eight unknowns and, accordingly, the matrix of planning has eight experiments N.

Matrix of planning in Table 2 is supplemented by a column in which the results of calculations of the response function are entered. To determine the values of s1max we turn to the value of the permissible values of the stresses of the dough. For a yeast dough for strawberries, the allowable stress for stretching is 40 – 50 MPa. We will accept the thickness of the dough δ = 0.125 m, and width b = 0.5 m. Then the cross-sectional area of the film is A = 0.0625 m^2 and per allowable stress for stretching [σ]p 40 MPa.

The next step involves defining the coefficients included in the regression equation. The experimental data array was processed using the application package "Statistica-12" for the computer. The coefficients of the regression equation or approximating function, provided the orthogonality and symmetry of the plan matrix of the planned factor experiment, were determined according to the standard method for known dependencies. The regression equation has the form:

$$y = -5095.9 + 0.29X_1 + 2632.05X_2 + 9130.19X_3$$

At the probability level p = 0.95 and the t-alpha criterion value of 2.365, the following statistics were obtained (Figure 5): the coefficient of multiple determination D = 0.842; coefficient of multiple correlation R = 0.917; standard deviation of estimation s = 0.802; Fisher's F-criterion is 7.087. Coefficient D is significant with probability p = 0.92863.

Graphic representation of the change in the adhesion strength of the dough according to experimental data (Figure 6), that is, the surface of the response of the functional change of the strength of adhesion of the dough as a functional

$$F_{ad} = f(f_T, \alpha)$$

It follows from this that all factors of influence are important in terms of the interests of increasing the tightening of the dough (tension) in contact with the plane, as the driving factor in this case is the adhesion force (condition (7)).

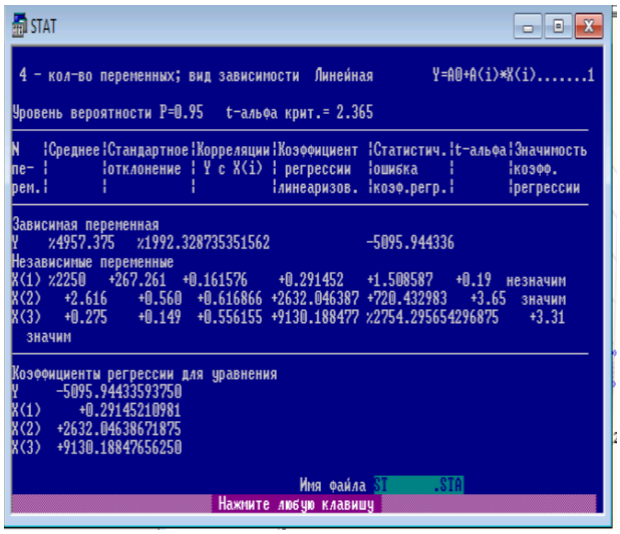

Figure 5 Statistical evaluation.

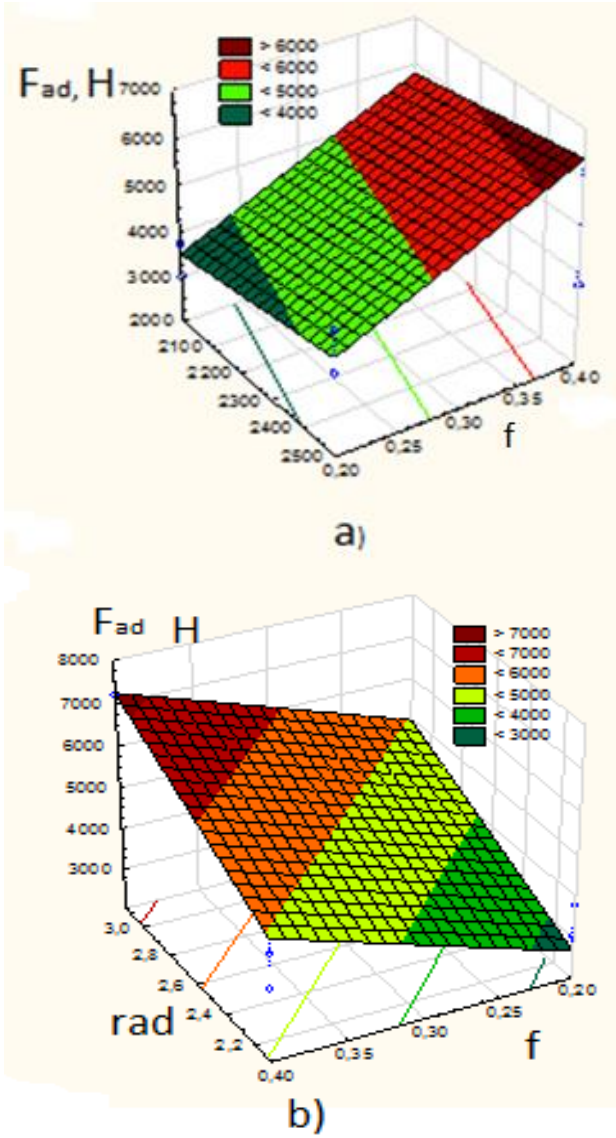

Figure 6 Dependency response graphs. Note: a) y from x_1 and x_2; b) y from x_2 and x_3.

CONCLUSION

The modern theoretical basis for the synthesis of technological machines on the basis of the interaction of working bodies with the environment combines the possibility of considering technological, economic requirements, indicators of high productivity, energy savings, restrictions of dynamic loads, etc. The achievement of the combination of these requirements is largely due to the use of adhesion bonds directly between the working bodies of technological machines and in the production lines. In systems of displacement of a viscous medium (dough) the use of friction working bodies prevails. Energy costs in the displacement systems of the test are related to the work of the driving forces against adhesion and friction and to the creation of flows of kinetic energy of the moving masses. On the basis of the analysis of the peculiarities of the construction of molding machines, mathematical formalization of transient adhesion bonding processes with the achievement of influential modes consisting of two effects on the basis of non-overlapping stages of acceleration of working bodies and in connection with the transfer of forces of inertia to the role of driving forces was proposed.

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THE EFFECT OF MELON AND WATERMELON CONCENTRATES ON CONSUMER PROPERTIES OF POLYCOMPONENT DAIRY DESSERT

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ABSTRACT

A full balanced nutrition is a necessary condition for a person's normal physical and mental development, resistance to the effects of adverse environmental factors and strengthening immunity, which is of particular importance in the bad ecological situation in the world. Providing the population with high-quality bio-functional food is an important state task, the fulfillment of which is the key to the health of the nation, and ultimately ensures the security of the country. One of the effective ways to prevent and treat various diseases is the development of a new generation of functional dessert products with dietary properties. As a basis, there has been a principle considered, i.e., the high nutritional, biological value and physiological activity of the product are predetermined by the high quality of the raw materials. The article analyzes the state of the dairy industry at the present stage. The efficiency of melon and watermelon concentrates in the production of multicomponent dairy dessert has been substantiated. The positive effect of the tested concentrates on the structural and mechanical properties of the jelly part of the dessert, organoleptic characteristics and nutritional value of the product has been established. The optimal concentration of the gelling agent in the jelly has been determined. There were some conducted experiments aimed at maximum reducing of the gelling agent's weight fraction in the product.

Keywords: milk dessert; whey; jelly; carrageenan; melon and watermelon

INTRODUCTION

At the present stage of the food industry development, the whey as the main raw material has been a research subject for a number of scientific schools in the USA, Great Britain and Japan **(Faryabi et al., 2009; Yang, Irudayaraj and Sakhamuri, 2001)**. Their research studies were mainly aimed at functional properties of whey and its feasibility, mainly in clinical nutrition **(Ghosh and Playford, 2003)**. Whey proteins are used to prepare baby foods, since their composition is more similar to the composition of mother milk proteins **(Gorlov et al., 2015)**.

Today, one of the promising areas for the introduction of whey products is the market for dairy desserts. It is considered to be one of the most dynamically developing and marginal. Over the four pre-crisis years, it grew by more than 30% **(Mgebrishvili et al., 2013a)**. The reason for this was not only the high demand for dairy desserts, but also the constantly expanding assortment. Original milk desserts enjoy high demand, their consumer properties are formed depending on the type and quality of the raw materials used **(Khramtsov and Vasilisin, 2004)**. In the Lower Volga region, there are considerable regional resources of watermelon and melon that may be involved in the production of dairy products with a predetermined composition and consumer properties **(Mgebrishvili and Gorlov, 2012)**. In solving this problem, a promising direction is the development of innovative functional complex dairy desserts with high nutritional and biological value.

The combined use of by-products of dairy processing production and regional plant raw materials offers exciting possibilities for creating new types of dairy products with preventive properties, provided their cost is reduced **(Mgebrishvili et al., 2013b)**.

The research conducted meets the current trends in the development of the dairy industry. The most important trend is the development of innovative technologies aimed at expanding the range and improving the quality of output products. An important aspect here is the use of secondary dairy raw materials, in particular, whey, which not only increases the biological value of the product, but also reduces its cost price **(Gorlov, Mosolova nad Korotkova, 2012)**. In relation to the problem, it becomes appropriate to use melon and watermelon concentrates as functional fillers in recipes for dairy desserts. New types of fillers have many useful properties, which makes the dessert functional.

Scientific hypothesis

We are expecting: watermelon and melon fillers has significant effect on the process of structure formation.

MATERIAL AND METHODOLOGY

The research material contained raw ingredients, namely, goat cheese, unsalted cheese whey, watermelon (*Citrúllus lanátus*), melon (*Cucumis melo*), sponge cake with flaxseed and produced milk dessert itself.

Evaluation of the quality of the raw materials and product was conducted according to the following generally accepted methods, i.e., titrable acidity by the titrimetric method according to **GOST 3624-92**; density by the areometric method according to **GOST R 54758-2011**; and weight fraction of dry matter by the drying method according to **GOST 3626-73**. The optimal dose of the thickener in the jelly part of the dessert was established on the basis of the system's viscosity data obtained by the method of viscometry using an SV-100 vibration viscometer. With respect to the task, a low-cost technology for the production of melon and watermelon concentrates based on the thickening method was proposed. This technology allowed promising use of inexpensive plant material in recipes of complex dairy products.

Milk dessert "Sankarini" consisted of three layers. The first layer was pieces of airy sponge cake enriched with flax seed additive **(Edwards, 2009)**. The implementation of the agenda aimed at ensuring healthy nutrition of the population in the country made scientists pay attention to flax seed as a source of biologically active substances **(Buldakov, 2001)**.

The second layer was a jelly layer made of whey divided into two figured parts **(Crus, Tinyakov and Fofanov, 1986)**. One part was filled with watermelon, the other one with melon. The color of the jelly was due to the natural pigments of the filler, i.e., concentrated mashed watermelon or melon pulp, condensed in the process of vacuum evaporation. Desalted whey was used as a base for jelly. Carrageenan that is a polysaccharide derived from red seaweed was used as a gelling agent **(Gomes et al., 2013)**. Pieces of the jelly phase had a delicate sweet taste with the aroma characteristic of watermelon or melon, respectively.

The third layer was the so-called "cheese cream" made from soft goat cheese **(Krupin, 2009)**. The functional properties of this component were confirmed by the well-known effect of goat milk that is inherently identical to female milk, as it contains a lot of β-casein. Goat milk contains more sialic acid that is included in the structure of the body's immunity barriers. The fat globules in goat milk are much smaller than in cow's milk, therefore they are better digested by the body **(Mgebrishvili et al., 2013c)**.

The textures and tastes of all three parts of the product were brilliantly combined with the undoubted functionality of both individual ingredients and the whole Sankarini dessert **(Ajewole et al., 1999)**. To analyze the effect of watermelon and melon concentrates in the production of a multicomponent dairy dessert based on whey, it was necessary to investigate changes in the structural and mechanical properties of jelly depending on the number of thickener doses applied.

Statistic analysis

Experimental studies presented in the article are processed by methods of variation statistics with the determination of the criterion of reliability of the difference according to the t-test Fisher criterion using the program STATISTIKA-10.

RESULTS AND DISCUSSION

At the first stage of experimental studies, three laboratory samples of jelly without fillers were obtained. The recommended amount of carrageenan needed for gelling varies in over the range of 0.02 – 0.1% **(Basiria et al., 2018)**.

The proportion of gelling agent in the samples obtained was 0.05, 0.075 and 0.1%. Dry carrageenan was injected into the whey. One hour later, which was required for gelling, the dynamic viscosity of each sample was determined. According to the data obtained, the jelly samples with a carrageenan concentration of 0.05 and 0.075% at a temperature of about 20 °C had a coefficient of dynamic viscosity of water of 0.15 Pa.s, that is, they were not defined as a viscous system. So, only the sample with a concentration of 0.1% carrageenan was identified as a viscous system.

Today, scientists in different countries found that watermelon and melon contain high levels of fiber and pectin **(Balaghi and Senge, 2014; Sethi et al., 2016; Gul et al., 2016; Karnopp et al., 2017)**. In this regard, it was suggested that using watermelon and melon concentrates as fillers would not only add preventive properties to the product, but also minimize the concentration of carrageenan and also make maximum use of secondary raw materials.

At the second stage of the experimental studies, two model samples of jelly were developed, i.e., with watermelon and melon concentrates. The concentration of carrageenan in these samples was reduced to 0.075%. The values of the dynamic viscosity coefficients were obtained and best corresponded the jelly structure (Table 1).

When comparing the values of the dynamic viscosity coefficients for jelly samples without melons concentrates and with the concentrates, it could be seen that the fillers helped not only reduce the carrageenan concentration, but also increase the stability of the jelly system. These models are non-linear, statistically significant ($F_{observe} > F_{table}$) high decreasing linear relationship and a good value of the determination coefficient, characterizing a high proportion of the variance of the effective feature (viscosity), can be explained by regression to the total variance. Show almost the same close linear relationship and viscosity rate. (Figure 1).

At the third stage of the experimental studies, to minimize the concentration of the gelling agent, locust bean gum was proposed for this purpose. This type of stabilizers in combination with carrageenan exhibits synergistic properties and enhances the effect of the latter. Similarly, to the previous test, two model samples of jelly were developed: with watermelon and melon concentrates. The gelling agents' (carrageenan and locust bean gum) concentrations in these samples were reduced to 0.025%. These models are also linear and statistically significant ($F_{observe} > F_{table}$) with a high decreasing linear relationship and a good value of the determination coefficient characterizing the high proportion of the variance of the resultant trait (viscosity), explained by regression, in the

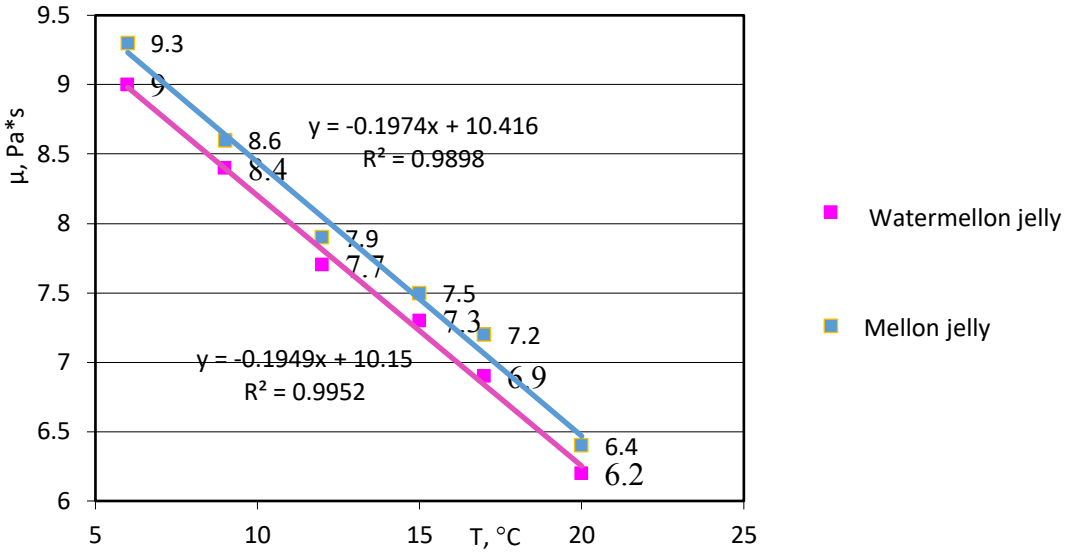

Figure 1 Influence of watermelon and melon fillers on the viscosity of jelly samples with a concentration of 0.075% carrageenan.

Table 1 Dynamic viscosity of jelly with fillers, with carrageenan concentration being of 0.075%

Experience #	Sample's temperature, °C	Dynamic viscosity coefficient [eta], Pa.s	
		melon jelly	watermelon jelly
1	6	8.9	8.6
2	9	8.54	8.2
3	12	7.82	7.44
4	15	7.36	6.98
5	17	6.9	6.66
6	20	6.2	6

Table 2 The coefficients of dynamic viscosity jelly with fillers, with gelling mixture concentration being of 0.025%.

Experience #	Sample's temperature, °C	Dynamic viscosity coefficient [eta], Pa.s	
		melon jelly	watermelon jelly
1	6	9.26	9.02
2	9	8.6	8.44
3	12	7.9	7.66
4	15	7.46	7.34
5	17	7.1	6.86
6	20	6.4	6.2

Table 3 Organoleptic characteristics of the dessert's jelly part.

Indicator	Feature
Appearance	Figured transparent pieces with glitter, keeping the shape
Color	Pink, yellow, characteristic of fillers
Smell	Pleasant, characteristic of fillers
Consistency	Dense, resilient, gelatinous
Taste	Sweet, characteristic of fillers

total variance. Show almost the same close linear relationship and viscosity rate.

The values of the dynamic viscosity coefficients were obtained and best corresponded to the jelly structure (Table 2). When comparing the values of the dynamic viscosity coefficients for jelly samples with a carrageenan concentration of 0.075% and a gelation mixture concentration of 0.025%, it was clear that the data from the two experiments were almost identical with a considerable decrease in the proportion of gelling agent in the second case (Figure 2).

Table 4 Physical and chemical indicators of jelly samples.

Indicator	Feature	
	melon jelly	watermelon jelly
Weight fraction of dry substances,%, not less than	40 ±0.5	38 ±0.5
Acidity, °T, no more than	20	20

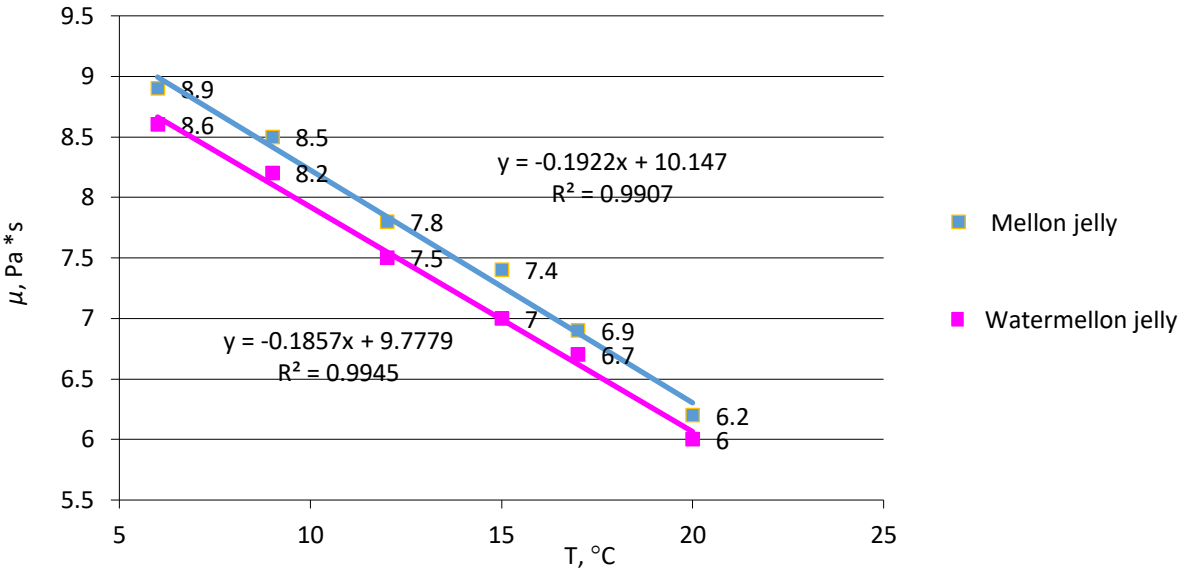

Figure 2 Effect of watermelon and melon fillers on the viscosity of jelly samples with the concentration of the mixture of gelling agents 0.025%.

At the fourth stage of the experimental studies, an organoleptic assessment of the jelly phase of the milk dessert was made in terms of appearance, color, smell and texture. The objects of the study were model samples of jelly with watermelon and melon fillers obtained using a mixture of carrageenan and locust bean gum in an amount of 0.025% (Table 3).At the fifth stage of the experimental studies, the physicochemical parameters of the jelly part of the dessert, in particular the acidity and weight fraction of dry substances, were determined. For this purpose, four laboratory samples of jelly were obtained, two with watermelon and two with melon fillers. Physicochemical characteristics of the jelly samples are shown in Table 4.

CONCLUSION

The positive effect of melon concentrates on the process of structure formation of whey is scientifically substantiated and practically proved.

Studies have found that to ensure optimal conditions of structure formation in a mixture of whey and melon concentrates, a dose of a mixture of gelling agents in an amount of 0.025% of the total weight is required.

To optimize the technological parameters in the direction of ensuring maximum yield of jelly, it is advisable to partially replace carrageenan with carob gum, which in this combination shows synergistic properties.

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EFFECT OF PLANT GROWTH REGULATORS ON BIOCHEMICAL COMPOUNDS OF TANGERINE (*CITRUS UNSHIU* MARC.)

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ABSTRACT

We investigated the effect on tangerine of new generation plant growth regulators. The use of drugs in the period of fruit ripening has led to increased 2.0 – 3.7 times abscisic acid (AA) and 1.9 – 4.7% of Indole-acetic acid (IAA) acid in the leaves. Studies have shown that Indole-acetic acid and abscisic acid beginning of a sharp accumulation of their hormones coincides with action of stress factors and growth dormancy period. The use of the regulators had an impact not only on their content in leaves but also on fruit quality. For example, treatment Indole-acetic acid and Obstaktin led to an increase in the fruit of vitamin C. After treatments with plant growth regulators has been a significant decline in the total number of organic acids (up to 2.35% at the option of Melaphen and to 2.50% at Obstaktin, LSD ($p \leq 0.05$) = 0.06). By reducing the content in the fruits of organic acids to all variants increased the sugar-acid index. After each spraying tangerine on the treatment options plant growth regulators has been a significant increase the dry matter. Thus, the positive effect of plant growth regulators on all the quality characteristics of tangerine was shown. In the summer period, the treatment by regulators may have a protective effect, increases the content in plants the content of Indole-acetic acid. The plant growth regulators of new generation have a positive effect on quality of dwarf tangerine. Given that the plants of tangerine in the subtropical zone of Russia each summer have to drought and are losing not only in yield, fruit quality too, new regulators may exert a protective effect, because increases the content in plants is Indole-acetic acid, which activates gene expression of drought resistance.

Keywords: *Citrus unshiu* Marc.; growth regulator; spray application; dry matter; total acidity; total sugar; ascorbic acid

INTRODUCTION

Study of the effect of growth regulators on the activity of agriculture plants (in particular fruit) have been studied by many scientists. A significant contribution to the study of this problem made **Chaylakhyan (1977)**; **Sheveluha et al. (1998)**; **Gudkovskij (1999)**; **Sergeeva, Nen'ko and Kiseleva (2013)**; **Doroshenko et al. (2017)**; **Tacken (2014)**; etc. They identified a list of the most commonly used regulators, for example, on apple and pear to reduce of lost harvest fruits are often used such substances as alpha-naphthaleneacetic acid (NAA) and its potassium salt, which in parallel leads to increased fruit set, growth and ripening, as well as, to increase marketable qualities **(Agafonov and Phaustov, 1972; Edgerton, 1983; Gomez-Cadenas et al., 1996; Rath et al., 2006; Yuan and Carbaugh 2007; Doroshenko et al., 2017)**. Research of influence of regulators on fruit plants was showed not only an increase in crop yields, but also increase their resistance to adverse environmental factors **(Liholat, 1983; Zhuchenko, 2001; Chumakov, 2013; Trebichalský et al., 2016; Trebichalský et al., 2017)**. In the conditions of Krasnodar region (on the basis of Russian Research Institute of Floriculture and Subtropical Crops) was conducted to study the effect of chlor- chlorine- chloride (CCC) on plants of pear, grape and dwarf tangerine **(Gorshkov, 1976; Mikhailyuk, 1979)** and influence of micro nutrition's on dwarf tangerine and tea **(Belous, 2013; Abilfazova and Belous, 2016; Ryndin et al., 2017)**. The high responsiveness of the culture of the pear on used regulator are a reduced length growth of shoots, increased leaf formation, increased quality and break bud , increased leaf area, resulting in improved productivity. In addition, was increased drought resistance and resilience of trees pears to diseases (scab, phomopsiosis) **(Mikhailyuk, 1979)**. The effectiveness of the regulator to increase the yield of tangerine was shown. Studies have shown increase the sustainability of the culture of the vine to mildew **(Lepilov, 1985)**.

Thus, considering the above, it can be noted that the researchers made an enormous contribution to the study of influence of biologically active substances on features of functioning of agricultural plants. Currently, however, some growth regulators which used previously, or prohibited for use, or have a number of significant limitations. The nature of the impact of drugs of new generation for resistance to abiotic factors, the crop and fruit quality are not well understood, hampering their application in the practice of gardening. At the same time, the Black sea coast area (and, namely, the area of the Big Sochi) refers to the resort and adjacent to the biosphere reserve, so the use of many old

regulators banned because of their danger to endemic communities. In this regard, there is an urgent need to study the mechanism of the effect on tangerine of new generation regulators with subsequent development of evidence-based recommendations for their effective use. Soil and climatic conditions of the Black sea coast of Krasnodar region is humid subtropical, however, and in these conditions systematically recorded detrimental effect on fruit plants the different climatic stress factors. For tangerine limiting abiotic stressors are cold with no snow cover, drought and elevated air temperature during summer. Thus, at the temperature of +35 °C, there is depression of vital processes of tangerine, which leads to uneven growth of the fruit, not simultaneous maturation, and deterioration of their colour and reduce palatability **(Gorshkov, 1976; Abilfazova, 2017)**.

Scientific hypothesis

The use of growth regulators has become an important component of agro-technical procedures for most of fruit plants, namely, tangerine. The plant hormones are important components in the integration of plants developmental activities. Environmental factors often exert inductive effects on metabolism and distribution of hormones within the plant. Apart from it, they also regulate expression of intrinsic genetic potential of plants. In this review, we focus on the plant growth regulators of new generation. We thinks that plant growth regulators influence on quality of dwarf tangerine - namely, increase the content of dry matter, total sugars and vitamin C in fruits.

MATERIAL AND METHODOLOGY

Field experiments were carried out on tangerine plantations (*Citrus unshiu* Marc.) in accordance with the "Program and methods of variety investigation of fruit, berry and nut crops" **(Sedov, 1999)**. The object of study – plants of the dwarf tangerine varieties 'Miagawa Wase' grafted on *Poncirus trifoliata*, planting in 1986 at the plantation Institute. As plant growth regulators used the following regulators:

1. IAA (Heteroauxin or Indole-3-acetic acid) – production control, at a concentration of 0.02%;
2. Melaphen in concentration of $1.10^{-8} – 1.10^{-9}$ %;
3. Obstaktin in concentration of 0.05%.

Control - treatment of water. Repeated experience is 5- samples, for a single sample is a "tree-repetition". Repeated laboratory tests are three times. Foliar treatment was carried out three times: first in the phase "the closing of the sepals" (3rd decade of May), the second by size of the fetus "walnut" (3rd decade of June), the third for 30 days before harvesting the fruits.

Heteroauxin (Indole-3-acetic acid (IAA) has many different effects, such as inducing cell elongation and cell division with all subsequent results for plant growth and development. On a larger scale, IAA serves as signalling molecule necessary for development of plant organs and coordination of growth. IAA enters the plant cell nucleus and binds to a protein complex composed of ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, and a ubiquitin ligase, resulting in ubiquitination of Aux/IAA proteins with increased speed. Aux/IAA proteins bind to auxin response factor (ARF) proteins, forming a heterodimer, suppressing ARF activity. IAA inhibits the photo respiratory-dependent cell death in photorespiratory catalase mutants. This suggests a role for auxin signalling in stress tolerance. Obstaktin is phyto-regulators growth and the effect of auxin. Active substance is a potassium salt of 1-naphthylacetic acid. Melaphen is a regulator of growth and development of a new plants generation. Its distinguishing feature is the high efficiency and breadth of action at extremely low concentrations used. Application melaphen leads to significant increase in productivity and quality of the products. Laboratory analyses were made at Laboratory of biotechnology, biochemistry and plant physiology in triple repetitions: Ascorbic acid content was determined by iodometric method with 2% HCl, titrated 0.001 N solution of KIO_3; total acidity - titration with NaOH (0.1 mol.dm^3) in the presence of phenolphthalein indicator. The amount of sugar was determined by Bertran`s refractometric method. The method is based on the ability of sugars aldehyde group interact with Fehling's reagent and restore CuO to Cu_2O precipitated as a red solid **(Ermakov et al., 1972)**.

Statistic analysis

Statistical processing of the experimental data was carried out using the ANOVA package in STATGRAPHICS Centurion XV (version 15.1.02, StatPoint Technologies) and MS Excel 2007. Statistical analysis included univariate analysis of variance (method of comparing averages using variance analysis, *t*-test) and variance analysis (ANOVA). The significance of difference between the means of the least significant difference (LSD) results with $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate and the values were expressed as mean ±*SD*. The differences between the samples were assessed using unpaired *t*-test. Correlation analysis with calculation of pair correlation coefficient, for establish the dependence of parameters on abiotic factors was used.

RESULTS AND DISCUSSION

Studies have shown that the use of a number of drugs in the period of ripening (30 days before harvest) led to an increase in 2.0 – 3.7 times abscisic acid (AA) and 1.9 – 4.7% indole-3-acetic (IAA) acid in the leaves (Figure 1). As you know, the content of AA increases in the state the growth dormancy, under the action of stress factors (for example, water deficit), during the period of abscission of fruits etc. **(Pustovoitova, Zhdanova and Zholkevich, 2004)**. Studies have shown that IAA and AA involved in signal transmission of stress and activate gene expression of drought resistance; the beginning of a sharp accumulation of these hormones coincides with the loss of plant turgor **(Levitt, 1985; Sergeeva, Nen´ko and Kiseleva, 2013; Madzhar, 2015; Doroshenko et al., 2017)**. In our case, there was a combination of two processes - the action of stress factors coincided with the growth dormancy period.

The use of the regulators had an impact not only on their content in leaves but also on fruit quality (Table 1). We have determined that ascorbic acid content in fruits on average in the variant of experience over the entire study period was 93 – 111 mg.100g^{-1}.

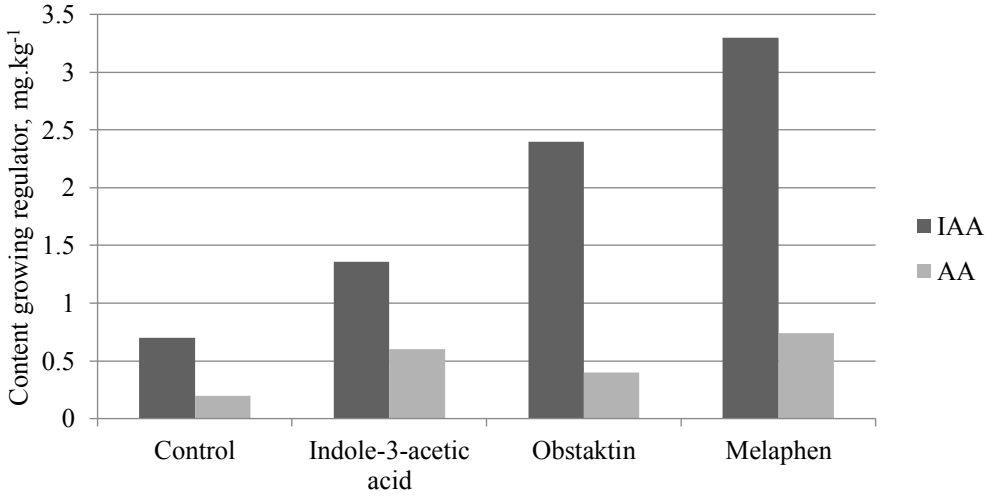

Figure 1 Influence of growth regulators on the their content in leaves, the analysis was performed 30 days after treatment.

Table 1 Dynamics of chemical composition of fruit after spraying with regulators.

Variant	Ascorbic acid, mg.100g^{-1}	Total sugar, %	Total acidity, %	Sugar-acid ratio, unit	Dry matter, %
1 spray application					
Control	94.92 ±0.97	10.76 ±0.39	3.03 ±0.02	3.56 ±0.12	14.30
Indole-3-acetic acid	107.30 ±1.15	10.36 ±0.29	2.79 ±0.03	3.71 ±0.07	30.30
Obstaktin	83.84 ±1.02	11.00 ±0.25	2.83 ±0.02	3.89 ±0.09	31.70
Melaphen	91.17 ±1.23	11.40 ±0.18	2.60 ±0.08	4.39 ±0.69	31.70
***LSD* ($p \leq 0.05$)**	**2.07**	**0.54**	**0.08**	**0.69**	**-**
2 spray application					
Indole-3-acetic acid	103.78 ±1.02	11.19 ±0.07	2.14 ±0.03	5.24 ±0.05	29.4
Obstaktin	106.13 ±1.76	10.90 ±0.06	2.56 ±0.07	4.26 ±0.12	28.5
Melaphen	85.59 ±1.02	10.69 ±0.30	2.32 ±0.05	4.61 ±0.12	25.5
***LSD* ($p \leq 0.05$)**	**2.33**	**0.47**	**0.09**	**0.5**	**-**
3 spray application					
Indole-3-acetic acid	89.12 ±1.34	11.68 ±0.11	2.35 ±0.02	4.98 ±0.05	29.8
Obstaktin	111.99 ±1.02	9.89 ±0.31	2.11 ±0.01	4.69 ±0.15	28.9
Melaphen	98.97 ±1.59	10.83 ±0.33	2.12 ±0.02	5.10 ±0.18	27.5
***LSD* ($p \leq 0.05$)**	**2.92**	**0.45**	**0.04**	**0.19**	**2.65**

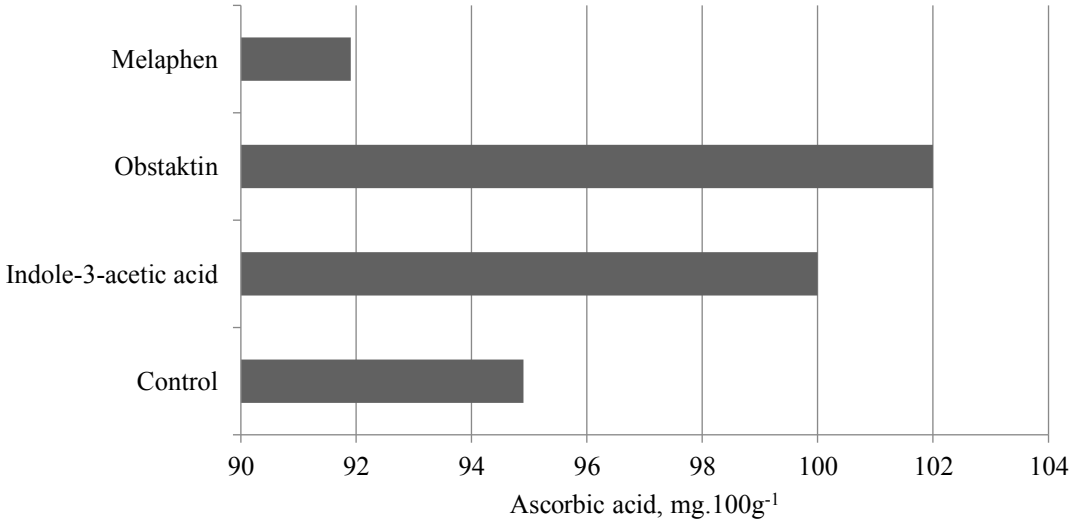

Figure 2 Content of ascorbic acid in the fruits under the treatments with regulators, LSD ($p \leq 0.05$) = 0.49.

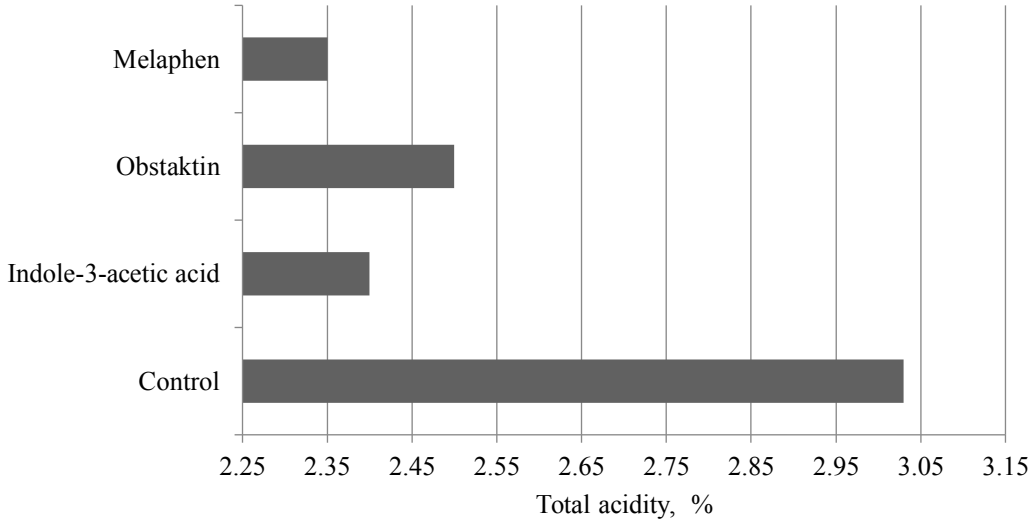

Figure 3 Content of titratable organic acid in the fruits under the treatments with regulators, LSD ($p \leq 0.05$) = 0.06.

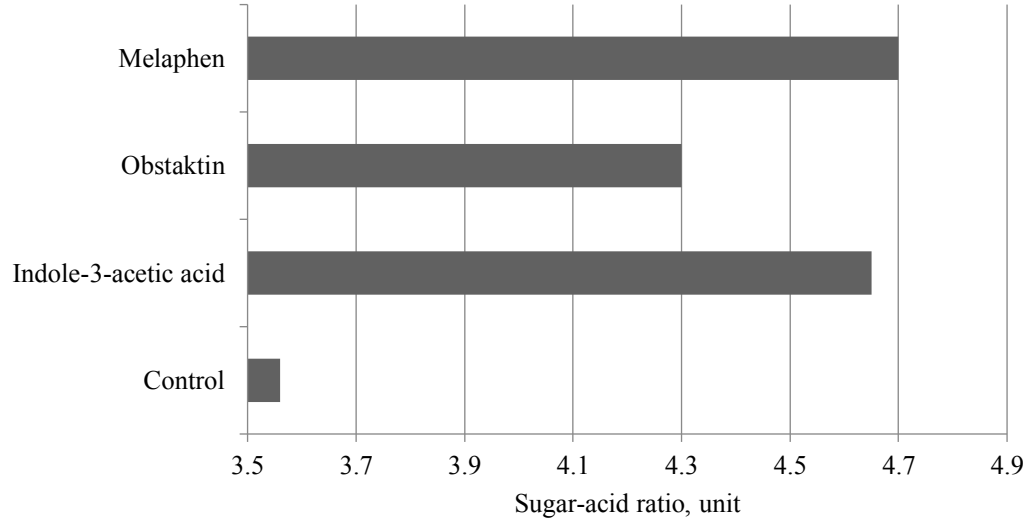

Figure 4 Sugar-acid ratio in the fruits under the treatments with regulators, LSD ($p \leq 0.05$) = 0.44.

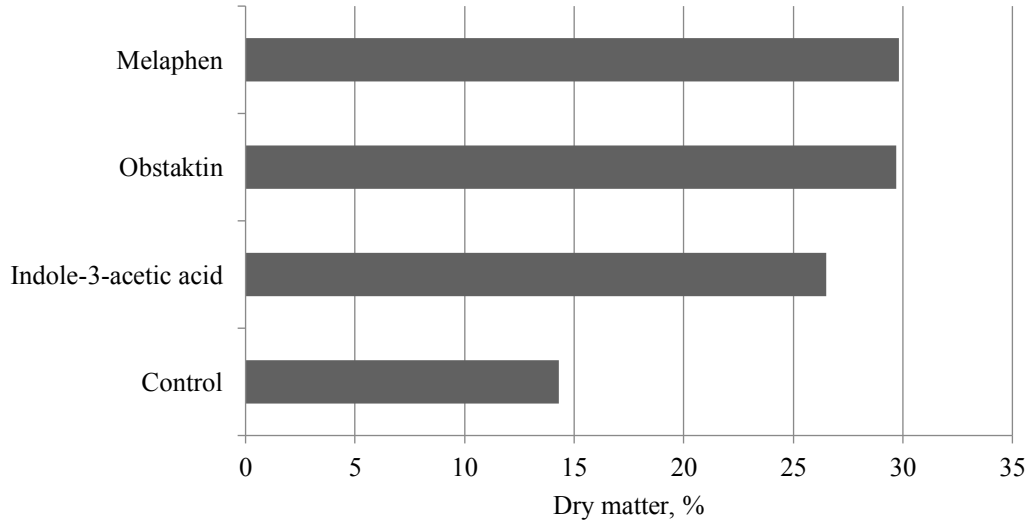

Figure 5 Content of dry matter in the fruits under the treatments with regulators, LSD ($p \leq 0.05$) = 0.65.

Moreover, an active accumulation of ascorbic acid was observed after the second and after the third spraying (Table 1). Thus, at the time of harvest fruit the variants "Indole-3-acetic acid" and "Obstaktin" were more nutritious than others (Figure 2).

The treatment of plants with growing regulators did not affect the total content of sugars, which for the entire period of studies on the variants of the experiment was in the range of 10 – 11%. Some reduction of sugars quality compared with the control was observed only after the third processing of Obstaktin (Table 1). In contrast to the sugar content, the total number of titratable organic acids in fruits has significantly changed after treatments of growing regulators (Figure 3). So, for period of studies the content of organic acids was on average 2.57%, but after each treatment there was a significant (LSD (p ≤0.05) = 0.06) decline to 2.35% (Melaphen) – 2.50% (Obstaktin) compared with the control (3.03%).

The taste quality of the fruit determines not only the content of sugars and acids, but in the first place their ratio - sugar-acid index. The higher index is the better dessert quality of the fruit. In our study, this indicator ranges from 3.56 (Control) to 4.70 (Melaphen) with an average value of 4.30 units (Figure 4). As can be seen from Figure 4 all variants with spraying of phytohormones significantly exceeded the control (LSD (p ≤0.05) = 0.44). Moreover, if after the first treatment, the increase in sugar-acid index was observed only at the option Melaphen (4.39 units), after the second and third treatment with phytohormones, sugar-acid index has risen on all variants relative to the control due to the reduction in the fruit organic acids (Table 1).

One of the most important indicators determining the quality of plant raw materials is the content of dry substances in the fruits. The amount of solids in the fruit's ranges from 10% to 20%. In some cases (subtropical fruits, for example, feijoa, persimmon etc.) it can reach 25% and above. In our case, the dry matter content ranged from 14.30 to control, to 29.83 – on option Melaphen, with an average value of 25.52% (Figure 5). The study showed that a significant increase of dry matter in tangerine under used hormones occurred after each spraying (Table 1).

CONCLUSION

The plant growth regulators of new generation have a positive effect on quality of dwarf tangerine - namely, increase the content of dry matter, total sugars and vitamin C in fruits. The plant growth regulators (Indole-3-acetic acid, Melaphen and Obstaktin) proved to be highly effective, as it had a positive effect on all quality characteristics of fruit. Given that the plants of tangerine in the subtropical zone of Russia each summer have to drought and are losing not only in yield, fruit quality too, new regulators may exert a protective effect, because increases the content in plants is Indole-acetic acid (IAA), which activates gene expression of drought resistance.

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PLANTS OF *NEPETA CATARIA* VAR. *CITRIODORA* BECK. AND ESSENTIAL OILS FROM THEM FOR FOOD INDUSTRY

Nataliia Frolova, Anatoliy Uktainets, Olga Korablova, Volodymyr Voitsekhivskyi

ABSTRACT

Nepeta cataria var. *citriodora* Beck. (catmints) is a source industrial production of citral and attractive raw material for food industry and cooking. Aerial part of *Nepeta* are characterized by high antimicrobial activity and fungicidal action against mold fungi, used in folk medicine, as ingredient in recipes for sausages, liqueurs and soft drinks, vegetable and fruit canned food, in the manufacture of vermouth. Ukrainian variety 'Melody' was created specifically for growing in the Forest-Steppe zone, and variety 'Peremozhets' – in the Steppe zone. Data on the yield aerial part and essential oil *Nepeta* was determined. The dry aerial part of plants *N. cataria* we used to create a dry spicy mixture for sweet dessert dishes. Quantitative content and qualitative composition of essential oil of plants by organs and phases of vegetation are presented in the article. In our research we used essential oils obtained by hydro distillation procedure for 2 h using Clevendger-type apparatus from the flowering parts of plants *N. cataria* 'Peremozhets' and 'Melody'. Investigate of components was carried out by high effective gas chromatography with HP 6890 chromatograph coupled with HP 5972 mass selective detector. The most abundant components of *Nepeta* essential oil was citral, geraniol, as well as nerol, citronellol, citronellal, carvacrol, camphor, eugenol. We proposed fractional distillation of essential oils to obtain a line of flavors with stable sensory and physicochemical indicators for food industry. The separation of essential oils into fractions was carried out on a pilot installation of fractional distillation DFD (Device of Fractional Distillation). Calculations of parameters controlled dispersal of essential oils (residual pressure, temperature regimes, number of theoretical plates, reflux number) were carried out. During fractionation of essential oil of *N. cataria* four fractions were obtained with a content of 96 ±0.5% to the total mass of samples. Sensory and physicochemical analysis of aromatic fractions announced them as promising flavours for food industry.

Keywords: *Nepeta cataria*; yield; essential oil; fraction; flavour

INTRODUCTION

The total number of aromatic and spicy-aromatic plants of the world flora is estimated at 2500 – 3000 species. The main families, which include a large number of aromatic plants, are *Lamiaceae, Apiaceae* and *Asteraceae* **(Tkachenko, 2011)**. *Nepeta cataria* var. *citriodora* Beck. (common name – catnip, catmints) is a source of industrial production citral and attractive raw material for the food industry and cooking, it is widely used in the world due to the very pleasant aroma of essential oil **(Burt, 2004; Bhattacharya, 2016)**. The plant originates from the Mediterranean and the Western Asia. It is distinguished by its high ecological plasticity. *N. cataria* is characterized by a significant intravivid variability, which results to selection of high-yielding samples – source forms for breeding. One of the ways to intensify production raw materials of aromatic plants in Ukraine for the production of essential oils is the creation of new high-yielding varieties **(Korablyova, 2001)**. Variety *Nepeta cataria* 'Melody' was created specifically for growing in the Forest–Steppe zone of Ukraine and the variety 'Peremozhets' – for growing in zone Steppe.

Essential oil of *N. cataria* is a colourless, mobile liquid with a pleasant herb-citrus aroma with tones of geraniums. The main components of essential oil *Nepeta cataria* are citral – 12%, geraniol – 25%, as well as nerol, citronellol, citronellal, carvacrol, camphor, eugenol. The composition of the oil includes the main classes of compounds: monoterpene alcohols – 71.2%, monoterpene aldehydes – 19.3%, sesquiterpenes – 2.5%. Nepetalactone accumulates in this essential oil 0.8 – 1.9% **(Ríos, 2016)**. It should be noted that in literary sources data are given on the composition of essential oils *N. cataria* with significant differences, even with the content of key components. These are citronellol and geraniol, the content of which varies for citronellol from 8 to 70%, citral from 0 to

18.33%, and geraniol – from 0 to 28%. World production of essential oils is more than 5000 tons per year **(Franz, 2006)**. Most often, essential oils are used in the production of perfumery and cosmetic products, hygiene products, in the form of drugs. The average annual outputs of such products in the world are fixed at 7.5 million units **(Khan and Abourashed, 2011)**.

Plants of *N. cataria* are used in folk medicine **(Naghibi et al., 2005; Adiguzel et al., 2009)**. The broth from them has immunomodulatory properties, normalizes the work of the cardiovascular, nervous and respiratory system, and stimulates appetite. It is used for malignancy, cough, liver disease, jaundice, intestinal atony, hysteria, headache, gynecological diseases, as well as anti-helminthes agent **(Gilani et al., 2009)**. At a fairly low concentration of essential oil as aerosols in the air, blood pressure is rapidly normalized.

Essential oil of *N. cataria* is characterized by high antimicrobial activity and fungicidal action in relation to mold fungi: *Mucor*, *Penicillium*, *Aspergillus* **(Zomorodian et al., 2012; Foltinová, Tančinová and Císarová 2017; Tančinová et al., 2018; Stević et al., 2014)**, like as potent source of nematicidal compounds for use in phytopathology and entomology **(Pandey et al., 2000; Peterson and Ems-Wilson, 2003; Tworkoski, 2002; Juglal, Govinden and Odhav, 2002; Amer and Mehlhorn, 2006)**.

As a flavouring *Nepeta* is an ingredient in recipes for sausages, liqueurs and soft drinks, vegetable and fruit canned food, dry spicy mixtures. It is used fresh and dry in the confectionery industry, in the manufacture of vermouth, tea and different cheeses, as tea and drinks additives **(Korablova and Rakhmetov, 2012; Shanaida et al., 2018)**. Natural flavours – this is primarily the essential oils, as well as individual aromatic components that are derived from essential oils by physical methods. GC-FID is the traditional method for essential oils quantification while GC-MS is the most common analytical method for qualitative analysis **(Smelcerovic et al., 2013; Baranauskiene et al., 2003; Frolova et al., 2005; Ukrainets and Frolova, 2009)**.

In food technologies, with considerable demand for natural aromatic substances, using of essential oils are quite limited **(Utami et al., 2011)**. Objective obstacle is a rather narrow range of commercial essential oils, which is associated with the difficulties of their qualitative selection and storage **(Tkachenko, 2011)**. The component composition of the essential oils varies depending on the climatic conditions of cultivation, and the terpene components of the oil when in contact with air during storage are rapidly oxidized **(Méndez-Tovar et al., 2016)**.

This leads to a change in the colour and organoleptic characteristics of the oil. Hardly controlled changes in the quality of essential oils make producers find alternatives to flavouring products, use synthesized substitutes **(Surburg and Panten, 2006)**. Distribution of the release of synthetic sources of aroma is considered the main subjective reason for the narrow use of essential oils in food technologies.

Essential oils in their natural form practically do not use. A significant amount of them is processed by different technologies. The most effective technologies are the following: the receipt of monofractions with subsequent chemical transformation **(Utami et al., 2011)**, deterpening, which releases essential oils from the group of terpene components **(Fantin et al., 2010)**, aims to separate the fractions by distillation, extraction, membrane technology **(Brose et al., 1995)**. Studies of recent years extend to the study of the possibilities of fractionation of essential oils by supercritical fluids **(Chiyoda et al. 2011)**.

We considered the experience of medicine, the perfume and cosmetics industry regarding the mechanisms and practices of fractional disintegration of organic mixtures and proposed fractional distillation of essential oils to obtain a line of flavours of stable sensory and physicochemical indicators for the food industry.

Essential oils are not technologically processed in Ukraine. A common practice is the combination of solutions of essential oils in ethyl alcohol, propylene glycol, as well as in so-called "heavy" ethers. Such mixtures are the basis for the receipt of perfumery and cosmetic products **(Peshuk, Bavkina and Demidov, 2007)**.

Getting natural flavours of improved stability from essential oil *Nepeta cataria* with the development of parameters for its processing into separate fractions (flavouring) of various flavours and their use in formulations of composite flavours for the food industry has urgency, social and industrial request.

Scientific hypothesis

Growing conditions and varietal characteristics of *N. cataria* can affect the productivity of green mass and the composition of the essential oil. Separating different oil fractions will allow us to create flavours with a planned smell.

MATERIAL AND METHODOLOGY

The work was carried out at the National University of Food Technologies and M. M. Gryshko National Botanical Gardens of NASU, located on the border of the Forest-Steppe zone and the Polissya of Ukraine**.** The object of the study was plants of Ukrainian varieties *N. cataria* 'Peremozhets' and 'Melody' **(Korablova and Rabotyagov, 2007)**. The crop accounting was carried out during the period of mass flowering of plants in 2011 – 2015 by the method of field experiments **(Dospekhov, 1986)**. The raw material was cut by hand and immediately weighed. The yield of the above-ground mass was calculated by weighing the raw material from the whole plot.

In our researches essential oils were obtained by water distillation procedure for 2 h using Clevendger apparatus, according to the method described by **El-Seedi et al. (2008)** from the flowering parts of plants *N. cataria*.

Investigate of components was carried out by high effective GC-MS-Analysis with HP 6890 chromatograph coupled with HP 5972 mass selective detector. Injection volume – 1 μL. Inlet: temp – 250 °C, split ratio 10:1. Column – JW DB-5MS, Kat.Nr. 122-5532, length – 30.0 m, nominal diameter – 250 μm, nominal film thickness – 0.25 μm. Carrier gas – He. Mode was constant flow. Initial flow – 1.3 $mL.min^{-1}$. Nominal init pressure – 11.06 psi. Average velocity – 42 $cm.sec^{-1}$.Temperature of the thermostat was linearly programmed from 50 °C (5 min) up to 280 °C (2 min)with speed 3 $ºC.min^{-1}$. MSD Transfer Line – 280 °C. Solvent Delay – 6 min, scan range: 40 – 550 AMU, threshold – 20. Sample – 3

(0.91 Scan.sec^{-1}). The chromatograms represent the total ion current. Identification of the components according their mass spectra carried out using the data bases NIST and Wiley 275 and their retention indices **(Adams, 2001; McLafferty, 1989; NIST, 1994)**.

Fractioning of essential oil *Nepeta cataria* was performed on a pilot universal automatic facility – DFD (Device of Fractional Distillation). This chromatographic method of investigation was developed in the research laboratory National University of Food Technologies **(Frolova et al., 2005; Frolova and Korablova 2016)**. The type of column – three-section; number of real plates, pcs – 20; number of side - bars, pcs – 3; diameter of refractive part 30 mm; head type – full condensation; regulation of the reflux ratio and temperature in a cube from the control unit; control of temperature – automatic. Facility elements are made of inert material – heat-resistant glass produced by Simex (Kavalierglass, USA).

Statistic analysis

The mathematical processing of results the experimental studies was carried out using dispersion statistical methods under the program Microsoft Excel-2010 and the package of programs of statistical analysis in crop production "AGROS" **(AGROS, 2000)**. All experiments determinations were performed in triplicate and the values were expressed as mean ±*SD*.

RESULTS AND DISCUSSION

Nepeta cataria var. *citriodora Beck.* – a perennial grassy plant of a pale green colour, strongly short-cut. Leaves are a pubescent silvery-gray (Figure 1). The root is rod-shaped, long. Stems are quadrilateral, pubescent, from the base branched, up to 80 cm high, with plenty of leaves. Each lateral branch ends with a dense spike-shaped inflorescence from the strongly converged unreal, multi-threaded crests. Flowers are small five-membered, double-haired, corolla white, coupled with the tiny twists of tubular flowers. Fruit – dark brown nuts. The quality of aromatic plants is primarily determined by the content of essential oils (Table 1).

The research has established that the amplitude of the variability the mass fraction of essential oil *N. cataria* varies not only over the years. The mass fraction of essential oil increases in the process of development starting from the vegetative growth phase, reaching the maximum to period of full flowering, and the ending of flowering is accompanied by its reduction (Table 2).

So, the maximum of essential oil of the *N. cataria* during flowering is associated with its high content during this period in the flowers. We have found that the essential oil of flower-lemon flavour in the plants is contained throughout the growing season and is accumulated both in the generative and in the vegetative organs (Table 3).

The maximum amount of essential oils contains fresh inflorescences in the phase of mass flowering. In dried raw materials it contains 3.3 times less essential oils than in fresh inflorescences, which proves the inexpediency of drying the grass before processing. We recommend adhering to the optimum cut height of the upper part of the plant stem at the level up to lower fresh leaves (25 – 35 cm). The dynamics of essential oil in the aboveground mass of the whole plant is determined by the aggregate of its content in separate parts at a definite phase of development. It has been established that essential oil is contained in all organs of *N. cataria*, but is distributed unevenly, and its amount varies in the process of ontogenetic development.

In the structure of the *N. cataria* yield dominates the fate of stems, in which the content of essential oils is very small compared with leaves and inflorescences. This fact greatly affects the overall collection of essential oils.

The component composition of essential oil *N. cataria* was investigated according to the developed by us method of gas chromatographic analysis oxygen-containing components of sources aromatic substances. Chromatogram of essential oil *N. cataria* is shown in Figure 2.

Sample of essential oil was injected into a chromatograph in amount 100 μL oil + 500μL CH_2Cl_2. The main components that determine the qualitative indices of essential oil from the *N. cataria* are citral (neral and geranial), nerol, geraniol and citronellol, as well as geranylacetate (Table 4). Depending on the predominance of these components, several chemotypes of the *N. cataria* are can be distinguished. Quality indicators of the samples essential oil of *N. cataria* are shown in Table 5.

It was established that the amplitude of the variability the mass fraction of essential oil of *Nepeta cataria* under the conditions of Forest-Steppe zone of Ukraine varies by cultivars and organs ranging from 0.795% to 2.863% of the absolutely dry mass of plant material.

The quantitative relations of the *Nepeta cataria* essential oil components are represented with Figure 3, and it is allowed estimate typicalness of the essential oil.

As a result of biochemical research, we have recommended aromatic plants *Nepeta cataria* var. *citriodora* as component of spicy seasonings that are suitable for use in the food industry. Spicy mixtures were prepared by mixing dried and crushed parts of spicy plants in different combinations and quantities.

According to the results of the organoleptic evaluation of the various mixtures and the tasting of dishes with the *Nepeta cataria,* dry plants was included to seasoning ‘Citrina’ as the most abundant component. Seasoning ‘Citrina’ is a loose spicy powder without lumps, with lemon tones, beige-greenish colour. It was proposed to use the mixture ‘Citrina’ for prepare sweet and dessert dishes.

The most abundant component of *N. cataria* was geraniol (Figure 3). His content for cultivar 'Melody' was 23.26% of the total oil, as well as nerol – 22.37%, citronellol – 11.36%, geranial – 9.43%, neral – 7.41%, nepetalactone – 6.08%. Content of geraniol for cultivar 'Peremozhets' was 24.41% of the total oil, as well as nerol – 22.04%, citronellol – 12.32%, nepetalactone – 10.62%, geranial – 9.57%, neral – 7.72%. It was established that the second among the main components of essential oil of the *N. catariai*s the citral (the sum of its cis- and trans isomers – neral and geranial). A group of components of flowery note aroma is citral, citronellol, geraniol, geranyl acetate.

The key components have different contents and their own original aroma. It was affected on the sensory characteristics of the fractions. Due to that each from four fractions has their own unique aroma and can be an independent flavour.

Table 1 Yield of the herb and essential oil of *N. cataria* under condition of Forest-Steppe zone of Ukraine.

Sample	Direction of use	Yield green mass. $t.ha^{-1}$	Essential oil. $kg.ha^{-1}$
Nepeta cataria 'Peremozhets'	Perfumery	26.5 ±0.05	89.6 ±0.1
Nepeta cataria 'Melody'	Food	28.5 ±0.05	146.2 ±0.1

Table 2 Essential oil content depending on plant organs and their humidity in of *N. Cataria* 'Peremozhets'.

Samples	Amount of sample	Contents essential oil	
	g	g	%
Fresh inflorescences	200	2.57 – 2.67	1.06 ±0.01
Fresh inflorescences with stems	200	0.72 – 0.92	0.46 ±0.01
Dried inflorescences	200	1.78 – 2.05	1.03 ±0.01
Dried inflorescences with stems	200	0.52 – 0.64	0.31 ±0.01

Table 3 The content of essential oil in the plants *N. cataria* by phases of vegetation. %.

Samples	Regrowth phase	Flowering phase	
	leaves	leaves	inflorescence
Nepeta cataria 'Peremozhets'	0.811 ±0.01	1.374 ±0.01	2.572 ±0.02
Nepeta cataria 'Melody'	0.795 ±0.01	1.485 ±0.01	2.863 ±0.02

Table 4 Composition of essential oil of *N. cataria* cultivars 'Peremozhets' and 'Melody'. % of total oil

Component	Content	
	'Melody'	'Peremozhets'
Neral	7.41 ±0.03	7.72 ±0.03
Geranial	9.43 ±0.04	9.57 ±0.04
Nerol	22.37 ±0.05	22.04 ±0.05
Citronellol	11.36 ±0.04	12.32 ±0.04
Geraniol	23.26 ±0.05	24.41 ±0.05
Nepetalactone	6.08 ±0.03	10.62 ±0.04
6-Methyl-5-heptene-2-one	1.2 ±0.02	1.5 ±0.02
Citronellyformate	0.5 ±0.01	0.4 ±0.01
Geranylformate	0.5 ±0.01	0.4 ±0.01
Geranylacetate	1.6 ±0.02	1.5 ±0.02
Ethylgeranate	1.1 ±0.02	0.7 ±0.01
Caryophyllene oxide	1.4 ±0.02	1.1 ±0.02
Total	86.21	92.28

Table 5 Quality indicators of *N. cataria* essential oil

Quality indicator	Norms of quality indicators	Analysisresults
Raw material for production	Dried plants	Fresh plants
Method of production	Rejection by pair	Hydrodistilation
Appearance	Liquid yellow color	Liquid of amber color
Scent	Pleasant, appropriate for this plant	Grassy-Citrus with floral tint
Density at 20 ºC, $g.cm^3(d_4^{20})$	0.900 – 0.9980	0.962 ±0.015
Refraction index at 20 ºC, (n_D^{20})	1.4700 – 1.500	1.4861 ±0.012
Acid index, mg $KOH.g^{-1}$	not more than 30.0	19.44 ±2.45
Solubility 1 volume EO in 70% ethyl alcohol	in three volumes	corresponds
Availability of water	is not allowed	corresponds

Table 6 Parameters of separation binary systems of *Nepeta cataria* essential oil.

System	Dispersion temperature, ˚C	Pressure, kPa	Relative volatility, α	The degree of separation, $\mathbf{n_{min}}$	Reflux number, $\mathbf{V_{work.}}$
mircen – cineol	70.5 – 74	1.32	3.85	2.6	1:5
cineol – linalool	76 – 79	0.96	3.45	2.8	1:4
linalool – citral	94 – 104	0.33	3.3	3.1	1:7
citral + nerol-citronellol	115 – 120	0.33	1.36	7	1:7
citronellol + geraniol + geranylacetate (cubic balance)	142 – 147.5	0.33	1.32	7.5	–

Figure 1 Plants of *Nepeta cataria* var. *citriodora* (1) and their inflorescences (2).

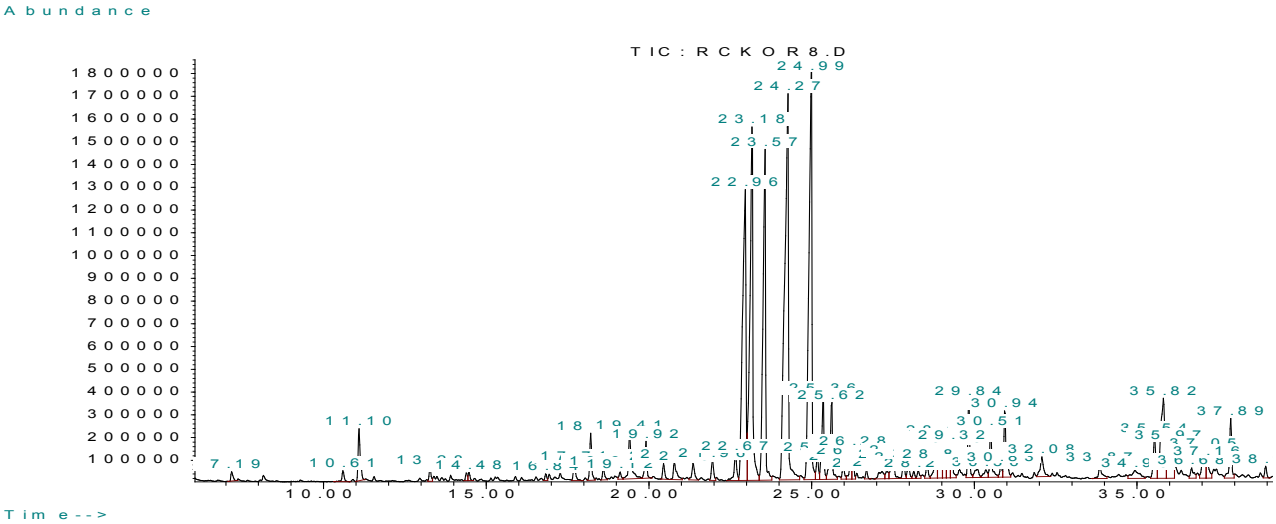

Figure 2 Chromatogram of essential oil *N. cataria* 'Melody'.

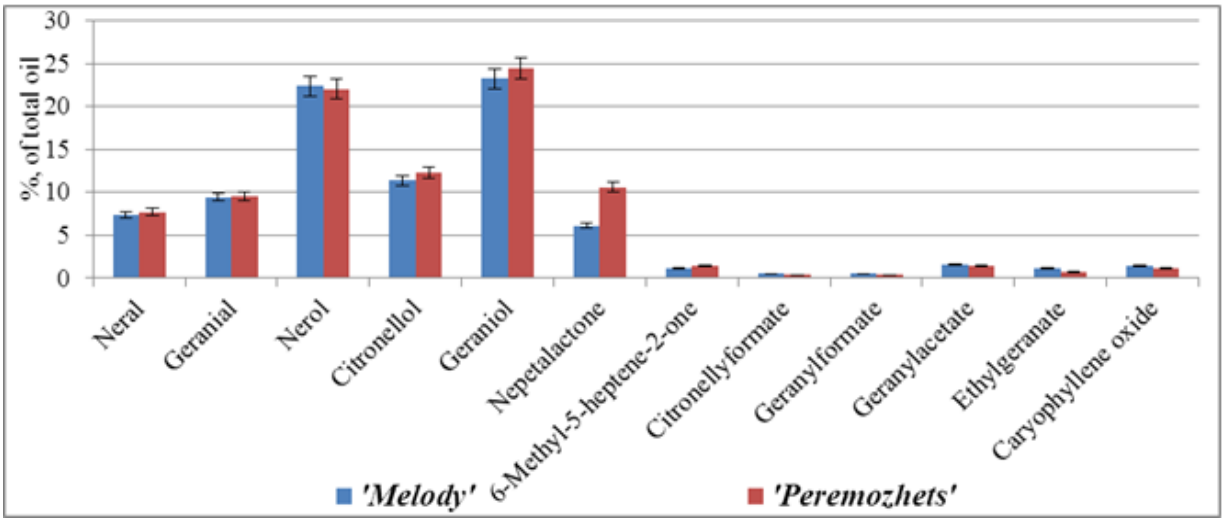

Figure 3 The quantitative relations of the *Nepeta cataria* essential oil components.

Group of components α-pinene, β-pinene, camphene, α-phellandrene, cineol – add a pine note to the general aroma; group β-myrcene, d-limonene, β-phellandrene, n-cymene – have intensive citric note; and group d-camphor and l-borneol – have camphor aroma.

The development parameters of fractional dispersal essential oil of *Nepeta cataria* was aimed to obtaining fractions with the above-mentioned concrete tonality of flavours. It was used the concept of 'key components' – the boundaries, between which fractionation is carried out. By the key components method, *Nepeta cataria* essential oil was considered as the sum of five binary systems – 4 fractions and distillation residue. The modes of distillation essential oil are presented in Table 6.

Calculations of parameters controlled dispersal of essential oils (residual pressure, temperature regimes, number of theoretical plates, reflux number) were carried out according to the rules of distillation and data of quantitative composition.

The process of obtaining a fraction occurs with the enrichment of the most volatile key component, as well as components with similar boiling temperatures. Such components greatly affect the aroma tone of the fractions.

After the discontinuation of the distillate selection at the initial temperature, the temperature of the cube was raised, thereby achieving new equilibrium conditions and receiving a new fraction. Similarly, the following fractions were collected. The fractions were selected from the top of the column. During the fractionation of essential oil *Nepeta cataria* were obtained 4 fractions with a total content of 96 ±0.5% of the total mass essential oil.

Loss due to incomplete capture of low boiling components was 3 ±0.5%.

The fractioning process allows concentrating the key aromatic components and receiving highly concentrated flavours of original pure notes. Results of the study **(Frolova and Korablova, 2016)** indicate the possibility to combine the components of the fractions for use in the product to provide the desired flavour notes.

The flavours not only give products a special scent, but also are characterized by the orientation of the physiological action, saturation, and improved stability. Application of flavour enables to expand assortment of developed products to improve them tasting properties.

CONCLUSION

The formation and accumulation of essential oils occurs in all overground organs of plants *Nepeta cataria* var. *citriodora*. The analysis of the component composition of essential oil *Nepeta cataria* var. *citriodora*, grown in the Kyiv region, have had made it possible to identify several basic components, the most valuable of which is citral. Dedicated fractions of *Nepeta cataria* essential oil had been offered to the food industry as natural flavours for functional and dietary dishes. Seasoning "Citrina", the main component of which is *Nepeta cataria*, is included in the Ukrainian standard "Dry seasoning with spicy aromatic plants". Our research confirms that *Nepeta cataria* var. *citriodora* and their essential oil are promising aromatic raw materials for use in the food industry as flavours.

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INVESTIGATING ON THE EFFECT OF FREEZING IN DIFFERENT TIME PERIODS ON THE CHEMICAL AND QUALITATIVE CHANGES OF FISH (GIANT TREVALLY)

Ali Aberoumand, Saladin Ayoubi

ABSTRACT
Changes in proximate composition of the Giant trevally were carried out for 0 days, 7 days, 14 days, 21 days, 28 days and 35 days at a freezing temperature. The moisture, ash, protein and fat contents were measured using standard methods. Based on obtained results, moisture content was decreased during different periods of freezing and its amount in fresh fish before freezing was (74.72%) that were decreased to 73.5%, 71.74%, 70.4%, 69.06% and 67.72%, respectively for 7, 14, 21, 28 and 35 days of freezing. The fat content in the fresh fish before freezing was (8.13%) that were decreased to 7.02% and 5.93%, and 5.33%, and 4.73%, and 4.13%, respectively, at 7, 14, 21, 28, and 35 days of freezing, respectively. The amount of protein in the fresh fish before freezing was (20.02%) that were decreased to 18.23% and 16.99%, and 15.75%, 14.51% and 13.27%, at 7, 14, 21, 28 and 35 days of freezing, respectively. Ash content in fresh fish before freezing was (1.21%) that decreased to 1%, 0.82% and 0.70%, and 0.58% and 0.46%, during 7, 14, 21, 28 and 35 days of freezing, respectively. it can be concluded that the best quality of frozen fish was obtained between 14 to 7 days of freezing.

Keywords: freezing; fish; Giant trevally; proximate nutrients; different time periods

INTRODUCTION
Freezing fish is an important method of fish processing. However, when seafood is frozen, and they are kept in frozen condition, they lose quality **(Haard, 1992)**. Losing the quality of frozen fish is mainly due to changes in the integrity of the muscles, proteins and fats **(Cappeln et al., 1999)**. Cell decomposition during freezing can cause lipid acidic hydrolysis and release of free fatty acids. Changes in fish muscle, proteins, lipids, and tissue properties have been studied for decades due to their economic importance **(Gandotra et al., 2012; Solanki et al., 2011)**. **Aberoumand and Jooyandeh (2010)** reported that for the different types of packaging storage at the appropriate temperature of freezing and characteristics of freezing of fish various species cause to great effects on the quality of the fish. They showed fish if need to freeze for a short time to maintain the taste and taste, it causes that protein and fat keep the optimum level. Freezing has been known as the best way to maintain food for long time. Freezing methods can affect the quality of fish, although there is a discrepancy in this issue. Hence, some authors observed that frozen fish over three months cannot be different compared to fresh fish species in colour, flavour and various parameters. However, other studies have shown that fishing for long periods of time may cause undesirable structural changes in muscle **(Nielsen and Jessen, 2007; Makri, 2009; Boodhoo et al., 2009)**. Protein denaturation is responsible for changing the properties of muscle, which ultimately causing changes in the characteristics of the tissue. In this sense, it has been reported that myosin protein accumulation cause to hardens and reduces the storage capacity of water (WHC) in frozen fish **(Ramirez et al., 2000; Ayala et al., 2005)**. The result is production of hard, dry, and low-quality fish. Additionally, the muscle tissue is more sensible to degradation, which, after freezing, causes rapid damage. These effects are maximized when the chain changes occur during the freezing period, such as air temperature, freezing speed. Some studies have confirmed that the increase in temperature through freezing during storage cause unpleasant changes in muscle of the fish, which causes more changes in the nature of the fish **(Zhou and Li-Chan, 2009)**. Frozen storage is an important method for processing of fish. However, when seafood is frozen and stored in frozen state they necessarily lose quality. Loss in quality of frozen stored fish is mainly due to changes in muscle integrity, proteins and lipids **(Marwa, 2015)**. Aim of this research was to study on influence freezing in time difference periods on quality and changes of proximate nutrients and energy values in fish Giant trevally.

Scientific hypothesis

Freezing has an effect on the nutrient composition (fat, protein, ash, moisture) and nutritional values (energetic value) of fish.

MATERIAL AND METHODOLOGY

Sample preparation

We were bought a fish with weight 843.5 g from Behbahan Fish Market, southern Iran and we transferred it to the Fisheries Laboratory of Khatam-Al-Anbia University of Technology. We washed out the fish and separated the fillet with weight 388 g and then chopped it and we stored it in a freezer. Fish fillets were extracted from the freezer after 0, 7, 14, 21, 28, 35 days, and analysed separately.

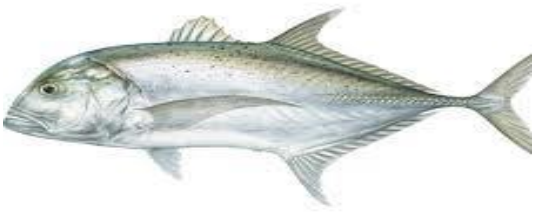

Figure 1 Giant trevally fish.

Ash analysis method

We took out a frozen fillet slice from the freezer and placed it in suitable place for melting. After thawing the fillets, fillets with weight 2 g by a digital scale with a precision of 0.1 (ek-5000 max: 5000-0.1, made in Japan), then they were individually placed in Chinese bushes and they were put in the furnace (made in Iran). The temperature of the furnace was set to 500 °C, which was not steady and was changed from 490 to 510 °C. When the furnace temperature reached to 414 °C, the furnace started to smoke out of the smoke. After the furnace temperature reached 500 °C, we started taking time and the sample kept in the furnace for two hr. After two h, we turn off the furnace and open the door by taking safety precautions and took out the bushes. If the sample was dark grey with dark spots, this indicated that the organic material of the sample was not completely converted into ash and we again placed the sample in a furnace for 1 h extra. The temperature was same the initial temperature of the furnace (500 °C). We started the timing after reaching a temperature of 500 °C. then, we turn off the furnace and separated sample from inside, so that the sample turned out to be bright grey, indicating the loss of all the organic matter in the Chinese bushes. Then we obtained the amount of ash **(AOAC 2005).**

Moisture analysis method

We were taking a large fish Giant trevally from the Behbahan market, Iran and we transferred it to the Fisheries Laboratory of Khatam-Alanbia University of Technology. We washed the fish and filtrated and stripped the fillet and kept it in freezer for freezing. We kept the fillet in the freezer for 0 day, 7 days, 14 days, 21 days, 28 days and 35 days, then we took it from the freezer for moisture analysing.

We brought out a frozen fillet piece for the test from the freezer and placed it in the suitable place for melting. After thawing the ice, we measured the fillets to 2 g with a digital scale of 0.1 precision (ek-5000 max scale: 5000-0. Made in Japan).

We put the measured samples to pre-weighed petri dishes and then we put them in an oven (Model: Memmert of Germany) at 110 °C for 1.5 h. We started timing after reaching the temperature of the oven to 110 °C. then, the petri dishes took out from the oven and then the samples were placed in the dictator to stabilize the weight and temperature, then samples weighed separately, and then samples again were put into the oven for 15 min, to ensure that all water inside the sample has been evaporated, if this sample weighed after 15 minutes was equal to the weight after 1.5 hr., it means that all the water inside the sample has been evaporated, otherwise the samples again was placed in the oven for another 15 min. For measuring fat, from moisture **(AOAC 2005)**. We used from below formula:

$$\text{Percentage of moisture} = (94\text{-}80.1) \times \text{fat percentage}$$

Different of moisture maximum and moisture minimum in fatty and low-fat fishes are numbers in brackets which are constant numbers.

The ash, fat and moisture contents were deducted from 100 will be calculated the amount of protein.

Energy evaluation

The food energy was calculated from the values of the proximate determination assuming that protein, and fat yield 4, and 9 calories respectively per g **(Iwe and Onuh, 1992)**.

Chemical analysis

Proximate analysis for moisture, fat, protein, ash contents were determined according to **AOAC (2005)** while the carbohydrate content of samples was obtained in form of difference between 100 and the sum of moisture, protein, fat and ash values.

Statistic analysis

Average values per each sample were determined and analysed using descriptive statistics using SYSTAT Version 6.0. The data obtained were compared with standard values as reported in the literature. Results are expressed as mean of triplicate trials. Data were analysed by one-way analysis of variance on the means of values (p <0.05).

RESULTS AND DISCUSSION

Results obtained has been showed in Figure 1, Figure 2, Figure 3, Figure 5 and Table 1.

Figure 1 showed a decreasing pathway of moisture to 67.72% on day 35 (from 74.72% to 67.72%), which has a significant difference in all the steps mentioned (p <0.05). In the research conducted on the Caspian Sea fish (*Rutilus frisi kutum*), they found that the moisture content in this fish decreased from 75.9% to 72.3% after 12 months of storage in the refrigerator at 18 °C, that it agreed with our study (p <0.05).

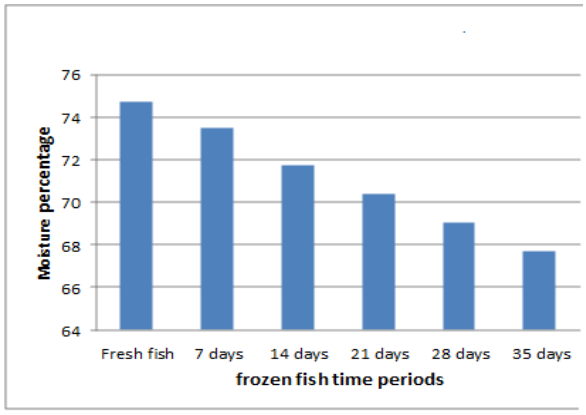

Figure 1 Moisture contents in the fish freezing different time periods.

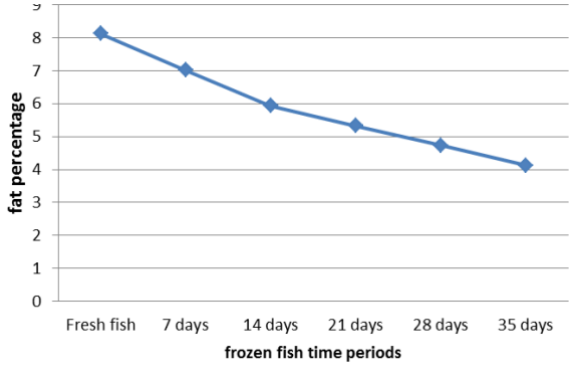

Figure 2 Fat contents in the fish freezing different time periods.

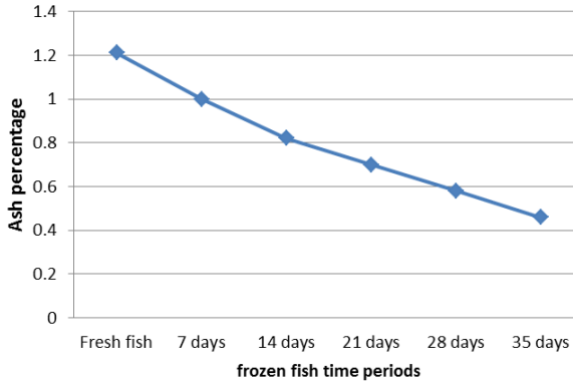

Figure 3 Ash contents in the fish freezing different time periods.

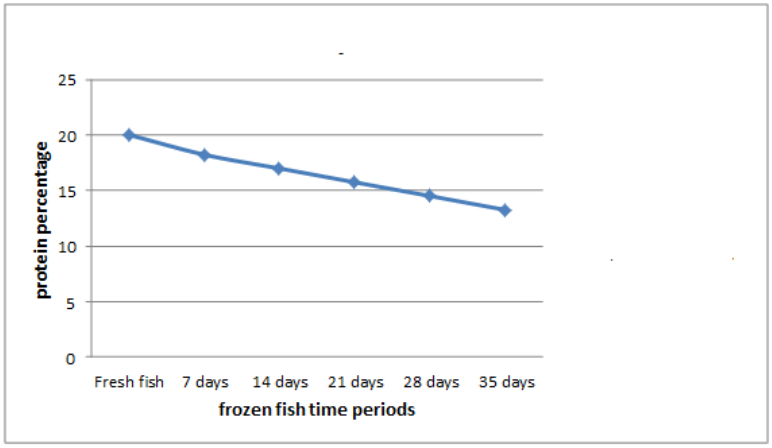

Figure 4 Protein contents in the fish freezing different time periods.

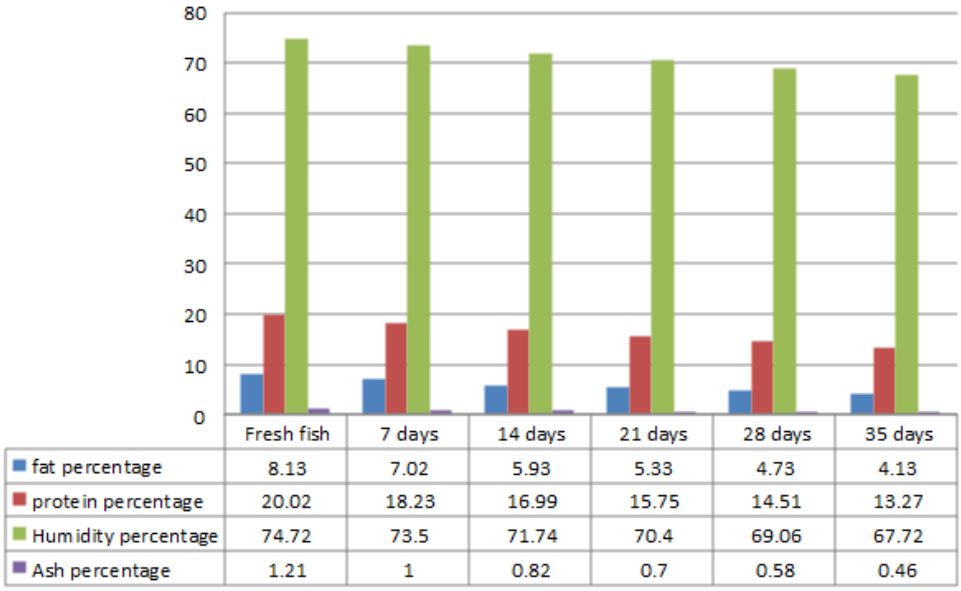

Figure 5 Comparison of proximate nutrient contents in the fish freezing different time periods.

Table 1 Proximate nutrients and energy values in fresh and frozen fish in different time periods.

	Fat	Protein	Ash	Moisture	Energy (KJ)	Energy (Kcal)
Fresh fish	8.13	20.02	1.21	74.72	660.45	157.25
7 days after freezing	7.02	18.23	1.00	73.50	571.62	136.10
14 days after freezing	5.93	16.99	0.82	71.74	509.59	121.33
21 days after freezing	5.33	15.75	0.70	70.40	479.30	114.12
28 days after freezing	4.73	14.51	0.58	69.06	434.74	103.51
35 days after freezing	4.13	13.27	0.46	67.72	390.18	92.90

In the two species of *Upeneuts molluccensis* and *Mullus suranuletus* moisture contents were reported to be 79.41% and 73.14% respectively after freezing **(Öksüz and Küver, 2011)**. Also, it was found in Atlantic Ocean Herring fish (*Clupea harengus*), Macrel fish (*Scomber scombrus*) 68.6 and 65% **(Olagunju et al., 2012)**. The highest water content is fillet, was about 80% in low fat and fat free fish and about 70% in fatty fish, so the amount of water in fish muscle was different (p <0.05).

According to the test carried out, Figure 2 showed that during the storage period of the fish fillet in the freezer, a decrease in fat was observed.

The main reasons for reducing fat during storage in freezer depends on freezing conditions (freezing of low and fast), and formation of ice crystals during the storage period of the samples, which, depending on the diameter of the particles, which may cause damage to the frozen fish tissue., the formation of the ice crystals not only causes tissue rupture in the samples, but also the removal of these crystals in a thawing time in the form of droplets of water from the tissue is separated, which together with the fat and other soluble materials, which causes to reduce the percentage of final fat in treatments.

Fat oxidation was another factor in reducing fat during storage in freezer, which can reduce fat percentage at the end of the shelf life. The final reduction is the total fat in the measured samples because of the enzymes that are effective on the hydrolytic spoilage of the fat, especially the cold-resistant lipases and its conversion to free fatty acids. The result of an increase in temperature at the surface causes that the moisture at food surface quickly evaporates. The outer surface of product dried and crust is formed. Gradually, the internal moisture content of the product also becomes steam and a positive slope of the vapor pressure is created. The vapor penetrates through the vents and causes canals at the surface of cells and membranes. As the operation progresses, the oil is sticked to the surface of the product and penetrates the product through holes and channels created as a result of evaporation of water. In this mechanism, there is a linear relationship correlation between reducing moisture and absorbing oil during frying. For example, the food contains higher moisture also absorbs more oil. The fat content in *Orcynopsis unicolor*, *Euthynnus affinis* and *Liza dussmieri*, were different **(Aberoumand, 2012)**. In Harring and Macrel fat contents has been reported 11.14% and 12.33% respectively **(Olagunju et al., 2012)**.The amount of fat in fish body muscle varies according to environmental conditions of fish.

Due to long time storage of the samples in freezer, the ice crystals join together and become big and cause a rupture of the cell membrane, which results in the release of ell materials that contain minerals and nutrients in thawing time, so that causes reducing the minerals exist in ash. Figure 3 showed this subject.

According to Figure 3, the ash content of fresh fish (zero day) was 1.21% that decreased to 0.46% in 35 days. This reduction was due to the increase in exit of water during freezing. According to some studies, the amount of ash in

Shagh fish and Mackerels were 1.6% and 0.7% respectively **(Emadi, 2008)**. Ash contents in Cod fish was 1.2% and in Yellow Ribbon 1% **(Razavi Shirazi, 2007)**. Ash contents in the *Orcynopsis tricolor*, *Euthymults affinis*, and *Liza dussmieri* were 2%, 3.27% and 1.36% **(Aberoumand, 2012)**, Ash contents in *Clupea harengus*, *Scomberus scomber* reported 1.51% and 1.79% **(Olagunju et al., 2012)**. The reason of the difference in ash contents in the fish species were type of feeding, gender, age, habitat and the method of measurement.

According to the Figure 4, the percentage of protein decreased from 20.02% in fresh fish (zero day freezing) to 13.27% on 35 days.

The reason for decreasing the protein percentage in the sample during the storage period in freezer was release of amine compounds. If the freezing time increases, the amount of nitrogen released also will be increased and it is removed from the main components of the protein chain and leads to decrease protein contents during freezing. This decrease occurs at higher temperatures (above zero) and leads to a further reduction in protein content, and if the frozen temperature of product was less than -18 °C (i.e. 35 °C), this decrease occurs less **(Chiba et al., 1991)**. Loss quality during frozen storage is inevitable, and to obtain satisfactory results, fish for freezing must be of good quality. The freezing of fish influenced the proximate composition of their muscles also the quality of fish samples during storage revealed the decreasing of the taste with increasing duration of storage **(Marwa, 2015)**.

From the results of Table 1, it can be concluded that fillet energy value in treatment 7 days freezing period was the best compared to the other freezing periods. Table 2 showed that the amount of proximate nutrients in each freezing period related to the previous period were constant from the 21 days freezing period , which indicates the lack of effect of freezing on nutrients. However, in the 14 days freezing period related to the previous period, all nutritional compounds were decreased, which indicates the effect of freezing on proximate nutrients. Freezing from the 14 days period did not have any effect on the amount of protein since its amount was constant. The amount of energy decreased from the 14 days freezing period to that of the previous period and fresh fish.

CONCLUSION

The results of this research showed that the best quality of frozen fish found between 7 days and 14 days of freezing and the quality of fish was best in fresh item. The rate of loss quality was accelerated during frozen storage time. The protein denaturation, lipid hydrolysis and oxidation increase as the storage period increase. The freezing of fresh fish leads to decrease in protein %, lipid %, ash % and moisture % compared with fresh muscle fish. The increases of pH value for imported frozen. However, in the 14 days freezing period related to the previous period, all nutrient compounds were decreased, which indicates the effect of freezing on proximate nutrients. Freezing from the 14 days period did not have any effect on the amount of protein because its amount was constant. The amount of energy decreased from the 14 days freezing period to that of the previous period and fresh fish. Recommendation was eating fresh fish which is most benefit for human health.

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OPTIMIZATION OF INFRARED DRYING CONDITION FOR WHOLE DUKU FRUIT USING RESPONSE SURFACE METHODOLOGY

Laila Rahmawati, Daniel Saputra, Kaprawi Sahim, Gatot Priyanto

ABSTRACT

Duku (*Lansium domesticum*), tropical exotic fruit, was successfully preserved by drying using exposure to infrared radiation emitters. Response surface methodology (RSM) is used to optimize independent variables (IRE distance of 6 cm and 10 cm, IRE temperature of 200 °C, 300 °C, 400 °C, and IRE exposure time of 50 s, 60 s, 70 s, and to produce response variables (weight loss, fruit firmness, titratable acidity, total soluble solid, and browning index). It could be concluded from the optimization performed that drying duku skin in a whole fruit by exposing the fruit to the infrared emitter resulted in a duku fruit with a relatively good physical and chemical conditions and still be consumable. The IRE distance of 6 cm gave a desirability value of 0.80 while the IRE distance of 10 cm gave a desirability value of 0.92 however the IRE distance of 6 cm gave a better storage time. The IRE distance of 6 cm has an optimum value of weight loss 2.2%; optimum value of fruit firmness of 40.92 N; optimum value of total soluble solid of 17.48 brix; optimum value of titratable acidity of 0.33%; and optimum value of browning index of 0.9. The fitting model base on RSM resulted from this research indicated that this study could be used as the basis for alternative process in food processing of duku but still need further research to increase the shelf life and a better result in the chemical and physical characteristics of duku.

Keywords: Duku; infrared; optimization; response surface methodology

INTRODUCTION

Food processing such as thermal and non-thermal processes could affect changes in structure and composition of the food **(Mercier et al., 2011)**. The process of food processing with a thermal method, could cause a chemical and organoleptic properties damage and reduce nutrition or nutritional bioavailability. An example of food processing technology with thermal is drying. Drying is one of the food processing method to prolong shelf life or preserve grains, fruits, vegetables and food in all varieties. The quality of dried fruits depends on the conditions of drying process. One type of drying processes is by using infrared radiation. Infrared drying has been widely implemented in the food process because of its several advantages including to reduce water content in food, low energy consumption, short time in processing and also maintain and ensure product quality conditions **(Pan et al., 2009)**. The advantages of infrared radiation could inhibit the pathogens in products which include mold, yeast, bacteria and spore by controlling some parameters such as power on the heater **(Hamanaka et al., 2000)**, temperature of sample **(Sawai et al., 2003)**, wavelength and the target wave in a wide range **(Krishnamurthy et al., 2008)**, sample thickness **(Sawai et al., 2000)** and sample water content **(Hamanaka et al., 2006)**. As with other electro magnetics wavelengths such as microwaves and radio frequencies, infrared radiation has a unique characteristic in the design of its spectral distribution and energy intensity which could be be controlled by using optical filters. Furthermore, the unique characteristic of infrared radiation to the product is the heat energy from the emitter only affected the surface of food in a short time without raising the inside temperature of material **(Li and Pan, 2014a)**. Infrared radiation is divided into three different categories, namely near-IR (NIR) with a spectrum scale in the range of 0.75 – 1.4 μm, mid-IR (MIR) with a spectrum scale in the range of 1.4 – 3 μm, and far-IR with a spectrum scale in the range of 3 – 1000 μm (FIR) **(Sakai and Hanzawa, 1994)**. Infrared radiation has a longer wavelength than visible light, but is shorter than terahertz radiation and a microwave. The infrared radiation spectrum has a range of 750 nm up to 100 μm and widely used in food processing in several ways including food processes involving heating processes, spectroscopic measurements of chemical composition (food analytical applications), and measurement of non-contact food temperature.

The use of infrared has been carried out in previous research for drying the skin of fresh duku. The design of this research was a multi-variate process involving many factors

that affected the efficiency and the effectiveness of drying process on duku fruit. Previously, classical method was used to determine the optimum condition by using one parameter and it was time-consuming, laborious and the result mostly could not be guaranteed **(Wernimont, 1985)**. On the other hand, experiments with many factorial combinations and variables were not effective and efficient because of the large number of experiments needed **(Haaland, 1989)**. To overcome this problem, a method that often used in optimization process is response surface methodology (RSM). RSM is a good statistical technique to optimise a complex processes with several factor variations simultaneously. This model have the advantages of reducing the number of trials, increasing statistical interpretation and efficient **(Gan and Latiff, 2011; Jiang et al., 2011)**. RSM has been used for more than 60 years since its development in 1951. Initially used in the area of Chemistry and chemical engineering, but since then has been used in many areas to optimize the variables used in an experiment **(Hill and Hunter, 1966)**. Some of the designs used in RSM are Full Factorial Design (FFD), Box-Behnken Cesign (BBD), and Central Composite Design (CCD). Box-Behnken design (BBD) is more efficient and easier to arrange and to interpret the result of experiment than other designs **(Ferreira et al., 2007)**. RSM is better than the elimination process on multiple variable and more informative in interpreting and analysing the result **(Ozdemir et al., 2008)**. RSM could help in determining and solving multivariate problems simultaneously which describe the effect of test variables and determine the correlation among variables and its combined effect **(Erbay and Icier, 2009)**. 3D RSM and 2 D contour plot could help in visualization the relationship among variables and type of interactions between variables. The shapes of the contour plots indicate the significant of interactions among variables **(Muralidhar et al., 2001)**. RSM is more useful than the artificial neural network because RSM is directly showing the interaction between different components and determining the relationship among variables. RSM could provide statistically acceptable result with fewer experiments **(Youssefi, Emam-Djomeh and Mousavi, 2009)**.

The use of the RSM method has been widely used in food processing in optimizing various processes such as extraction, drying, blanching, enzymatic hydrolysis and clarification, production of microbial metabolites. RSM also has been used in the formulation of food products such as the optimization of food drying process including spray drying of guava powder **(Vaibhav et al., 2014)**. The optimization of pink guava had been performed using central composite face-centred design to optimize the spray drying parameters of the inlet temperature, maltodextrin concentration (MDC) and feed flow (FF) **(Sishir et al., 2016)**; the optimization of the extraction of phenolic compounds and antioxidant potential of *Berberis asiatica* fruits **(Belwal et al., 2016)**, the optimization of the extract of flax seed oil **(Ondrejovič et al., 2011)**, and species determination of common carp **(Bajzík et al., 2011)**. The main goal of this study was to optimize the results of the drying process of the skin of whole duku fruit which was exposed to infrared radiation and to select the optimum combination of analytical response variables of the physical and chemical test results with the dependent variables of distance of emitter, exposure time and temperature of the emitter using RSM.

Scientific hypothesis

The hypothesis of this research was that the response surface methodology (RSM) could optimize the drying process of the skin of the whole of duku fruit.

MATERIAL AND METHODOLOGY

Sample of duku

The material used in this research was the local exotic fruit *Lansium domesticum cor.* or Duku. The sample of duku used in this study was taken from the area of South Sumatra. Sample selection includes physical (a diameter selection of fruit was 2.5 to 3.5 cm) and chemical (microbial attack) were selected and checked before the drying process.

Infrared Expose for Drying Process

The fruit was exposed using infrared radiation which has two emitters (245 mm x 60 mm size; 1000w for each). The emitter was adjusted for 6 cm and 10 cm (X1) and the temperature of infrared radiation was turned into 200 °C, 300 °C and 400 °C (X2), while the temperature of emitter was arranged to 50, 60, 70 and 80 seconds according to the exposure time (X3). After exposing time, the fruits were stored in controlled temperature (15 °C ±2 °C) and then the chemical and physical effects of infrared were analysed continuously (every two days). The chemical and physical properties have been discussed in previous research **(Rahmawati et al., 2018).**

Experimental design

A Box and Behnken design (BBD) on Design Expert Program (DEP)TM Version 11 (Stat-Ease, Inc., Minneapolis, US) with three variables was used in this research to obtain the optimum IR heating to maximize the process. The independent variables in this design were the Infrared emitter distance (X_1, cm), the temperature of infrared emitter (X_2, °C), exposure time (X_3, s), storage time (X_4, days), while the response variables were weight loss (Y_1, g), fruit firmness (Y_2, N), total soluble solid (Y_3, %), titratable acidity (Y_4, °brix), and browning index (Y_5). The preliminary single factor test was used to determine the range for each variable. To analyse the response pattern and to establish the models for this research, seventeen experiments was conducted randomly. A second-order polynominal and regression coefficients were calculated for the experimental data. The quadratic equation to predict the optimum point of this research was explained as follows (Eq. 1):

$$Y = a_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} a_{ij} X_i X_j \quad (1)$$

where Y is the dependent variable; a_o, a_i, a_{ii} and a_{ij} are coefficients estimated by the model, X_i, X_j are levels of the independent variables. They represent the linear, quadratic and cross product effects of the X_1, X_2, X_3, and X_4 factors on the response, respectively. **Design Expert Program Version 11 (2018)** (DEP)™ software was used to determine the response of the independent variables and to plot the

response surface graphs. The result of the fitted equation was expressed in three-dimensional and two-dimensional surface graphs in order to illustrate the relation between the response variables and independent variables.

Determination of the response variable goals.

The point of optimization in this research was employed in order to optimize the independent variables to maximize the result of the response variables. The goals of the response variables were chosen from the sensory test of consumers (Table 1). The optimum combination of response variables would also be the optimum point of storage times of duku fruit after exposure using infrared radiation.

Statistic analysis

The statistical analysis of the optimation were analysed statistically by analysis of variance (ANOVA) for each response and significance was judge by P-value on model which calculated from the data by (DEP)™ software.

RESULTS AND DISCUSSION

Statistical analysis and Fitting of response surface model

RSM is a collection of statistical and mathematical methods used to improve, develop and optimize a process with multivariable data simultaneously. The result of ANOVA is shown in Table 2. The model adequacy were obtained by calculating the parameters including *R-square*, and Adequate precision. The model *p*-value from Table 2 shows that all of the response variables were less than 0.05 except fruit firmness and index browning on Infrared emiter (IRE) distance of 10 cm, and titratable acidity on IRE distance of 6 cm.

A small P value shows that the data is significant **(Guo et al., 2010)**. The time of exposure, temperature, and IRE distance had a significant effect (p <0.05) on all response variables except fruit firmness and browning index on IRE distance of 10 cm and also the titratable acidity on IRE distance of 6 cm. The insignificant effect means that the interaction between the different factors did not influence the response variables (fruit firmness and browning index). The fit of the model was checked by determination of the coefficient *R-square* which was calculated from 0.54 to 0.98. The high value of *R-square* was an indication that the model is well adapted to the response variables.

High stability and insignificant variability of the model were implied by low value of *C.V*. All the *PRESS* value on Table 2 show that the model used could predict the independent variable quite well. The precision of the model used also shown by the relatively low value of adequate precision. Those values indicate a good signal to-noise ratio. The signal-to-noise ratio greater than 4 of adequate precision is desirable (**Myers and Montgomery, 2002**). It was concluded that by the ANOVA shown on Table 2 that the models could be used for optimization and navigating the design space of the research.

Table 1 The goals of the response variable of duku's quality.

Name	Goals	Lower Limit	Upper Limit
A:Temperature	in the range of	200	400
B:Exposure Time	in the range of	50	80
C:Storage Time	in the range of	1	25
Weight loss	minimize	6.97	19.41
Texture	maximize	19.26	56.25
Titratable Soluble Solid	maximize	8.9	20.2
Total Acidity	minimize	0.35	0.67
Index Browning	minimize	0.06	0.26

Table 2 Result of the model design on the response variables Weight loss (Y_1), Fruit firmness (Y_2), Titratable acidity (Y_3), Total soluble solid (Y_4), and Browning index (Y_5).

Sources	Response Variables									
	Y_1 Weight loss (gram)		Y_2 Fruit firmness (N)		Y_3 Titratable acidity (TA) (%)		Y_4 Total soluble solid (TSS) (brix)		Y_5 Browning index (BI)	
	6cm	10cm	6cm	10cm	6cm	10cm	6cm	10cm	6cm	10cm
Model (*P*-Value)	0.0002*	<0.0001*	<0.0001*	0.15	0.10	0.005*	0.0002*	0.0006*	0.0098*	0.14
R-Squared	0.99	0.98	0.82	0.54	0.73	0.91	0.97	0.96	0.90	0.75
Adjust R-Square	0.99	0.97	0.78	0.27	0.38	0.80	0.93	0.90	0.76	0.43
C.V %	5.41	12.25	13.99	28.81	11.69	13.48	4.2	11.44	16.86	97.37
PRESS	8.74	103.38	751.88	2515.2	0.45	2.84	56.22	262.80	0.08	10.32
Adequate precision	66.35	23.91	15.53	5.67	5.35	11.24	18.48	12.45	9.35	6.35

Note: (*) Significant effect (p <0.01).

Adjust R-Square, Coefficient of Variation (*C.V*), PRESS

Table 3 Optimization result of independent variables and response variables on duku with design expert program.

IRE distance (cm)	Independent variables		Response variables[1]					Desirability
	Temperature of IRE (°C)	Exposure time of IRE (s)	ML	FF	TSS	TA	BI	
6	400	80	2.2	40.9	17.4	0.3	0.09	0.80
10	300	80	1.3	31.8	16.8	0.6	0.30	0.92

Note: [1]ML = Weight Loss (%); FF= fruit firmnes (N); TSS = Total Soluble Solid, (°Brix); TA = Titratable Acidity (%); BI = Browning Index (Abs).

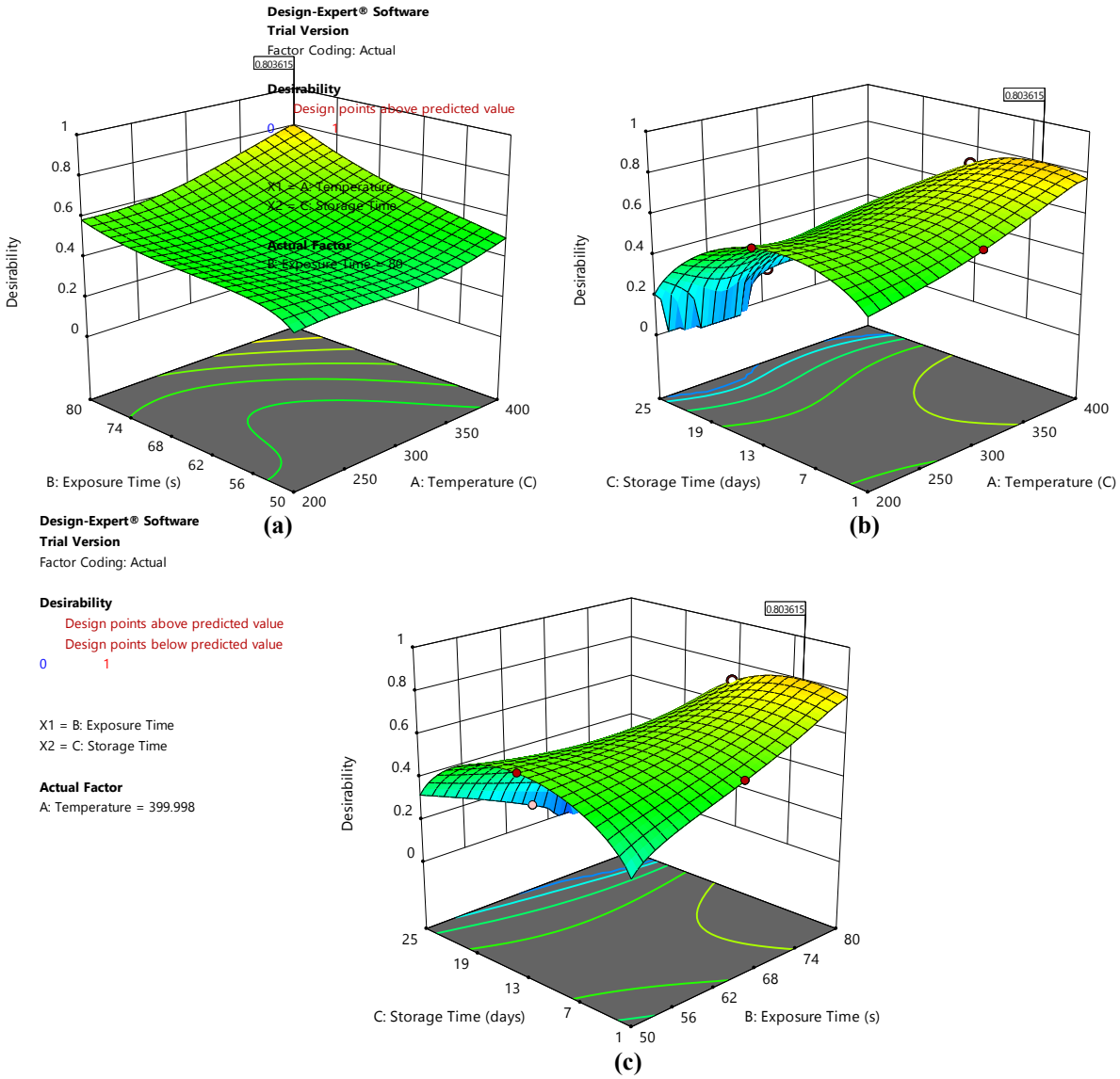

Figure 1 Response surfaces (3D) showing the desirability at an IRE distance of 6 cm. Note: The correlation between exposure time and IRE temperature (a), storage time and IRE temperature (b), and storage time and exposure time (c), respectively.

Optimization of drying condition for whole duku fruit using infrared emitter radiation

The determination of the optimum value of response variables on the drying process by exposing to IRE is performed by plotting the response surface of variables against the independent variables. The purposes of the optimization process in this research are selected based on the consumers and seller point of view. For the consumers, the most important sequence in choosing fruit is a visual appearance (good texture), a minimum browning colour, a relatively high sweetness (TSS) and a relatively low sour taste (TA). For the seller, in order to get high profit, the fruit with a relatively low in weight loss is more desirable.

The desirability value for the optimization process were in the range of 0.80 at an IRE distance of 6 cm and 0.92 at an IRE distance of 10 cm (Table 3). The range of desirability value usually in the range of zero to one. The desirability value equal to one, represents an ideal case, which is the optimization selection results according to the goals

specified, while the zero value represents that the value of one or several variable responses was not in accordance with the goals. Table 3 shows that the desirability produced have a significant difference (p <0.05).

The response surface had an optimum point within the experimental range of the independent variables. The coordinates of the three independent variables were obtained by numerical optimization analysis.

By using the second-order polynominal shown on equation (1) with the response variables of weight loss (Y1, gr), fruit firmness (Y2, N), total soluble solid (Y3, %), titratable acidity (Y4, °brix) and browning index (Y5), with the independent variables of IRE distance, temperature

$$Y_1 = 14.1665 + 1.27X_1 + 0.48X_2 + 5.49X_3 + 0.688X_1^2 + 0.161X_2^2 + 3.22X_3^2 - 0.188X_1X_2 + 1.091X_1X_3 + 0.804X_2X_3 \quad (2)$$

$$Y_2 = 36.165 + 2.05X_1 + 8.26X_2 + 11.7X_3 \quad (3)$$

$$Y_3 = 17.33 - 0.023X_1 - 0.007X_2 + 0.027X_3 - 0.0091X_1^2 - 0.0337X_2^2 + 0.0113X_3^2 - 0.068X_1X_2 - 0.0065X_1X_3 + 0.039X_2X_3 \quad (4)$$

$$Y_4 = 0.51 - 0.704X_1 - 1.0713X_2 - 1.17X_3 - 0.79X_1^2 + 1.41X_2^2 - 1.557X_3^2 - 0.85X_1X_2 - 2.27X_1X_3 - 2.257X_2X_3 \quad (5)$$

$$Y_5 = 0.1455 - 0.0083X_1 + 0.028X_2 + 0.0269X_3 + 0.44X_1^2 + 0.045X_2^2 + 0.0008X_3^2 + 0.7X_1X_2 + 0.07X_1X_3 + 0.72X_2X_3 \quad (6)$$

Where:
Y_1 = Weight loss,
Y_2 = Fruit firmness,
Y_3 = Total soluble solid,
Y_4 = Titratable acidity, and
Y_5 = Browning index.

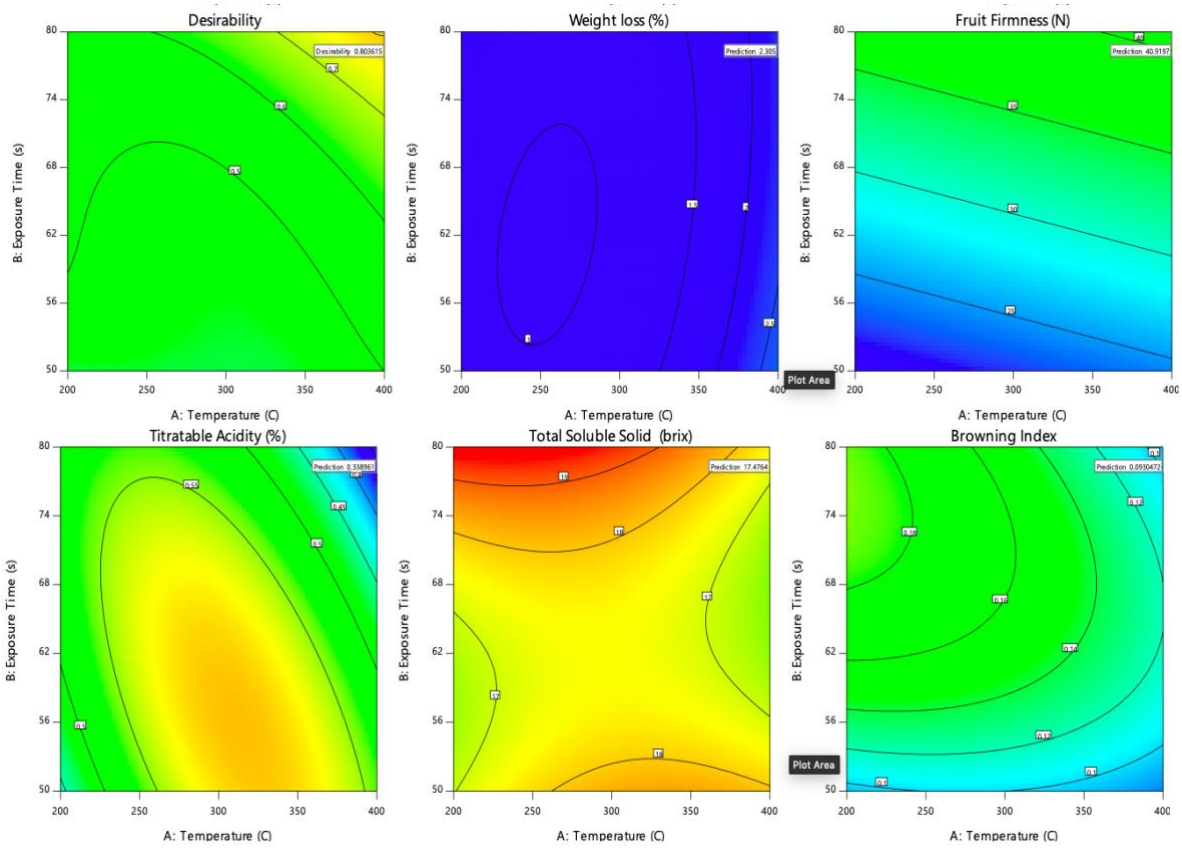

Figure 2 Response surfaces (2D) at 6 cm distance of emitter infrared for the optimization points of exposure time, and temperature on response variables.

(X1, °C), exposure time (X2, s), the RSM was performed to find the optimum point (maximum) of the fitted model as shown on Equation (2), (3), (4), and (5).

The results of the above equation were derived from equation (1) by calculating the optimization on drying with the IRE distance of 6 cm. The equations (2), (3), (4), and (5) show that the results of the model in the response variable could be used to determine the correlation between each factor.

All the equation (2) to (5) except equation (2) which represent the fruit firmness show that all the dependent variable was affected by the three independent variables linearly, quadratically, and its interactions which were the hypotheses RMS using A Box and Behnken design (BBD). However, the fruit firmness only affected by independent variable linearly or the quadratic and its interaction have no significant contribution to the fruit firmness. Nevertheless, the model could be used for the calculation on the optimization of the drying process, especially on duku (exotic fruit) although the optimal shelf life produced was not significantly different from previous research using modified atmosphere **(Saputra and Pratama, 2013)**.

Based on the equation and Figure 1 for the IRE distance of 6 cm show that the cross of IRE temperature (400 °C) and exposure time (80 seconds) gave a desirability value of 0.8; IRE temperature of 400 °C and storage time 7 days gave a desirability of 0.8; and storage time of 7 days with the exposure time of 80 second gave a desirability of 0.8. The IRE distance of 10 cm show that the IRE temperature of 300 °C and exposure time of 80 seconds gave a desirability value of 0.92; IRE temperature of 350 °C and storage time of 5 days gave a desirability value of 0.92; and the exposure time of 80 seconds and storage time of 1 days gave the desirability value of 0.92. Although the IRE distance of 10 cm gave a higher desirability value than the IRE distance of 6 cm, the IRE distance of 6 cm gave a better shelf life value of 7 days.

A response surface could also shown on a 2D contour plot (Figure 2) which were a graphical representations of the model equation. The contour plot was an interaction between independent variables **(Muralidhar et al., 2001)** and the prediction of the maximum value presented in the smallest ellipse in the contour **(Tanyildizi et al., 2005)**. The main purpose of the plot was to determine the optimal value of the response variable so that the response was maximized. The design plot was a function of two factors at a time. The results of a 2D plot graph show that the weight loss on duku fruit after being exposed to infrared and stored for 25 days has an optimum value 2.2% at IRE distance of 6 cm. The weight loss would increase with not significantly different from previous research using modified atmosphere **(Saputra and Pratama, 2013**).

Based on the equation and Figure 1 for the IRE distance of 6 cm show that the cross of IRE temperature (400 ^{o}C) and exposure time (80 seconds) gave a desirablity value of 0.8; IRE temperature of 400 ^{o}C and storage time 7 days gave a desirability of 0.8; and storage time of 7 days with the exposure time of 80 second gave a desirability of 0.8. The IRE distance of 10 cm show that the IRE temperature of 300 ^{o}C and exposure time of 80 seconds gave a desirability value of 0.92; IRE temperature of 350 ^{o}C and storaget time of 5 days gave a desirability value of 0.92; and the exposure time of 80 seconds and storage time of 1 days gave the desirability value of 0.92. Although the IRE distance of 10 cm gave a higher desirability value than the IRE distance of 6 cm, the IRE distance of 6 cm gave a better shelf life value of 7 days.

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CONCLUSION

It could be concluded from the optimization performed that drying duku skin a whole fruit by exposing the fruit to the infrared emitter resulted in a duku fruit with a relatively good physical and chemical conditions and still be consumable. The IRE distance of 6 cm gave a desirability value of 0.80 while the IRE distance of 10 cm gave a desirability value of 0.92 however the IRE distance of 6 cm gave a better storage time. The IRE distance of 6 cm has an optimum value of weight loss 2.2%; optimum value of fruit firmness of 40.92 N; optimum value of total soluble solid of 17.48 brix; optimum value of titratable acidity of 0.33%; and optimum value of browning index of 0.9. The fitting model base on RSM resulted from this research indicated that this study could be used as the basis for alternative process in food processing of duku but still need further research to increase the shelf life and a better result in the chemical and physical characteristics of duku.

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IS EDIBLE INSECT AS A NOVEL FOOD DIGESTIBLE?

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ABSTRACT

This work deals with the digestibility of a selected species of edible insect - mealworm (larvae) as novel food in dependency on its culinary treatment. The aim of this work was to find suitable thermic culinary treatment of mealworm larvae considering its optimum digestibility by human. The digestibility of materials from whole insect and extracted nitrogenous substances was determined using three different culinary treatments - without culinary treatment (freshly killed), dried insect and roasted insect. The digestibility was determined by gravimetric in vitro method using pepsin and pancreatin enzymes and their combination. The total nitrogen content of the insect samples was determined by the Kjeldahl method. The digestibility of the whole homogenized larvae using the combination of pepsin and pancreatin enzymes, thus simulating human digestion in-vitro, ranged from 81% for roasted specimens to 91.5% for culinary unprocessed insect. Similarly, the digestibility of nitrogenous substances of homogenized insect samples using this combination of enzymes ranged from 24.2% for roasted specimens to 80.2% for culinary unprocessed samples. The work showed the dependence of the digestibility of the mealworm larvae on the culinary treatment - the increasing heat load of the sample reduced the digestibility. Furthermore, it proved the effect of the digestive enzyme on the digestibility of the insect sample.

Keywords: digestibility; mealworm; culinary treatments; enzymes; nitrogenous substances

INTRODUCTION

Digestion is a physiological process in which nutrients contained in food are decomposed into a resorbable form. Nitrogenous substances, fats and carbohydrates have to be split up so that they can pass through the intestinal wall into the blood. The blood will transport them further to the necessary places in the organism where they are utilized **(Mišurcová et al., 2010)**. Digestibility is most commonly determined as protein digestibility. To a large extent, this digestibility is influenced by the culinary treatment. Culinary treatment, especially cooking and frying, improves sensory quality of food, and induces formation of flavours, attractive colours and textures. Cooking also improves hygienic quality by inactivating some pathogenic microorganisms, improves digestibility and increases the bioavailability of certain nutrients in the gastrointestinal tract **(Bognár, 1998)**.

At present, many studies **(Megido et al., 2018; Grabowsky and Klein, 2017; Klunder et al., 2012; Vandeweyer et al., 2017)** deal with the hygiene and food safety conditions applicable in the European food industry for edible insect, but only a few studies deal with the influence of culinary treatment on the edible insect nutritional value. This creates an information gap for everyday consumers, chefs, cookbooks authors, etc., who have minimal access to information about a safe and healthy way to cook edible insect **(Megido et al., 2018)**. Due to the increasing demand for commodities of animal origin, focusing on protein sources and their digestibility, consumer pressure is also increasing to fill this information gap **(Mlček et al., 2014; Tan, Berg and Stieger, 2016; Adámková, 2017)**. In addition, the availability of this information may reduce fears in the part of the European public about the consumption of edible insect **(Yen, 2009)**.

During the heat treatment of food, proteins are denatured, amino acids modified or destroyed and Maillard reaction occurs. In the heat treatment, proteins may also interact with other proteins or with oxidizing agents, sugars, polyphenols, tannins or solvents **(Finot, 1983)**. Denaturation at higher temperatures results to better enzymatically digestible proteins due to cleavage of developed polypeptide chains or inactivation of antinutritional compounds **(Finot, 1983; Opstevedt et al., 2003)**. On the other hand, the digestibility of proteins may be reduced by reacting with each other and by reacting with amino acids which cannot subsequently be hydrolysed by digestive enzymes **(Opstevedt et al., 2003)**.

The question of the use of edible insect as part of feed in livestock and pets (dogs, cats, etc.) has been dealt with by several studies **(Bosch et al., 2014; McCusker et al.,**

2014; De Marco et al., 2015; Panini et al., 2017). In spite of these data, the knowledge about digestibility of edible insect in humans is minimal. The reason is physiological differences and differences in the composition of digestive juices, therefore the digestibility of this commodity may be different in man and animal **(Bussink et al., 2007)**. Due to the inclusion of edible insect in the "novel food" category in European countries, the solution to this issue becomes important when a complex view of edible insect is needed, concerning not only nutritional or sensory properties, but also the digestibility.

For this reason, this study focused on digestibility of edible insect, which assumes that digestibility is different for different culinary treatments of insect. The aim was to find a suitable heat culinary treatment of the mealworm in terms of its optimum digestibility by man. Because of the inclusion of edible insect in the novel food category, comparison is also required with other commodities of animal origin. For this reason, this study focused on digestibility of edible insect, which assumes that digestibility is different for different culinary treatments of insect

Scientific hypothesis

Scientific hypothesis is: the digestibility of edible insect materials is dependent on culinary treatments. The aim was to find a suitable heat culinary treatment of the mealworm in terms of its optimum digestibility by man. Because of the inclusion of edible insect in the novel food category, comparison is also required with other commodities of animal origin.

MATERIAL AND METHODOLOGY

Material

For the analysis, samples of mealworm larvae (*Tenebrio molitor*) were used for analysis. Samples were purchased at a pet store. Prior to analysis, insect samples were treated as follows: mealworm larvae in the last and penultimate stages were taken from the breed and left to starve for 24 hours. Subsequently, the insect was killed with boiling water
(100 °C) and dried with a warm air stream at a temperature of 75 °C ±5 °C for 30 s. Samples of killed and wiped larvae were divided into three experimental groups with the following treatment procedures:

1. no treatment – freshly killed insect with no further culinary treatment
2. dried insect – killing, subsequent drying for 2 minutes at 120 °C and then drying for 5 – 7 minutes at 70 – 80 °C
3. roasted insect – killing, subsequent roasting for 4 minutes at 160 °C.

After treatment, all samples were homogenized and stored in cooling box at 4 – 7 °C until analysis.

Dry matter digestibility determination

Determination of digestibility was performed by gravimetric in vitro method using a Daisy incubator (ANKOM Technology, USA). For digestion, pepsin EC 3.4.23.1 from porcine gastric mucosa (activity: 0.7 FIP-U.g^{-1}) and pancreatin from pancreas (protease activity: 350 FIP-U.g^{-1}, lipase activity: 6000 FIP-U.g^{-1}, amylase activity: 7500 FIP-U.g^{-1}) were used. Both enzymes were supplied by Merck (Darmstadt, Germany).

Enzymatic hydrolysis involved hydrolysis by pepsin (0.5 g enzyme per g sample), pancreatin (0.5 g enzyme per 1 g of sample) and combined hydrolysis with pepsin and subsequently with pancreatin. In case of hydrolysis by pepsin, digestibility was measured after 30 minutes. For pancreatin hydrolysis, digestibility was determined after 6 hours. In the case of combined hydrolysis, the pepsin enzyme was left to function for 30 minutes, followed by the pancreatin enzyme treatment for 6 hours. Samples were evaluated 3 times. The determination was carried out according to the modified methodology **(Mišurcová et al., 2010; Mišurcová, 2008)**.

For determination of digestibility, 0.5 g of sample was weighed into F57 filter bags with a porosity of 25 μm (ANKOM Technology, USA). The bags were sealed, placed in incubation flasks containing 1.7 liters of the appropriate solution (in the case of pepsin 0.1 M HCl, in the case of pancreatin pH 7.45 phosphate buffer), conditioned to 40 °C and added to adequate amount of the corresponding enzyme to meet the above requirement of 0.5 g of enzyme per 1 g of sample. Together with the samples, a sealed control bag without a sample was placed in the incubation bottle. This was followed by hydrolysis for the time intervals mentioned above. After the hydrolysis was complete, the bags were washed with distilled water, dried for 24 hours at 103 °C and weighed. In the case of combined hydrolysis, the samples were first hydrolysed with pepsin, and hydrolysis with pancreatin was initiated immediately after completion of the pepsin hydrolysis and washing of the bags in distilled water **(Mišurcová et al., 2010; Mišurcová, 2008)**.

Determination of nitrogenous substances digestibility

To determine the digestibility of nitrogenous substances, the nitrogen content of the non-hydrolysed samples and the nitrogen content of the samples enzymatically hydrolysed with pepsin, pancreatin and combined – pepsin and then pancreatin – had to be evaluated. Enzymatic hydrolysis was carried out as described above. The total nitrogen content of both hydrolysed and non-hydrolysed insect samples was determined by the Kjeldahl method using an automatic distillation unit Pro Nitro A (JP Selecta S.A., Spain). The results were expressed as a percentage in the form of the coefficient of digestibility of the nitrogenous compounds.

The coefficient of digestibility of nitrogenous compounds (KS) can be calculated according to the equation below (1). To calculate the digestibility coefficient, the nitrogen content of the non-hydrolysed samples (NLN) from equation (2) and the nitrogen content of the hydrolysed samples (NLH) from equation (3) **(Mišurcová, 2008)** must be determined. Samples were measured 2 times.

$$K_S = \frac{NL_N - NL_H}{NL_N} \cdot 100 \quad (1)$$

$$NL_N = \frac{N_N}{m_{NL}} \cdot f \cdot 100 \quad (2)$$

$$NL_H = \frac{N_H}{m_{NL}} \cdot f \cdot 100 \quad (3)$$

where:

K_S digestibility coefficient (%),
NL_N content of nitrogenous substances in non-hydrolysed samples (%),
NL_H content of nitrogenous substances in hydrolysed samples (%),
N_N content of nitrogenous substances determined by Pro Nitro in non-hydrolysed samples (mg),
N_H content of nitrogenous substances determined by Pro Nitro in hydrolysed samples (mg),
m_{NL} sample weight (mg),
f conversion factor (f = 6.25).

Statistic analysis

Data was evaluated using Excel 2013 (Microsoft Corporation, USA) and STATISTICA Cz version 12 (StatSoft, USA). The results were expressed by average ± standard deviation. Kruskal-Wallis test (α = 0.05) was used to compare of samples.

RESULTS

The samples were hydrolysed with pepsin, pancreatin, and their combination (marked as "PePa"). The digestibility of the dry matter for each sample is shown in Table 1.

The highest digestibility was found in untreated samples. With processing, the digestibility decreased. The lowest was found for roasting, which can produce enzymatically unprocessable complexes. For the pepsin enzyme and dried and roasted samples, this value decreased by more than 35%. The pancreatic enzyme and combination of enzymes did not make such difference - for pancreatin, it was less than 15% and less than 11% for enzyme combination. The dried sample hydrolysed by the combination of pepsin and pancreatin enzymes has an average value just slightly below the level of the sample hydrolysed only by the pancreatin, and it seems that the above trend cannot be applied.

In the case of monitoring the dependence on the type of hydrolysis after the same heat treatment, it was found that the lowest digestibility values were determined for the pepsin enzyme, Figure 1. The reason is the chosen hydrolysis time (30 min). On the other hand, despite this hydrolysis time, the digestibility of unprocessed insect was more than 85%. In hydrolysis by the pancreatin enzyme, where the hydrolysis time was longer, the digestibility was determined to be up to 30% higher. The highest digestibility values were reached by the combination of pepsin and pancreatin. In this case, digestibility was over 80% for all culinary treatments (no processing, drying and roasting). This combined hydrolysis is most similar to human digestion from the hydrolysis types used in this work.

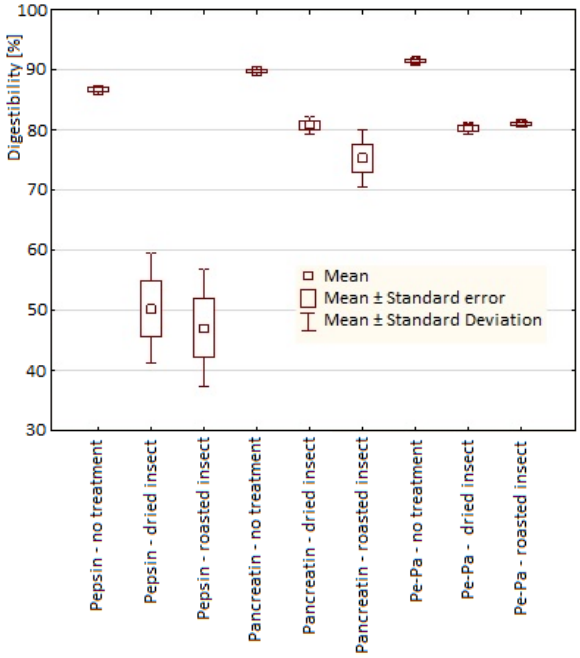

Figure 1 Digestibility of samples enzymatically hydrolyzed with pepsin, pancreatin and combined – pepsin and then pancreatin (marked as "PePa").

Due to the non-compliance with the homogeneity condition for some sample sets, the Kruskal-Wallis test and the multiple comparison of the *p-values* were selected for the comparison of the groups. The results of comparison of the groups are shown in Table 2. In this table a statistically significant difference between roasted and untreated samples by pepsin hydrolysis can be seen. A statistically significant difference (p <0.01) between unprocessed and roasted samples can also be found in pancreatin hydrolysis. In hydrolysis by the combination of these enzymes, a statistically significant difference was found between the dried and untreated samples. No other statistically significant difference was found in this study, although some differences can already be traced from the chart.

For each sample gained by hydrolysis the content of crude protein was analysed, Table 3. This value was used to calculate the digestibility of the nitrogenous substances. From the measured values of nitrogenous substances for individual samples, their digestibility was determined, Table 4. In this table, a significant decrease in the digestibility of nitrogenous substances in hydrolysed

Table 1 The digestibility of samples [g.100g^{-1}].

	no processing		dried insect		roasted insect	
	M	SD	M	SD	M	SD
Pepsin	86.7	0.8	50.4	9.2	47.2	9.8
Pancreatin	89.8	0.7	80.8	1.4	75.3	4.7
Pe-Pa	91.5	0.6	80.3	0.9	81.0	0.5

Note: PePa – combined hydrolysis using pepsin and pancreatin.

Table 2 Multiple comparison of the *p-values* for different culinary treatments and hydrolyses with pepsin, pancreatin and their combination.

Dependent value	Pepsin Multiple comparison of the *p-values* (both sides) Kruskal-Wallis test: H =7.423077; *p* = 0.0244		
	No treatment	Drying	Roasting
No treatment		0.072337	0.042684
Drying	0.072337		1.000000
Roasting	0.042684	1.000000	
Dependent value	**Pancreatin** Multiple comparison of the *p-values* (both sides) Kruskal-Wallis test: H = 9.846154; *p* = 0.0073		
	No treatment	Drying	Roasting
No treatment		0.349993	0.005106
Drying	0.349993		0.349993
Roasting	0.005106	0.349993	
Dependent value	**Pepsin + Pancreatin** Multiple comparison of the *p-values* (both sides) Kruskal-Wallis test: H = 8.000000; *p* = 0.0183		
	No treatment	Drying	Roasting
No treatment		0.018119	0.149581
Drying	0.018119		1.000000
Roasting	0.149581	1.000000	

samples with culinary treatment can be seen. It is believed that the decline in digestibility is due to the formation of enzymatically unprocessable complexes due to the increasing heat effect of heat culinary treatment.

DISCUSION

Several parameters can affect digestibility, e.g. chitin content, phytate content, interaction of individual nutrients, oxidative changes, etc. The results are simulated in vitro, so they can be different from real digestive processes **(Svačina, 2010)**. **Poelaert et al. (2016)** determined the digestibility of unprocessed mealworm dry matter by in-vitro method (IVDMD) 76.2%. This result is lower than in this work. Similarly, this was also the case with thermal effects on commodities, where **Poelaert et al. (2016)** declared an 18% lower digestibility than that measured in this work. However, the trend is similar in both researches. In accordance with this work, **Poelaert et al. (2016)** noticed reduced protein digestibility when using a heat processing of up to 13% when samples were autoclaved.

When comparing with mealworm, **Poelaert et al. (2016)** declared up to 23% lower digestibility of the house cricket

Table 3 Nitrogenous substances content in samples [g.100g^{-1}].

	no processing		**dried insect**		**roasted insect**	
	M	**SD**	**M**	**SD**	**M**	**SD**
No hydrolysis	204.2	1.7	739.4	24.8	488.0	2.1
Pepsin	58.8	4.4	668.8	0.8	184.2	2.5
Pancreatin	54.0	3.2	618.9	8.6	171.9	1.4
Pe-Pa	40.5	1.2	560.3	11.0	149.2	0.9

Note: PePa - combined hydrolysis using pepsin and pancreatin.

dry matter depending on the heat treatment. However, protein digestibility (IVCPD) is comparable in both species. **Poelaert et al. (2016)** also reports a comparison with commodities of plant origin (beans, lentils, peas, soybean), where the digestibility is mostly lower in raw state and the significantly increases with raising temperature - the lentils had an increase in digestibility by up to 28%. Generally, however, the digestibility of dry matter in these commodities of plant origin is up to tens of % lower than determined by **Poelaert et al. (2016)** in their work for a mealworm or than the values in this study.

In terms of nutritional values, however, the more important is the digestibility of crude proteins determined in vitro (IVCPD). Besides **Poelaert et al. (2016)** also **Marono et al. (2015)**, **Caparros Megido (2017)**, and **Panini et al., (2017)** dealt with it. **Panini et al., (2017)** for his research on "alternative protein source for Pacific white shrimp" reported a 45.9% dry matter digestibility and 76.1% protein digestibility for "mealworm meal". **Marono et al. (2015)** declared the protein digestibility of "insect meals" from different suppliers ranging from 65.5% to 66.7%. These values are comparable to the values (59.5% – 72.5%) reported by **Poelaert et al. (2016)** and values measured in this work but, are lower than the values (85.0% – 91.5%) reported by **Megido et al. (2018).** Although the difference in digestibility between **Poelaert et al. (2016)** and **Megido et al. (2018)** was 13% for a crude insect sample, **Poelaert et al. (2016)** declared it as the highest, and **Megido et al. (2018)** as the lowest. From the results reported by **Megido et al. (2018)**, therefore, the trend is the increasing protein digestibility with raising the temperature. On the contrary, **Poelaert et al. (2016)** show the opposite trend - heat treatment reduces protein digestibility. This trend can also be seen for the results in this work. However, the specific values are not completely comparable, due to different experimental methodology (e.g. time and temperature of hydrolysis, selected enzyme

Table 4 Digestibility of nitrogenous substances after culinary treatment and hydrolysis with the selected enzyme.

Sample	Nonhydrolyzed sample [g.100g^{-1}]	Hydrolyzed sample [g.100g^{-1}]	Digestibility [%]
No treatment			
Pepsin	204.2	58.8	71.2
Pancreatin	204.2	54.0	73.5
Pepsin and pancreatin	204.2	40.5	80.2
Drying			
Pepsin	488.0	184.2	62.3
Pancreatin	488.0	171.9	64.8
Pepsin and pancreatin	488.0	149.2	69.4
Roasting			
Pepsin	739.4	668.8	9.5
Pancreatin	739.4	618.9	16.3
Pepsin and pancreatin	739.4	560.3	24.2

type, correction). For this reason, it is possible to compare only culinary treatments between themselves and the influence of a particular enzyme.

When comparing digestibility with samples of animal origin, **Megido et al. (2018)** pointed out the match of their results with other commodities - beef (89%), pork (90%), turkey meat (78%) and salmon (85%) **(Bodwell, Satterlee and Hackler, 1980)**. They declared the differences from other studies were due to the different "raw materials" and the use of various "different batches of mealworms" with different fat or antinutritional factors content. At higher temperatures, digestibility is reduced as a result of the formation of difficult-to-digest protein complexes with oxidized fats. In addition, digestibility can be reduced by, for example, reacting with mineral substances and reacting minerals with one another. Reagents, such as phosphorus and calcium, form an insoluble complex (phytates) that reduces the digestibility of proteins and makes them inaccessible **(El Hassan et al., 2008).**

Similar to other commodities, the heat can not only positively affect the properties, but can also lead to a reduction in nutritional value, e.g. by oxidation of amino acids or by changing or losing essential amino acids, or even creating substances that are undesirable from the point of view of health (toxic, carcinogenic or mutagenic effects substances). Highly dangerous substances can arise from proteins of animal origin (i.e. insect), and therefore all excessively browned to blackened portions of the food should be removed. Insect, in our case, mealworm is a specific biological material. Despite being regarded a farm animal after being included into novel foods by EFSA, it has a different anatomy and physiology of the body than ordinary livestock (mammals). Therefore, it should be borne in mind that, from the nutritional point of view, this commodity contains, in addition to fat and crude protein, a considerable amount of chitin **(Adámková et al., 2017)**. However, the European consumer does not have enough chitinase to digest it.

CONCLUSION

The digestibility of edible insect, on which this work was focused, is dependent on subsequent culinary treatments. In terms of the digestibility of the dry matter, the highly in-vitro digestible sample of the mealworm is thermally untreated and the most difficult for digesting is sample after roasting. However, for the safety reasons, it is not possible to recommend the consumption of unprocessed mealworm meal by humans. However, insect can be used both as dried and uncooked (freshly killed) as feed for farm animals. Even in the case of nitrogen digestibility analysis, the highest digestibility value was detected for thermally unprocessed insect. From a safety point of view, the heat treatment by drying is more suitable, which reduces the digestibility of nitrogenous substances, but not so much as in the case of roasting. The practical use of this work lies in the contribution of knowledge that could enable the fortification of food by the addition of commodity from edible insect ideally roasted. However, due to the possible formation of dangerous roasting complexes (Maillard reaction), further analyses are needed in this area.

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DETERMINATION OF HMW – GS IN WHEAT USING SDS – PAGE AND LAB-ON-CHIP METHODS

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ABSTRACT

SDS-PAGE is widely used to determine the amounts of the different gluten protein types. However, this method is time-consuming, especially at early stages of wheat breeding, when large number of samples needs to be analyzed. On the other hand, LoC (Lab-on-Chip) technique has the potential for a fast, reliable, and automatable analysis of proteins. Benefits and limitations of Lab-on-Chip method over SDS-PAGE method in gluten proteins evaluation were explored in order to determine in which way LoC method should be improved in order to make its results more compliant with the results of SDS-PAGE. Chip electrophoresis provides a very good reproducibility of HMW-GS patterns. Moreover this approach is much faster than the conventional SDS-PAGE methods requiring several hours for an analysis. Another advantage over traditional gel electrophoresis is lower sample and reagent volume requirements, as well as specialized protein standards for accurate reproducibility and quantification. In the present study, we identified novel complex allele located at the locus *Glu-1B*.

Keywords: wheat; HMW-GS; SDS-PAGE; LoC

INTRODUCTION

Wheat (*Triticum*) is one of the key crops for nutrition, being the second most grown crop in 2016 with a global production of 749.3 million tons **(FAO, 2017)**. Mainly hexaploid bread wheat or common wheat (*Triticum aestivum* L.) is used as forage and for bread making pointing out the economic importance of these varieties as well as durum wheat (*Triticum durum* L.), a tetraploid species, especially used for pasta **(Cauvain and Young, 2003)**. It is generally known, that wheat gluten has a major effect on the end-use quality of baking industry products, since it is responsible for the visco-elastic properties of the dough. This protein macromolecule is composed of two components, gliadins and glutenins. Gliadins are responsible for the extensibility of dough, whereas glutenins for the dough elasticity **(Payne et al., 1984)**. Glutenins are classed as high molecular weight (HMW) encoded at *Glu*-1 loci and low molecular weight (LMW) encoded at *Glu*-3 loci. HMW glutenin subunits are further subdivided into high M_r x – type with 80 – 88 kDa and low M_r y – type with 67 – 73 kDa subunits **(Payne, Holt and Law, 1981)**.

Correlations and genetic studies of HMW-GS **(Payne et al., 1987)** established subunits with both positive (5 +10) and negative (2 +12) effects on bread making quality. Other allelic variant pairs showed similar results **(Payne et al., 1987)**. In general, a null at *Glu-1A* locus, subunit 6 +8 encoded at *Glu-1B* and 2 +12 at *Glu-1D* are negatively related with the quality parameters **(Weegels, Hamer and Schofield, 1995)**. The highest polymorphism of HMW – GS is regularly detected on 1B chromosome **(Li et al., 2010; Gregová et al., 2011; Hernández et al., 2012; Kuťka Hlozáková, Gregorová and Gálová, 2015)**. A scoring system for HMW-GS has been developed **(Payne et al., 1987)** as the sum of the contributions of each of the three HMW-GS loci. The breeder particularly needs this information to predict the dough-strength potential of the large numbers of lines involved at the earliest stages of breeding, thus to ensure that the poor-quality lines are not unnecessarily propagated. In officially accepted food testing procedures **(Wrigley, 1992)**, SDS – PAGE is used to assess this presence and quantity.

However, the traditional SDS-PAGE method for analysing glutenin subunit composition has several disadvantages. For instance, SDS-PAGE is time-consuming and includes a number of necessary manual steps, such as staining, destaining, imaging, analyzing **(Hsieh and Chen, 2007)**. Quantification can also be difficult **(Hou and Ng, 1995)** and one of the used chemical is acrylamide, a potential neurotoxin. The new, promising, fast electrophoretic technique for protein examinations is a microfluidic or Lab-on-Chip (LoC) method, which allows the integration of electrophoretic separation, staining, destaining, and fluorescence detection into a single process which can be combined with data analysis. This new technique is comparable to time consuming

SDS-PAGE stained with standard Coomassie in sensitivity, sizing accuracy and reproducibility **(Kuschel et al., 2002)**. However, the sizing accuracy of SDS-PAGE and chip – based analysis depend on the protein characteristics and may therefore vary for individual proteins. Some proteins may not migrate according to their molecular weight. In general, the sizing reproducibility of the LoC method is excellent, commonly achieving a sizing reproducibility of 5% or better **(Kuschel, 2000)**.

Scientific hypothesis

This aim of this work was to explore the possibilities of application of novel LoC method for analysis of glutenin subunits in comparison to SDS-PAGE, especially for HMW-GS, isolated from European common wheat varieties, where novel HMW-GS were found.

MATERIAL AND METHODOLOGY

Plant material

The European wheat genotypes used in this study were obtained from the collection of wheat genetic resources maintained in the Gene Bank of the Slovak Republic (National Agricultural and Food centre, Plant Production Research Center, Piešťany).

Protein extraction and SDS-PAGE

Seed storage proteins were isolated from the endosperm of intact, dry and mature single seeds. There were analysed one hundred individual grains from each genotype. Seed homogenization was carried out by grinding. Glutenins were extracted by standard referee method ISTA and were performed by discontinuous PAGE based on ISTA methodology **(Wrigley, 1992)** using the electrophoretic unit Protean II (BioRad). Protein fractions were stained by Coomassie Brilliant Blue R – 250. The separate gluten subunits were identified by the nomenclature of **Payne and Lawrence (1983)**.

Protein separation on chip electrophoresis

The commercial Agilent Protein 230 analysis kit and the 2100 Expert software were used for the glutenin fractionation. The Agilent automated electrophoresis system applies a combination of microfluidic separation technology and sensitive fluorescent detection of proteins. It automatically performs all steps of gel-based electrophoresis (sample separation, staining, destaining, imaging, band detection, data analysis). The analysis of each chip takes 30 min for a set of ten samples. The 2100 Expert software displays the separation results in both the electrophoreogram (peak) and simulated gel views.

RESULTS AND DISCUSSION

Protein content and composition are key parameters related to various aspects of end-use quality of bread wheat *(T. aestivum* L*).* The classification of these proteins into solubility groups is used in the nomenclature for a long time. The UPOV method **(Wrigley, 1992)** for wheat variety differentiation is based on the comparison of Coomassie-stained polyacrylamide gels after one-step grain extraction of weight-matched single grains. For protein band assignment a system according to **Payne and Lawrence (1983)** is accepted where molecular weights of the subunits were determined by SDS-PAGE and the largest HMW-GS, in terms of molecular weights, got the lowest number (Figure 1a, Figure 1b). Typically, only HMW-GS with a molecular weight larger than 100 kDa are considered for variety assignment.

Protein sizing with the chip-based protein analysis system was performed by running the protein sizing standard on each chip from a designated well. Following the analysis of this sizing standard, the software generated a standard curve of the measured migration time versus the known molecular weight of each standard protein which was used to determine the size of each of the proteins detected within the sample **(Kuschel et al., 2002)**. Internal standards, the lower and upper marker, were included in each sample. The protein 230 assay exhibited clear protein patterns in the desired molecular weight range between 14 and 230 kDa which was moreover similar to the SDS-PAGE pattern. Distinct molecular weights could be assigned to interesting protein signals (Figure 2). Therefore, the protein 230 assay was chosen for all further experiments. It is obvious that color intensity of protein bands is caused by concentration of individual HMW-GS.

In this study, the HMW-GS composition is known for all samples and Table 1 lists the names of the wheat varieties together with all HMW-GS. Nevertheless, it has to be mentioned that the determined molecular weights were, in all cases, higher than those derived from SDS-PAGE. Six protein bands corresponding to HMW-GS were in a molecular weight range from 100 to 220 kDa which is in accordance with findings **Marchetti-Deschmann et al. (2011)**; **Balázs et al. (2011)** and **Chňapek et al. (2015)**. This fact was already observed for the first generation of CGE-on-a-chip protein assays (protein 200+) **(Uthayakumaran, Batey and Wrigley, 2005; Uthayakumaran et al., 2006)** where the authors could assign certain HMW-GS to distinct peaks based on the analysis of wheat mutants lacking certain alleles. The determination of higher molecular weights for the extracted proteins can only be explained by a lower electrophoretic mobility of the HMW-GS in the given capillary-based gel system caused maybe by interaction of the proteins with the capillary walls or hindered mobility of protein aggregates in miniaturized systems. Additionally, some of the HMW-GS are migrating in a different order in comparison to SDS-PAGE.

Figure 1a a Figure 1b show the inverted migration order of HMW-GS numbers 5 and 1. This finding corroborates the assumption that the electrophoretic mobility of HMW-GS is highly affected by the chosen capillary-based separation system. One possible explanation can be the fact that glutenins are glycine- and glutamine-rich proteins (>50% of amino acids composition) with average isoelectric points of 5 to 6. It is a known fact that acidic proteins tend to bind SDS poorly **(Eley et al., 1979)** which subsequently affects electrophoretic mobility adversely. The phenomenon of changed migration order in electrophoresis was also already observed for non-miniaturized CGE systems **(Weegels, Hamer and Schofield, 1995; Sutton and Bietz, 1997; Bean and Lookhart, 1999)**.

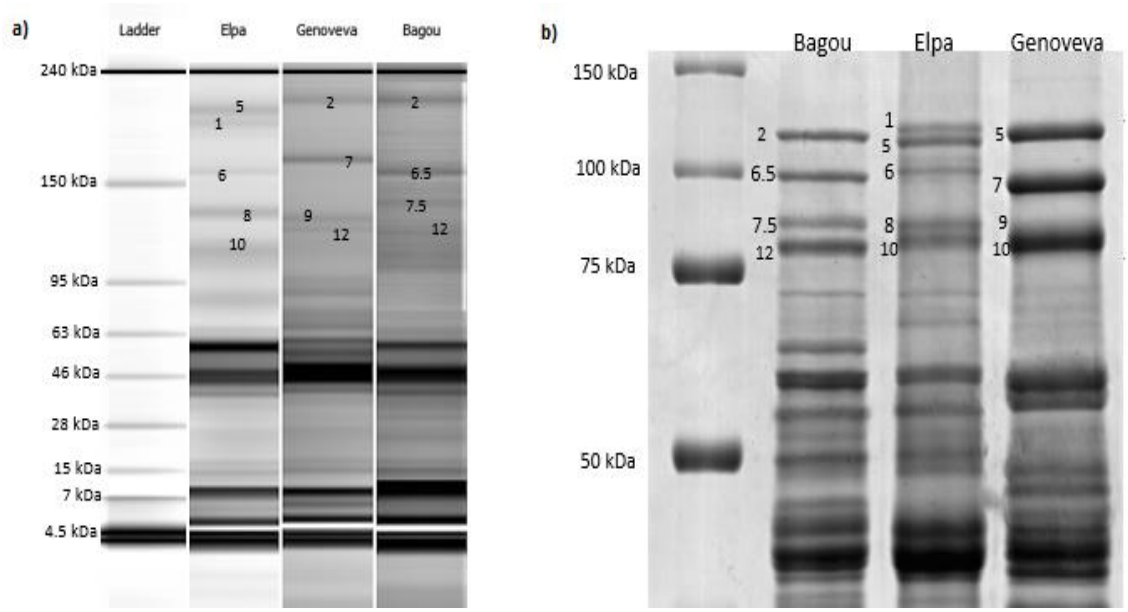

Figure 1 Comparison of the protein patterns of chosen varieties from chip electrophoresis (a) and SDS-PAGE (b).

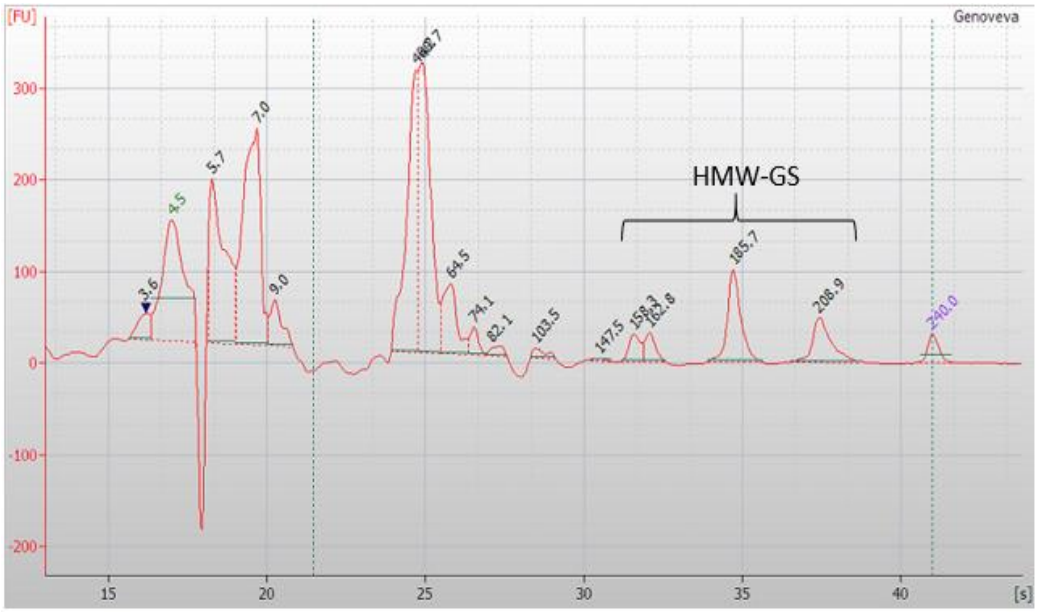

Figure 2 Electrophoreogram of chosen wheat variety Genoveva with HMW-GS identification.

Table 1 Determined molecular weights for proteins extracted by single-grain extraction by means of SDS-PAGE and Lab-on-chip analysis.

HMW – GS	SDS – PAGE (kDa)	LOC (kDa)
1	113	206 – 212
2	108	214 – 216
2*	108	205
5	105	216 – 224
6	100	180
6.5	99	159
7	98	169 – 188
17	90	171
18	89	138
7.5	88	146
8	86	148 – 153
9	83	130
10	82	126 – 134
12	80	129 – 131

Several authors used LoC method for identification and quantification HMW-GS in different wheat varieties **(Uthayakumaran, Batey and Wrigley, 2005; Uthayakumaran et al., 2006; Marchetti-Deschmann et al. 2011)**, whereas **Balázs et al. (2011)** confirmed that influence of environmental conditions on quantity of wheat protein subunits could be monitored using this method. **Chanvrier, Uthayakumaran and Lillford (2007)** followed the polymerization of protein of wheat gluten under processing such as extrusion, while **Maforimbo et al. (2008)** studied the interaction of glutenin subunits and soy proteins by LoC method. Furthermore, molecular weight and concentration of different compounds which are involved in biochemical processes such as nicotinamide adenine dinucleotide phosphate NAD(P)+ isolated from pea, soybean, and wheat proteins **(Nagaoka, 2003)** and Kunitz trypsin inhibitor in soybean varieties **(Torbica et al., 2010)** can be determined by the LoC method.

CONCLUSION

The aim of this study was to evaluate a Lab-on-chip system for fast and reliable wheat variety control. During the study it turned out that the on-chip reproducibility of LoC system in terms of molecular weight determination and protein quantification is very good. Nevertheless, the fast analysis of one-step one grain extracts showed protein pattern which can directly be compared to SDS-PAGE performed according to UPOV method, the internationally accepted method for wheat control. This suggests that this method could be used as a high-throughput alternative for the time- and labour- consuming SDS-PAGE.

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The monitoring of biogenic amines in the raw food

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ABSTRACT

The aim of this work was to evaluate microbial quality and the presence of biogenic amines in raw bars. This study was focused on microbiological research in order to determine the presence of selected indicator groups of microorganisms depending on the composition of raw food. Identification of microorganisms was carried out by MALDI-TOF MS. In the second part of the experiment, biogenic amines and polyamines were analyzed using high performance liquid chromatography with UV/VIS detection. An increased incidence of mold has been reported in the samples, which is associated with a risk of mycotoxin production. After identifying microorganisms, it was found out that genera *Micrococcus*, *Bacillus* and *Staphylococcus* were the most represented. The highest concentration of biogenic amines (tyramine 42.2 ±4.8 mg.kg^{-1}; putrescine 54.0 ±2.9 mg.kg^{-1}) was found in a sample containing the vegetable component. The average concentration of biogenic amines in the tested raw bars was <30 mg.kg^{-1} and therefore they do not pose a serious health hazard to a consumer.

Keywords: raw food; biogenic amines; UHPLC; microorganisms

INTRODUCTION

Raw foods consist mainly or entirely of raw uncooked food. In the Czech Republic, we can also see the name "live diet", and supporters of this trend are called "vitarians" **(Cunningham, 2004; Červenka, Brožková and Fišerová, 2016)**. Food is considered raw unless it has undergone a heat treatment of more than 48 °C. Generally, there is no single specific temperature in the literature, but the range is between 38 – 48 °C **(Cunningham, 2004; Červenka, Brožková and Fišerová, 2016)**. Food meets raw food parameters unless it is refined, pasteurized, treated with pesticides or otherwise processed industrially. Instead, this diet approves a variety of food modifications like mixing, lyophilization, soaking and germination. Raw diet is based on eating a plant-based diet, especially fruits, vegetables, nuts and seeds. Cereals and legumes are also allowed, but usually they are first soaked **(Cunningham, 2004)**.

Among the positive effects of eating raw food is the high intake of fiber, minerals and water-soluble vitamins **(Craig, 2009)**. The controversial effects of eating raw food are reducing the risk of cancer, especially women-specific types of cancer **(Lanou and Svenson, 2011; Tantamango-Bartley et al., 2013)** when mortality decreases compared to people, who consumed animal-based food **(Orlich et al., 2014)**. Raw food has a positive effect on the composition of the intestinal microflora, which exhibits a protective effect **(Glick-Bauer and Yeh, 2014)**. **Ling and Hänninen (1992)** describe a significant decrease in the activity of some precarcinogenic enzymes formed by the intestinal microflora when the raw foods were eaten for a week. Consuming raw food also seems useful in terms of intake of protective nutrients and photochemicals and also of minimizing intake of substances that are involved in many chronic diseases **(Dewell et al., 2008)**.

Limiting the animal fat in the diet decreases the intake of saturated fatty acids and cholesterol. These aspects lead to a lower incidence of cardiovascular disease. At the same time, HDL cholesterol is also reduced, as well as insufficient intake of vitamin B12 **(Dewell et al., 2008; Koebnick et al., 2005)**. In the long term, insufficient intake of vitamin B12, iron, zinc, essential fatty acids and essential amino acids is considered to be the major drawback of this nutritional trend. The largest natural sources of vitamin B12 are meat, offal, seafood, eggs, milk and dairy products **(Watanabe, 2016)**.

Deficiency of B12 may result in megaloblastic anemia, which causes interruption of the cell division process. Clinical manifestations are consequently fatigue, weakness, paleness and decreased muscle activity **(Aslinia, Mazza and Yale, 2006)**. Another type of anemia associated with the raw diet is sideropenic anemia, which is associated with reduction of iron in the blood **(Sahovic, Vukobrat-Bijedic and Sahovic, 2012)**. The best sources of iron in the raw diet are mainly nuts (cashews, almonds, hazelnuts) and legumes (lentils, beans, peas), which are first recommended to be soaked or germinated. In the long term, intake of raw foods leads to weight loss, but also to underweight in many cases. Increased consumption of raw

food is therefore usually associated with low values of BMI **(Koebnick et al., 1999; Craig, 2009)**. Eating raw food results in low intake of protein, calcium and vitamin D. Low density of bone tissue and increased risk of osteoporosis **(Fontana et al., 2005)** are often manifested in people following this diet. **Ganss, Schlechtriemen and Klimek (1999)** reported an increased incidence of tooth enamel erosion. Its decay is associated with excessive consumption of fruit, which contains easily fermentable sugars. Eating raw foods leads to insufficient intake of polyunsaturated fatty acids necessary for normal function and further development of the brain, especially in children and adolescents who are still growing **(Fonseca-Azevedo and Herculano-Houzel, 2012)**.

Eating raw food is also associated with worse digestibility of plant proteins, correlated with reduced nutrient utilization due to the presence of antinutrients. Antinutrients act on the activity of some enzymes, vitamins and minerals. In legumes and cereals, lectins, protease inhibitors, saponins and phytic acid are found to be destroyed only by heat treatment of foods **(Soetan and Oyewole, 2009)**. Protease inhibitors, which were found in soybeans and peanuts, prevent proteolysis and subsequent protein utilization. The body reacts to the resulting amino acid deficiency by producing pancreatic proteases. In adolescents, these substances can cause stop in growth and further development **(Kvasničková, 1998)**.

Also, it is importat to report increased risk of food intoxications, which stems from inadequate heat treatment of foods **(Cunningham, 2004)**.

Biogenic amines are one of the substances involved in the food quality. They are low molecular weight organic nitrogen compounds. Biogenic amines exist in living organisms, where they fullfil a number of metabolic and physiological functions **(Silla-Santos, 1996; Košmerl, Šućur and Prosen, 2013; Cunha, Lopes and Fernandes, 2016)**. Biogenic amines are essential for all humans. But in high concentrations, they may cause health problems. Histamine and tyramine belong among the most toxicologically relevant biogenic amines **(Shalaby, 1996; Buňková et al., 2013)**. The most common manifestation of the occurrence of biogenic amines are respiratory problems, nausea, palpitations, irregular heartbeat, erythema, swelling and headaches **(Santos et al., 2003; Li et al., 2013)**.

The maximal limit permitted by European legislation is defined only for histamine. According to European Commission of Regulation (EC) nu. 2073/2005, the maximum histamine content in fish and fishery products is set at less than 100 $mg.kg^{-1}$. A number of biogenic amines in foods of plant origin have been described by some authors **(Halász et al., 1994; Nishibori, Fujihara and Akatuki, 2007; Pleva et al., 2018)**. However, according to available literature, the determination of these substances in raw food has not been carried out yet.

Scientific hypothesis

Biogenic amines can be present in raw bars and their content is variable.

MATERIAL AND METHODOLOGY

Isolation and identification of the microorganisms:

Ten grams of the fermented raw food sample (Figure 1) was weighed out, aseptically removed and put into 90 mL of sterile physiological solution that was subsequently homogenised for 10 min (using a stomacher). The raw bars were then subjected to routine microbiological analysis. The total microorganism counts were assessed according to **ISO 4833−1 (2013)**, the *Enterobacteriaceae* bacteria family according to **ISO 21528−2 (2017)**, yeasts and moulds according to **ISO 6611 (2004)** and halotolerant microorganisms (staphylococci) according to **Chapman (1945)** on mannitol salt phenol red agar after cultivation at 37 °C for 2 days. The selected colonies were isolated into BHI broth and cultivated for 24 – 48 h at 25 °C (yeasts), 37 °C (*Enterobacteriaceae*, *Staphylococcus*) or 30 °C (other microorganisms). Each raw bar product sample was microbially analysed 3 times. Identification of the microorganisms was performed via the MALDI−TOF MS method using a Bruker Autoflex Speed (Bruker Daltonics, Bremen, Germany) and the Biotyper 3.1 database (Bruker Daltonics) after preliminary classification of isolates into individual microorganism groups. Visualisation of the protein profiles was performed via mMass 5 **(Strohalm et al., 2010)**. The individual identifications were performed in at least two independent experiments in two parallels **(Pleva et al., 2018)**.

Figure 1 Various types of raw bars. Note: (top left – raw sesame bar, top right – raw stick with cashew, left bottom – raw chocolate florentines, bottom right – raw apple ball).

Table 1 Composition of the product.

code	product	composition of the product
B1	Raw balls tropical mix	dates, almonds, dried mango, dried pineapple, almond paste, uncooked cocoa beans, raw syrup of agave, orange peel, ethereal orange oil
B2	Raw balls coconut	coconut grated, raisins Sultana, dates, sunflower seed
B3	Raw balls Jamaica	dates, unroasted cocoa beans, almonds, ground vanilla, spices
B4	Raw chocolate marokánka	dates, figs, raw cashews, almonds
B5	Raw bars with cashew	cocoa powder, agave syrup, orange peel, almonds, walnuts, dates, raisins, pecans, sunflower seeds, pumpkin seeds, coconut, apples, ground cinnamon, ground cardamom, Himalayan salt pink
B6	Raw sesame bars	cashews, raisins, sunflower seeds
B7	Raw vegetable bars	date, sesame
B8	Raw vegetable bars	Brazil nuts, dried tomatoes with sea salt, garlic, onion, Sultana raisins, hemp seeds, Roman cumin, marjoram, chilli minced
B9	Raw apple balls	raisins, sunflower seeds of the core, apple pulp powder, cinnamon
B10	Raw bars with red beet	dates, raisins Sultana, sunflower seed, beet powder, extra virgin olive oil, lemon essential oil
B11	Raw cocoa balls	raisins, dates, cocoa, coconut, chia seeds, sunflower
B12	Raw protein bars with banana	dates, banana, rice protein, coconut
B13	Raw protein hazelnut bars	dates, hazelnuts, rice protein (heat-unprocessed protein from whole-grain brown rice, rice oligodextrin, stevia, xanthan gum, sea salt, pectin), sunflower seeds, raw cocoa mass, chia seeds
B14	Raw apple bars	date, activated sunflower seed, dried apples, Sultana raisins, cinnamon
B15	Raw plum bars	dates, cashew, beetroot, plums, cocoa beans, poppy

Table 2 Viable counts (log CFU.g^{-1}) of the main microbial groups (first day) in raw bars in the Czech republic.

	log CFU.g^{-1}							
Sample	**VRBA**	**MSA**	**MRS**	**SB**	**M17**	**CHYGA**	**RCA**	**BHI**
B1	3.9 ±0.3	3.8 ±0.2	3.2 ±0.1	3.7 ±0.4	2.8 ±0.1	3.6 ±0.1	2.6 ±0.4	3.8 ±0.3
B2	3.5 ±0.3	5.2 ±0.1	3.5 ±0.2	-	3.9 ±0.5	2.3 ±0.1	5.0 ±0.2	3.9 ±0.3
B3	7.3 ±0.4	7.6 ±0.3	2.7 ±0.1	4.1 ±0.2	5.0 ±0.2	3.5 ±0.1	5.3 ±0.1	9.2 ±0.4
B4	3.6 ±0.2	3.0 ±0.1	3.0 ±0.2	3.7 ±0.2	3.5 ±0.3	3.3 ±0.1	6.1 ±0.2	2.9 ±0.2
B5	3.0 ±0.3	2.9 ±0.2	-	3.2 ±0.3	6.4 ±0.2	3.6 ±0.3	3.3 ±0.3	3.3 ±0.1
B6	4.3 ±0.5	3.6 ±0.2	4.8 ±0.3	-	5.1 ±0.2	3.0 ±0.1	3.4 ±0.2	5.2 ±0.2
B7	6.7 ±0.4	3.2 ±0.3	3.9 ±0.3	3.2 ±0.1	3.7 ±0.4	3.6 ±0.2	5.0 ±0.2	7.5 ±0.2
B8	3.7 ±0.3	3.2 ±0.2	4.6 ±0.4	4.0 ±0.3	2.6 ±0.1	3.3 ±0.2	5.7 ±0.2	3.5 ±0.1
B9	3.4 ±0.3	3.4 ±0.1	-	-	3.7 ±0.2	2.4 ±0.1	-	3.7 ±0.2
B10	4.2 ±0.5	5.3 ±0.4	6.7 ±0.2	-	4.4 ±0.1	-	-	3.4 ±0.4
B11	2.7 ±0.3	4.0 ±0.2	6.3 ±0.3	3.0 ±0.7	6.1 ±0.4	3.2 ±0.2	4.9 ±0.3	3.6 ±0.2
B12	2.9 ±0.4	4.0 ±0.2	-	-	3.4 ±0.2	4.0 ±0.1	-	4.2 ±0.3
B13	3.6 ±0.3	3.7 ±0.3	3.8 ±0.1	3.5 ±0.2	3.7 ±0.3	2.5 ±0.1	2.3 ±0.2	3.7 ±0.1
B14	3.3 ±0.2	4.7 ±0.4	-	-	4.8 ±0.1	-	-	4.6 ±0.2
B15	3.0 ±0.3	4.1 ±0.1	-	-	4.1 ±0.2	3.6 ±0.2	-	4.4 ±0.3

Preparation:

Lyophilised raw bar products were used for the biogenic amine (BA) and polyamine (PA) analysis. Triple extraction of BA and PA from the lyophilised samples was carried out using a perchloric acid solution (0.6 mol.L^{-1}). Three independent extractions were performed on each raw bar sample. The filtrated extract (filter porosity 0.45 μm) was then used directly for derivatisation and a following determination of BA/PA content **(Dadáková, Křížek and Pelikánová, 2009; Buňková et al., 2013)**.

Biogenic amine detection by HPLC:

The concentrations of eight present biogenic amines, such as histamine (HIM), tyramine (TYM), phenylethylamine (PHE), tryptamine (TRY), putrescine (PUT), cadaverine (CAD), spermine (SPE) and spermidine (SPD), were analysed via high performance liquid chromatography (HPLC) (LabAlliance, USA and Agilent Technologies, Agilent, Santa Clara, California, USA) after derivatisation using dansylchloride. The dansylchloride sample derivatisation procedure was performed according to **Dadáková, Křížek and Pelikánová (2009).** 1,7−heptandiamine was used as the internal standard. Chromatographic separation (ZORBAX Eclipse XDB−C18, 50 9 3.0 mm, 1.8 lm; Agilent Technologies) and detection (spectrophotometric λ = 254 nm) were performed according to **Buňková et al. (2013)**. Each extract was derivatised twice after cultivation, and each derivatised mixture was applied to the column twice. Each raw bar sample was analysed 12 times (3 extractions, 2 derivatisations, 2 applications to the column). Detection limits for the individual amines were in the range 0.24 – 1.39 mg.kg^{-1}. Given the significance of biogenic amines to human health and food safety, monitoring their content in foodstuffs is very important. Currently, HPLC based methods are the most suitable for the analysis of fermented food **(Pleva et al., 2018)**. The reliability and sensitivity of these methods render them useful as important techniques to determine the concentrations of all biogenic amines in fermented food **(EFSA, 2011)**.

Statistic analysis

The obtained experimental data were analysed using Statistical software Unistat 6.5 (Unistat, London, UK). The significance level of all statistical tests was set at p <0.05. The Kruskall-Wallis and Wilcoxon tests were used to evaluate the data obtained.

RESULTS AND DISCUSSION

Microbial analysis

Raw bars are ideal media for the growth and survival of a variety of fungi and bacteria.

The results of the microbial analysis are given in Table 2. The amount of microorganisms cultured in BHI ranged from 2.9 to 9.2 log CFU.g^{-1}. Although there is no hygienic limit for this type of product in the current legislation, the log boundary of 6.0 CFU.g^{-1} is considered to be safe

(Červenka, Brožková and Fišerová, 2016). This hygienic limit was not crossed by all tested samples, except for samples B3 (9.2 log CFU.g^{-1}) and B7 (7.5 log CFU.g^{-1}). The number of yeasts and moulds (CHYGA) ranged from 2.3 to 4.0 log CFU.g^{-1}. A similar result was obtained by **Červenka, Brožková and Fišerová (2016)**, who reported the amount of moulds in raw foods ranging from 1.8 to 3.7 log CFU.g^{-1}. Yeasts and moulds occurred in almost all samples, despite the fact that the antimycotic agent hexamidine at a concentration of 50 mg.L^{-1} was applied to B9, B10, B12, B14 and B15 samples. An increased number of moulds can be caused by contamination of feedstocks or by used processing technology. Later, isolated moulds were microscopically identified as *Aspergillus* and *Penicillium*, which are responsible for the production of mycotoxins. Many authors have reported an increased occurrence of mycotoxins, especially ochratoxin A and aflatoxins, in dates **(Ragab, Ramadan and Abdel-Sater, 2001; Azaiez et al., 2015)** or in raisins **(Azaiez et al., 2015)**, which form a substantial part of the raw bars. Mycotoxins were also found in other raw materials, that raw bars are made of. For example mainly in figs **(Azaiez et al., 2015)**, dried plums **(Engel, 2000; Azaiez et al., 2015)**, but also peanuts **(Hoeltz et al., 2012; Schwartzbord and Brown, 2015)**, cashews **(Milhome et al., 2014)**, coconut **(Saxena and Mehrotra, 1990)** and sunflower seeds **(Jiménez et al., 1991)**.

The number of coliform bacteria (VRBA) ranged from 2.7 to 7.3 log CFU.g^{-1}. However, an increased number of coliform bacteria was observed in samples B3 (7.3 log CFU.g^{-1}) and B7 (6.7 log CFU.g^{-1}). The presence of coliform bacteria can be caused by fertilization of bio food with faecal matter, insect vector transport or by contaminated water. **Červenka, Brožková and Fišerová (2016)** reported that the content of coliform bacteria was in the interval from 1.9 to 4.4 log CFU.g^{-1} in samples of raw food but, in two samples, such increase was not noticed due to the antimicrobial effect of young barley. In their study, **Brožková et al. (2016)** presented the contents of coliform bacteria in raw materials for the production of raw bars, such as hazelnuts (2.9 log CFU.g^{-1}), goji (2.8 log CFU.g^{-1}), cashew (<1 log CFU.g^{-1}), chia seed (<1 log CFU.g^{-1}) and linseed (5.9 log CFU.g^{-1}). Although the microbicidal effect of essential oils was described in citruses **(Oikeh et al, 2016)**, these compounds did not have a significant effect on the number of coliform bacteria in B1 and B10 samples. The number of staphylococci (MSA) was found to range from 2.9 to 7.5 log CFU.g^{-1}, with the highest concentration being detected in samples B3 (7.5 log CFU.g^{-1}), B10 (5.3 log CFU.g^{-1}) and B2 (5.2 log CFU.g^{-1}). Enterococci (SB) were recorded in 8 samples with numbers ranging from 3.0 to 4.13 log CFU.g^{-1}. Streptococci (M17) and lactobacilli (MRS) were observed in all samples, the number of streptococci ranged from 2.6 to 6.4 log CFU.g^{-1} and the number of lactobacilli (MRS) ranged from 2.3 to 6.1 log CFU.g^{-1}.

Out of 15 samples cultivated on 8 selectively diagnosed soils, 68 species of bacteria and yeast were isolated and identified by the MALDI-TOF MS method. The following microorganisms were identified: *Acinetobacter pittii* (B6, B10), *Bacillus cereus* (B4, B10, B12, B15), *Bacillus safensis* (B9, B14, B15), *Bacillus thuringiensis* (B4), *Cronobacter sakazakii* (B6), *Enterococcus casseliflavus* (B15), *Micrococcus luteus* (B1, B5, B6, B7, B8, B10, B11, B12, B13), *Pseudomonas oryzihabitans* (B6, B10), *Rhodotorula mucilaginosa* (B12), *Serratia fonticola* (B3), *Serratia marcescens* (B1, B3), *Staphylococcus aureus* (B3), *Staphylococcus hominis* (B1, B13), *Staphylococcus pasteuri* (B9), *Staphylococcus warneri* (B2, B4, B7).

Biogenic amine and polyamine analysis

The selected results of the chromatographic analysis of biogenic amines and polyamines are summarized in Figure 2. These biogenic amines were detected: PEA (8.14 − 37.78 mg.kg^{-1}), HIM (2.14 − 18.92 mg.kg^{-1}) and TYM (1.98 − 42.23 mg.kg^{-1}).

The highest concentration of PEA was observed in samples B4 (37.8 ±2.4 mg.kg^{-1}) and B9 (35.0 ±2.3 mg.kg^{-1}) and the lowest was in B13 (8.1 ±0.8 mg.kg^{-1}) and B3 (8.2 ±1.6 mg.kg^{-1}). In case of HIM, the highest concentration was detected in samples B1 (18.9 ±1.2 mg.kg^{-1}) and B13 (11.9 ±2.0 mg.kg^{-1}), on the other hand, the lowest was in B3 (2.1 ±0.8 mg.kg^{-1}) and B2 (2.9 ±1.1 mg.kg^{-1}). The highest concentration of TYM was observed in samples B8 (42.2 ±4.8 mg.kg^{-1}) and B4 (31.7 ±3.4 mg.kg^{-1}), the lowest was in samples B6 (2.0 ±0.5 mg.kg^{-1}) and B14 (3.3 ±1.6 mg.kg^{-1}). Based on the statistical analysis, statistically significant differences ($p \leq 0.05$) were found in the BA content of individual raw bars.

PEA is a natural component of cocoa beans **(Halász et al., 1994; Silla-Santos, 1996)**. PEA (<20 mg.kg^{-1}) was reported in non-cured cocoa beans, however, higher concentrations were detected in roasted beans. Higher concentrations are related to the decarboxylation of phenylalanine to PEA as a result of roasting **(Halász et al., 1994)**. No increased concentrations of PEA were recorded in samples containing cocoa beans or cocoa powder. The measured HIM concentration was below 20 mg.kg^{-1}. The highest TYM content was recorded in samples containing dried tomatoes (B8), bananas (B12) and plums (B15). **Halász et al. (1994)** also reported an increased incidence of TYM in tomatoes, plums and bananas.

The total polyamine content ranged from 6.88 to 28.32 mg.kg^{-1}, PUT from 8.31 to 53.95 mg.kg^{-1}, SPD from 0.76 to 11.23 mg.kg^{-1} and SPM from 9.24 to 30.73 mg.kg^{-1}.

The highest concentration of CAD was observed in samples B8 (28.3 ±3.8 mg.kg^{-1}) and B7 (25.7 ±2.4 mg.kg^{-1}), while the lowest was in B6 (6.9 ±1.1 mg.kg^{-1}) and B2 (10.1 ±0.8 mg.kg^{-1}). The most of PUT was contained in samples B8 (54.0 ±2.9 mg.kg^{-1}) and B4 (39.4 ±2.6 mg.kg^{-1}) and the lowest concentration was recorded in samples B3 (8.3 ±0.6 mg.kg^{-1}) and B14 (10.1 ±1.7 mg.kg^{-1}). The highest volumes of SPD were observed in samples B6 (11.2 ±0.9 mg.kg^{-1}) and B7 (10.3 ±0.9 mg.kg^{-1}) but the lowest SPD content was in samples B12 (0.8 ±0.3 mg.kg^{-1}) and B10 (2.8 ±0.2 mg.kg^{-1}). In case of polyamine SPM, the highest volumes were detected in samples B3 (30.7 ±2.4 mg.kg^{-1}) and B6 (26.3 ±1.8 mg.kg^{-1}), the lowest were in samples B10 (9.2 ±0.4 mg.kg^{-1}) and B9 (10.8 ±0.7 mg.kg^{-1}).

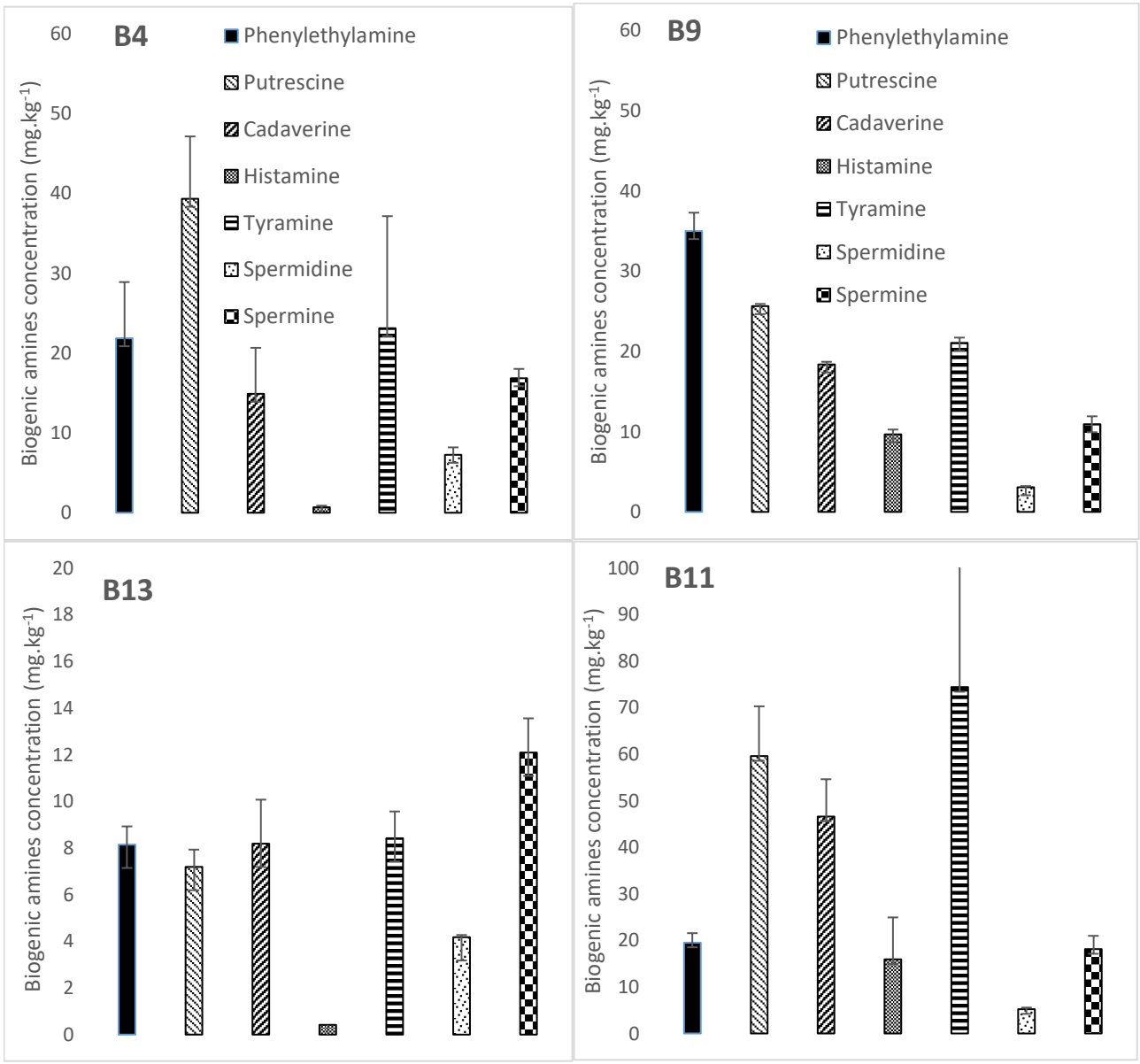

Figure 2 Biogenic amines content in selected raw bar samples (B4, B9, B11 and B13) (mg.kg^{-1}).

Nishibori, Fujihara and Akatuki (2007) reported the amount of polyamine SPM to be 13.6 mg.kg^{-1} in almonds and 24.1 mg.kg^{-1} in cashews. Results from this study correspond with our results because the samples B3 (almonds) and B6 (cashews) contained the highest SPM concentration in the raw bars. The highest measured PUT content was in sample B8 (54.0 ±2.9 mg.kg^{-1}) containing the vegetable component. However, this result is different compared with results achieved by **Nishibori, Fujihara and Akatuki (2007)** who reported lower amounts of PUT in tomato (5.9 mg.kg^{-1}), raisins (0.1 mg.kg^{-1}), garlic and onion (each 2.3 mg.kg^{-1}).

CONCLUSION

The first part of this study concerned the characteristics of raw food, its microbial quality and the problematics of biogenic amines. 15 types of raw bars with various content composition were selected for this experiment (Table 1). These foodstuffs were subjected to a microbial analysis with a goal to find indicator groups of microorganisms (facultative anaerobic mesophilic microorganisms, enterobacteria, staphylococci, yeasts, moulds, and lactic acid bacteria). The highest concentration of biogenic amines was recorded in the sample of the raw bar containing vegetable components with this product containing, beside others, a biogenic amine tyramine in concentration 42.23 mg.kg^{-1} and a polyamine putrescine in concentration 53.95 mg.kg^{-1}. More than a half of the samples did not exceed the limit of concentration of biogenic amines 15 mg.kg^{-1}; two thirds of the samples did not exceed the limit 20 mg.kg^{-1}. Identification of present microorganisms proved that the most represented genus were *Micrococcus*, *Staphylococcus* and *Bacillus*, which a decarboxylase activity was observed in. Taking this fact into account, it is important to consider the content of individual biogenic amines in the tested samples. The achieved results of this study show that raw bars contain various microorganisms according to their content composition. It is necessary to pay attention to the content of individual types of foodstuff and their microflora, especially in relation to human health. Even though it was not a primary goal of this study to focus on presence of

moulds, the occurrence of mycotoxigenic genus *Aspergillus* and *Penicillium* in the studied samples is alarming. The presence of mycotoxins is very probable in these products and that is why it would be suitable to focus the studies of raw bars this way. The amounts of biogenic amines in the tested samples were not high. However, it is important to consider a "cocktail effect" of these substances and to consume raw bars in moderate amounts.

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EVALUATION OF GENETIC DIVERSITY OF EDIBLE HONEYSUCKLE MONITORED BY RAPD IN RELATION TO BIOACTIVE SUBSTANCES

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ABSTRACT

The aim of this study was clarifying the relation between genetic diversity of edible honeysuckle (*Lonicera kamtschatica*) and the major group of biologically active substances as total polyphenols content (TPC) including antioxidant activity (AO). Fruits of edible honeysuckle becomes more and more popular, especially in Europe. The current status of research on polyphenolic compounds in the berries of edible honeysuckle and their biological effects, including recommended utilization, are reviewed.The biological material including 14 cultivars of the edible honeysuckle (´Zoluška´, ´Amfora´, ´Pruhonický 44´, ´Vasilijevsky´, ´Moskovskaja´, ´Vojtek´, ´Sinoglaska´, ´Altaj´, ´Lipnická´, ´Kamčadalka´, ´Sinaja Ptica´, ´Fialka´, ´Modrý Triumf´, and ´Leningradský velikán´) originated from Czech republic (Žabcice near Brno). The content of TPC and AO were determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysuckle acessions, too. DPPH method was used to analyze AO and Folin-Ciocalteu method was used to determine TPC. The results of experiment showed that the highest value of AO was determined at the cultivars ´Zoluška´ (81.04 mg.L^{-1}) and the lowest was measured in ´Kamčadalka´ (54.122 mg.L^{-1}). On the contrary, the highest content of TPC was determined at the cultivar ´Kamčadalka´ (51.09 mg.L^{-1}) and the lowest value was measured at the cultivar ´Pruhonický 44´ (21.65 mg.L^{-1}). Phylogenetic trees were similar in genetic distance. The content of TPC and AO were not statistically significant in relation to cultivar. The analyzed cultivars of the edible honeysuckle were separated in 4 clusters according to used primers. In both gel images, the amplicon size ranged from 100 to 1,500 bp. We found that genetic diversity was partially related to content of total polyphenolic substances and antioxidant activity. Based on phylogenetic trees we have stated that variety ´Lipnická´, ´Sinoglaska´, ´Altaj´, ´Leningradský velikán´, ´Modrý Triumf´, ´Sinaja Ptica´ and ´Kamčadalka´ were grouped in the similar cluster. The highest genetic distance was determined at the variety ´Vasilijevskaja´ and ´Amfora´. In the same way, there were variety ´Vojtek´, ´Fialka´ and ´Zoluška´.

Keywords: honeysuckle; RAPD; DPPH; Folin-Ciocalteu; total polyphenols content

INTRODUCTION

Fruits of edible honeysuckle, despite their valuable qualities have been less well-known fruits species in the territory of Slovakia. Edible honeysuckle come from the territory of the Russian Federation. From the point of view of the soil-climatic conditions of the locality, the al plants are not demanding. Among their precious properties in terms of growing conditions are high freezing resistance as well as resistance to diseases and pests (the incidence of plant diseases affected by diseases and pests is very low). Furthermore, their growing importance based on the early flowering period, which also result in earlier planting of the plants, thus significantly reducing the length of the growing season **(Matuškovič et al., 2003)**. Moreover, the fruit of edible honeysuckle have been rich in phenolic acids, flavonoids (quercetin, rutin, anthocyanins) and ascorbic acid content too **(Juríková et al., 2012)**.

The genetic aspect and content of biologically active substances of *Lonicera kamtschatica* varieties (Sevast.) Pojark, have been still only a little explored **(Naugžemys et al., 2014)**.

The existence of multiple taxonomic classifications means that several names are used for the same taxa. According to **Handa et al. (2006)**, *L. kamtschatica* is considered a separate species. Phylogenetic analyzes of cultured plants are very important in terms of their taxonomy.

Plants produce bioactive substances as secondary metabolites in their defense, which have considerable fungicidal, bactericidal and biocidal activity, such as e.g. protect the embryo from harmful UV radiation. The action of antioxidants in the human body protects the body from the effects of exogenous and endogenous free radicals. In addition to endogenous low-molecular-weight antioxidants (glutathione, uric acid, coenzyme Q, etc.), substances of natural origin are also at the center of attention, ie those substances that are taken up by the body through food. Above all, they are vitamins like C, E and carotenoids. Other polyphenolic substances, along with these, occur in

vegetables, fruits, teas, wines, and, last but not least, in aromatic and medicinal plants **(Kaczmarska et al., 2015)**.

Nowadays, plant breeding has been focused on producing large-fruited varieties with regular fertility and high polyphenolic content in combination with vitamin C. In recent years, different breeding programs on *Lonicera kamtschatica* were conducted in Europe, US and Canada **(Becker et al., 2019)**. Over the course of several millennia, refinement of crops has only been done by selecting the most viable and fastest growing plants. The selection should then influence on morphological and quantitative properties of crops. After identifying DNA as a carrier of heredity and describing its chemical structure, studies have focused on more detailed DNA properties, its association with enzymes that are present in cells of living organisms. Studies have also led to revelations of mechanisms such as a gene that is stored in a DNA molecule can encode a visually detectable attribute. The development of this scientific field has also had a profound impact on modern breeding methods **(Holubec et al., 2019)**. Authentication of raw plant materials are required and necessary for the standardization of functional foods and medicaments **(Heinrich, Švarcová and Valentová, 2008; Jiang et al., 2013)**. Identification of honeysuckle with DNA-based molecular tools has been used to obtain promising genotypes in terms of flavonoids, phenolic acid content and high antioxidant activity (DNA barcoding - **Sun et al., 2011**; ITS sequencing - **Hu et al., 2012)**. For example, characterization of *Lonicera caerulea* by ISSR markers **(Kaczmarska et al., 2015)**, specific SCAR markers developed from the high GC-RAMP-PCR products of *Lonicera japonica* **(Cheng et al., 2016)** or the quality marker concept and a set of integrated strategies used to improve the two chemical markers of *Lonicera japonica flos* (LJF) and *Lonicera flos* (LF) often confused in the management of chemical marker quality **(Ding et al., 2017)**.

The aim of this study was clarifying the genetic diversity of selected cultivars of edible honeysuckle in relation to content of the predominant group of the biologically active ingredients sumed up as total polyphenols content (TPC) and antioxidant activity (AO) of fruits.

Scientific hypothesis

The content of bioactive substances are determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysukle acessions, too.

MATERIAL AND METHODOLOGY

In this study, we evaluated the genetic variability of edible honeysuckers, the content of bioactive substances, and the relation between these two attributes.

Biological material - characteristics of the studied plants

The assayed biological material included the cultivars of species *Lonicera kamtschatica* (Sevast.) Pojark and *Lonicera edulis* Turcz. Ex Freyn. Plant material was originated from the Czech Republic. The experimental area Lednice (Czech Republic) is located at an altitude of 177 m above sea level with a long-term average annual temperature of 9.7 °C and an annual average rainfall of 525 mm. The warm weather at the end of March accelerated the onset of the growing season.

In the research, the following cultivars of edible honeysuckle were selected:
˝Zoluška˝, ˝Amfora˝, ˝Pruhonický 44˝, ˝Vasilijevsky˝, ˝Moskovskaja˝, ˝Vojtek˝, ˝Sinoglaska˝, ˝Altaj˝, ˝Lipnická˝, ˝Kamčadalka˝, ˝Sinaja Ptica˝, ˝Fialka˝, ˝Modrý Triumf˝, and ˝Leningradský velikán˝.

The collection of biological material necessary for the individual analyses was carried out from Žabčice- Brno, Czech Republic at the end of June. To obtain representative samples for the determination of the content of bioactive substances (TPC and AO), it was necessary to collect berries from different parts of the plants (i.e., top, middle and down). ˝Altaj˝ is a foreign-born variety bred by the crossing of *Lonicera kamtschatica* x *Lonicera turczaninowii*. The fruits are elongated with a pointed tip and weigh about 0.7 to 1 g. The colour of the fruit is dark blue and has a sweet-sour taste. ˝Amfora˝ is a self-pollinating variety created by loose pollination of the ˝Roksana˝ variety. The fruits have a smooth surface and weigh from 0.9 to 1.2 g. The colour of the fruit is purple and the fruit has a sweet and aromatic taste. The variety ˝Fialka˝ was achieved by the same way as the ˝Amfora˝ variety, the fruit are cylindrical with average weight 0.7 – 0.9 g. Fruits weigh about 0.8 g and have a sweet-sour taste. The variety ˝Leningradský velikán˝ is a partially self-pollinating variety with ovate fruit. Fruits are cylindrical in shape and reach a weight of about 1 g. They are dark blue with uneven surfaces and have a distinctive scent. Together with cultivar Sinnaja Ptica were obtained from Research Institut in Sankt Peterburg in 1999. ˝Sinnaja Ptica˝ fruit are oval shaped and medium sized 0.7 – 0.9 g.˝Kamčadalka˝ belongs to the first generation of bred varieties in the Russian Bakcari breeding station. The fruit are elongated-oval 0.7 – 0.9 g. Moskovskaja was the next accesiom originated from Russian Federation and cultivated in Žabčice in 2011. ˝Pruhonicky 44˝ is represented genotype achieved from botanical expedition of researches from VŠÚO (Czech Republic) in Kamčatka, the fruit are dark blue with smooth surface, the average weight is 0.7 g. ˝Sinaja Ptica˝ and ˝Zoluška˝ were selected in NII of (Novosibirsk Institut of fruit production in Sibir). The fruit of ˝Sinaja ptica˝ are dark grey with smooth surface, elongated, medium sized 0.87 g. ˝Zoluška˝ fruit are medium sized the average weight of fruit is 0.71 g. ˝Sinoglaska˝ represented cultivar with sour taste and elongated oval fruit, on the other hand ˝Vasiljevsky˝ can be characterized by cylindrical sweet fruit 0.7 – 0.9 g. ˝Vojtek˝ represented Polish variety with tart sweet taste of fruit 1 – 1.5 g reminded blueberry. ˝Lipnická˝ is cultivar of *Lonicera kamtschatica* originated from Czech Republic with cylindrical fruit medium weighted 0.7 – 0.9 g.

DNA extraction

For the molecular laboratory for the RAPD method, the leaves were harvested without visible damage. DNA from fresh young plant leaves was isolated using the CTAB protocol by **Rogers and Bendich (1994)**.

RAPD amplification

RAPD-PCRs were carried out in volumes of 15 µL, containing 50 ng of DNA, 7.5 µL Combi mastermix, 1 µL primer and 5.5 µL water.

The thermal cycler (My Cycler BioRad) was programmed for one cycle of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, and finally by one cycle of 5 min at 72 °C.

Amplicon analysis

Amplification products were separated by electrophoresis (BioRad) in 6% PAGE (30% acrylamide, 5xTBE, 10% APS, TEMED). Gels were stained with GelRed, visualized by Transilluminator UVP with documentation system G-Box SynGene and analytic software GeneSnap, SynGene. Marker GeneRulerTM DNA Ladder Mix (MBI Fermentas) was used to determine the size of the DNA fragments. Fourteen plants including *Lonicera* were analysed using 2 RAPD primers (ACCGCGAAGG and GGACCCAACC). DNA fragments detected not in all accessions profiles were considered as polymorphic. Amplicon analysis were similar as in study **Vivodík et al. (2019)**.

Fihure 1 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Altaj´ **(Mlček, 2013)**.

Fihure 2 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Fialka´ **(Mlček, 2013)**.

Fihure 3 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Kamčadalka´ **(Mlček, 2013)**.

Fihure 4 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Leningradský velikán´ **(Mlček, 2013)**.

Determination of content of total polyphenols content (TPC) and antioxidant activity (AO)

Determination of antioxidant activity by DPPH method, which consists in reaction of test substance with DPPH (stable free radical 1,1-diphenyl-2-picrylhydrazyl) by method **Quiros et al. (2010)**. The measurement took place at $\lambda = 515$ nm.

Determination of total polyphenolic content by Folin-Ciocalteu method was performed with Folin-Ciocalteu reagent, 1.5 mL 20% Na_2CO_3. Methodology for the determination of this method is implemented by **Paulová, Bochořáková and Táborská (2004)**.

Statistic analysis

Gel images were analyzed using Gel-Pro Analyzer 2010a (Media Cybernetics, L.P, USA). The values we acquired were recalculated using Neighbor-Joining using PHYLIP software (University of Washington, Seattle, version 3.696). Through the clustering method, we transformed data to create a phylogenetic tree. Distancematrix is used as input. In this method we also used the Q-matrix method. For this study there has been used UPGMA statistic method by **Nei and Li (1979)**. We used the Dendroscope V 3.5.9 software to construct the dendrogram of analysed accessions. The values of AO and TPC content were analyzed by statistical methods correlation analyse and ANOVA. The results have been shown in constructed trees.

RESULTS AND DISCUSSION

In the study **Kucharska et al. (2017)** were identified 50 compounds included 15 iridoids, 6 anthocyanins, 9 flavonols, 2 flavanonols (dihydroflavonols), 5 flavones, 6 flavan-3-ols, and 7 phenolic acids. 8-*epi*-Loganic acid, pentosyl-loganic acid, taxifolin 7-*O*-dihexoside, and taxifolin 7-*O*-hexoside were identified in honeysuckle berries for the first time.

Our results of determination of AO and TPC of the selected 14 cultivars of edible honeysuckle are given in Figure 5 and 6.

The TPC values for different *Lonicera kamtschatica* cultivars originated from territory of Czech Republic ranged from 57.50 to 90.30 mg/GAE/l FW **(Rop et al., 2011a)** that represented lower values with assayed cultivars in the same conditions of cultivation . The highest content of TPC was determined at the cultivar ´Kamčadalka´ (51.09 $mg.L^{-1}$) and the lowest value was measured at the cultivar ´Pruhonický 44´ (21.65 $mg.L^{-1}$).

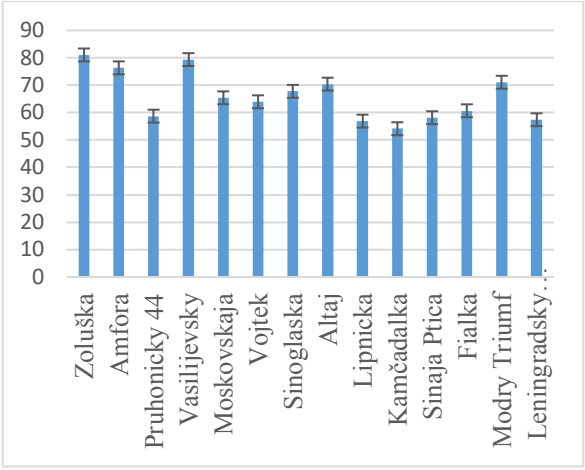

Figure 5 Average AO content (equivalent to TROLOX mg.l^{-1}).

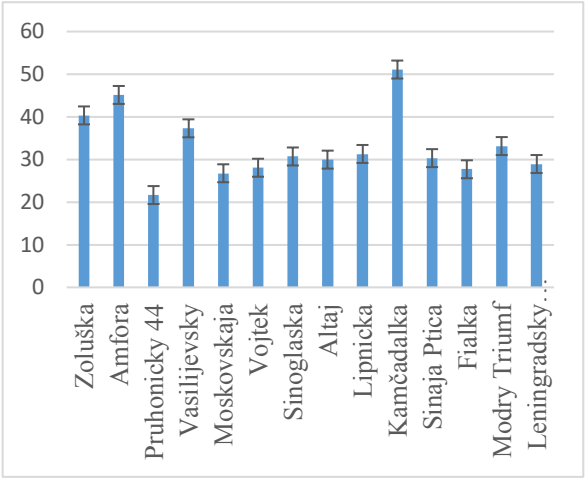

Figure 6 Average content of PP (polyphenols to gallic acid mg.l^{-1}).

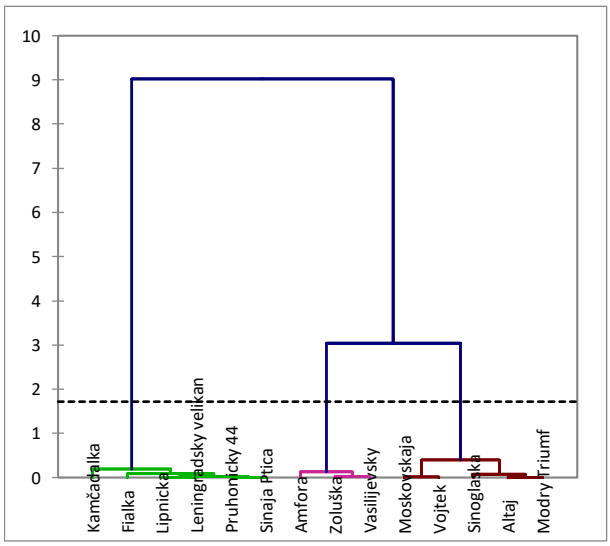

Figure 7 Average AO content (equivalent to TROLOX mg.l^{-1}). Note: According to AO cultivars were separated into 3 class:
Cluster 1: ´Zoluška´, ´Amfora´, ´Vasiljevský´;
Cluster 2: ´Pruhonický 44´, ´Lipnická´, ´Kamčadalka´, ´Sinaja Ptica´, ´Fialka´, ´Leningradský velikán´;
Cluster 3: ´Moskovskaja´, ´Vojtek´, ´Sinoglaska´, ´Altaj´, ´Modrý Triumf´ .

The results of determination of AO showed that the highest value was determined at the cultivar ´Zoluška´ (81.04 mg.L^{-1}) and the lowest values were measured at the cultivar ´Kamčadalka´ (54.122 mg.L^{-1}) and Lipnicka (56.88 mg.L^{-1}). Similarly, in the study research of **Juríková et al. (2014)** compared Russian cultivars of *Lonicera kamtschatica* ´Lipnická´achieved the lowest value of AO. In fresh honeyberry fruits, high values of analyzed bioactive compounds (vitamin C, TPC, TFC, TNFC and TAH) and antioxidant capacity were observed in the study of **Žlabur et al. (2019)**. The total phenol content (TPC) in FHs samples was 6.209 g GAE.100g^{-1} DM.

Average value of total content of AO was 65.8 mg.L^{-1}. Average value of total content TPC reached up 33.025 mg.L^{-1}. **Gazdík et al. (2008)**, who studied 21 clones of *Lonicera kamtschatica*, pointed to a statistically significant positive weak correlation between anthocyanin and ascorbic acid content in samples studied in 2008.

As Figure 3 and figure 4 showed antioxidant activity (AO) and total content of polyphenols (TPC) were similar except for ´Kamčadalka´, ´Lipnická´. They were extended separated as group 5. In the same way, ´Sinoglaska´, ´Altaj´, and ´Modrý Triumf´ were extracted as group 4. In the same way ´Vasiljevský´ and ´Leningradský velikán´ created the separated groups in TPC content in the study of **Sochor et al. (2014)**. On the other hand, ´Amfora´, ´Vasiljevský´ were group into one cluster together with ´Leningradský velikán´ and ´Altaj´.

Because of high degree of similarity in AO and TPC content, the correlation analysis was provided as well. By using statistical method correlation we have found that all represented pairs of values lay on a single line and the function has a rotating character. The coefficient was equal to +1, thus showing a greater degree of interdependence, and the observed values reflect a higher degree of interdependence. It means that there has been positive correlation between the content of TPC and AO (r = 1). In the same way **Matuškovič et al. (2009)** found a statistically significant positive strong correlation in the same samples of *Lonicera kamtschatica* cultivars in 2009. **Sochor et al. (2014)** found out the statistically significant correlation between TPC and AO assayed 20 cultivars of *Lonicera kamtschatica* originated from territory of Žabčice (r^2 = 0.998). Another study by **Rop et al. (2011b)** using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test in particular cultivars of *Lonicera kamtschatica* introduced into the conditions of the Czech Republic pointed to high antioxidant activity of fruit ranged from 6.59 – 10.17 g of ascorbic acid equivalent/kg of fresh mass significantly correlated to TPC content. The antioxidant activities were well correlated with the total phenolic and total anthocyanin contents in the study **Zhao et al. (2015)**.

The analyzed cultivars of the edible honeysuckle were separated in 4 clusters according to used primers. In both gel images, the amplicon size ranged from 100 to 1,500 bp. In the similar way, both phylogenetic trees were similar in genetic distance. Based on phylogenetic trees we have stated that variety ´Lipnická´, ´Sinoglaska´, ´Altaj´, ´Leningradský velikán´, ´Modrý Triumf´, ´Sinaja Ptica´ and ´Kamčadalka´ were grouped in the similar cluster. The highest genetic distance was determined at the variety ´Vasilijevskaja´ and ´Amfora´. In the same way, there were variety ´Vojtek´, ´Fialka´ and ´Zoluška´.

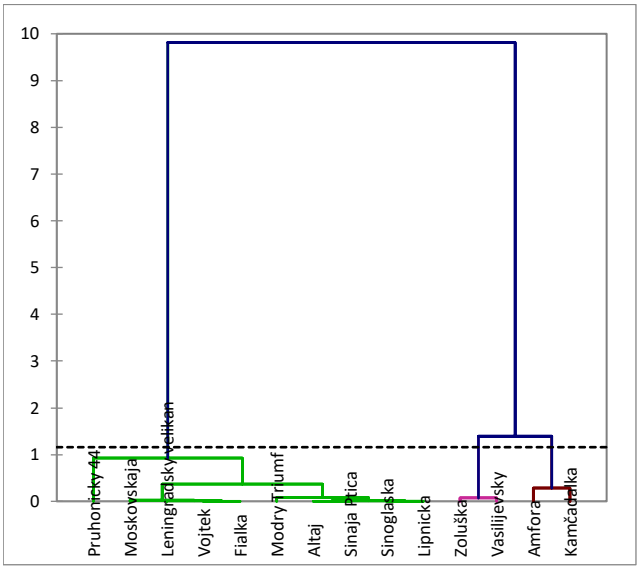

Figure 8 Average content of TPC (polyphenols to gallic acid mg.L^{-1}). Note: Based on TPC content the cultivars were distinguished into 5 clusters:
Cluster 1: ´Zoluška´, ´Amfora´, ´Vasiljevský´;
Cluster 2: ´Pruhonický 44´, ´Sinaja Ptica´, ´Fialka´, ´Leningradský velikán´;
Cluster 3: ´Moskovskaja´, ´Vojtek´;
Cluster 4: ´Sinoglaska´, ´Altaj´, ´Modrý Triumf˝;
Cluster 5: ´Lipnická´, ´Kamčadalka´.

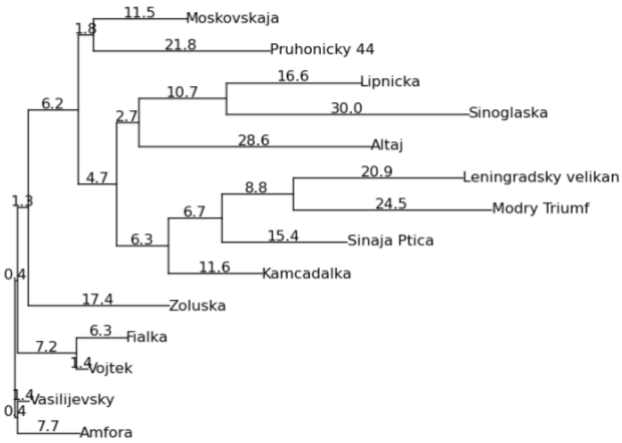

Figure 9 Phylogenetic tree of 14 varieties *Lonicera sp.* (used primer ACCGCGAAGG).

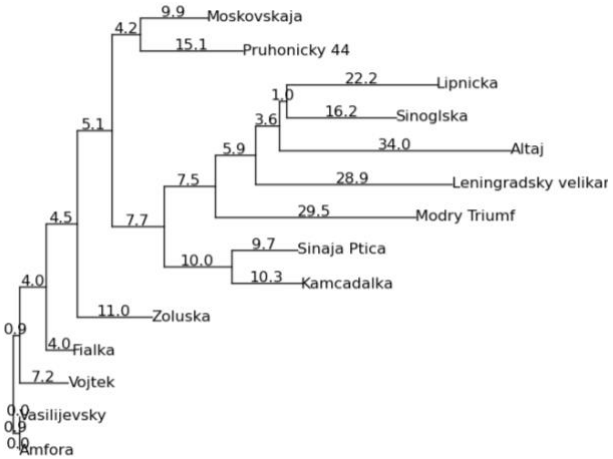

Figure 10 Phylogenetic tree of 14 varieties *Lonicera sp.* (used primer GGACCCAACC).

We found out there has proved only partial similarity in relation between dendrograms of total antioxidant activity and polyphenolic content compared to phylogenetic trees. The polyphenols content and antioxidant activity of *Lonicera* fruit has been partially influences by genetic background of plants and conditions of cultivation **(Juríková et al., 2012)** that has been proved in our study too. This examination was proved by ANOVA evaluation, in which the content of TPC and AO were not statistically significant in relation to cultivars (p ≥0.05). A combination of enviromental conditions (temperature, precipation, light intensity led to different accumulation of secondary metabolites in honeyberry fruit **(Senica et al., 2018)**.

The markers in RAPD allow the identification of species or isolates, and the construction of dendrogram from the computed distances **(Williams et al., 1990)** although there are some problems with this technique. One of the main limitations of this technique is the low level of repeatability of band pattern if the amplification reactions are not optimized **(Fu et al., 2013)**. Usually the number of bands produced by RAPD primers is independent of the size of the genome, with an average number of five bands per reaction. Genetic diversity of different *Lonicera* species influenced by geographic distance, has been reported previously **(Lima et al., 2011)**. However, to characterize the genetic feature of *L. kamtschatica*, more samples from a wide range of geographical regions should be compared. Also other molecular markers may also be combined with RAPD analysis for more accurate authentications **(Fu et al., 2013)**.

CONCLUSION

The content of TPC and AO were determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysukle acessions, too. Based on constructed phylogenetic trees and dendrograms, there was high statistically approved results. The content of TPC and AO was not statistically significant in relation to cultivars (p ≥0.05). We found that genetic diversity was partially related with content of total polyphenolic substances and antioxidant activity of fruit.

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MORPHOLOGICAL AND ANTIRADICAL CHARACTERISTICS OF RUGOSA ROSE (*ROSA RUGOSA* THUNB.) FRUITS CANNED IN DIFFERENT KIND OF HONEYS AND IN BEVERAGES PREPARED FROM HONEY

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ABSTRACT

The aim of the work was to determined the basic morphological and morphometric traits of rugosa rose (*Rosa rugosa* Thunb.) and antiradical activity of fruit pulp canned in different kind of honeys and in beverages prepared from honey. In experiments there were used 4 genotypes of roses originated from arboretum Mlyňany (Slovakia). The evaluation of 11 morphometric traits of fruit showed that the average weight of the fresh fruit without pedicle reached up 5.14 – 5.46 g, the weight of pedicle was 0.05 – 0.08 g, weight of pulp and seeds 4.80 – 5.13 g, weight of calyx 0.25 – 0.31 g, length and width of fruit (16.10 – 18.13 mm, 21.38 – 22.46 mm), the number of seeds in fruit 48.45 – 71.05, thickness of pulp 2.63 – 2.97 mm. Separated fruit pulp was canned at 40 °C and 80 °C and premixed in robinia honey and honeydew honey. Beverages were prepared by mixture of fruit pulp in honey (15 g) with cold water (150 mL). Antiradical activity was determined by DPPH method in fruit pulp (in methyl alcohol and water extracts), in honeys (black locust honey and honeydew honey) and beverages. There had been confirmed statistically significant differences in morphological traits, especially in colour and shape of fruit. Antiradical activity of fresh fruit pulp in methyl alcohol extract was determined 94.59%, in water extract 89.71%. Antiradical activity of black locust honey was 7.63%, honeydew honey 6.54%. Antiradical activity was determined also adding honeydew honey and black locust honey to fresh pulp of fruit prepared at 80 °C (33.55% and 77.58%). In beverages prepared from fresh pulp, honey and water it was investigated the higher values of antiradical activity in samples with addition of honeydew honey (81.81 – 83.86%) in comparison with robinia honey (75.57 – 79.96%).

Keywords: *Rosa rugose*; antioxidant activity; DPPH radical; honey; morphology

INTRODUCTION

In recent years, there has been increased interest in utilisation of plants in relation of prevention and health support especially in the area of chronic diseases and healthy diet. At the same time it has been noticed the progress in scientific knowledge about less-known and less used species such as *Asimina triloba* L., *Elaeagnus multiflora* Thunb., *Cornus mas* L., *Diospyros virginiana* L., *Morus nigra* L., *Pseudocydonia sinensis* Schneid., *Sambucus nigra* L., *Ziziphus jujuba* Mill. **(Brindza et al., 2007; Grygorieva et al., 2014; Grygorieva et al., 2018a; Grygorieva et al., 2018b; Monka et al., 2014; Kucharska et al., 2015; Klymenko, Grygorieva and Brindza, 2017; Ivanišová et al., 2017; Horčinová Sedláčková, Grygorieva and Brindza, 2018; Horčinová Sedláčková et al., 2018; Brindza et al., 2019)** especially in case of bioactive components displayed health benefits including *Rosa rugosa* Thunb. (rugosa rose, beach rose, Japanese rose, or Ramanas rose). Moreover, mentioned species displayed significant antioxidant potential **(Capcarova et al., 2012)**.

Rugosa rose belong to family *Rosaceae* Juss. a genus *Rosa* L. The species is native to eastern Asia, in China, Japan, Korea and south-eastern Siberia (Kamtschatka). It has been cultivated for ornamental purposes and especially valued for fragrant flowers with exotic colour in another part of world, especially in Asia and Europe **(Buchwald et al., 2007)**. In China the species has been utilised in folk medicine and food industry for about thousand years. *Rosa rugosa* is a suckering shrub 1.5 – 2 m with the bright crinkled leaves typically turn yellow before falling in autumn **(Bruun, 2005; Jung et al., 2005)**. Rugosa rose is very adaptable, with heat and drought tolerance, cold hardiness endured very low temperatures up to -45 °C. It tolerates full sun and partial shade **(Strobel, 2006)**. Moreover, it has high degree of pests and diseases

resistance not accumulated heavy metals **(Calzoni et al., 2007)**. On the other hand, it is sensitive to flooding and underfooding soils. It gives regular yields, size of fruit and high degree of utilised parts of plants **(Procházka, 2007)**.

The fruit are bright, smooth red hips. The hips are large (3 – 3.5 cm), shorter in relation to fruit diameter (2 – 2.5 cm) with maximum length more than 5 cm **(Novák and Skalický, 2007)**. Hips contain 21.5% dry matter, about 1200 mg.100 g^{-1} ascorbic acid, 6.4% sugars, vitamines A, B_1, B_2, E, K and elagic acid. Fruit are valued for β-caroten, lycopen, tocopherol, bioflavonoids, organic acids, tannins, pectines, aminoacids and essential fatty acids content (**Novák and Skalický, 2007; Olech et al., 2012)**. The proportion of pulp takes 70 – 75% **(Najda and Buczkowska, 2013)**. In food industry fruit are valued for bioactive compounds – essential oils, flavonoids, polysaccharides, pigments and terpenoids **(Lu and Wang, 2018)**. Phenolic compounds represent the major group of biologically active substances present in rosehips, these include tannins, flavonoids, phenolic acids, and anthocyanins **(Najda and Buczkowska, 2013)**.

Rugose rose has become the widely cultivated species all around the world especially for production of flowers, fruit and other parts of plant. All parts represent the source of well known and lesser explored biologically active substances. The utilization of plant is given by the high content of bioactive substances – especially flavonoids with high antioxidant activity play an important role in prevention and treatment of cancer and diabetes. Tannins displayed antimicrobial activity. Triterpenoids (eusaphic acid, tomentic acid) revealed the pharmacological effect associated with anti-inflammatory properties. High content of ascorbic acid has been used for treatment of infectious diseases, cardiovascular diseases and metabolism disorders **(Olech et al., 2012; Lu and Wang, 2018)**. The fresh fruits are valuable for canning industry for preparation of jams, juices, sauces, syrups, wines and jellies. The regular consumption of rose hip increases the resistance of human organism against diseases. The fruit are suitable for drying and preparation of tea **(Ercisli, 2007)**. Petals are rich in essential oils, utilised in perfumes, cosmetics, aromatherapy, spices, and nutrition (tea, jams, wines and juices) **(Ma et al., 2004; Mabellini et al., 2011)**. The most important components and antiradical activity of rose hip in comparison with another rose species are given in Table 1 and Table 2. Except for rose hip very important uses has also *Rosa acicularis* Lindl. **(Sabarajkina and Brindza, 2017)**.

The rose hip has an enormous etnopharmacologic utilisation. Traditionally it has been used in treatment of *diabetes mellitus* and chronic inflammatory diseases. In Korea it has been utilised in prevention of cancer **(Cho, Yokozawa and Rhyu, 2003)**.

Methanol extract of rugose rose has been utilised in treatment of prostate cancer. **Medeiros et al. (2008)** found out that methanolic extract synergically increases the antagonistic effect of medicine from the group of anti-androgenes used in treatment of advanced stage of prostate carcinoma. Interaction of extract and medicine can give more significant effect in comparison with monocomponent utilisation **(Lee et al., 2008)**. Selective cytotoxic effects on cervical (HeLa) and breast cancer (T47D) cell lines were proved by **Olech et al. (2017a)** and **Olech et al. (2017b)**.

Antioxidant contents, antioxidant (antiradical) and anti-inflammatory properties of rosehips studied and evaluated many research works **(Gao et al., 2000; Daels-Rakotoarison et al., 2002; Böhm, Fröhlich and Bitsch, 2003; Uggla, Gao and Werlemark, 2003; Ercisli, 2007; Chrubasik et al., 2008; Barros, Carvalho and Ferreira, 2011; Guo et al., 2011; Andersson et al., 2011; Andersson et al., 2012; Zhong et al., 2016; Al-Yafeai, Bellstedt and Böhm, 2018; Al-Yafeai, Malarski and Böhm, 2018)**.

Because of unique physico-chemical and antioxidant properties the rugose rose has a good potential for processing industry. In Europe it has been mostly utilised rose hip and rugose hip in food industry **(Henker, 2000)**. Biological pecularities, broad utilisation of all part of plants, biochemical composition and verified therapeutically effect has ranged rugose hip into potential species in socio-economical development especially family farms. The plant has great importance and potential for medicine and human health and pharmaceutical and food products as well **(Olech et al., 2017a; Olech et al., 2017b)**.

This was the reason why rugose hips became the goal of research. The present study aimed at evaluating and comparing of the basic morphological, morphometric properties of rugose hip genotypes and antiradical activity of fruit pulp and especially honeys and combination of prepared products for food industry.

Scientific hypothesis

Variability of evaluated morphometric traits of rugosa rose in collection of genotypes is high.

Antiradical activity of preserved and separated pulp of matured fruit achieved by heating with addition of honey is higher *versus* pulp of matured fruit.

MATERIAL AND METHODOLOGY

Experimental material

The 4 genotypes of rugose rose originated from arboretum Mlyňany (Slovakia) in altitude 167 above sea level, with average year temperature 9.5 °C. Fruit were collected in physiological stage of maturity at the end of september. Genotypes were marked according to latin name *Rosa rugosa* (RR) from RR-01 up to RR-04.

Determination of morphometric traits

The representative sample was 30 fruit. In the collection of fruit were measured the following morhometric traits: weight of fruit (g), weight of stalk (g), weight of fruit pulp with seeds (g), weight of calyx (g), weight of seeds (g), length of stalk (mm), length of calyx (mm), length of fruit (mm), width of fruit (mm), thickness of pulp (mm) and number of seeds in fruit. Separation of individual parts was provided by mechanical separation.

Weight was measured using analytical scales (Kern ADB-A01S05, Germany) with an accuracy of 0.01 mg, length and thickness were measured using by Metrica 10002 Digital calliper with an accuracy of 0.01 mm.

Conservation of fruit pulp of rugose rose with honeys

The pulp of fruit of mature rugose rose were separated by heating at 40 °C (variant I) and 80 °C (variant II). The seeds from overcooked fruit were separated mechanically. For the preparation of samples of pulp 25% proportion from each genotype were used. The pulp was mixed with black locust (*Robinia pseudoacacia* L.) honey (M1) and honeydew honey (M2) in variable proportion of fruit pulp and honey (Table 3).

Beverages from canned fruit pulp in honey

The bevarages were prepared by mix of 15 g of samples 1 – 8 (Table 3) with 150 mL of water.

Determination of antiradical activity of fruit

The antiradical activity of fresh fruit pulp of rugose rose was determined in methanolic (RRM) and water extract (RRW). The samples 1 g in 25 mL water/methyl alcohol were mixed for 12 hours and antiradical activity was determined after filtration of samples.

In the frame of antiradical activity (ability to eliminate the free radicals) was tested the capacity of rugosa rose to remove DPPH$^{\bullet}$ radicals (2.2-diphenyl-1-picrylhydrazyl) using methods of **Brand-Williams, Cuvelier and Berset (1995)** and of **Sánchez-Moreno, Larrauri and Saura-Calixto (1998)**. Absorbance at 515 nm has been registered in regular time intervals until the reaction equilibrium was reached – using the GENESYS 20 Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). First was measured the DPPH$^{\bullet}$ (Sigma Aldrich, USA) absorbancy without antioxidant substance (control). The inhibition of DPPH$^{\bullet}$ radicals was calculated in percent of free DPPH$^{\bullet}$ radicals in the samples using the method of **Von Gadow, Joubert and Hansmann (1997)**:

$$\% \text{ of inhibition} = [(A_{C0} - A_{At})/A_{C0}] \times 100;$$

Where: A_{C0} is absorbance of control in time t = 0 min (DPPH• solution), AAt is absorbance in the presence of antioxidant in time t min, the result is in % of DPPH• radicals inhibition.

Statistic analysis

The degree of variability was determined by values of variation coefficients **(Stehlíková, 1998)**. Probability of differencies among genotypes was tested by Tukkey test in programme Statistica 13.1. Correlation analyses explored relation between fruit weight and assayed morphometric traits of fruit.

RESULTS AND DISCUSSION

Morphometric traits

Investigations of morphology of roses are still relatively few numbers **(Khapugin, 2015)**. Fruit weight represents the very important economic trait in respect of the practical utilisation of fruit. Fruit presents potential raw material for food industry. This is a reason for selection of cultivars with high proportion of pulp and lower amount of seeds and another part of fruit **(Najda and Buczkowska, 2013)**. Fruit of rugose rose are presented on Figure 1.

Weight of the fruit, pulp and seed

The results of morphometric analyses showed that the average weight of fruit ranged from 5.14 g (RR-02) up to 5.46 g (RR-01), weight of pulp with seeds from 4.8 (RR-02) to 5.13 g (RR-01). Variation coefficients confirmed the average and high degree of variability in the both evaluated traits (Table 4). It means that on shrubs are fruit of different weight. These facts were verified within tested collection by analysis of variance. There has not been proved statistically significant differencies among genotypes (Table 4). **Najda and Buczkowska (2013)** evaluated the Polish genotypes of rugose rose and found out the average weight 1.58 ± 0.16 g that represent lower value in comparison with our assayed cultivars (Table 4). The average fruit weight is very variable and influence by many factors such as cultivar, conditions of cultivation etc.

The average weight of seed of rugose rose were determined in range from 0.55 g (RR-02) up to 0.72 g (RR-03) as we can see at Table 6. Variation coefficients pointed to high degree of variability (27.47 – 50.24%). Analyse of variance confirmed statistically significant differences among genotypes with the highest value in RR-03 genotype. This genotype seems to be suitable in respect of extraction of high quality oil. **Najda and Buczkowska (2013)** found out lower values of average weigh of seed 0.19 ±0.1 g among Polish genotypes.

Shape of fruit

In the population of rugose rose has been noticed the variability in fruit shapes. Shape of fruit can be characterised by shape index, it means the ratio of length and width of fruit. The larger value of the parameter, the fruit is more elongated. The shape index of assayed genotypes varied from 0.73 – 0.84 (Table 5) that represent lower value in comparison with Polish genotypes 1.66 ±0.11% **(Najda and Buczkowska, 2013)**. The most important shapes (predominantly spherical and oval shape) are presented on Figure 1.

The evaluation of another morphometric traits

Secondly, variability of another morphometric traits (weight of fruit stalk, weight of calyx, weight of seeds, thickness of fruit pulp, average number of seeds in fruit) were determined (Table 6). Results of morphometric traits showed the high variability, so the scientific hypothesis have been proved.

Correlation analyses

The correlation coefficients were calculated among evaluated morphometric traits of rugose rose (*Rosa rugosa*) (Table 7). It has been proved positive correlation between weight of fruit and weight of pulp with seeds that is crucial for selection of fruit on maximum weight of fruit with the highest proportion of pulp with seeds (r = 0.976). It is also significant correlation between weight of fruit and weight of seeds (r = 0.613) that make possible the production of seeds with the content high quality oil. Negative correlation was evaluated between fruit weight, length of fruit and thickness of pulp. The increased size of fruit lead to the decreased thickness of pulp (r = -0.564) that was presented in Table 4 (genotype RR-03).

Table 1 The overview of total polyphenols, ascorbic acid, antiradical activity of fruit of selected species of roses **(Najda and Buczkowska, 2013)**.

Species	Total polyphenols (mg.100g^{-1} FM)	Ascorbic acid (mg.100g^{-1} FM)	DPPH (µM TE.g^{-1} FM)
Rosa californica	161.03 ±0.14^{b}	863 ±0.1^{b}	59.7 ±0.01^{b}
Rosa villosa	192.56 ±0.25^{a}	706 ±0.4^{c}	51.3 ±0.07^{c}
Rosa rugosa	**215.14 ±0.18^{a}**	**974 ±0.1^{a}**	**74.5 ±0.05^{a}**
Rosa spinosissima	121.38 ±0.05^{c}	845 ±0.2^{b}	61.2 ±0.08^{b}
Rosa damascene	109.67 ±0.15^{c}	932 ±0.3^{a}	70.4 ±0.11^{a}

Note: FM – fresh mass; DPPH – antiradical activity (ability to eliminate the free radicals) was tested the capacity to remove DPPH$^{\bullet}$ radicals (2.2-diphenyl-1-picrylhydrazyl). Statistical significance in case of different letters.

Table 2 The acidity, the content of extract and sugars in selected species of roses **(Najda and Buckowska, 2013)**.

Species	Acidity (%)	Extract (%)	Sugars (%)
Rosa californica	2.07 ±0.13^{a}	18.2 ±0.01^{b}	20.3 ±0.06^{b}
Rosa villosa	0.89 ±0.02^{c}	17.0 ±0.03^{b}	17.1 ±0.03^{b}
Rosa rugosa	**1.18 ±0.07^{c}**	**20.1 ±0.01^{a}**	**32.6 ±0.02^{a}**
Rosa spinosissima	1.29 ±0.09^{b}	19.7 ±0.04^{a}	27.5 ±0.05^{a}
Rosa damascene	1.21 ±0.18^{b}	18.6 ±0.07^{b}	21.9 ±0.01^{b}

Note: Statistical significance in case of different letters.

Table 3 Assayed samples of fruit pulp of rugose rose canned with honey.

Number of sample	Abbrevation	Pulp of fruit (g) + honey (g)
	pulp from matured fruit at 40 °C	
1	RR–M_1	50 +100
2	RR–M_2	50 +100
3	RR–M_1	50 +150
4	RR–M_2	50 +150
5	RR–M_1	50 +50
6	RR–M_2	50 +50
	pulp from matured fruit at 80 °C	
7	RR–M_1	50 +100
8	RR–M_2	50 +100

Note: Genotypes marked according to latin name *Rosa rugosa* (RR) from RR-01 up to RR-04; M1 – black locust honey, M2 – honeydew honey.

Table 4 Variability of weight of fresh fruit and weight of pulp with seeds among assayed genotypes of rugose rose (*Rosa rugosa* Thunb.).

Genotypes	n	Fresh weight of fruit (g)					Weight of pulp and seeds (g)					Proportion of weight of pulp and seeds (%)
		min	max	x	s_x	V%	min	max	x	s_x	V%	
RR-01	30	3.40	8.41	5.46^{a}	0.35	28.73	3.10	8.01	5.13^{b}	0.34	29.63	93.58
RR-02	30	2.08	7.33	5.14^{a}	0.27	24.08	1.83	6.74	4.80^{a}	0.26	24.50	93.38
RR-03	30	3.85	7.62	5.43^{a}	0.22	18.74	3.38	7.12	5.03^{a}	0.22	19.86	92.63
RR-04	30	2.91	9.09	5.18^{a}	0.30	26.63	2.68	8.71	4.88^{a}	0.30	27.53	94.20

Note: n – total number of evaluated fruit; min – minimal value; max – maximal value; x – mean value of set of genotypes; s_x – standard error of the mean; V % – variation coefficient in %; proportion of weight of pulp and seeds from total weight of fruit (%) – percentual proportion of weight of separated parts of fruit in relation to average weight of fruit.

Table 5 Variability of lenght and diameter of fruit (mm) among genotypes of rugose rose (*Rosa rugosa* Thunb.).

Genotypes	n	Length of fruit (mm)					Width of fruit (mm)					Length/width ratio (L/W)
		min	max	x	s_x	V%	min	max	x	s_x	V%	
RR-01	30	12.23	22.14	16.42[a]	0.60	16.52	18.57	27.50	22.46[a]	0.54	10.76	0.73
RR-02	30	14.28	26.26	18.13[a]	0.68	16.78	15.58	24.27	21.58[a]	0.42	8.70	0.84
RR-03	30	12.89	20.01	16.10[b]	0.45	12.48	17.75	25.80	21.38[a]	0.49	10.31	0.75
RR-04	30	12.05	20.42	16.41[a]	0.48	13.22	17.78	27.04	21.42[a]	0.51	10.68	0.76

Note: n – total number of evaluated fruit; min – minimal value; max – maximal value; x – mean value of set of genotypes; s_x – standard error of the mean; V % – variation coefficient in %.

Table 6 Variability of another selected morphometric traits of rugose rose (*Rosa rugosa* Thunb.) in collection of genotypes.

Genotypes	Weight of fruit stalk (g)		Weight of calyx (g)		Weight of seeds (g)		Thickness of fruit pulp (mm)		Average number of seeds in fruit (pcs)	
	x	V%	x	Cv%	x	V%	x	V%	x	V%
RR-01	0.05[b]	29.32	0.27[b]	19.89	0.70[a]	46.16	2.67[a]	24.37	11.78[c]	40.79
RR-02	0.05[b]	77.21	0.28[a]	16.42	0.55[b]	50.24	2.97[a]	18.15	15.10[a]	40.87
RR-03	0.08[a]	47.47	0.31[a]	18.07	0.72[a]	27.47	2.67[a]	16.35	14.80[a]	23.85
RR-04	0.05[b]	38.76	0.25[b]	24.29	0.71[a]	37.90	2.63[a]	15.39	13.38[b]	35.37

Note: x – mean value; V% – variation coefficient in %; pcs – pieces; Statistical significance in case of different letters. Genotypes marked according to latin name *Rosa rugosa* (RR) from RR-01 up to RR-04.

Table 7 Correlation analyses among morphometric traits of rugose rose (*Rosa rugosa* Thunb.).

r	s_r	CO	r^2	t-test	Probability
		weight of fruit (g) : weight of stalk (g)			
0.491	0.014	$-0.890 \leq r \geq 0.986$	0.241	0.797	0.509
		weight of fruit (g) : weight of fruit pulp and seeds (g)			
0.976	0.041	$0.229 \leq r \geq 0.999$	0.952	6.264	0.024
		weight of fruit (g) : weight of calyx (g)			
0.459	0.031	$-0.898 \leq r \geq 0.985$	0.211	0.732	0.541
		weight of fruit (g) : weight of seeds (g)			
0.613	0.079	$-0.846 \leq r \geq 0.990$	0.376	1.099	0.386
		weight of fruit (g) : length of stalk (mm)			
-0.475	1.640	$-0.986 \leq r \geq 0.894$	0.226	0.764	0.524
		weight of fruit (g) : lenght of calyx (mm)			
0.772	1.742	$-0.734 \leq r \geq 0.994$	0.595	1.714	0.228
		weight of fruit (g) : length of fruit (mm)			
-0.689	0.817	$-0.993 \leq r \geq 0.8054$	0.4748	1.344	0.311
		weight of fruit (g) : width of fruit (mm)			
0.541	0.524	$-0.875 \leq r \geq 0.988$	0.292	0.908	0.459
		weight of fruit (g) : number of seeds (pieces)			
0.949	3.826	$-0.130 \leq r \geq 0.999$	0.902	4.291	0.050
		weight of fruit (g) : thickness of fruit pulp (mm)			
-0.564	0.161	$-0.989 \leq r \geq 0.867$	0.3180	0.966	0.436

Note: r – correlation coefficient, s_r – standard error of the correlation coefficient, CO – correlation, r^2– coefficient of determination.

Antiradical activity of fruit pulp

Extracts of fruit pulp were prepared in two versions: water and methanolic. Previous studies **(Dudra et al., 2015; Olech et al., 2017a; Olech et al., 2017b)** confirmed that extracts of *Rosa rugosa* displayed strong antioxidant and antiradical activity (up to EC50 0.85 $mg.mg^{-1}$ DPPH) indicating widespread utilisation of rugose rose as natural antioxidant in food and pharmaceutical industry. In same way our results pointed to strong antiradical activity of aqueous 90.22 – 90.84% and methanolic extract as well 92.87 – 94.59% (Table 8). The notable antioxidant activity was also confirmed *Rosa dumalis* Bechst., *R. dumetorum* Thuill. and *R. sempervirens* LM **(Nadpal et al., 2018)**.

There has not been proved statistically significance between assayed extracts. **Dudra et al. (2015)** compared antioxidant activity of *Rosa canina* fruit in relation to type of solvent and found out that ethylacetate and water extract showed lower values of antioxidant activity than methanolic and diethyleter extracts. Similarly, **Olech and**

Nowak (2012) noticed the significant influence of extraction technique on antiradical activity of rugose rose petals. According to results of experiment they reccomended polar organic solvents. High efficacy of 15% methanolic extract of rugose fruit was proved in respect of antiradical activity in studies of **Lee et al. (2008)**.

In study of **Al-Yafeai, Bellstedt and Böhm (2018)** different degrees of ripeness affected the bioactive compounds as well as the antioxidant capacity in the fruit of *R. rugosa* (hips). The maximum concentration of carotenoids was observed at late harvesting. The maximum concentration of both vitamin E and vitamin C was obtained in the orange hips and total phenolic contents were determined in the mature hips (red colour) with significant difference. The highest hydrophilic and lipophilic Trolox equivalent antioxidant capacity (TEAC) values were determined in the mature red hips, whereas oxygen radical absorbance capacity (ORAC) showed significantly lower activity in the mature hips. **Andersson et al. (2012)** found that amounts of total tocopherols and vitamin E activity being decreased in rose hips during ripening. According to **Uggla, Gao and Werlemark (2003)** the contents of vitamin C in rosehips ranged from 200 to 2800 mg.100g^{-1}, rosehips are considered the most abundant source of natural vitamin C. **Ercisli (2007)** found that contents of ascorbic acid (vitamin C) in the fresh fruits of rose species were between 706 and 974 mg.100g^{-1}. According to **Al-Yafeai, Bellstedt and Böhm (2018)** the contents of ascorbic acid in *R. rugosa* hips at different ripening degrees ranged between 798 and 1090 mg.100g^{-1}. The medicinal value of rosehips depends largely on the vitamin C contents.

Table 8 Antiradical activity (%) of pulp of rugose rose (*Rosa rugosa* Thunb.).

Sample	min	max	x	s_x	V%
RRW	90.22	90.84	90.47^a	0.19	3.65
RRM	92.87	94.59	93.73^a	0.49	9.15

Note: min – minimal value; max – maximal value; x – mean value of set of genotypes; s_x – standard error of the mean; V % – variation coefficient in %; RRW – fresh fruit pulp of rugose rose in water extract; RRM – fresh fruit pulp of rugose rose in methanolic extract.

Table 9 Antiradical activity (%) of assayed honeys.

Honey	n	min	max	x	s_x	V%
Black locust	5	6.35	8.35	7.63^a	0.645	14.62
Honeydew	5	6.11	7.28	6.54^a	0.373	9.85

Note: n – number of samples; min – minimal value; max – maximal value; x – mean value of set of genotypes; s_x – standard error of the mean; V % – variation coefficient in %.

Antiradical activity of honeys

Black locust honey and honeydew honey were used for conservation of fruit pulp of rugose rose. Antiradical activity of black locust honey and honeydew honey represented 6.35 – 8.35% and 6.11 – 7.28% respectively. There have not been statistically significant differences between assayed honey types (Table 9).

Antiradical activity of fruit pulp preservated in honey

The average values of antiradical activity of fruit pulp preserved in honey ranged from 33.55% (sample 8) up to 77.58% (sample 1) as it has been showed at Table 10. Addition of honey caused decrease antiradical activity of fruit pulp. On the other hand, honey displays unique antimicrobial properties **(Israili, 2014; Faustino and Pinheiro, 2015)**. The addition of honey to samples leads to stabilization of final products and play an important role as natural sweetener. The high temperature 80 °C during separation and mix of fruit pulp cause the degradation of thermolabile compounds that generally lead to decrease of antiradical activity after addition of preservants by 36% (black locust honey) and by 41% (honeydew honey). The analyse of variance confirmed the statistically significant

Table 10 Antiradical activity (%) of pulp and beverages with fruit pulp of *Rosa rugosa* Thunb. preserved in black locust honey and honeydew honey.

Number of sample	Sample	Honey	Fruit pulp (g) + honey (g)	x	s_x	V%	x	s_x	V%
	Preserved and separated pulp of matured fruit achieved by heating at 40 °C with addition of honey						**Beverages**		
1	RR-M_1	black locust	50 + 100	77.58^a	2.26	5.06	79.96^b	4.08	8.85
2	RR-M_2	honeydew	50 + 100	57.79^b	3.28	9.84	83.63^a	2.50	5.18
3	RR-M_1	black locust	50 + 150	56.90^b	3.26	9.94	79.25^b	1.86	4.06
4	RR-M_2	honeydew	50 + 150	66.86^a	2.95	7.65	81.92^a	3.88	8.20
5	RR-M_1	black locust	50 + 50	65.38^a	0.88	2.34	76.90^b	5.47	12.32
6	RR-M_2	honeydew	50 + 50	70.96^a	4.18	10.20	83.29^a	3.53	7.34
	Preserved and separated pulp of matured fruit achieved by heating at 80 °C with addition of honey						**Beverages**		
7	RR-M_1	black locust	50 + 100	48.98^c	3.76	13.30	75.57^b	0.89	2.05
8	RR-M_2	honeydew	50 + 100	33.55^d	0.74	3.86	81.81^a	0.97	2.05

Note: n – total number of evaluated fruit; min – minimal value; max – maximal value; x – mean value of set of genotypes; s_x – standard error of the mean; V % – variation coefficient in %; M1 – black locust honey, M2 – honeydew honey. Statistical significance in case of different letters.

Figure 1 Fruit of rugosa rose (*Rosa rugosa* Thunb.).

differences among assayed samples, variation coefficient ranged from 2.34 – 13.30% pointed to low up to high degree of variability.

The antimicrobial activity of bee honey is one of its most studied biological properties. The specificity of this activity, as well as the others honey's bioactivities such as antitumor, anti-inflammatory, antioxidant and antiviral properties, depends on honey's components, which vary according to its floral, geographical and entomological origin **(Kačániová and Almeida-Aguiar, 2016)**. **Kačániová et al. (2009)** study the antimicrobial activity of honey samples against *Candida species*. The antimicrobial activity was determined as an equivalent of the inhibition zones diameters (in millimetres) after incubation of the cultures for 48 hours. There were not seen an inhibition zones against the yeasts investigated in the 25 and 50% concentration of honey samples. In another study **Kačániová et al. (2011)** the antifungal activities of honey samples were tested by 10, 25 and 50% (by mass per volume) concentration against fungi *Penicillium crustosum, P. expansum, P. griseofulvum, P. raistrickii* and *P. verrucosum* and by the agar well diffusion method. The solutions containing 10% (by mass per volume) of honey did not have any effect on the growth of fungi. The strongest antifungal effect was shown by 50% honey concentration against *P. raistrickii*.

Antiradical activity of beverages with pulp preserved in honey

Beverages were prepared by mixing of 15 g separated fruit pulp preserved in honey with 150 mL of water. The antiradical activity of beverages reached up 75.57% (sample 7) up to 83.63% (sample 2). The samples 7 and 8 displayed statistically significantly lower variability in antiradical activity in comparison with the rest of samples. Variation coefficients (Table 10) pointed to low or medium degree of variability (2.05 – 12.32%). Samples with addition of honeydew honey displayed the antiradical activity about 80%. Antiradical activity of beverages with pulp preserved in honey displayed higher values of antiradical activity in comparison with maturated fruit. The second hypothesis has been verified.

Total water-soluble antioxidants were also significantly varied among Rosa species. This value was significantly higher (up to 2 times) for the fruit of *Rosa rugosa* (p <0.05) **(Czyzowska et al., 2015)**.

Horčinová Sedláčková, **Grygorieva and Brindza (2018)** determined antioxidant activity of activated beverages of dilute/aqueous honey prepared from fresh inflorescences elderberry (*Sambucus nigra* L.) in the saccharide extract. It was determined antioxidant activity in the range from 16.81 to 24.16%. The enlarging of activation positively correlated with an increasing in antioxidant activity.

CONCLUSION

Rugose rose belong to prospective species for cultivation in monoculture especially in case of small and family farmers contributed to social-economic development. This species give a very valuable fruit – hips that can be utilized in preparation of teas, jams, alcoholic and non-alcoholic beverages, oils and another products. Morphological evaluation of results proved statistically significant differences in colour and shape of fruit. In the fruit pulp there has been determined a higher values of antiradical activity in methanolic extract (94.59%) than in water extract (89.71%). The average value of antiradical activity of black locust honey reached up 7.63%, and honeydew honey 6.54%. The average values of antiradical activity of fruit pulp ranged from 33.55% (canned in honeydew honey at 80 °C) up to 77.58% (in black locust honey at 40 °C). In beverages from pulp, honey and water there have been evaluated the higher values of antiradical activity in samples with addition of honeydew honey (81.81 – 83.86%) in comparison with addition of black locust honey (75.57 – 79.96%).

Fruit pulp contains the large number of bioactive components with positive nutritional and phytotherapeutic effect that has been confirmed by results of antiradical activity of fruit. In technologies of jams, syrups and other products preparations there have been utilised a high temperatures leading to degradation of thermolabile bioactive compounds. This was the reason why our research work was aimed at evaluation of different methods of hip's pulp separation to minimalize the losses. Results of determination of antiradical activity confirmed the high retention of valuable substances. Separated fruit pulp represents the initial material for production of

valuable food commodities. At the same time it has been confirmed the value of traditional beverage based on water with honey that has been utilised a long time ago by ancestors. Water mixed with honey has been represented the unique natural beverage with the significant source of energy and valuable nutritional and therapeutic compounds of honey. The nutritional and therapeutic effect of honey and beverages can be increased by conservation of rugosa rose pulp without thermic treatment. Blocking of fermentation process after the addition of pulp into the honey can be solved by appropriate portion of honey and pulp.

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STUDY OF THE INFLUENCE OF BREWING WATER ON SELECTED ANALYTES IN BEER

Lenka Punčochářová, Jaromír Pořízka, Pavel Diviš, Václav Štursa

ABSTRACT

Brewing water is one of the basic raw materials for beer production and knowledge of its composition and pH is essential for the proper conduct of the entire brewing process. In this study, it was observed how the composition of water influences OG values, content of B vitamins, organic acids and iso-α-acids. For brewing, synthetic water was prepared by adding chemicals to deionized water. Models of hard (pH 8.47 ±0.08) and soft (pH 7.68 ±0.23) synthetic water were used for brewing pale bottom-fermented lager beers. Samples of wort, hopped wort, young beer and beer were collected during beer production. HPLC-DAD was used for B vitamins and iso-α-bitter acids quantification. Determination of organic acids was done by ion chromatography with conductivity detector. Obtained data were statistically processed with ANOVA (Analysis of Variance) and interval of confidence was set to 95%. According to the statistical analysis, water composition affects analytes content during beer production and in the final product. Hard water seemed to be a better extraction buffer and its composition (pH) positively affected some processes during brewing technology. One of them was obtaining higher OG values compared to soft water. The beer made from hard water also contained more B vitamins. Composition of brewing water had no influence neither on concentration of organic acids nor on iso-α-acids in conditions of homebrewing.

Keywords: brewing water; homebrewing; B vitamins; organic acids; iso-α-acids

INTRODUCTION

Beer is composed of about 94% of water, so water becomes an essential, but often neglected ingredient in beer production **(Comrie, 1967)**. Water has a significant effect on the chemical and sensory characteristics of beer. Therefore, knowledge of brewing water composition (liquor) is important for breweries.

However, the water must accomplish certain parameters to be used in brewing. The liquor requirements may be grouped as „aesthetic“ (colour, turbidity, odour and taste), microbiological standards (particularly the absence of pathogens), the levels of organic and inorganic materials that are in solution and the presence of radioactive materials **(Briggs et al., 2004)**.

Discussions of brewing water composition often involve the total hardness. The total hardness is defined as the sum of all alkaline-earth ions (calcium, magnesium, strontium and barium ions) **(Kadlec, 2002; Eßlinger, 2009)**. It is divided in carbonate and non-carbonate hardness. Common counter ions for non-carbonate (permanent) hardness are sulfate, nitrate and chloride and these remains in solution when the water is boiled **(Briggs et al., 2004; Eßlinger, 2009)**. Carbonate or temporary hardness is caused chiefly by calcium and magnesium bicarbonates and is so-called because if the water is boiled the bicarbonate is converted to the carbonate, which precipitates leaving the clarified water “softened” **(Kadlec, 2002; Briggs et al., 2004)**. Soft water contains low concentrations of dissolved salts, particularly salts of calcium and salts of magnesium. Hard water contains high concentrations of salts, usually mainly calcium bicarbonate or calcium sulphate. The distinction is important if the liquor is to be used for mashing or, even more, for sparging **(Briggs et al., 2004)**. Calcium and magnesium salts are predominant elements in water **(Kadlec, 2002)**.

Other important parameters, which correlate with water hardness are pH and ionic strength **(Basařová et al., 2010)**. Apart from the legal requirements, additional quality criteria for brewing water need to be complied with, since water ions influence the pH value of mash, wort and beer, and thus enzymatic and non-enzymatic reactions. Consequently, these have a considerable influence on the acidity. Hydrogen carbonate ions count as acid destroying since they lead to an increase in the pH value. Calcium and magnesium ions are acidity supporting and lead to a pH decrease of the mash **(Eßlinger, 2009)**.

The dissolved salts are present in water at low concentrations, but significantly affect the sensory qualities of beer, enzymatic activity during mashing and regulate processes during boiling, cooling and

fermentation of the wort **(Comrie, 1967)**. For example, calcium ions serve several important functions in brewing. They stabilize the enzyme α-amylase (approximately 80 °C and higher temperatures cause inactivation of enzyme) during mashing and they interact with phosphate, phytate, peptides and proteins in the mash and during the copper boil, the pH values of the mash and the wort are usefully reduced. Ions in beer can influence its flavour and calcium ions in particular influence the mashing process **(Briggs et al., 2004; Ganbaatar et al., 2015)**.

Water hardness is important in assessing the quality of water used for brewing. The composition of water is conditioned by the place of occurrence. Historically, different regions became famous for particular types of beer and in part these beer types were defined by the waters available for brewing. One of them is Pilsner water, which is well-known as a very soft water with small proportion of inorganic compounds. It is suitable for pale and delicate lagers. Burton-on-Trent, with its extremely hard water, rich in calcium sulphate, is famous for its pale ales. Compared to that, Munich is well-known for its dark lagers **(Rudin, 1976; Basařová et al., 2010; Kábelová-Ficová et al., 2017)**.

It is now usual for breweries to adjust the composition of the brewing water they use **(Comrie, 1967; Briggs et al., 1981; Briggs et al., 2004)**. However, homebrewers are not used to treat brewing water, but if they do, they are often limited with availability of technology. Treatments may reduce levels of organic compounds in solution or adjust the ionic composition of the liquor **(Briggs et al., 2004)**.

Sodium chloride may be added to brewing liquors (75 – 150 $mg.L^{-1}$) to enhance "palate-fullness" and a certain sweetness. Sometimes potassium chloride is added instead, at low concentrations, to achieve a less sour flavour **(Briggs et al., 2004)**.

The aim of this study is to assess the impact of the composition of the brewing water on selected analytes in the technology of beer. For experiments, Pilsner type beers of soft and hard synthetic water were brewed, and samples of wort, hopped wort, young beer and beer were taken during production. Subsequently, the samples were analysed. HPLC was used for the assay. OG values were determined by refractometer and pH values were determined by pH meter. The assessment of differences between soft and hard water was provided by the statistical method of analysis of variance (ANOVA).

Scientific hypothesis

Precedent published studies dealt with composition of water and its effect on brewing. The aim of this study was to find out, whether composition of brewing water significantly influences selected analytes during beer brewing and in the beer in conditions of homebrewing.

MATERIAL AND METHODOLOGY

Pilsner type beers were brewed from soft and hard synthetic water and analysed as follows.

Synthetic water preparation and beer production

Synthetic water was prepared according to **Smith, Davison, and Hamilton-Taylor (2002)** by adding selected compounds to distilled water. Thus, soft and hard model water was created.

For study of the influence of brewing water on selected analytes, beer was made from soft and hard synthetic model water in three replicates. These were the pale bottom-fermented beers also called lagers. The raw materials were pale Pilsner barley malt (Malt house Bernard, Rajhrad), Sládek hop pellets with alpha acid content of 8.08% and Saflager S-23 yeasts.

Milled barley malt was mixed with brewing water at 38 °C and then an infusion method was chosen for mashing process. Evaporated water was compensated with distilled water. The values of original gravity (OG) and pH were measured in wort after lautering. Then it was continued with wort boiling for 90 min. After this process, whirlpooling of wort was done. Also the values of OG and pH were measured. After that wort was cooled down and then it was inoculated and aerated and stored at 14 °C. The degree of attenuation was checked by measuring the OG value to confirm that yeasts completed fermentation. The OG value was in range of 7.0 – 7.3%. Values of OG and pH were measured in the samples of young beer. Then bottled beer was stored at 7 °C. The secondary fermentation and maturing last for three weeks.

Measurement of basic quantitative parameters of tested beers

The OG values were measured refractometrically (A. Krüss Optronic GmbH, Hamburg, Germany). The pH of synthetic water was measured with pH meter (WTW, Germany).

Determination of B group vitamins by HPLC analysis

The samples of wort, hopped wort, young beer and beer were degassed by sonication (Ultrasonic Compact cleaner PS03000A 2.5 L, PowerSonic s r.o.) and then diluted with distilled water. All samples were analysed by HPLC-DAD (Agilent 1260 Infinity, Agilent Technologies, USA) to determine the quantity of B vitamins. Specifically, it was focused on riboflavin, niacin, pyridoxine and cobalamin. B vitamins were separated on Polar C18 column (150 mm x 3.0 mm; particle size 2.6 μm) with set temperature to 40 °C. The mobile phase consisted of 10 mM ammonium formate (Sigma-Aldrich Inc., USA) and 0.1% solution of formic acid (solvent A) (Sigma-Aldrich Inc., USA) and acetonitrile (Sigma-Aldrich Inc., Germany) and 0.1% solution of formic acid (solvent B) (Sigma-Aldrich Inc., USA). Gradient elution was used for the analysis. The gradient was as follows: 0 min, 100% A; 12 min, 40% A; 15.10 min, 100% A and then held for another 7 min. The samples were detected with DAD detector (260 nm, 270 nm, 271 nm, 292 nm, 360 nm). The data were collected by the Agilent 1260 Infinity chromatographic data system.

Determination of organic acids by IC analysis

Ion chromatography (850 Professional IC, Metrohm, Switzerland) was used to determine lactic and acetic acid in the samples of wort, hopped wort, young beer and beer. Each sample was degassed by sonication (Ultrasonic Compact cleaner PS03000A 2.5 L, PowerSonic s r.o.) for 20 minutes. Then the samples were filtrated using filters

with 0.45 μm pore size (Labicom, Czech Republic) and diluted with distilled water in proportion 1:1.

Organic acids were separated on Metrosep Organic Acids column (250 mm x 7.8 mm; particle size 9 μm) with temperature set to 30 °C. The mobile phase was 0.5 mmol perchloric acid (Sigma-Aldrich Inc., USA). Isocratic elution was used and the flow of mobile phase was 0.6 $mL.min^{-1}$. The analytes were detected with conductivity detector.

Analysis of hop bitter acids

Extraction of iso-α-acids

Degassed samples of hopped wort, young beer and beer (10 mL) were transferred into a 50 mL centrifuge tube, then sample was acidified with orthophosphoric acid (0.5 mL) (Lach-Ner, s.r.o., Czech Republic). The mixture was extracted into isooctane (10 mL) (Sigma-Aldrich Inc., USA). Mixture was vortexed for 1 minute and methanol (1 mL) (Sigma-Aldrich Inc., USA) was added. Thereafter, the two-layered solution was centrifuged for 5 minutes at 2,000 rpm (Z 36 HK, HERMLE Labortechnik GmbH). Then 4 mL of supernatant was collected into a glass tube, evaporated under N_2 gas and redissolved in methanol (2 mL) (Sigma-Aldrich Inc., USA) to give the HPLC sample.

Determination of iso-α-acids by HPLC analysis

After filtration (filters with 0.45 μm pore size) (Labicom, Czech Republic), samples were subjected to quantitative HPLC analysis (Agilent 1260 Infinity, Agilent Technologies, USA). Iso-α-acid content (isohumulone, isocohumulone and isoadhumulone) in the samples were measured simultaneously using ICS-I4 as a calibration standard (Labor Veritas AG, Switzerland). Iso-α-acids were separated on Poroshell 120 EC – C18 column (4.6 mm x 50 mm; particle size 2.7 μm) with temperature set to 40 °C. Isocratic elution was chosen. The mobile phase consisted of 0.1% solution of H_3PO_4 (Sigma-Aldrich Inc., Germany) and 0.2 mM Na_2EDTA (solvent A) (Sigma-Aldrich Inc., France) and acetonitrile (solvent B) (Sigma-Aldrich Inc., Germany) in proportion A:B = 35:65. Flow of mobile phase was 1 $mL.min^{-1}$. The analytes were detected with DAD detector (270 nm). The data were collected by the Agilent 1260 Infinity chromatographic data system.

Statistic analysis

Data analysis and statistical evaluation was carried out by software XLSTAT (Addinsoft, USA). Influence of the brewing water on the selected parameters of beer was evaluated by Analysis of Variance (ANOVA). ANOVA was set to a confidence interval of 95%.

RESULTS AND DISCUSSION

Selected quantitative beer indicators

First, the pH of prepared synthetic model water was measured. The average pH value of soft water was 7.68 ±0.23, the average pH value of hard water was 8.47 ±0.08. Compared to theoretical pH, the measured values were lower. The theoretical model of soft water pH was 7.83 and 8.49 hard water **(Smith, Davison and Hamilton-Taylor, 2002)**.

Figure 1 shows difference between pH of soft and hard water during mashing. During mashing, the pH decreases due to malt enzymes activity, which releases phosphates from nucleic acids. In the process of wort boiling, the wort acidity is increased by precipitation of phosphates in the presence of calcium and magnesium ions. Hop bitter acids and Maillard reaction products further contribute to pH reduction. When fermenting, the pH declines by the activity of yeasts that consume amino acids and produce organic acids. The pH also changes because of presence of the carbon dioxide, which dissolves in the solution **(Basařová et al., 2010)**.

OG (original wort extract) and ABV (Alcohol by Volume) were selected as the basic quantitative indicators for beer brewing. The extract of the original wort was used to express the total carbohydrate content presented in the medium. Differences in beers brewed from different kind of synthetic water were observed. Both, original gravity and concentration of alcohol were statistically different. The mean value of wort OG prepared by using soft water was 12.6 ±0.1%, while wort OG of hard water was 13.05 ±0.05% (see Figure 2). Higher yields in hard water are probably due to higher amounts of Mg^{2+} and Ca^{2+} ions, which stabilize α-amylases and increase its activity **(Karbassi and Saboury, 2000; Saboury, Ghasemi and Umar Dahot, 2005)**. After wort boiling, the OG slightly increased, probably due to the extraction of chemical compounds in hop. A large decline occurred during fermentation when yeasts utilized carbohydrates. A small drop occurred during secondary fermentation and maturing in bottles because yeasts largely depleted the substrate and was no longer as vital as at the beginning. The process of fermentation of all samples is shown in Figure 3. As the yeast assimilated the substrate, the OG also declined and the ethanol concentration in the medium grew as expected. Complete fermentation of experimental beers took 70 hours.

Quantification of selected B vitamins

Determination of B2 (riboflavin), B3 (niacin), B6 (pyridoxine), B12 (cyanocobalamin) vitamins were done to assess the influence of the two different kind of experimental brewing waters on content of B vitamins. The concentration of the last two mentioned vitamins was below the detection limit in all samples. The results of the analysis of the determined B vitamins are shown in Table 1 and Table 2.

Statistically significant differences between hard and soft water were found in wort (p = 0.0007, F = 92.214). Hard water seemed to be a better extraction agent due to different pH and ionic strength. Content of B3 vitamin changed during the brewing. In the presence of yeast, the level of B3 vitamin declined rapidly as yeast used vitamin in the wort in biochemical processes and nicotinic acid synthesis did not occur **(Basařová et al., 2010)**. Relatively small, but significant difference in concentration of B3 vitamin was found in the final product (p = 0.0448, F = 8.3243). A slightly higher average concentration of vitamin B3 was determined in beer from hard water. This phenomenon is probably caused by releasing cell content during yeast autolysis.

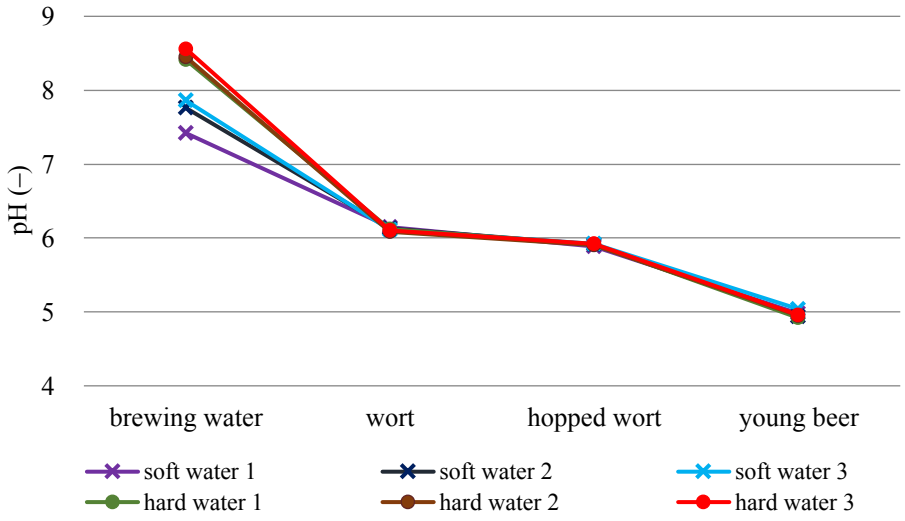

Figure 1 Changes of synthetic water pH during beer production.

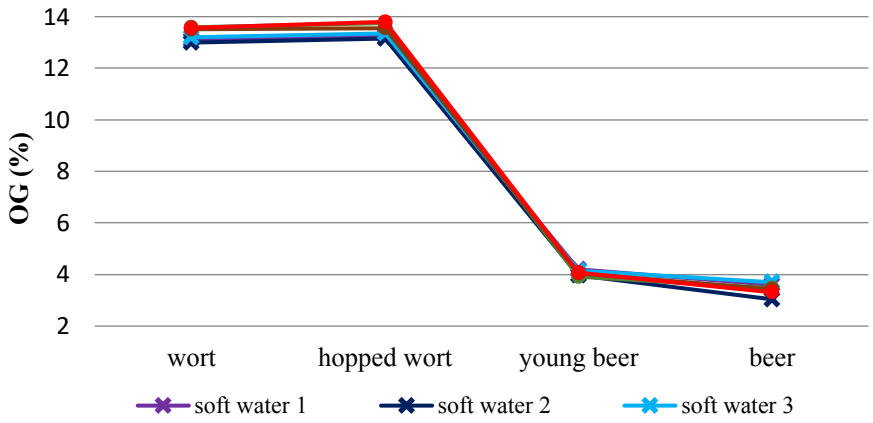

Figure 2 Changes of OG in individual phases of beer production.

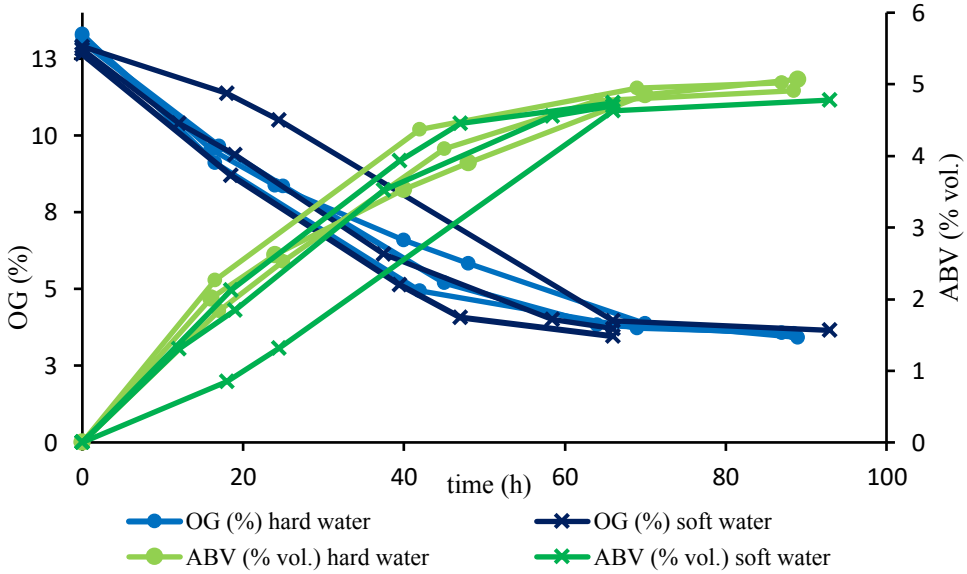

Figure 3 OG and ABV changes during primary fermentation.

Table 1 Quantification of B3 vitamin (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	31.68	30.07	33.18	0.0007	92.2144
	Hard	40.85	40.25	41.36		
Hopped wort	Soft	36.13	32.92	41.56	0.0674	6.2091
	Hard	43.05	42.14	43.97		
Young beer	Soft	8.97	6.70	10.59	0.7838	0.0861
	Hard	9.34	8.54	10.11		
Beer	Soft	8.99	7.97	10.02	0.0448	8.3243
	Hard	10.97	10.47	11.63		

Note: * All samples were made in triplicates.

Table 2 Quantification of B2 vitamin (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	0.90	0.84	0.93	0.0385	9.2210
	Hard	1.00	0.96	1.01		
Hopped wort	Soft	1.09	0.97	1.18	0.5513	0.4221
	Hard	1.14	1.11	1.16		
Young beer	Soft	0.29	0.27	0.33	0.0008	83.2461
	Hard	0.57	0.53	0.62		
Beer	Soft	0.77	0.70	0.90	0.3008	1.4099
	Hard	1.02	0.62	1.25		

Note: * All samples were made in triplicates.

Table 3 Quantification of acetic acid (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	34.00	33.64	34.18	0.7063	0.1640
	Hard	34.21	33.26	34.87		
Hopped wort	Soft	34.92	33.83	35.87	0.0998	4.5510
	Hard	36.51	35.61	37.02		
Young beer	Soft	87.91	86.40	89.94	0.0786	5.5162
	Hard	111.25	93.26	127.32		
Beer	Soft	122.42	105.56	143.49	0.5623	0.3982
	Hard	115.28	112.72	118.97		

Note: * All samples were made in triplicates.

Table 4 Quantification of lactic acid (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	94.49	93.35	95.95	0.6203	0.2874
	Hard	93.87	92.55	95.48		
Hopped wort	Soft	94.74	93.86	95.92	0.1181	3.9418
	Hard	98.04	95.33	100.67		
Young beer	Soft	225.55	179.34	271.00	0.0988	4.5894
	Hard	287.48	266.54	306.74		
Beer	Soft	300.06	299.22	304.71	0.1020	4.4690
	Hard	298.37	288.99	299.44		

Note: * All samples were made in triplicates.

Table 5 Quantification of isocohumulone (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	1.04	0.93	1.14	0.5836	0.3545
	Hard	1.07	1.05	1.10		
Hopped wort	Soft	0.57	0.45	0.75	0.6376	0.2590
	Hard	0.62	0.54	0.67		
Young beer	Soft	0.40	0.26	0.64	0.5619	0.3991
	Hard	0.49	0.35	0.59		

Note: * All samples were made in triplicates.

Table 6 Quantification of isohumulone (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	2.23	1.97	2.49	0.7584	0.1085
	Hard	2.29	2.15	2.41		
Hopped wort	Soft	0.74	0.42	1.13	0.9758	0.001
	Hard	0.75	0.52	0.94		
Young beer	Soft	0.50	0.31	0.76	0.7393	0.1273
	Hard	0.57	0.34	0.79		

Note: * All samples were made in triplicates.

Table 7 Quantification of isoadhumulone (F_{krit} = 7.71).

Type of sample	Type of water	Mean (mg.L^{-1})	Min (mg.L^{-1})	Max (mg.L^{-1})	*p*	F
Wort	Soft	44.63	39.83	50.44	0.8564	0.0372
	Hard	45.26	43.22	46.96		
Hopped wort	Soft	17.68	11.26	25.55	0.9472	0.0050
	Hard	17.35	13.33	20.68		
Young beer	Soft	10.36	6.02	17.15	0.5314	0.4681
	Hard	13.25	8.59	16.90		

Note: * All samples were made in triplicates.

Usual content of niacin in beer is around 5 mg.L^{-1} **(Basařová et al., 2010)**. The measured values are higher in comparison with the literature, approximately 8 – 12 mg.L^{-1}. High vitamin content can be caused by no further treatment after beer bottling, unlike commercial beers, which are filtered and often pasteurized.

Very similar trend was observed for B2 vitamin. Statistically significant differences were found in wort (p = 0.0385, F = 9.2210) and young beer (p = 0.0008, F = 83.2461) (shown in Table 2). Riboflavin was better extracted from barley malt during mashing in hard brewing water than in soft. This could be due to different pH of the water. Riboflavin is a thermostable vitamin, there were observed no loss of the vitamin in the process of wort boiling. During fermentation there was a certain decrease in vitamin content because riboflavin participates in many biochemical processes **(Hucker, Wakeling, Vriesekoop, 2016)**. The greatest difference was observed in soft water. In hard water, to thereby prevent high losses probably because riboflavin form stable complexes with Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+} **(Sheraz et al., 2014)**. In beer, the vitamin concentration was probably increased by the release of yeast autolysis **(Hucker, Wakeling, Vriesekoop, 2011)**.

Usual content of riboflavin in beer is approximately 0.25 mg.L^{-1} **(Olšovská, Jurková and Čejka, 2012)**. When comparing the values and literature the B2 vitamin concentration is several times higher – 0.6 to 1.2 mg.L^{-1}. Again, it is probably the reason for omitting post fermentation adjustments.

Quantification of organic acids

Determination of organic acids was done to assess the influence of soft and hard experimental brewing waters. Retention time of lactic acid was 11.17 min, acetic acid 14.22 min. The results are shown in Table 3 and Table 4.

The results have shown that there are no statistically significant differences in content of acetic acid in beer made from soft and hard brewing water (Table 3).

The content of acetic acid increases rapidly during fermentation **(South, 1996)**. The greatest changes between samples made from soft and hard brewing water were found in young beer, though Analysis of Variance did not prove statistically significant differences. This difference was probably due to a divergent ratio of minerals in hard and soft brewing water, which can either positively or negatively affects the level of organic acid synthesis.

Concentration of acetic acid in beer brewed from soft and hard brewing water varied from 106 to 144 mg.L^{-1}, whereas **Vontrobová et al. (2017); Coote and Kirsop (1974); Walker (1998); Zhang, Jia and Zhang, (2012)** indicate values ranging from 30 to 200 mg.L^{-1}.

Lactic acid consists of two isomers. D-lactate, which comes from malt and the content may vary during mashing, and L-lactate, which indicates bacterial activity. The D and L isomers of this acid have been reported in wort and beer on a number of occasions **(Coote and Kirsop, 1974)**. Lactate was excreted throughout the period of sugar utilization **(Coote and Kirsop, 1974; Whiting, 1976)**.

The results of determination of lactic acid are shown in Table 4. The difference in composition of brewing water had no influence on content of lactic acid. During wort boiling, it was observed no decline of acid, concentration slightly increased in hard brewing water. It could be possibly related with extraction of organic acids from hops. High production of acid occurred during fermentation. Higher amounts of lactic acid were obtained in young beer made from hard brewing water (p = 0.098 8, F = 4.589 4). On the contrary, higher concentration was determined in beer prepared from soft water (p = 0.102 0, F = 4.469 0), while the concentration in beer made from hard water remained the same.

Experimental beers content high amounts of lactic acid, the concentration was ranging between 289 – 305 mg.L^{-1}. **(Coote and Kirsop, 1974; Whiting, 1976)** present big range of lactic acid concentration in beer. Specifically, this span varies from 37 to 233 mg.L^{-1} in lagers, ales 44 – 276 mg.L^{-1} and dark strong beers 276 – 292 mg.L^{-1}.

Lactic acid bacteria (LAB) may be the reason of high concentrations of lactic acid. They may proliferate in wort, hopped wort and even during fermentation. They survive in pH 4 – 7. Optimal growth temperature is 20 – 30 °C, but psychrophilic strains occur, which may grow in conditions of low temperatures during fermentation and maturing of beer **(Basařová et al., 2010)**.

Quantification of iso-alpha-acid

The results of iso-α-acids analysis are in Table 5, Table 6 and in Table 7. The retention time of isocohumulone was 7.481 min, isohumulone 9.610 min and isoadhumulone 10.256 min.

Impact of brewing water composition was studied on the most represented analogues of iso-α-acids – isohumulone, isocohumulone and isoadhumulone. Analysis of variance did not prove statistically significant differences neither in soft nor hard brewing water. Isomers of α-acids are unstable and during beer production its concentration declined rapidly. Of the total amount of bitter compounds 30% at most will end in beer, whereas approximately 20% remains in the spent hops, 20 – 30% is captured in waste and 20 – 30% is captured in the head of foam on the top of the fermentation. Isomerization is also affected by time of wort boiling and OG value **(Basařová et al., 2010)**.

CONCLUSION

In conclusion, brewing water significantly influences some selected parameters. In the final product – beer – it was found out that hard brewing water is better for higher yields of OG values (OG value equals 13.05 ±0.05%) than soft water (OG value 12.6 ±0.1%).

Brewing water also affected the content of riboflavin and niacin. Statistically significant difference in concentration of B3 vitamin appeared between hard and soft water in wort (p = 0.0007, F = 92.214) because hard water was a better extraction agent due to different pH and ionic strength. Another significant difference in concentration of B3 vitamin was found in the stabilized beer (p = 0.0448, F = 8.3243). A higher concentration of B3 vitamin was determined in beer from hard water. It is probably caused by yeast autolysis when the cell content is released into solution. Very similar trend was observed for B2 vitamin. Statistically significant differences were found in wort (p = 0.0385, F = 9.2210) and young beer (p = 0.0008, F = 83.2461).

Unlike statistical analysis did not prove an effect on content of organic acids or iso-α-acids.

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COMPARATIVE STUDY OF SOME BIOACTIVE COMPOUNDS AND THEIR ANTIOXIDANT ACTIVITY OF SOME BERRY TYPES

Amina Aly, Rabab Maraei, Omneya Abou El-Leel

ABSTRACT
Berries are wealthy in bioactive compounds like phenolic compounds and flavonoids that are deemed antioxidants and are great important to health. This research was performed to examine, recognize and compare bioactive compounds in certain types of berries and their antioxidant activity. The data show that blue berry, black berry and Egyptian black mulberry contain the highest content of most bioactive compounds such as phenolic compounds, flavonoids and tannins, while long mulberry and red currant berry have the lowest content for most of these compounds. They therefore, contain the highest value of antioxidant activity. The chemical composition of the berries varies depending on cultivar, variety, location of growth, environmental conditions and harvest time, as well as post-harvest treatments therefore the composition differed from berry fruit to another. Thus, berry fruits are very useful in nutrition to protect the body from many diseases because of its containment of these compounds, which act as free radicals scavenger that harm the body and thus rid the body of many harmful toxins.

Keywords: berries; bioactive compounds; phenolic compounds; antioxidant activity

INTRODUCTION
Berries and small soft-fleshed colored fruits are consumed worldwide for their numerous health benefits. Mature fresh berry fruits contains a large amounts of phytochemical components; phenolic compounds, flavonoids, tannins, carotenoids and anthocyanins **(Skrovankova et al., 2015)**. These compounds used as functional food ingredients in the food industry **(Starast et al., 2007)**. In addition, fruits comprise flavonols and anthocyanins, discovered in big quantities, particularly in dark berries like black currant and bilberry **(Govindaraghavan, 2014)**. Some phenolic acids like *P*-coumaric acid and lignin related to ferulic acid or other cell wall elements can be discovered in berries fruits **(Andreasen et al., 2001)**. Many bioactive compounds are powerful antioxidants, acting as reactive oxygen inhibitors and free radical scavengers and display as anti-inflammatory, antiallergic, antihypertensive, anti-carcinogenic, antifungal, and antiviral agents **(Oszmianski and Lachowicz, 2016)**. The content of phenolic compounds, flavonoids and anthocyanins can be used to describe the antioxidant activity and thus the potential health benefits of berries fruit **(Anttonen and Karjalainen, 2005)**. Also, berry fruits contains a high content of sugars, diatery fiber and organic acids (oxalic, malic, citric, tartaric and fumaric acids) while, it contains a low content of calorie and fat **(Nile and Park, 2014)**. Berries are a wealthy source of vitamins like Vit. C and folic acid, so the most significant antioxidants in berry fruits are phenolic compounds and ascorbic acid **(Beekwilder et al., 2005)** influencing dietary importance, sensory characteristics and fruits quality **(Lachowicz et al., 2017)**. The chemical structure of berries is affected by many factors, including variety, environmental factors, maturity stage, harvest time and method, storage conditions and duration **(Bobinaitė et al., 2012)**. In latest years, interest in berries fruits has risen as an element of a good diet due to the elevated quantity of bioactive compounds.

Scientific hypothesis
Berry fruits contain a number of active compounds responsible for many biological activities that can be used to help protect against many diseases such as cardiovascular disease, cancer and diabetes. This research aims to investigate, identify and compare bioactive compounds (phenolic compounds, flavonoids and tannins) and their antioxidant activity in certain types of berries.

MATERIAL AND METHODOLOGY
Berry fruits
Eight types of berry fruits were inclusive in this study: crane berry (*Vaccinium macrocarpon*), long mulberry (*Morus nigra*), red currant berry (*Ribes rubrum*), Egyptian black mulberry (*Morus nigra* L.), raspberry (*Rubus idaeus*), black berry (*Rubus fructicosus*), blue berry (*Vaccinium corymbosum*) and strawberry (*Fragaria*

ananassa). Fruit samples were bought from Cairo-Egypt local market (Figure 1).

Chemicals

The chemicals and solvents used for this study were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of extracts

Under liquid nitrogen, fresh samples were homogenized and then lyophilized for 48 hours (Virtis model 10-324). Powder samples were extracted three times at room temperature using 80 percent ethanol for 24 h. Use rotary evaporator at 40 °C after filtration and solvent evaporation **(Aly et al., 2019)**. To determine the yield extract, the extracts obtained were weighed. These components have been dissolved in 80 percent ethanol and used for chemical analyses.

Phenolic content

Phenolic content was analyzed using the Folin-Denis reagent in accordance with **Shahidi and Naczk (1995)** technique. A one ml of sample extract was mixed with 0.5 mL of Folin–Denis and 1.0 mL of concentrated Na_2CO_3 solution, adding 3.0 mL of distilled water.

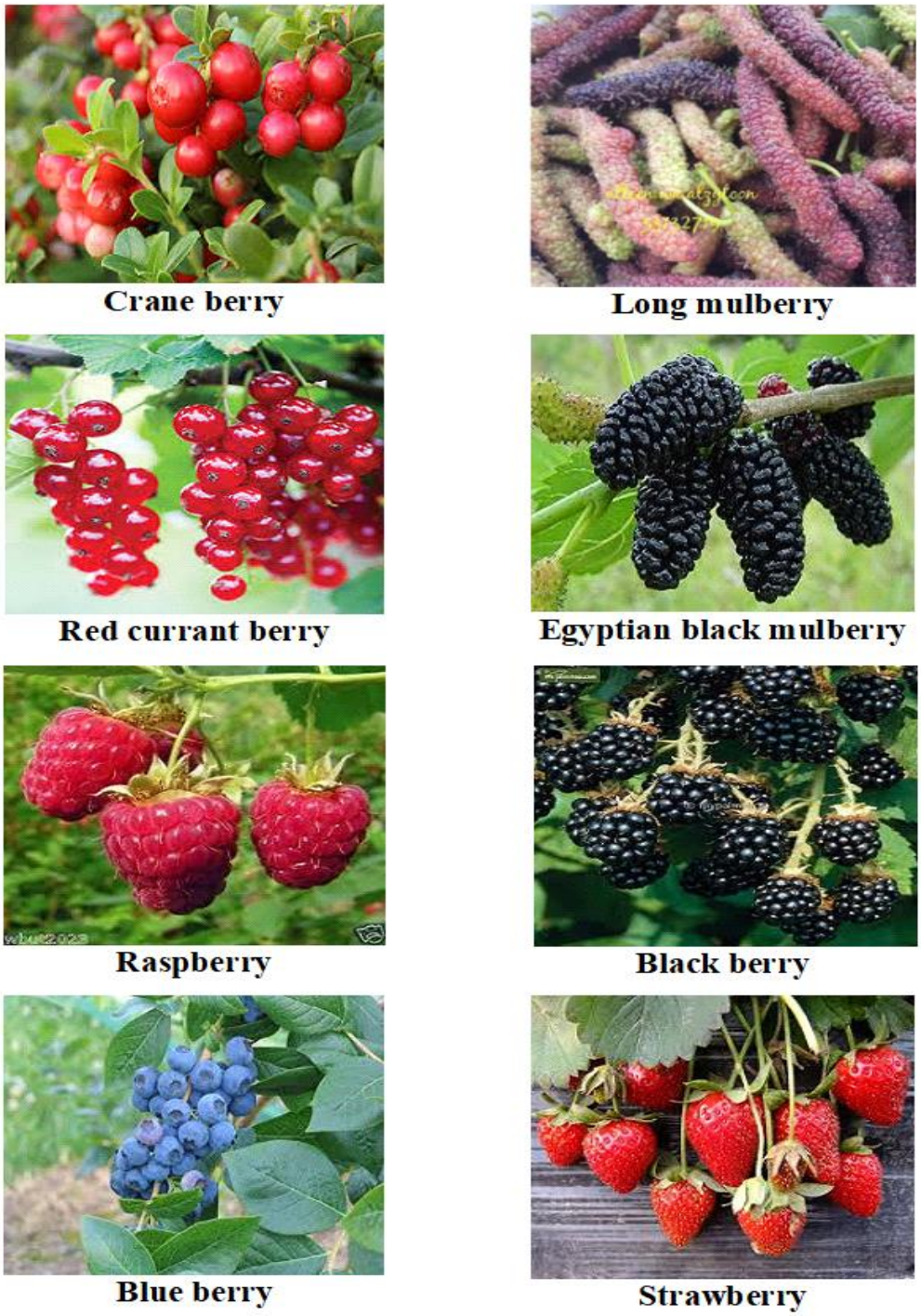

Figure 1 Photograph showing types of some ripe berry fruits used in this study.

The absorbance was evaluated at 725 nm against the blank after an hour. The results were expressed as mg of gallic (GAE) equivalent /g dry weight of extract.

Flavonoids content

Flavonoids content were determined in the extracts by the aluminum chloride colorimetric assay methods by **Marinova et al. (2005)**. Each ethanolic extract (1.0 mL) or conventional quercetrin solution has been added to 4.0 mL distilled water and 0.3 mL of 5% $NaNO_2$ has been introduced. Added 0.3 mL of 10% $AlCl_3$ after 5 min and added 2.0 mL of 1M NaOH after 6 min. The absorbance was evaluated at 510 nm against the blank. Total flavonoids were presented as mg quercetin (QE) equivalent /g DW of extract.

Tannins content

The content of tannins was according to a modified vanillin assay **(Price et al., 1978)** determined the quantity of tannins. One ml of extract was mixed with 5 mL of vanillin/HCl reagent (0.5 g vanillin in methanol 4% hydrochloric acid (v/v). Samples and control (without vanillin) were permitted to remain for 20 min in the dark and then the absorbance at 500 nm was read. Total tannin content was displayed as mg tannic acid equivalent /g DW of extract.

Flavonols content

Yermakov et al. (1987) determined flavonols. Added one ml of the sample, 2 mL (20 $g.L^{-1}$) of aluminum trichloride and 3 mL (50 $g.L^{-1}$) sodium acetate solutions. After 2.5 h at 20 °C at 440 nm the absorbance was read by spectrophotometer (Jasco V-530, Japan). The flavonol content was displayed as mg quercetin equivalent /g dry weight of extract.

Antioxidant activity by:

Scavenging activity on DPPH radical

The radical scavenging activity of extracts against 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical was determined as outlined by **Gulluce et al. (2004)**. Add 0.5 mL of each sample to 1.0 mL of DPPH (2 mM) ethanolic solution. The absorbance was read by spectrophotometer (Jasco V-530, Japan) at 517 nm against the blank after 30 min of the incubation period.

Lipid peroxidation (TBA test)

The level of lipid peroxidation in the berry samples was as outlined by **Buege and Aust (1978)**, the amount of lipid peroxidation in the samples was determined as reactive metabolites 2-thiobarbituric acid (TBA) mainly malondialdehyde (MDA). The pink color absorbance was assessed at 532 nm and adjusted by subtracting the absorbance at 600 nm for non-specific turbidity by spectrophotometer (Jasco V-530, Japan). The concentration of MDA was calculated based on A532 – A600 (Σ = 155 $mM^{-1}cm^{-1}$). The outcome has been displayed as μmol/g dry weight of the extract.

Reducing power

Reducing power was determined by **Oyaizu (1986)** technique. Each 0.5 mL sample was mixed with 0.2 M sodium phosphate buffer (2.5 mL) and 1% potassium ferricyanide (2.5 mL) and incubated at 50 °C for 20 min. Follwing the addition of 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged for 10 min at 200g. The upper layer (5.0 mL) was blended with 5.0 mL of deionized water and the test sample was read against the blank at 700 nm.

Metal chelating

Metal chelating activity was consequently evaluated by 0.1 mM $FeSO_4$ (0.2 mL) and 0.25 mM ferrozine (0.4 mL) in 0.2 mL of samples **(Chew et al., 2009)**. After 10 min of incubating at room temperature, absorbance of the blend was recorded against a blank at 562 nm by spectrophotometer (Jasco V-530, Japan). The lower absorption of the test sample indicated greater chelating capacity of ferrous ion. The control contained all the reagents except test sample.

Identification of bioactive compound by GC-MS

Berry fruits extracts was engaged for GC-MS analysis using a HP G1800A instrument. Operating with a capillary column HP-5 (length 30 m, i.d. 0.25 mm); carrier gas: helium; flow rate: 1 $mL.min^{-1}$; inlet temperature: 250 °C; detector temperature 280 °C; programmed temperature 100 °C for 3 min, then 10 – 250 °C, and 30 – 280 °C with a mass detector. Peak identification was carried out by comparing the retention times and mass spectra of eluting constituents with those of the Wiley library (Wiley7, NIST 0.1; Wiley, West Sussex, UK). In Agricultural Research Center, Giza, Egypt the GC-MS analysis was conducted.

Statistical analysis

The information was shown as the average ±*SD*. All the statistical analyzes were carried out using an ANOVA and we used the multiple range tests of Duncan **(Duncan, 1955)** to compare the results of the experiments ($p \leq 0.05$).

RESULTS AND DISCUSSION

Yield extracts percentage of berry fruits

Data in Figure 2 shows that the highest extract yield was 7.80% of red currant berry followed by blue berry 7.63% while strawberry gave the lowest yield 5.36%. Whereas, black berry gave 7.20% and Egyptian black mulberry 6.36% **(Aly et al., 2019)**.

Phenolic and flavonoids contents of berry fruits

Phenolic compounds are a big class of phytochemicals that exist as secondary metabolites present in crops. Most of them are phenolic acids, flavonoids, and tannins in human food. Besides contribution to sensory properties of food. Phenolic compounds also have a broad variety of biological and physiological tasks, similar to anti-allergenic, anti-inflammatory, antimicrobial and antioxidant operations that benefit human health **(Balasundram et al., 2006)**.

The findings showed that blue berry has the largest value of phenolic content (6.74 $mg.g^{-1}$ DW of extract) followed by black berry (5.98 $mg.g^{-1}$ DW of extract), but the lowest content (1.62 $mg.g^{-1}$ DW of extract) was obtained from long mulberry (Figure 3).

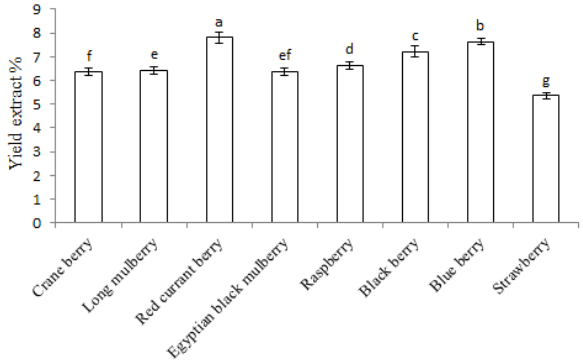

Figure 2 Yield extracts percentage of some different types of ripe berry fruits.

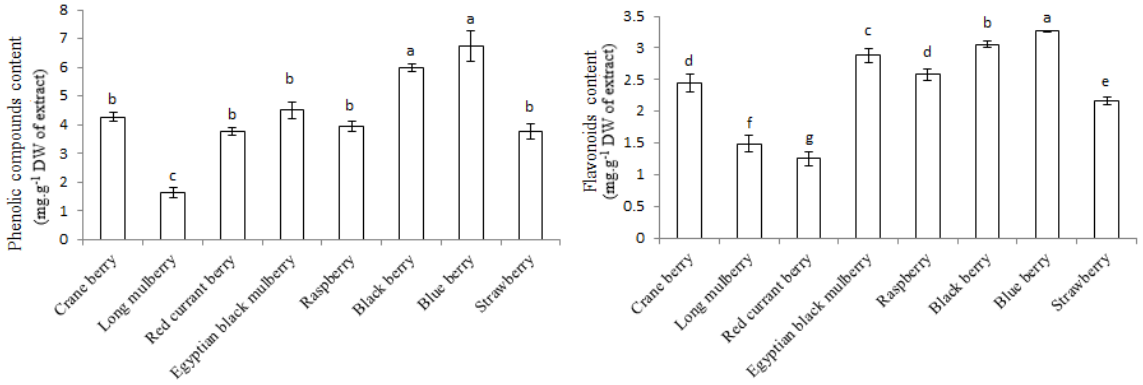

Figure 3 Phenolic compounds and flavonoid contents (mg.g^{-1} DW of extract) in some different types of ripe berry fruits.

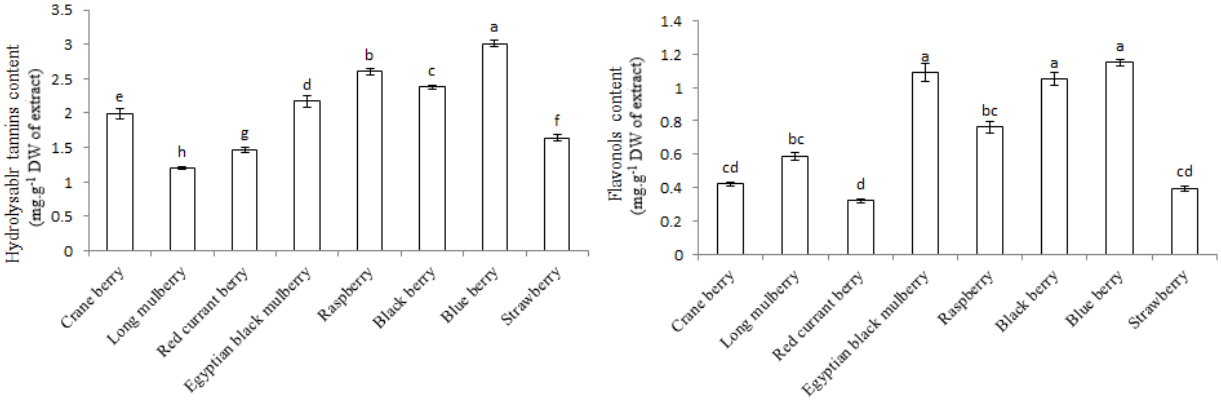

Figure 4 Hydrolysable tannins and flavonols content (mg.g^{-1} DW of extract) in some different types of ripe berry fruits.

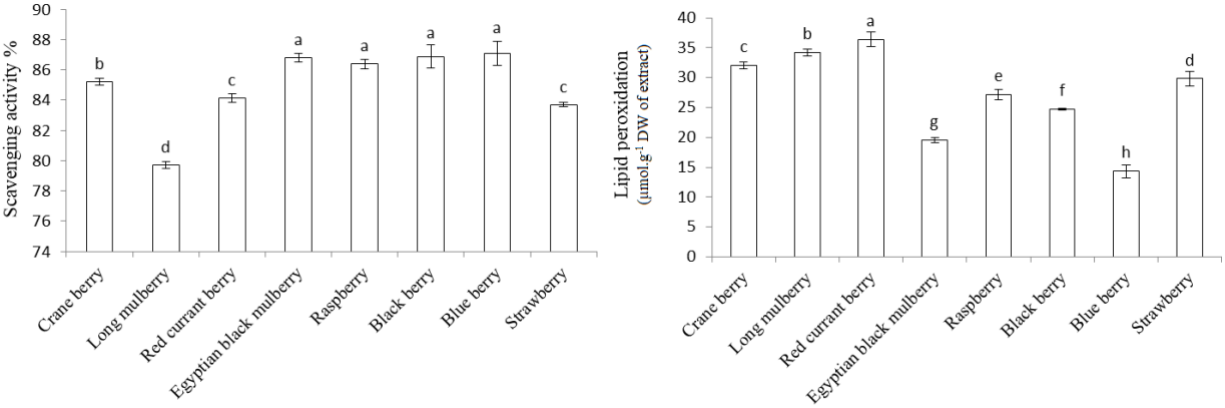

Figure 5 Scavenging activity percentage and lipid peroxydation (µmol.g^{-1} DW of extract) in some different types of ripe berry fruits.

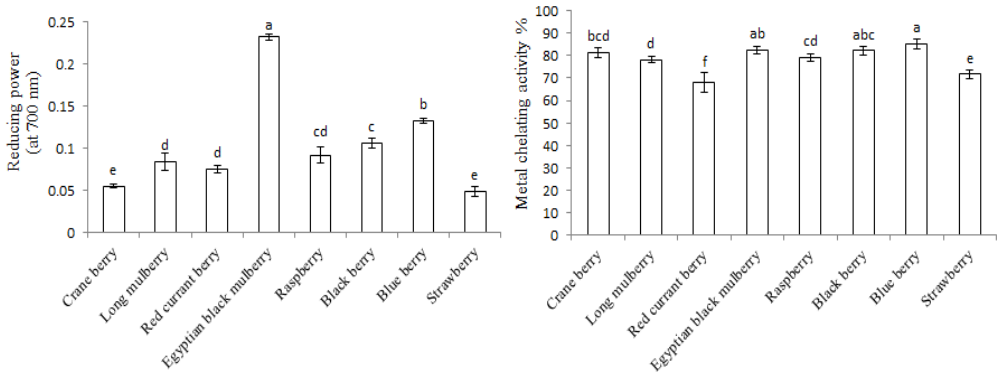

Figure 6 Reducing power and metal chelating activity percentage in some different types of ripe berry fruits.

For flavonoids, blue berry contains the highest content (3.26 mg.g^{-1} DW of extract) followed by black berry (3.05 mg.g^{-1} DW of extract), while red currant berry gave the lowest content of flavonoids (1.14 mg.g^{-1} DW of extract) (Figure 3).

These findings are consistent with **Huang et al. (2012)** who studied the total phenolic content, and total flavonoid content of methanolic extracts of three berry fruits (blue berry, black berry and strawberry) growing in Nanjing and found that total phenolic content was 9.44, 5.58 and 2.72 mg gallic acid.g^{-1} DW of blue berry, black berry and strawberry, respectively. Also, indicated that blue berry and black berry contain the highest content of flavonoids and strawberry contains the lowest content. The phenolic compounds content for analyzed berries ranged from 1.62 to 6.74%, these results are comparable to **Diaconeasa et al. (2015)** who indicated that the total phenolic compounds in some berries grown in Romania ranged from 200.3 to 678 mg GAE.100g^{-1} FW. Likewise, **Giovanelli and Buratti (2009)** found that the total phenolic compounds values between 251 – 310 mg GAE.100g^{-1} FW for some cultivated blue berries and between 577 and 614 mg GAE.100g^{-1} FW for wild Italian blue berries. However, higher contents in other blackberries cultivars have been observed and reported by **Deighton et al. (2000)**. The difference between the analyzed berries and others studies is due to differences in extraction method, environmental growth conditions, degree of maturity at harvest and genetic variations **(Zadernowski et al., 2005)**. As antioxidants, antimutagenic, anticarcinogenic and their capacity to alter gene expression, phenolic compounds are of excellent significance **(Marinova et al., 2005)**. Flavonoids are a big class of phenolic compounds discovered either free or as glycosides in various areas of the crop. It has many biological functions such as antimicrobial, antioxidant, anticancer, inhibition of protein kinase, etc **(Bhat et al., 2005)**.

Hydrolysable tannins and flavonols content of berry fruits

Tannins are secondary metabolites that are widely distributed throughout the plant world and distinguished by their water solubility. The results in Figure 4 showed that blue berry and raspberry gave the highest content of tannins (3.01 and 2.60 mg.g^{-1} DW of extract, respectively) while the lowest value was from long mulberry (1.20 mg.g^{-1} DW of extract). The results indicated that blue berry has the highest value of tannins. This result in agreement with **Diaconeasa et al. (2015)** who evaluated and compared some phytochemical compounds (phenolic compounds and tannins) in common fruits consumed in Romania (blueberry, blackberry, raspberry and cranberry) and found that the blue berry and raspberry contained the highest content of tannins.Concerning the content of flavonols, blue berry, black berry and Egyptian black mulberry gave the highest content of flavonols (1.15, 1.05 and 1.09 mg.g^{-1} DW of extract, respectively) followed by raspberry (0.762 mg.g^{-1} DW of extract), while red currant berry gave lowest value of flavonols content (0.321 mg.g^{-1} DW of extract, respectively) (Figure 4).

Antioxidant activity

Antioxidant can be widely described as any drug that retards or inhibits a target molecule's oxidative harm **(Yamagishi and Matsui, 2011)** and prevent biological molecules such as proteins, lipids, and other molecules from oxidation by reactive oxygen species (ROS) such as hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), etc. **(Brindza et al., 2019)**. These reactive oxygen species are produced in the body either as a by-product of normal cellular aerobic respiration or exposure to environmental factors such as herbicides, radiation, pollution, and cigarette smoke **(Alam et al., 2019)**. Also, antioxidant's primary characteristic its capacity to intercept free radical. Antioxidant compounds such as phenolic acids, polyphenols and flavonoids scavenge free radicals and prevent the oxidative harm resulting in many illnesses **(Wu et al., 2011)**. A great cause of antioxidants is vegetables and fruits. Results from this research showed that blue berry, black berry and Egyptian black mulberry yielded elevated antioxidant activity scores accompanied by raspberry (Figures 5 and Figure 6). Concerning scavenging activity the blue berry gave 87.1% of scavenging activity followed by black berry and Egyptian black mulberry (86.88 and 86.81% respectively). Additionally, they gave a high content of metal chelating activity but red currant berry was on the contrary, giving lower value of metal chelating activity (67.86%). There were slight differences in reducing power between berry fruits where Egyptian black mulberry gave the highest value in reducing power (0.232), but strawberry gave the lowest value of reducing power (0.049).

Table 1 Bioactive compounds in different types of some ripe berry fruits.

No.	RT (min)	Name	1	2	3	4	5	6	7	8
1	4.933	Sinapic acid	1.25	1.41	1.23	1.54	1.37	1.78	1.84	1.56
2	4.97	Vitamin E acetate	1.87	1.46	1.49	1.98	1.41	1.36	1.07	1.43
3	5.113	Trans-Borneol	10.54	11.27	11.17	10.79	11.8	10.91	11.25	10.83
4	5.62	3,4-Dimethoxycinnamic acid	10.09	10.85	10.93	10.83	11.25	11.68	11.5	11.05
5	6.78	Phenol, 2,6-dimethoxy-	13.54	14.62	12.44	10.35	11.39	11.49	11.32	13.20
6	7.27	Fumaric acid	3.01	5.96	4.3	5.89	6.04	5.86	5.01	5.47
7	7.5	Ascorbic acid	9.71	10.77	11.8	9.44	12.24	9.86	9.44	11.29
8	7.819	Phytol	2.61	2.53	2.5	3.72	2.23	3.17	3.7	2.39
9	7.9	2,5-Dihydroxybenzoic acid	1.12	1.4	1.19	1.52	1.21	1.87	1.39	1.32
10	8.63	Isoborneol	1.96	2.71	1.33	1.37	1.26	1.95	1.24	1.49
11	8.9	1,3,5-Benzenetriol	1.08	0.92	0.92	1.38	1.75	1.55	0.78	1.16
12	9.03	Carvenone	0.4	0.49	0.86	1.06	1.96	1.53	1.7	0.8
13	9.172	Citronellal	3.9	2.39	3.14	2.06	1.65	2.45	2.79	1.89
14	9.78	Terpinen-4-ol	1.08	2.03	1.12	0.27	1.26	1.2	1.22	2.18
15	10.2	(-)-Lavadulol	0.35	0.26	0.61	1.31	0.92	0.37	0.39	0.78
16	11.22	Quercetin 3-rhamnoside	1.42	1.18	1.01	2.45	1.74	2.11	2.16	1.64
17	11.67	Benzoic acid, 3,4,5-trihydroxy-, methyl ester	0.53	0.3	0.3	0.71	0.64	0.9	0.69	0.56
18	12.15	Adipic acid	10.92	10.38	11.9	13.02	10.9	11.15	11.22	9.37
19	12.41	Cyanidine cation	1.44	1.52	1.38	1.82	1.45	1.34	1.59	1.49
20	12.6	β-Cryptoxanthin	0.51	0.47	0.49	0.74	0.58	0.63	0.67	0.53
21	13.06	Methyl jasmonate	0.39	0.29	0.49	0.68	0.95	0.94	0.99	0.76
22	13.45	4-Chromanol	0.32	0.69	0.54	0.3	0.49	0.37	0.4	0.48
23	14.09	Nadolol	1.3	1.12	1.14	1.87	1.36	1.11	1.18	1.36
24	14.15	Glycitein	0.7	0.8	0.65	0.62	0.87	0.79	0.38	0.46
25	14.58	Flavone	0.56	0.28	0.27	0.82	0.63	0.72	0.77	0.49
26	15.4	Resveratrol	2.12	1.21	1.47	1.57	1.47	2.24	2.35	1.35
27	15.7	Fisetin	1.7	1.88	1.38	1.35	1.55	1.23	2.37	2.46
28	16.11	Apigenine 7-methyl ether	0.8	0.18	0.59	0.8	0.46	0.84	0.84	0.57
29	16.64	Trans-Geranylgeraniol	3.48	2.79	3.71	2.43	1.48	1.72	2.04	2.71
30	16.7	Patchoulanol	1.83	2.56	1.98	1.76	1.35	1.41	2.08	1.80
31	16.85	Kaempferol	0.78	0.36	0.65	0.96	0.86	0.95	0.94	0.64
32	16.95	Linoleic acid	1.49	0.77	0.25	0.41	1.32	0.38	0.56	0.33
33	17.07	Lycopene	0.5	0.37	0.43	0.26	0.43	0.26	0.29	0.55
34	17.27	Ethyl linolenate	0.82	0.33	0.45	0.28	0.42	0.34	0.24	0.48
35	18.37	Luteolin 6,8-C-diglucoside	2.29	2.16	2.15	1.23	1.47	1.45	1.25	2.00
36	20.6	α-Bisabolol	1.54	0.85	1.75	0.85	0.83	0.87	0.91	1.47
37	21.4	Apigenin	0.6	0.21	0.41	0.75	0.31	0.51	0.47	0.37
38	22.26	Phloretin	1.45	0.23	1.58	0.81	0.7	0.71	0.97	1.29

Note: 1: crane berry, 2: long mulberry, 3: red currant berry, 4: Egyptian black mulberry, 5: raspberry, 6: black berry, 7: blue berry and 8: strawberry.

This outcome is in agreement with **Diaconeasa et al. (2015)** who specified the greatest total phenolic compounds (678 mg.100g^{-1} FW and 422 mg.100g^{-1} FW respectively) also, blue berry and black berry had the greatest antioxidant activity. Also, **Huang et al. (2012)** indicated that the three berry fruits (blue berry, black berry and strawberry) grown in Nanjing manifest excellent antioxidant activity, the blue berry was the superior than black berry and strawberry. **Burdulis et al. (2009)** revealed that the blue berry cultivar (Berkeley) was the strongest antioxidant activity, and its scavenging activity was 82.13 ±0.51%. While scavenging activity in bilberry

specimens was 63.72 ±1.11%. Blue berry cultivar (Covillei) reported the smallest antioxidant content (51.30 ±0.72 percent). The fruits of blue berry and bilberry are wealthy in flavonoids (quercetin, kempferol, epikatechin, catechin, and myrcetin), phenolic acids, chlorogenic acid and ascorbic acid, that are antiradical characteristics **(Sellappan et al., 2002)**.

Anthocyanins and other polyphenolic compounds, the main contributors to the antioxidant activity in berries. Furthermore **Vuong et al. (2018)** discovered that blue berry ash fruit includes elevated content of phenolic compounds, flavonoids and proanthocyanidins that add to the antioxidant capacity of this fruit.

Identification of bioactive compound by GC-MS

The chemical composition of the berries varies according to cultivar, variety, location of growth, environmental conditions and harvest time, as well as postharvest treatments, so it is variable.

Gas chromatography–mass spectrometry (GC-MS) is an analytical technique that incorporates gas chromatography with mass spectrometry to recognize different substances in a test specimen.

Identification of components

Mass spectrometer was used to recognize the composition and the nature at distinct time periods. The heights of the distinct peaks show the comparative quantity in the different components current in the specimen. The compound's finger prints that can be recognized from the database of the library.

GC-MS analysis

Approximately 38 compounds were identified by GC-MS analysis in fruit extracts from berries. Table 1 presented the name of compounds with retention time (RT) and concentration (percent).

The prevailing compounds were Sinapic acid, Vitamin E acetate, Trans-Borneol, 3,4-Dimethoxycinnamic acid, Phenol, 2,6-dimethoxy-, Fumaric acid, Ascorbic acid, Phytol, 2,5-Dihydroxybenzoic acid, Isoborneol, 1,3,5-Benzenetriol, Carvenone, Citronellal, Terpinen-4-ol, (-)-Lavadulol, Quercetin 3-rhamnoside, Benzoic acid 3,4,5-trihydroxy- methyl ester, Adipic acid, Cyanidine cation, β-Cryptoxanthin, Methyl jasmonate, 4-Chromanol, Nadolol, Glycitein, Flavone, Resveratrol, Fisetin, Apigenine 7-methyl ether, Trans-Geranylgeraniol, Patchoulanol, Kaempferol, Linoleic acid, Lycopene, Ethyl linolenate, Luteolin 6,8-C-diglucoside, α-Bisabolol, Apigenin and Phloretin. The investigation concluded that there are a number of active constituents responsible for many biological activities in the ethanolic extract of berry fruit, so these can be used to help protect against many diseases like cardiovascular disease, cancer and diabetes **(van Breda, Briedé and de Kok, 2019)**. These active ingredients found in fruit extracts from berries include the following compounds: Sinapic acid has antioxidant **(Zou et al., 2002)**, anti-inflammatory **(Yun et al., 2008)**, anticancer **(Hudson et al., 2000)**, antimutagenic, antiglycemic, and antimicrobial activities **(Engels et al., 2012)**. Vitamin E acetate is used in dermatological products such as skin creams and provides protection against the sun's ultraviolet rays. Ascorbic acid performs a significant part in a variety of body tasks inclusive collagen manufacturing and may decrease the danger of certain diseases due to its antioxidant action. Phytol has antioxidant, antiallergic **(Santos et al., 2013)**, antinociceptive, antibacterial and anti-inflammatory activities **(Ryu et al., 2011)**. Also, it is an excellent stimulant for immunity **(Lim et al., 2006)**. 2,5-Dihydroxybenzoic acid is used as antioxidant in some pharmaceutical preparations. Isoborneol has antiviral properties. Citronellal has antifungal properties and is used in aromatherapy. Terpinen-4-ol has an antibacterial and antifungal effect. (-)-Lavadulol is an important compound in the cosmetics industry. Quercetin 3-rhamnoside has an anti-inflammation effect used to treat cardiovascular disease. Cyanidine has a role as a metabolite, a neuroprotective agent and an antioxidant. β-Cryptoxanthin acts as a chemopreventive agent against lung cancer. Methyl jasmonate is used in plant defense and various developmental pathways such as seed germination, root growth, flowering, fruit ripening, and senescence. Flavone is a metabolite and a nematicide. Resveratrol acts as an antioxidant to prevent cancer and heart disease in the body. Fisetin has an anticancer activity that inhibits the activity of many pro-inflammatory cytokines and free radicals of scavenges. It also regulates the synthesis of glutathione, an internal antioxidant, anti-hyperlipidemic and anti-inflammatory agent, thereby reducing the effect of age and disease on the central nervous system function **(Maher, 2015)**. Kaempferol acts as an antioxidant by decreasing oxidative stress and reducing the risk of various cancers **(Chen and Chen, 2013)**. Linoleic acid is used in prostaglandins and cell membranes biosynthesis. Lycopene is an antioxidant (protective substance from cell damage). Luteolin 6,8-C-diglucoside has anti-inflammatory properties. α-Bisabolol has anti-irritant, anti-inflammatory and antimicrobial properties. Apigenin has anti-tumor activity **(Sung et al., 2016)**.

CONCLUSION

Berry fruits contain large quantities of phytochemical compounds (phenolic compounds, flavonoids and tannins) that act as antioxidants. There have been variations in the amount of these compounds between analyzed berries, as blue berry, black berry and Egyptian black mulberry have a high content of most bioactive compounds (phenolic compounds, flavonoids and tannins), while long mulberry and red currant berry have the lowest content for most of these compounds. The results of this study showed that blue berry, black berry and Egyptian black mulberry yielded elevated antioxidant activity scores accompanied by raspberry. Concerning scavenging activity the blue berry gave the highest value of scavenging activity followed by black berry and Egyptian black mulberry. Additionally, they gave a high content of metal chelating activity but red currant berry was on the contrary, giving lower value of metal chelating activity. There were slight differences in reducing power between berry fruits where Egyptian black mulberry gave the highest value in reducing power, but strawberry gave the lowest value of reducing power.Also, fruit extracts from berries contain an amount of effective components accountable for many biological operations. Thus, many diseases such as

cardiovascular disease, cancer and diabetes can be treated with these components. This study therefore, reveals that berries are very nutritionally useful in protecting the body against many diseases.

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ALTERNARIA SPP. IN FOOD COMMODITIES OF SLOVAK ORIGIN: OCCURRENCE AND MYCOTOXIN PRODUCTION ABILITIES

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ABSTRACT

Various food commodities of Slovak origin were analysed for the occurrence of *Alternaria* species-groups. Totally we analysed 14 samples of grapes, 3 samples of barley, 2 samples of wheat, 17 samples of fruit, vegetable and fruit-vegetable juices, 6 samples of red kuri squash with macroscopically visible infection. Mycological analyses were performed by using plate dilution method, method of direct placing of berries or grains on the plates with dichloran, rose bengal and chloramphenicol agar or by direct inoculation by mycological needle to the identification medium (potato-carrot agar). In all grape, barley, wheat and squash samples the presence of representatives of this genus was detected (100% isolation frequency). In juices, 41% of the samples were positive for their occurrence. The highest relative density of *Alternaria* isolates was found in grape samples (87%). All detected strains were segregated into four morphological species-groups: *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. The most dominant species-group in grapes was *A. arborescens*, in barley and wheat *A. tenuissima*, followed by *A. alternata*, in juices only *A. alternata* and *A. arborescens* species-groups were detected and isolates of squashes were not classified to the species-groups. Randomly selected 67 isolates were analysed for the ability to produce mycotoxins alternariol (AOH), alternariol monomethylether (AME) and altenuene (ALT) by means of thin-layer chromatography. Of all tested isolates, AOH production was most frequently reported (70% of tested isolates). AME was produced by 60% and ALT by 49% of tested isolates. The largest share of the productive strains originated from the squashes, where all tested isolates produced ALT and AOH, followed by isolates of juices. From the viewpoint of individual species-groups, *A. arborescens* isolates and *Alternaria* spp. appeared to be the most productive in all mycotoxins tested.

Keywords: *Alternaria* spp.; cereals; grapes; juices; mycotoxin

INTRODUCTION

Genus *Alternaria* Ness is ubiquitous, including species found worldwide in association with a large variety of substrates. Many species are saprophytes, animal/plant pathogens or postharvest pathogens **(Polizzotto et al., 2012)**. They can infect a wide variety of crops in the field and in the postharvest stage causing considerable losses due to fruit and vegetable decay. They are the principal contaminating fungi in wheat, sorghum and barley. In addition to cereal crops, *Alternaria* species have been reported to occur in oilseeds such as sunflower and rapeseed, tomato, apples, citrus fruits, olives and several other fruits and vegetables. They grow at low temperature, hence they are generally associated with extensive spoilage during refrigerated transport and storage **(Ostrý, 2008)**.

In addition to spoiling a wide variety of foods, several *Alternaria* species are able to produce secondary metabolites considered as both phytotoxins, which play an important role in the pathogenesis of plants, and mycotoxins, which can be harmful to humans and animals **(Patriarca, Vaamonde and Pinto, 2014)**. *Alternaria* is one of the major mycotoxigenic fungal genera with more than 70 reported metabolites **(Escrivá et al, 2017)**. Alternariol (AOH), alternariol monomethylether (AME), tenuazonic acid (TeA), tentoxin (TEN) and altenuene (ALT) are the main *Alternaria* compounds thought to pose a risk to human health because of their known toxicity and their frequent presence as natural contaminants in food **(EFSA, 2011; Da Cruz Cabral, Fernández Pinto and Patriarca, 2016; Pose et al., 2010)**. However, food relevant *Alternaria* species are able to produce many more metabolites **(Ostrý, 2008)**, for which there are no reports on function, toxicity, and it is not known if they can be produced in the plants. Moreover, new compounds synthesized by this genus are constantly being discovered from *in vitro* fungal cultures in the search for new bioactive substances **(Patriarca, 2016)**.

Importantly, toxicological data are limited to the above mentioned major metabolites, and even these data are

incomplete, with neither good bioavailability studies nor long term clinical studies **(Andersen et al., 2015)**. Although little is known so far about their properties and toxicological mechanisms, bioavailability, and stability in the digestive tract, *Alternaria* toxins have been shown to have harmful effects in animals, including cytotoxicity, fetotoxicity, and teratogenicity. They are also mutagenic, clastogenic, and estrogenic in microbial and mammalian cell systems and tumorigenic in rats **(Ostrý, 2008; Logrieco, Moretti and Solfrizzo, 2009)**. Some *Alternaria* mycotoxins are known for induction of DNA strand break, sphingolipid metabolism disruption, or inhibition of enzymes activity and photophosphorylation **(Escrivá et al., 2017)**. AOH and AME are mutagenic and highly active in cell based assays, but data on whole animal studies is absent in the literature **(Prelle et al., 2013)**. In relation to human health, AOH and AME have been associated with high levels of oesophageal cancer in China, and TeA with a haematological disorder in Africa **(Patriarca, 2016)**. Only cytotoxic activity has been proved for ALT, and TEN is a phytotoxin causing chlorosis in the seedlings of many plants **(Da Cruz Cabral, Fernández Pinto and Patriarca, 2016)**.

Due to its high prevalence in many food commodities, and of their toxins in food and food by-products, there has been a bloom of scientific research on this fungal genus in recent years **(Patriarca, 2016)**. Its taxonomy is, up to the present time, under discussion, without a general consensus in the scientific community. There are no official methods for detection of its mycotoxins in food products, as well as not enough data of their natural occurrence in staples and commodities. The toxicity of their broad range of secondary metabolites needs to be thoroughly investigated. All these items should be covered in the next years to be able to develop sensible legislation on susceptible foods and to establish prevention strategies to control the health risk associated with this genus **(Patriarca, 2016)**. According to **Andersen et al. (2015)**, viewed in food safety perspective, the food safety agencies should prioritize some *Alternaria* metabolites (specifically alternariol, alternariol monomethylether, tenuazonic acid and its derivate, tentoxin and dihydrotentoxin, altenuene, altertoxins I – III, alternarienonic acid and pyrenochaetic acid) in their monitor/observation/review programme in order to establish if *Alternaria* contamination of food and feed products constitutes a risk and if statutory guidelines should be made. Additionally, cereal and cereal products should be monitored for 4Z-infectopyrone and phomapyrone A, since these commodities also can be contaminated with strains belonging to the *A. infectoria* species-group.

The purpose of this work was therefore to monitor the occurrence of the genus *Alternaria* in various food commodities of Slovak origin and to test the ability of isolates to produce selected known toxic metabolites of this genus.

Scientific hypothesis

The *Alternaria* genus is one of the most common genera of micromyctes occurring on food commodities. Most isolates have assumptions to produce many toxic metabolites.

MATERIAL AND METHODOLOGY

Samples

Various food commodities of Slovak origin were analysed for the occurrence of *Alternaria* spp. The list and commodity origin is shown in the Table 1. Totally we analysed 14 samples of grapes, 3 samples of barley, 2 samples of wheat, 17 samples of fruit, vegetable and fruit-vegetable juices, 6 samples of red kuri squash with macroscopically visible infection. The collection of grape samples took place in the time of their technological ripeness. The grapes were picked at random by the diagonal of the land and each sample was made up of around 3 kg of grapes. Samples were collected in sterile plastic containers, stored in a cool place and transported to the mycological laboratory for analysis up to 24 hours from the collection.

Samples of barley and wheat were collected during storage, at the latest 4 months after harvest. Samples were collected in paper bags in amount of about 500 g of weight. Only grains without visible damage were used for mycological examinations.

Juices obtained from the AgroBioTech SPU Research Centre were prepared using a juicer MAGIMIX Le Duo Plus XL at room temperature 21 °C. The raw materials used for the juice production were from the Botanical Garden SPU (fruit) and from the Department of Vegetable Production FZKI SPU (vegetables). Fruits and vegetables were processed maximum within 3 hours after the harvest. Sea buckthorn berries were harvested a day in advance and frozen at -40 °C. In a frozen state it was separated from the branches, thawed and after about 3 hours pressed. Prior the processing, all fruits were washed and used vegetables were peeled, washed, sliced and pressed. The samples purchased in the trading network were 100% juices, obtained by cold pressing and treated with flash pasteurization (high temperature and short time - HTST). Juices obtained from buffets were juiced and purchased at the points of sale shown in the Table 1.

The red kuri squashes (*Cucurbita maxima*) came from one garden of domestic production grown without the use of chemicals and stored in cold rooms for maximum 1 month. During storage, some pieces of pumpkins were molded. Visibly microbiologically damaged pieces were analysed for the presence of *Alternaria* species.

Mycological analyses

Mycological analyses were performed with respect to a particular commodity. Specific ways are given in the following subchapters. In all cases (except for red kuri squashes) we used DRBC agar plates (agar with dichloran, rose bengal and chloramphenicol) **(Samson et al., 2002a)** and cultivation lasted from 5 to 7 days in darkness at 25 ±1 °C. Grown micromycetes were classified into the genera. *Alternaria* spp. were isolated by re-inoculation on the identification nutrient media PCA – potato-carrot agar **(Samson et al., 2002a)**, cultured for 7 days at room temperature and natural light and identified through macroscopic and microscopic observation in accordance with accepted mycological keys and publications **(Lawrence, Rotondo and Gannibal, 2015; Woudenberg et al., 2013; Simmons, 2007; Andersen, Kroger and Roberts, 2002; Dugan and Peever, 2002; Andersen**

Kroger and Roberts, 2001; Simmons, 1994; Simmons and Roberts, 1993).

Grapes

Grape samples were investigated for a total and endogenous mycobiota. The total mycobiota was determined by the method of direct placing of grape berries on agar plates **(Samson et al., 2002b)**. Exactly 50 berries from each sample were placed on DRBC plates**.** The endogenous mycobiota was determined by the method of direct placing of superficially sterilized berries on agar plates **(Samson et al., 2002b)**. More than 50 pieces of undamaged berries from each sample were superficially sterilized with chloramine solution, prepared from 10 mL of distilled water and 5 g of chloramine. Sterilization was carried out 2 minutes.

Grains were rinsed 3 times with sterile distilled water and dried on sterile filter paper. Exactly 50 berries from each sample were placed on DRBC plates.

Barley and wheat

The barley and wheat grain samples were analysed on exogenous and endogenous mycobiota. The exogenous mycobiota was determined by using the plate dilution method. Homogenized sample of whole grain in amount of 20 g was added to 180 mL of peptone water containing 0.02% Tween 80. Prepared suspensions were shaken on a horizontal shaker for 30 minutes. Dilutions 10^{-1}, 10^{-2} and 10^{-3} were in the triple repetition surface-inoculated in amount of 0.1 mL on DRBC agar plates.

The endogenous mycobiota was determined by the method of direct placing of superficially sterilized grains on agar plates **(Samson et al., 2002b)**. More than 100 pieces of undamaged grains from each sample were superficially sterilized with chloramine solution, in the same way as in the case of grapes. Exactly 100 grains from each sample were placed on DRBC plates.

Table 1 Overview of food commodities of Slovak origin analysed for the occurrence of *Alternaria* spp.

Food commodity	Origin	Year
Grapes - Green Veltliner	Vrbové, Small Carpathian vineyard area	2018
Grapes - Feteasca Regala		
Grapes - Chardonnay		
Grapes - Rheinriesling		
Grapes - Welschriesling		
Grapes - Sauvignon		
Grapes - Pálava		
Grapes - Pinot Blanc		
Grapes - Irsai Oliver		
Grapes - Müller Thurgau		
Grapes - Dornfelder		
Grapes - Blaufränkisch		
Grapes - Alibernet		
Grapes - Cabernet Sauvignon		
Barley 1	Štefanov	2018
Barley 2	Hrochoť	
Barley 3	Kolíňany	
Wheat 1	Kolíňany	2018
Wheat 2		
Red kuri squash	Nitra	2018
Juice - Carrot 1	Nitra, ABT RC	2017
Juice - Purple carrot		
Juice - Yellow carrot		
Juice - Beetroot 1		
Juice - White grape		
Juice - Red grape		
Juice - Apple 1		
Juice - Pumpkin		
Juice - Sea buckthorn		
Juice - Apple 2	Trademark, after flash pasteurization	
Juice - Carrot 2		
Juice - Apple 3		
Juice - Apple + beetroot 1		
Juice - Apple 4	Trnava, buffet	
Juice - Carrot 3		
Juice - Beetroot 2	Nitra, buffet	
Juice - Apple + beetroot 2		

Note: ABT RC - AgroBioTech SPU Research Centre.

Fruit, vegetable and fruit-vegetable juices

Samples were mycologically analysed within 1 hour of their preparation and the plate dilution method was used. Undiluted sample (10^0) and dilutions 10^{-1} and 10^{-2} were in two repetitions surface-inoculated in amount of 0.1 mL on DRBC agar plates.

Red kuri squashes

The squashes from which the isolates were obtained were visibly infested with filamentous microscopic fungi. The isolates were simply obtained by mycological needle from many different rotten places of the squash and multiply inoculated directly into a PCA nutrient medium. Grown micromycetes belonging to the genus *Alternaria* were subjected to mycotoxicological analyses.

Mycotoxicological analyses

For the determination of toxigenicity we used thin-layer chromatography according to the **Samson et al. (2002a)**, modified by **Labuda et Tančinová (2006)**. A total of 67 randomly selected strains of the genus *Alternaria* have been re-inoculated on yeasts extract sucrose agar (YES), cultured in the dark at a temperature of 25 ±1 °C for 7 – 14 days and then tested for the ability to produce mycotoxins alternariol (AOH), alternariol monomethylether (AME) and altenuene (ALT) by means of thin-layer chromatography. From the grown colonies we cut squares of the approximate size 2 x 2 cm and placed them into the Eppendorf tube with 0.5 mL of extraction solution chloroform : methanol, 2:1 (Reachem, SR). The content of the tubes was stirred for 5 minutes by Vortex Genie® 2 (MO BIO Laboratories, Inc. – Carlsbad, CA). The obtained extracts were applied to silica gel chromatography plate (Alugram® SIL G, Macherey – Nagel, Germany). Subsequently, we used developing solution toluene:ethyl acetate:formic acid, 5:4:1 (toluene – Mikrochem, SR; ethyl acetate and formic acid – Slavus, SR). After elution and drying, the mycotoxins have been confirmed by visual comparison with the standards of mycotoxins (ALT, AME – Merck, Germany) under UV light with a wavelength of 254 nm and 366 nm. The identity of AOH was determined on the device QTrap 4000 LC/MS/MS with TurboIonSpray ESI source and 1100 Series HPLC system. Chromatographic separation was performed at 25 ±1 °C by Gemini 5 µ C18, 150 mm x 4.6 mm (Phenomenex, USA).

Statistical analysis

The obtained mycological results were evaluated and expressed in isolation frequency (Fr) and relative density (RD) at the genus and species level. The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam, Sharma and Bhadauria, 2009)**. These values were calculated according to **González et al. (1996)** as follows:

$$Fr\ (\%) = (ns / N)\ x100 \qquad RD\ (\%) = (ni / Ni)\ x100$$

Where: ns = number of samples with a species or genus; N = total number of samples; ni = number of isolates of a species or genus; Ni = total number of isolated fungi.

RESULTS AND DISCUSSION

The study focused on the monitoring of *Alternaria* spp. occurrence in various food commodities of Slovak origin, such as grapes, barley, wheat, various fruit, vegetable or fruit-vegetable juices and red kuri squashes. Analyses have shown that *Alternaria* is an important part of the mycobiota of these commodities. An overview of the occurrence of this genus is given in the Table 2. In all grape, barley and wheat samples the presence of representatives of this genus was detected. In juices, 41% of the samples were positive for their occurrence. The highest relative density of *Alternaria* isolates was found in grape samples. Also, **Swart and Holz (2017)** demostrated that the mature grape bunches were asymptomatic despite high levels of *A. alternata* recovered from triple-sterilized bunch tissue.

Isolated *Alternaria* strains were examined morphologically according to the extended keys and sporulation definitions in the identification manual by **Simmons (2007)**. Our isolates have been identified in so-called species-groups. **Simmons (1992)** defined informal species-group as a group of taxa with similar patterns of sporulation and sharing a high degree of conidial morphological characters. The species-group concept was defined, in order to simplify classification **(Patriarca, 2016)**. Following morphological analyses, strains in our study were grouped according to the colony characteristics and to their three-dimensional sporulation pattern on PCA (potato-carrot agar). All detected strains were segregated into four morphological species-groups: *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. *A. alternata* species-group isolates were characteristic by short primary conidiophores and chains that mainly branch from the conidial body. *A. arborescens* species-group isolates formed long distinct primary conidiophores bearing branching chains of conidia. The first conidium in a branch was often longer than the others. *A. infectoria* species-group isolates were typical by short primary conidiophores and conidia in branched chains with long secondary conidiophores. They formed smooth, light coloured conidia. *A. tenuissima* species-group isolates had conidia in unbranching chains, borne on short primary conidiophores. Formation of branching conidial chains was infrequent. If branching occurred in these strains, short simple secondary conidiophores would usually originate from the conidial body.

Recent phylogenetic studies have made significant changes to the systematic taxonomy (the accurate identification of a taxon or group of taxa) within *Alternaria* by elevating 26 clades to the subgeneric taxonomic status of section **(Lawrence, Rotondo and Gannibal, 2015)**. Due to lack of molecular variation, a molecular study of **Lawrence et al. (2013)** pooled the *A. arborescens* and *A. tenuissima* species-groups with *A. alternata* into one section, called *Alternaria* sect. *Alternaria*. This section consists of approximately 60 of the common small-spored species. *A. infectoria* species-group belongs to *Alternaria* sect. *Infectoriae*, consists of

approximately 25 species **(Lawrence, Rotondo and Gannibal, 2015)**.

Grapes

Within the total mycobiota of grapes samples we recorded *A. alternata*, *A. arborescens* and *A. tenuissima* species groups. With the highest isolation frequency (100%) we recorded isolates of the *A. arborescens* species-group. They represented the largest part (48%) of all *Alternaria* isolates.

A similar representation of *Alternaria* spp. was recorded within the endogenous mycobiota. *A. arborescens* species-group occurred with the highest isolation frequency (100%) and the number of isolates represented 59% of all *Alternaria* isolates. In addition, two isolates of the

Table 2 Isolation frequency (Fr) and relative density (RD) of *Alternaria* spp. isolated from various commodities of Slovak origin.

Commodity	Analysed mycobiota	Fr [%]	RD [%]
grapes	total	100	87
	endogenous	100	81
barley	exogenous	33	3
	endogenous	100	38
wheat	exogenous	0	0
	endogenous	100	52
juices	total	41	nd

Note: nd – not determined.

Table 3 An overview of the ability of tested *Alternaria* isolates obtained from food commodities of Slovak origin to produce mycotoxins altenuene (ALT), alternariol monomethylether (AME) and alternariol (AOH) by thin-layer chromatography (TLC) – according to commodities.

Source	*Alternaria* species group	Number of tested isolates	Number/% of positive tests		
			ALT	AME	AOH
Grapes	*A. alternata*	9	1/11	1/11	1/11
	A. arborescens	15	9/60	12/80	13/87
	A. infectoria	1	0/0	0/0	0/0
	A. tenuissima	16	4/25	9/56	11/69
	∑	**41**	**14/34**	**22/54**	**25/61**
Barley	*A. alternata*	2	2/100	2/100	2/100
	A. arborescens	1	0/0	0/0	0/0
	A. tenuissima	5	3/60	3/60	3/60
	Alternaria sp.	1	1/100	1/100	1/100
	∑	**9**	**6/67**	**6/67**	**6/67**
Wheat	*A. alternata*	1	1/100	1/100	1/100
	A. arborescens	1	0/0	0/0	0/0
	A. tenuissima	4	4/100	4/100	4/100
	∑	**6**	**5/83**	**5/83**	**5/83**
Juice - Beetroot	*A. alternata*	1	0/0	1/100	1/100
Juice - White grape	*A. arborescens*	1	1/100	1/100	1/100
Juice - Red grape	*A. alternata*	1	0/0	1/100	1/100
Juice - Sea Buckthorn	*A. alternata*	1	0/0	0/0	1/100
Juice - Apple	*A. arborescens*	1	1/100	1/100	1/100
	∑	**5**	**2/40**	**4/80**	**5/100**
Red kuri squashes	*Alternaria* spp.	6	6/100	3/50	6/100
Total number of tested isolates		**67**	**33/49**	**40/60**	**47/70**

Table 4 An overview of the ability of tested *Alternaria* isolates obtained from food commodities of Slovak origin to produce mycotoxins altenuene (ALT), alternariol monomethylether (AME) and alternariol (AOH) by thin-layer chromatography (TLC) – according to isolated species-groups.

Species-group	Nr. of tested isolates	Number/% of positive tests		
		ALT	AME	AOH
A. alternata	15	4/27	6/40	7/47
A. arborescens	19	11/58	14/74	15/79
A. infectoria	1	0/0	0/0	0/0
A. tenuissima	25	11/44	16/64	18/72
Alternaria spp.	7	7/100	4/57	7/100

A. infectoria species-group were identified.

Similar results we reached in 2011, where *Alternaria* spp. colonized grapes on the surface and inside with an isolation frequency of 100%. Their relative density was 44.9% (unsterilized grapes), 57.9% (sterilized grapes). With the highest isolation frequency and relative density occurred *A. tenuissima* species-group, followed by *A. alternata* and *A. arborescens* species-groups **(Mašková et al., 2013)**.

Our grape samples were without visible growth of micromycetes, but on the other hand the authors **Kakalíková, Jankura and Šrobárová (2009)** published the first report of the *Alternaria* bunch rot on grapevines in Slovakia, which occurred during unusually hot summer weather in 2007 and 2008.

Barley and wheat

The analysis of the exogenous mycobiota of barley and wheat has produced unexpected results. On the agar plates only relatively low numbers of micromycetes have grown (from 1.4 x 10^2 CFU.g^{-1} to 4.8 x 10^3 CFU.g^{-1}). No *Alternaria* spp. were isolated from the wheat surface. On barley, *Alternaria* spp. occurred with isolation frequency 33% and only *A. tenuissima* species-group representatives were isolated.

Within the endogenous mycobiota the situation was different. The isolation frequency of *Alternari*a spp. in barley and wheat was 100%. The most common isolated species-group in both commodities was *A. tenuissima* – 61% in barley samples, 63% in wheat samples. The second most isolated species-group was *A. alternata* (more than 20% in both commodities). Less than 10% represented *A. arborescens* and *A. infectoria* species-groups. **Andersen et al. (2015)** claimed, that the *A. infectoria* species-group was unique to cereals.

Tančinová and Labuda (2009) mycologically analysed wheat bran of Slovak origin and isolated *Alternaria* spp. with frequency 62.5%. Authors detected *A. alternata* and *A. tenuissima* species-groups. **Tančinová, Kačániová and Javoreková (2001)** reported, that the low amount of fungal contamination of wheat and the high frequency of *Alternaria* occurrence suggest good storage conditions in the examined agriculture farms.

Fruit, vegetable and fruit-vegetable juices

Totally, we analysed 17 samples of different juices, of which 7 samples (41%) were positive for the presence of *Alternaria* genus isolates. All isolates were grouped into two species-groups: *A. alternata* and *A. arborescens*.

Out of 9 juices prepared in AgroBiotech, juice from yellow carrot, beetroot, white and red grapes, pumpkin and sea buckthorn were positive for the presence of *Alternaria* isolates. The best results of mycological quality were found in 4 juices purchased on the merchant network, presented as 100% cold pressed juices. These juices were heat-treated by flash pasteurization, resulting in a zero occurrence of filamentous microscopic fungi. Out of 4 juices that were produced and subsequently purchased in the buffet, only in an apple juice the presence of the *Alternaria* spp. was detected.

In a previous study **(Mašková et al., 2013)** 100% of the grape stum samples were positive for the presence of *Alternaria* genus. Relative density of this genus was 6.35%.

Red kuri squashes

A total of 6 isolates of the genus *Alternaria* were isolated from moldy squashes. In this case, due to improper storage *Alternaria* spp. caused a visible damage to the squashes. Closer identification of the isolates has not been carried out. The isolates were only tested for the production of selected mycotoxins.

Mycotoxin production

A total of 67 isolates were randomly selected for the detection of the ability to produce mycotoxins altenuene (ALT), alternariol monomethylether (AME) and alternariol (AOH) by thin-layer chromatography (TLC). The results of the analyses are processed in the Table 3. Of all tested isolates, AOH production was most frequently reported (70% of tested isolates). AME was produced by 60% and ALT by 49% of tested isolates.

Similar results have been obtained by **Andersen et al. (2015)**. The analyses in the study showed that at least 75% of the Argentinean strains are able to produce compounds (potential mycotoxins) commonly associated with *Alternaria*, such as the AOHs, altertoxins (ATXs), tenuazonic acid (TeA) and tentoxins (TENs). Less commonly produced mycotoxin was ALT (69%).

Due to the ubiquitous occurrence of *Alternaria* ssp. their mycotoxins are frequently found in a large range of foodstuff commodities. For example, AOH and AME have been detected in fruit juices **(Lau et al., 2003)**, wines **(Asam et al., 2009)** and beer **(Prelle et al., 2013)**, ALT and TeA in apple juice **(Prelle et al., 2013)**.

The largest share of the productive strains originated from the squashes, where all tested isolates produced ALT and AOH, followed by isolates of juices. On the other hand, the lowest (but not omissible) number of isolates which showed the production potential originated from grapes. The grapes from which the samples we analysed in our study were later used for wine production and according to **Zwickel et al. (2016)**, the winemaking is known to be non-effective in eliminating mycotoxins.

From the viewpoint of individual species-groups, *A.arborescens* isolates and *Alternaria* spp. appeared to be the most productive in all mycotoxins tested. An overview of the species-groups production abilities is listed in the Table 4.

Only one isolate of the *A. infectoria* species-group (from grapes) was tested and as expected, the production of the analysed metabolites has not been confirmed. The same result was recorded in previous studies **(Mašková et al., 2011; Mašková et al., 2012)**. This suggests that isolates of *A. infectoria* species-group found in food are of lesser concern than members of the *A. alternata*, *A. arborescens* and *A. tenuissima* species-groups. However, other studies have shown that some members of the *A. infectoria* species-group are able to produce altertoxin-like metabolites **(Andersen et al., 2009; Andersen and Thrane, 1996)**.

However, *Alternaria* spp. produce a variety of other metabolites for which there are no reports on function,

toxicity or if they are produced in the plants **(Andersen et al., 2015)**.

CONCLUSION

Alternaria represents an ecologically diverse fungal genus recovered worldwide as ubiquitous agents of decay of natural and artificial substrates, as confirmed in our study. Representatives of the genus *Alternaria* appeared in monitored food commodities with the high isolation frequency, especially in grapes, barley and wheat samples. *Alternaria* isolates were detected in all tested samples of mentioned commodities. The highest relative density of *Alternaria* isolates was found in grape samples (87%). All detected strains were segregated into four morphological species-groups: *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. The most dominant species-group in grapes was *A. arborescens*, in barley and wheat *A. tenuissima*, followed by *A. alternata*, in juices only *A. alternata* and *A. arborescens* species-groups were detected and isolates of squashes were not classified to the species-groups.

In addition, the tested isolates have been shown to have a relatively high potential of the production of tested mycotoxins. Randomly selected 67 isolates produced mycotoxins alternariol (70% of tested isolates), alternariol monomethylether (60% of tested isolates) and altenuene (49% of tested isolates). From the viewpoint of individual species-groups, *A. arborescens* isolates and *Alternaria* spp. isolated from squashes appeared to be the most productive in all mycotoxins tested. However, food-relevant *Alternaria* species are able to produce many more metabolites including that known as emerging *Alternaria* mycotoxins described as potentially hazardous. Therefore, it is necessary to provide a toxicological risk assessment for agricultural products for human consumption, with regard to this genus.

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DIVERSITY OF MICROORGANISMS IN THE TRADITIONAL SLOVAK CHEESE

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ABSTRACT

The aim of the present study was to describe the microbial groups of the traditional Slovak cheese Parenica during rippening. The microbial group included the total bacterial count, coliform bacteria, enterococci, lactic acid bacteria, and microscopic filamentous fungi, which may affect the organoleptic characteristics of this product. A total of 42 cheese samples were collected from four different farms during three months. The total bacterial counts were cultivated on Plate count agar at 30 °C, lactic acid bacteria (LAB) on MRS, APT and MSE at 37 °C, coliform bacteria on VRBL at 37 °C. Gram-positive and Gram-negative isolates were identified by MALDI-TOF MS profiling. *Bacillus* sp. and *Enterococcus faecium* were the most frequently identified species of bacteria. *Candida kefyr* was the most distributed yeast according to microbiological methods. Lactic acid bacteria group was represented by *Lactobacillus helveticus, L. jensenii, L. alimentarius, L. crispatus, L. curvatus, L. fermentum, L. suebicus, L. delbrueckii* ssp. *lactis, L. paracasei* ssp. *paracasei, Lactococcus lactis* ssp. *lactis, Leuconostoc lactis* and *Le. mesenteroides* ssp. *mesenteroides* . This report describing the indigenous microbiota of the traditional raw milk cheeses from Slovakia. Our results provide useful information on occurrence of valuable microbial strain for the industrialization of producing of the traditional dairy products in Slovakia.

Keywords: diversity; microbiota; smoked and non-smoked cheese; mass spectrometry

INTRODUCTION

Cheese is one of the oldest fermented foods **(Oyetunji and Adebisi, 2018; Franke and Cwiková, 2019)**. The history of cheese lasts for thousands of years with changed related to the technical, social and economic conditions in different parts of the globe. Therefore the cheese fermentation process is attributed to culture and tradition **(Štefániková et al., 2019)**. Especially cheese making traditions are pronounced in rural households and village communities. There are around 1000 types of (most artisanal) cheese known worldwide. Cheeses are very different in textures, aromas, visual presentations and flavours that is attributed to microbial activity. The microorganisms are present and growing in cheese-making process in large numbers and which degrade the components of the curd **(Montel et al., 2014)**.

Diversity of sensory characteristics as flavor, smell, and texture of cheese are linked to microbiological activity in the product. Microorganisms show large metabolic capacities, and contribute the sensory parameters through the production of digestive enzymes and small molecules. Different cheese production technologies can lead to the growth of different microbial groups or microorganisms. The source and raw milk treatment (raw or pasteurized) used for cheesemaking can result in different microbiota of cheese. Changes in product characteristics during aging can significantly influence the cheese-associated microbiota with pH, salt, moisture, and temperature of cheese reveal the biggest impact on microorganisms **(Button and Dutton, 2012)**.

The microbiota of cheese rinds vary from simple to complex with *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, yeasts and moulds are represented. The abundance and diversity of the microorganisms depend on cheese ripening process, type of rind (bloomy, washed or natural) and the processing technology (soft, hard or semi-hard). The lactic acid bacteria (LAB) from starter cultures are predominant in the initial stages of cheese ripening. Later, during ripening, the yeasts and/or moulds colonize the cheese surface and the yeast cell count may reach about 6 – 8 $\log_{10}$ cfu.cm^{-2}. Those counts remains unchanged until the end of ripening. The growth of yeasts and/or moulds lead to lower pH of the cheese surface that favours the development of acid-sensitive, salt-tolerant bacteria. The final bacterial counts were 1 – 2 $\log_{10}$ higher than that for yeast counts **(Cogan et al., 2014)**.

From a technological point of view, the contamination of processed cheese by Gram-positive spore-forming rod-shaped bacteria of the genera *Bacillus, Geobacillus*, and *Clostridium* is the most significant problem in cheese-making. The shelf-life and microbiologicl quality of processed mostly are affected by the microbiological quality of the raw material, hygienic conditions during the manufacturing process, type of packaging materials and

storage conditions. Other parameters such as the water activity, pH, salts and emulsifying salts and fat in the product influence the overall quality of processed cheese as well **(Buňková and Buňka, 2017)**.

The aim of our study was to detect the microbiota of the traditional Slovak cheese „Parenica" and to identify the microorganisms by mass spectrometry.

Scientific hypothesis

Are there different bacteria and yeast species present in smoked and non-smoked traditional Slovak cheese?

Are microscopic filamentous fungi presented in traditional Slovak cheese?

MATERIAL AND METHODOLOGY

Samples of cheese

In our study, 42 samples of Slovak traditional cows cheese "Parenica" were examined during three moths. The cheese samples included non-smoked cheese (n = 21) and smoked cheese (n = 21). Additionally, 42 cow milk cheese samples from the western and middle Slovak producers were collected (Bánovce nad Bebravou, Liptovský Mikuláš, Červený Kameň, Važec). Samples were collected in sterilized sample containers and brought to laboratory in icebox for microbiological investigations. Samples were kept in a refrigerator (4 ±1 °C) until the testing began.

The primary dilution of cheese were made by adding of 5 g of sample to 45 mL of 0.87% sterile saline. Then, the serial dilutions (10^{-2} to 10^{-4}) were done and 100 µL of each dilution was plated out onto agars.

Detection of total bacterial counts (TCB)

Plate count agar (PCA, (Sigma-Aldrich®, St. Louis, USA) for total count bacteria enumeration was used. Inoculated plates were incubated for 48 – 72 h at 30 °C and then examined for the presence of bacterial colonies.

Isolation of coliform bacteria (CB)

Violet red bile lactose agar (VRBGA, Sigma-Aldrich®, St. Louis, USA) for enumeration of coliforms bacteria was used. Inoculated plates were incubated at 37 °C for 24 – 48 h and examined for the presence of typical colonies.

Isolation of enterococci (E)

Enterococcus selective agar (ESA, Sigma-Aldrich®, St. Louis, USA) for enumeration of enterococci was used. Inoculated plates were incubated at 37 °C for 24 – 48 h and examined for the presence of typical colonies.

Isolation of lactic acid bacteria (LAB)

MRS (Main Rogose agar), MSE (Mayeux, Sandine and Elliker) and APT (All Purpose TWEEN® agar, Sigma-Aldrich®, St. Louis, USA) agars were used for cultivation of lactic acid bacteria. Inoculated plates were incubated at 37 °C for 72 h anaerobically and then the bacterial growth was evaluated.

Isolation of microscopic fungi and yeasts (MFY)

Malt extract agar (Sigma-Aldrich®, St. Louis, USA) and acid base indicator bromocresol green (Sigma-Aldrich®, St. Louis, USA) (0.020 $g.l^{-1}$) were used for microscopic fungi and yeasts identification. Inoculated plates were incubated at 25 °C for 5 days aerobically and then the growth was evaluated.

The colonies from total bacterial counts, *Enterobacteriales*, enterococci and lactic acid bacteria were selected for further confirmation with MALDI-TOF. Selected colonies were cultured overnight on TSA agar (Tryptone Soya Agar) aerobically or anaerobically and used for identification.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial isolate was transferred into an Eppendorf tubes and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13,000 g, 2 min). After removal of supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial pellets were resuspended in 20 – 50 µL of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13,000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

Statistic analysis

All experiments were carried out in triplicate. Standard deviations were calculated for replications. The experimental data were subjected to analysis of variance (Duncan's test) at the 95% confidence level of 0.05 (software XL STAT, 2019).

RESULTS AND DISCUSSION

The results on non-smoked and smoked cheese bacterial counts, including coliform bacteria, enterococci, total bacterial count, lactic acid bacteria and microscopic filamentous fungi are shown in Table 1. Coliforms bacteria were not found in samples with exception of sample no.6, where coliform bacteria counts ranged from 3.17 to 3.43 log $CFU.g^{-1}$. Number of enterococci ranged from 2.09 till 4.32 log $CFU.g^{-1}$. Total count of bacteria in cheese samples ranged from 3.17 till 4.36 log $CFU.g^{-1}$. Microscopic filamentous fungi ranged from 2.26 till 3.38 log $CFU.g^{-1}$. How we can see in our results the worst results were found in sample number 6. The composition of the cheese surface microbiota has been studied for

several decades via the application of conventional, culture-based analyses **(Valdés-Stauber, Scherer and Seiler, 1997; Maoz, Mayr and Scherer, 2003; Viljoen, Khoury and Hattingh, 2003; Feurer et al., 2004; Mounier et al., 2005, Mounier et al., 2009; Callon et al., 2006; Florez and Mayo, 2006; Larpin et al., 2006; Lopandic et al., 2006; Rea et al., 2007; Goerges et al., 2008; Bleicher et al., 2010; Roth et al., 2010; Larpin-Laborde et al., 2011; Amato et al., 2012; Lavoie et al., 2012; Panelli et al., 2012; Gori et al., 2013; Cogan et al., 2014; Gkatzionis et al., 2014)**.

From the genus Acinetobacter 4 different strains were isolated: *A. baumannii, A. dijkshoorniae, A. johnsonii* and *A. junii. Acinetobacter* were found in 33.33% of samples (Table 2). Candida was also widelt distributed and *C. kefyr, C. parapsilosis, C. rugosa, C. guilliermondii, candida zeylanoides* and *C. lusitaniae* were isolated. From Enterobacteriaceae *Enterobacter cloacea, Hafnia alvei, Citrobacter braakii, C. freundii, Klebsiella oxytoca* and *Serratia ureilytica* were found. The most abundant genus was Enterococcus with three species were detected: *E. durans, E. faecium* and *E. faecalis*. Among yeasts *Kluyveromyces, Saccharomyces* and *Yarrowia* were identified.

Secondary microflora such as yeasts could be frequently isolated from different types of cheeses with counts from $10^4 – 10^6$ cfu.g^{-1} to $10^7 – 10^8$ cfu.g^{-1}. The presence of yeasts in cheese could be attributed to the favourable conditions for their growth and the wide distribution in dairy environment **(Wyder, 2003; Alessandria et al., 2010; Mirzaei, 2011)**. The role of the presence of yeast depends on the particular type of cheese **(Wyder, 2003; Colombo, Borgo and Fortina, 2009)**. Yeasts are important for bacterial development in cheese but their influence on bacteria may vary **(Mounier et al., 2009)**. Yeasts can cause sensory defects as excessive gas production and cheese blowing, bitter taste, fruit flavours, changes in acidity and texture profile **(Wyder, 2003)**. Nevertheless, the yeasts can alter the unique characteristics of several cheeses due to their lipolytic and proteolytic activities, formation of aromatic compounds, and degradation of the lactic acid **(Wyder, 2003; De Freitas et al., 2009)**.

Among lactic acid bacteria the following species were isolated: *Lactobacillus helveticus, L. jensenii, L. alimentarius, L. crispatus, L. curvatus, L. fermentum, L. suebicus, L. delbrueckii* ssp. *lactis, L. paracasei* ssp. *paracasei Lactococcus lactis* ssp. *lactis, Leuconostoc lactis* and *Leu. mesenteroides* subsp. *mesenteroides* (Table 2).

Lactic acid bacteria are the most studied microorganisms in milk fermentation **(Olson, 1990; Urbach, 1995; Maragkoudakis et al., 2006)**. The LAB in milk fermentation can be either as contaminants or content starter cultures. Milk itself serve as one of the natural source of LAB **(Delavenne et al., 2012; Wouters et al., 2002)**. Under spontaneous fermentations, the growth of LAB can not be controlled, but this procedure has been done during traditional cheese production. Backslopping also is often used in traditional cheese production and under this procedure fermented milk products as artisanal cheese klila **(Mennane et al., 2007),** kumis **(Chaves-López et al., 2011),** iben **(Ouadghiri et al., 2008)** and kurut **(Sun et al., 2010)** are produced. In general, the technology of milk fermentation is relatively simple and cost-effective. The large-scale production of standardized fermented milk products in controlled conditions require the industrial application of LAB as starter cultures. There are significant differences in the composition of industrally produced cheese with additional of starter culture from naturally fermented product with high microbial diversity in the traditional, naturally fermented products **(Widyastuti, Rohmatussolihat and Febrisiantosa, 2014)**.

Among staphylococci *Staphylococcus warneri, S. epidermidis, S. saprophyticus subsp. saprophyticus, S. sciuri subsp. carnaticus, S. cohnii, S. xylosus* and *S. hominis* were isolated.

Three *Staphylococcus* species were isolated previously with distribution of certain species in particular cheese: *S. equorum* was unique to ardrahan and milleens cheeses, *S. saprophyticus* to gubbeen cheese, and *S. epidermidis* to durrus cheese. *S. equorum* and *S. saprophyticus* have been

Table 1 Microbial counts in traditional Slovak cheese.

	CB	E	TCB	LAB	MFY
Non- smoked cheese 1	0.00 ±0.00	2.46 ±0.05^{def}	3.67 ±0.06^{cde}	3.77 ±0.16^{a}	2.72 ±0.14^{bc}
Smoked cheese 1	0.00 ±0.00	2.29 ±0.07^{fgh}	3.54 ±0.09^{ef}	3.47 ±0.20^{bcde}	2.30 ±0.13^{ef}
Non- smoked cheese 2	0.00 ±0.00	2.60 ±0.07^{d}	3.62 ±0.09^{de}	3.87 ±0.09^{a}	2.79 ±0.05^{b}
Smoked cheese 2	0.00 ±0.00	2.23 ±0.02^{hi}	3.17 ±0.05^{h}	3.52 ±0.06^{bcd}	2.46 ±0.14^{cde}
Non- smoked cheese 3	0.00 ±0.00	2.41 ±0.15^{efg}	3.74 ±0.15^{cd}	3.64 ±0.10^{abc}	2.52 ±0.07^{cd}
Smoked cheese 3	0.00 ±0.00	2.17 ±0.02^{hi}	3.24 ±0.04^{gh}	3.46 ±0.19^{bcde}	2.26 ±0.05^{f}
Non- smoked cheese 4	0.00 ±0.00	2.55 ±0.02^{de}	3.70 ±0.14^{cde}	3.70 ±0.17^{ab}	2.75 ±0.10^{b}
Smoked cheese 4	0.00 ±0.00	2.32 ±0.11^{fgh}	3.34 ±0.08^{gh}	3.40 ±0.17^{cdef}	2.43 ±0.09^{def}
Non- smoked cheese 5	0.00 ±0.00	2.44 ±0.11^{defg}	3.88 ±0.09^{c}	3.51 ±0.19^{bcd}	2.72 ±0.17^{b}
Smoked cheese 5	0.00 ±0.00	2.09 ±0.04^{i}	3.41 ±0.13^{fg}	3.23 ±0.09^{ef}	2.38 ±0.15^{def}
Non- smoked cheese 6	3.17 ±0.03^{b}	4.32 ±0.23^{a}	4.36 ±0.14^{a}	3.37 ±0.09^{def}	3.38 ±0.12^{a}
Smoked cheese 6	3.43 ±0.10^{a}	3.53 ±0.06^{b}	4.08 ±0.03^{b}	3.19 ±0.07^{f}	3.28 ±0.06^{a}
Non- smoked cheese 7	0.00 ±0.00	2.84 ±0.09^{c}	3.60 ±0.07^{de}	3.68 ±0.10^{ab}	2.75 ±0.09^{b}
Smoked cheese 7	0.00 ±0.00	2.27 ±0.06^{gh}	3.27 ±0.03^{gh}	3.50 ±0.14^{bcd}	2.37 ±0.05^{def}

Note: mean ± standard deviation; different letters in column mean that the differences were significant.

Table 2 Microorganisms isolated from non-smoked and smoked cheese "Parenica".

Sample	Isolated microorganisms
1N	*Enterococcus faecalis, Enterococcus faecium, Candida kefyr, Lactobacillus helveticus, Lactobacillus jensenii, Lysinibacillus boronitolerans Sphingomonas parapaucimobilis*
1U	*Candida kefyr, Kocuria kristinae, Lactococcus lactis* subsp. *lactis, Lactobacillus alimentarius, Lactobacillus jensenii, Lactobacillus crispatus, Lactobacillus curvatus, Lysinibacillus boronitolerans, Staphylococcus epidermidis, Staphylococcus saprophyticus* subsp. *saprophyticus, Streptococcus lutetiensis, Sphingomonas parapaucimobilis,*
2N	*Bacillus megatherium, Bacillus safensis, Bacillus* sp., *Candida kefyr, Candida parapsilosis, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Lysinibacillus boronitolerans, Staphylococcus saprophyticus* ssp. *saprophyticus, Staphylococcus warneri,*
2U	*Acinetobacter dijkshoorniae, Bacillus badius, Candida kefyr, Enterobacter cloacae, Enterococcus faecalis, Enterococcus faecium, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus suebicus, Lysinibacillus boronitolerans, Micrococcus luteus, Paenibacillus lactis, Raoultella ornithinolytica, Staphylococcus warneri*
3N	*Bacillus* sp., *Candida kefyr, Lactobacillus delbrueckii* subsp. *lactis, Leuconostoc lactis, Enterococcus faecium*
3U	*Bacillus* sp., *Candida kefyr, Lactobacillus delbrueckii* subsp. *lactis, Leuconostoc lactis, Staphylococcus hominis*
4N	*Enterococcus durans, Enterococcus faecalis, Klebsiella oxytoca, Lactobacillus oligofermentans, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Lysinibacillus fusiformis, Serratia ureilytica, Stenotrophomonas acidaminiphila, Staphylococcus sciuri* ssp. *carnaticus*
4U	*Bacillus safensis, Bacillus* sp., *Candida kefyr, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Lysinibacillus boronitolerans, Proteus hauseri, Staphylococcus saprophyticus* subsp. *saprophyticus,*
5N	*Bacillus* sp., *Candida parapsilosis, Cupriavidus metallidurans, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Kluyveromyces lactis, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Leuconostoc mesenteroides* subsp. *mesenteroides, Micrococcus luteus, Staphylococcus epidermidis*
5U	*Bacillus* sp., *Bacillus safensis, Enterococcus durans, Lactobacillus paracasei* subsp. *paracasei, Sphingomonas parapaucimobilis*
6N	*Acinetobacter baumannii, Acinetobacter junii, Bacillus infantis, Bacillus sp., Candida parapsilosis, Candida rugosa, Citrobacter braakii, Chryseobacterium ureilyticum, Enterococcus faecalis, Enterococcus durans, Hafnia alvei, Leuconostoc lactis, Paenibacillus lactis, Raoultella ornithinolytica, Staphylococcus cohnii, Staphylococcus saprophyticus* subsp. *saprophyticus, Staphylococcus xylosus, Yarrowia lipolytica*
6U	*Acinetobacter johnsonii, Bacillus infantis, Bacillus safensis, Bacillus subtilis, Candida parapsilosis, Candida rugosa, Candida zeylanoides, Citrobacter freundii, Enterococcus durans, Enterococcus faecalis, Enterobacter cloacae, Hafnia alvei, Kluyvera cryocrescens, Lactobacillus suebicus, Raoultella ornithinolytica, Saccharomyces cerevisiae*
7N	*Acinetobacter baumannii, Alcaligenes faecalis* subsp. *faecalis, Bacillus* sp., *Candida guilliermondii, Candida zeylanoides, Enterococcus faecium, Lactobacillus paracasei* subsp. *paracasei, Sphingomonas parapaucimobilis, Streptococcus lutetiensis*
7U	*Bacillus infantis, Bacillus* sp., *Enterococcus faecalis, Candida guilliermondii, Candida lusitaniae, Enterococcus faecium, Gluconacetobacter liquefaciens, Hafnia alvei, Streptococcus lutetiensis, Sphingomonas parapaucimobilis*

isolated previously from the surfaces of traditional French cheeses **(Irlinger et al., 1997)**.

Bacillus megatherium, Bacillus sp., B. infantis, B. safensis, B. subtilis, B. badius were representatives of the genus Bacillus.

CONCLUSION

This study was designed to evaluate coliform bacteria, enterococci, yeasts and LAB population's in Slovak traditional non-smoked and smoked cheese. Our results show that the traditional Slovak cheese contains very diverse microbiota. In our study, the mass spectrometry method allowed the accurate identification of microorganisms and this method was reliable and easy done for identification in comparison with molecular methods.

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FACTORS INFLUENCING INTEREST OF SLOVAK CONSUMERS' IN ORGANIC DAIRY PRODUCTS

Iveta Ubrežiová, Tatiana Kráľová, Jana Kozáková

ABSTRACT
The aim of the article is to analyse the dependency of selected factors (age category, level of income and gender) on consumers' willingness to buy organic dairy products. The primary research based on the electronic interview survey carried out on the sample of 203 Slovak respondents of all ages, in different social situations and with different views on the issue. The questionnaire consisted of seven sorting questions and six questions addressed consumers' perception of organic dairy products and the reasons for their purchase or rejection. For evaluation the Chi square test of square contingency was used. Results were sorted into three parts. The aim of the first part of research was to find out whether there is a dependency between the age category of the respondents and whether they are buying organic dairy products. Results showed that the age category of the respondents and purchase of organic dairy products are independent. The second part of the research based on the examination of the dependency between the level of income of the respondents and their willingness to pay for organic products. In this case we confirmed the dependency between the customers' average income per month and their willingness to pay for organic dairy products. Last but not least, the dependency between the reasons that would discourage consumers from buying organic dairy products and their gender was examined. The results of analysis clearly showed that these two variables are independent. Despite generally persisted opinions that food of daily consumption in bio quality (organic) is mainly bought by women of specific age categories (joung independent woman after graduation, mothers on maternity leave) we can confirm just the significance of the impact of customers' average income per month on their willingness to pay for these high quality and therefore expensive products.

Keywords: organic dairy products; Slovakia; age category; gender; level of income

INTRODUCTION
Production of food is one of the main mission of the manufacturing and processing industry of every country. According to the Food and Agriculture Organization of the United Nations (FAO): "all people, at all times, should have physical, social, and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life". This principle is fundamental for food security and it concerns both the production of foodstuffs of plant and animal origin as well **(Cafiero et al., 2016)**. However, ensuring food security and sufficient consumption of different types of food is often not just the problem of developing countries. In Slovakia, present consumption of milk and dairy products is at the 70% of the recommended level. Consumption of cow´s milk is alarmingly low, covering only about 50% of the recommended milk consumption, while consumption of cheese, curd and sour-milk products has risen in recent years and slightly exceeds the recommended consumption intakes **(Kubicová, Predanocyová and Kádeková, 2019)**. Problem is not just on the demand side, there are some issues on the supply side as well. According to **SOSR (2019)**, milk production in Slovakia slightly decrease in last decade. While in 2007 we produced 96 422 000 ton per year in 2017 it was just 82 589 000 ton, which is just over 0.5% of total milk production in European Union (EU). Current situation is a consequence of previous cases and production quotas. In 1984, after several years of large overproduction of milk and dairy products (such as skimmed milk powder or butter), the Common Agricultural Policy (CAP) announced quotas for milk production in the European Union. After the CAP control in 2009, EU decided for a change and prepared the end of the milk quotas in so-called "soft landing" way. The impacts of quota removal on world markets were limited and demand context for meat and dairy products had a much more significant impact on agricultural markets than this removal alone **(Salou et al., 2017)**. However, at the time of prodution quotas, Slovakia did not produced even its maximum allowed production volumes. The reason is comparative disadvantage compared with other EU countries, in which milk production is more supported. Therefore, dairy production in the country is not increasing at the same pace as in other EU countries, despite fact highlited by **Michalek, Ciaian and Pokrivčák (2018)** who states that

the Rural Development Programme (RDP) supports the establishment of POs in Slovakia with the aim to enhance the bargaining position of farmers in the supply chain, and thus to contribute to the improvement of their value added and economic viability. Consequently, out food producers also have a significant problem in establishing at foreign markets. **Mura and Buleca (2014)** for example states no significant correlation between acceptance of internationalization as the current trend in a globalizing world, and the development of the volume of sales to foreign markets in the Slovak food producers. They also confirmed a difference in perception of the factors that give rise to international business activities of business subjects.

Despite mentioned problems, the Slovak market with milk and dairy products is still affected by globalization and development on international markets. Our producers have to face strong international competition. Even if they are not active at foreign markets, they still have to challenge with foreign competititors, who penetrate our domestic market. **Ubrežiová and Horská (2011)** confirmed, that one of the main global trend which affected competition internationaly is implementation of Corporate Social Responsibility (CSR). **Carroll and Buchholtz (2008)** state, that the main idea of CSR is that the enterprise has not only legal and economic obligations, but also other responsibilities to society that extend these obligations. CSR is taking into account actions of company on society. Corporate Social Responsibility includes economic, legal, ethical and philanthropic expectations that society has placed to the organization in a given point of a time **(Ubrežiová and Gurská, 2012)**. In the EU, the Lisbon Summit in 2000 was a fundamental step towards the implementation of CSR, with a commitment to support across the EU and make it capable of sustainable economic growth **(Madrakhimova, 2013)**. In addition, milk and dairy products have a great potential to reach not just the sustainable economic growth, but ecological as well. The main instrument of implementation of ecological principles into primarily agricultural production is organic farming since it **(Wachter and Reganold, 2014)** relies on the integration of a diversity of farm components, the cycling of nutrients and other resources, and stewardship of soil and environment. For a food product to be certified as organic, it must satisfy a comprehensive series of requisites on aspects of production that, in the case of milk, range from livestock management and feed to the labeling of the final product **(Ferreiro, Gayoso and Rodríguez-Otero, 2015)**. Consumer´s reaction on these products are positive, since their characteristics fulfill the need for more naturalness and sustainability **(Janssen, 2018; Sogari et al., 2015)**. They also believe that organic food is healthier, has a better taste, is better for the environment, and shows more respect for animal welfare, as well as enshrining important human and cultural value **(Hamzaoui Essoussi and Zahaf, 2009)**. In addition, **Kurajdová, Táboreckà-Petrovičová and Kaščáková (2015)** and **Benda-Prokeinová et al. (2017)** generally recommends to emphasize product attribute together with positive health impacts and demonstrate practical utilization of milk in households (e.g. through recipes on the package) to reinforce segment of purchasers.

Scientific hypothesis

According to previous data analysis we identified 3 main factors influencing the interest of Slovak consumers' in organic dairy products (age category, level of income and gender).

Factor 1 – Age category

The first part of research based on the expectation that there is a dependency between the age category of the respondents and their willingness of buying organic dairy products. According to this expectation the hypotheses was set:

- Hypothesis H0: The age category of the respondents and purchase of organic dairy products are independent.
- Hypothesis H1: There is a dependency between the age category of the respondents and purchase of organic dairy products.

Factor 2- Level of income

This part of research based on the expectation that there is a dependency between the level of income of the respondents and their willingness to pay for organic products. According to this expectation, following hypotheses was set:

- Hypothesis H0: The average income per month and the willingness to pay for organic dairy products are independent.
- Hypothesis H1: There is a dependency between the average income per month and the willingness to pay for organic dairy products.

Factor 3 – Gender

The last part of the research was connected with the expectation that there is a dependency between the reasons that would discourage consumers from buying organic dairy products and their gender. According to third expectation, the hypotheses was set:

- Hypothesis H0: The gender of respondents and the reasons that would discourage consumers from buying organic dairy products are independent.
- Hypothesis H1: There is a dependency between the gender of respondents and the reasons that would discourage consumers from buying organic dairy products.

MATERIAL AND METHODOLOGY

The primary research was conducted from February 19, 2019 to March 20, 2019, attended by 203 respondents from Slovakia. The results were obtained from a survey conducted in electronic form. It was filled by respondents of all ages, in different social situations and with different views on the issue.

The questionnaire consisted of 13 questions, divided into 2 categories:

- Questions 1 – 7 dealt with the characteristics of the respondents (their gender, age, status, etc.).
- Questions 8 – 13 addressed consumers' perception of organic dairy products and the reasons for their purchase or rejection.

For the collection of data was used Google form of questionaire. The results of the survey were processed using Microsoft Excel 2016 MSO: 16.0.4266.1001.

Statistic analysis

Chi square test of square contingency is used to summarize the existence of a statistically significant relationship between qualitative characters. The test consists of a comparison of empirical and theoretical frequences, this means, what would emperical frequences be, if characters A and B were independent. The first step is formulation of hypotheses, followed by calculation of test statistics, and the last step is evaluation of results and acception of null or alternate hypothesis **(Moore, 2006)**.

Calculation of theoretical frequencies is based on the theorem of independence of random characters A and B:

$$(a_i b_j)_0 = \frac{(a_i) * (b_j)}{\mathrm{n}}$$

Calculation of test statistics:

$$x^2 = \sum_{i=1}^{m} \sum_{j=1}^{k} \frac{((a_i\, b_j) - (a_i\, b_j)_0)^2}{(a_i\, b_j)_0}$$

Where:
(aibj) – the empirical frequency,
(aibj) 0 – the theoretical frequency,
m – number of categories of the first character,
k – number of categories of the second character,

Evaluation of the hypotheses:

After calculation of test statistics for the respective Chi square with selected degrees of freedom and compare it with the critical value. To calculate the critical value, we chose a level of significance at 0.05 level (5% level).
X2 < critical value → H0 is accepted, there is independence between characters examined.
X2 > critical value → H0 is rejected, there is dependence between characters examined.

RESULTS AND DISCUSSION

The composition of respondents by gender shows that three quarters of respondents were women who are probably more concerned with this issue and also buy food products more often than men. Specifically, 151 women and 52 men answered to the questions in the questionnaire.

The majority of respondents were between 26 and 40 years old (92 respondents, which is 42%). The second largest age group is 18 – 25 years (78 respondents, which is 39). This group consists mainly of students. The third largest group is people aged 41 – 60 years (25 respondents, which is 12%). The remaining two age groups are under 18 and over 60 (both only 4 respondents).

The number of respondents living in the city or in the countryside is more or less balanced (116 live in the city which is 57% and 87 in rural areas people which is 43%).

More than half of respondents had a university degree (110 respondents, which is 54%). The second largest group consists of respondents with secondary education with school-leaving exam (78 respondents, which is 38). Less than 3% belong to the group with specialized secondary education (6 respondents), 5 respondents had secondary education without school-leaving exam and 4 respondents only primary level of education.

According to the status, people from all possible categories appeared among results. Almost half of respondents were employed people (97 respondents, which is 48%). They are followed by students with (55 respondents, which is 27%), entrepreneurs and housewifes (both 18 respondents, which is 9%). Groups, with share less than 2%, were also employed students (5 respondents), pensioners (4 respondents), unemployed (3 respondents), invalids (2 respondents) and one voluntary unemployed person.

Most respondents, come from a household with 4 members (69 respondents). The second largest is the group with 3 household members (51 respondents). They are followed by a group with two members (42 respondents, which is 21%), and more than 4 members (26 respondents, which is 13%). The remaining 7% are people living alone (15 respondents).

Over half of respondents (123 respondents, which is 61%) have an average monthly income per household member from 500 – 1,000 EUR. Following groups are people with income less than 500 EUR (33 respondents), from 1,001 – 1,500 EUR (32 respondents), from 1,501 – 2,000 EUR (7 respondents) and income higher than 2,000 EUR per month (8 respondents).

Research based on the statistical verification of the influence of three choosen factors on the interest of Slovak consumers' in organic dairy products, or their willingness to buying these products.

We included age category as a first analysed factor and statistically connect it with the answers to the question no. 8 (Do you buy organic (BIO, ECO) dairy products?). The aim of this part of the research was to find out whether there is a dependency between the age category of the respondents and whether they are buying organic dairy products. The results showed that $\chi 2 = 8.89 < \chi$ таб. = 15.51. Therefore, we have accepted null hypothesis, and thus, the age category of the respondents and purchase of organic dairy products are independent (Table 1). Since customers' willingness to buy organic products is not connected with their age and thus wiew of life associated with generation, we were looking for other couses.

Respondents who do not buy organic dairy products have had the opportunity to say (question no. 9) why they chose to do so (If you answered "No" to previous question, please state the reason). Almost half of respondents do not trust the quality of organic dairy products on sale. 31% of respondents consider these dairy products to be more expensive and cannot afford such expenses. 15% think that there are few such products on the market. 4 respondents do not see the difference between organic and non-organic dairy products. One respondent has even added own version of answer and he is not buying it due to a short shelf life, he does not want to throw away unconsumed food (Figure 1).

Table 1 The dependency between age category of the respondents and their willingness of buying organic dairy products – Factor 1.

Empirical frequencies						
Age Category	18 – 25	26 – 40	41 – 60	Less than 18	More than 60	Total
Yes, some dairy products	35	58	14	2	2	111
Yes, all dairy products	1	4	1	0	0	6
No	42	30	10	2	2	86
Total	78	92	25	4	4	203
Theoretical frequencies						
Age Category	18 – 25	26 – 40	41 – 60	Less than 18	More than 60	Total
Yes, some dairy products	42.65024631	50.30541872	13.66995074	2.187192118	2.187192118	111
Yes, all dairy products	2.305418719	2.719211823	0.738916256	0.118226601	0.118226601	6
No	33.04433498	38.97536946	10.591133	1.694581281	1.694581281	86
Total	78	92	25	4	4	203
Statistics						
Age Category	18 – 25	26 – 40	41 – 60	Less than 18	More than 60	Total
Yes, some dairy products	1.372237527	1.176942417	0.007968757	0.016020947	0.016020947	-
Yes, all dairy products	0.739179403	0.603269794	0.092249589	0.118226601	0.118226601	-
No	2.427161451	2.06687603	0.03299347	0.055046397	0.055046397	-
Test statistics	8.897466329					
Critical value	15.50731306					

Note: Source: Own processing.

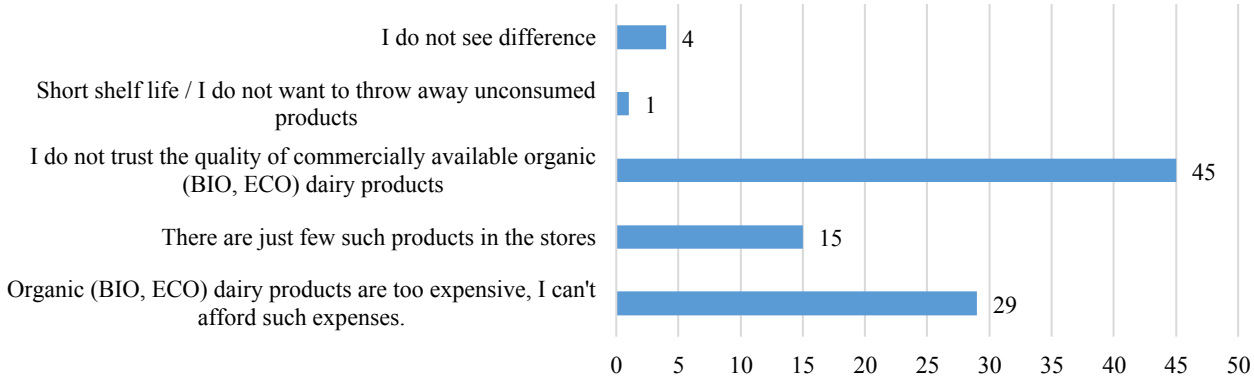

Figure 1 Reasons why customers do not buy organic dairy products (number of answers).
Note: Source: Own processing.

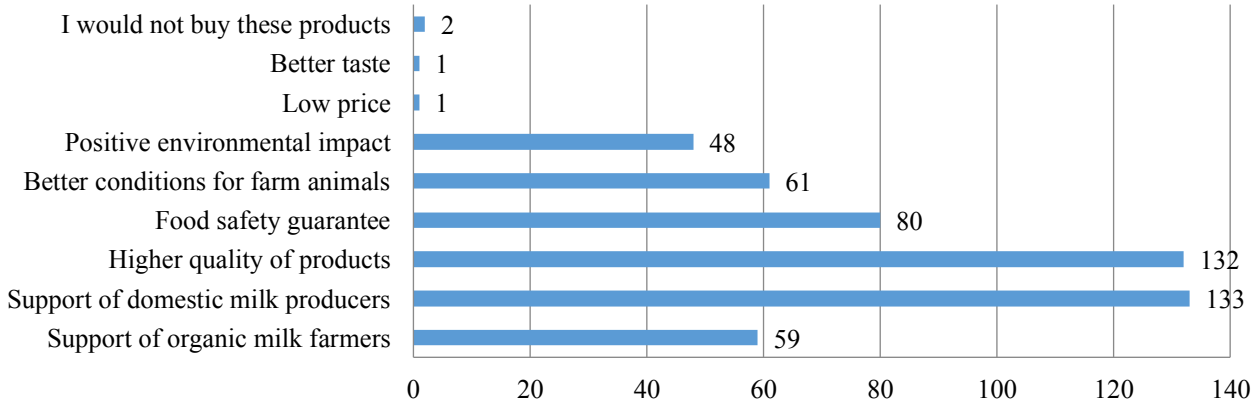

Figure 2 Reasons why customers buy organic dairy products (number of answers).
Note: Source: Own processing.

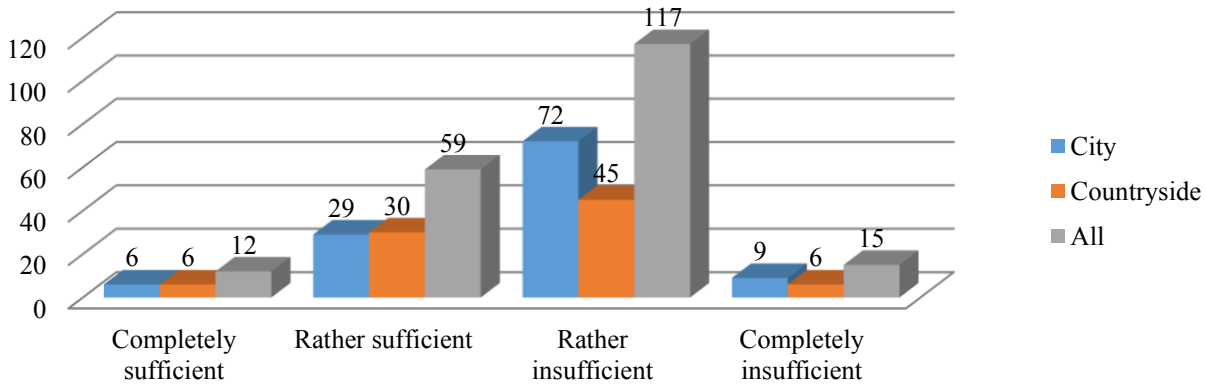

Figure 3 Customers consideration of suffiency of the range of organic dairy products in their region (number of answers).
Note: Source: Own processing.

Table 2 The dependency between the level of income of the respondents and their willingness to pay for organic products – Factor 2.

Empirical frequencies						
Income range	0.2	0.4	0.6	Less than 20%	More than 60%	Total
1000 – 1500€	19	3	0	10	0	32
1500 – 2000€	4	1	1	1	0	7
500 – 1000€	55	12	1	54	1	123
Less than 500€	8	5	0	20	0	33
More than 2000€	4	4	0	0	0	8
Total	90	25	2	85	1	203
Theoretical frequencies						
Income range	0.2	0.4	0.6	Less than 20%	More than 60%	Total
1000 – 1500€	14.18719212	3.9408867	0.315270936	13.39901478	0.157635468	32
1500 – 2000€	3.103448276	0.862068966	0.068965517	2.931034483	0.034482759	7
500 – 1000€	54.5320197	15.14778325	1.21182266	51.50246305	0.60591133	123
Less than 500€	14.63054187	4.064039409	0.325123153	13.81773399	0.162561576	33
More than 2000€	3.54679803	0.985221675	0.078817734	3.349753695	0.039408867	8
Total	90	25	2	85	1	
Statistics						
Income range	0.2	0.4	0.6	Less than 20%	More than 60%	Total
1000 – 1500€	1.632678229	0.2246367	0.315270936	0.862250072	0.157635468	-
1500 – 2000€	0.259003831	0.022068966	12.56896552	1.272210953	0.034482759	-
500 – 1000€	0.004016091	0.654124715	0.037025912	0.121114417	0.256317834	-
Less than 500€	3.004952646	0.21555456	0.325123153	2.766040586	0.162561576	-
More than 2000€	0.057909141	9.225221675	0.078817734	3.349753695	0.039408867	-
Test statistics	37.64714603					
Critical value	26.2962276					

Note: Source: Own processing.

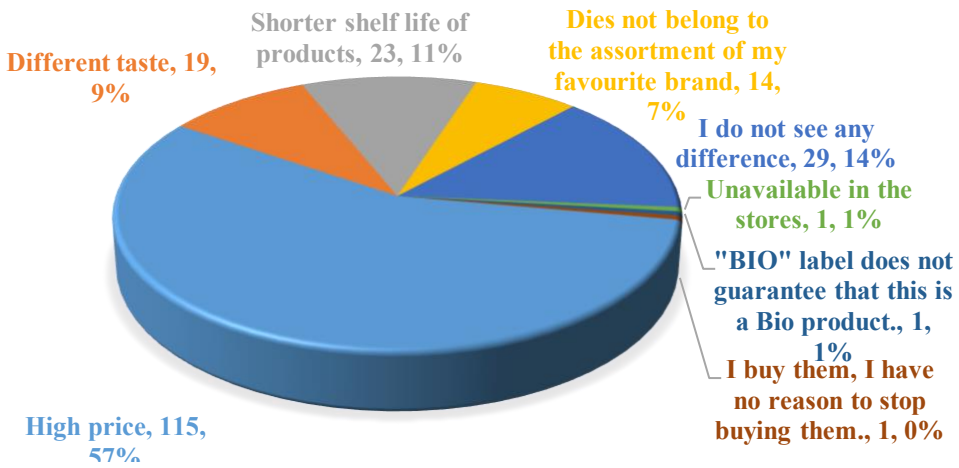

Figure 4 Reasons which would discourage customers from buying organic dairy products (number of answers).
Note: Source: Own processing.

Table 3 The dependency between the reasons that would discourage consumers from buying organic dairy products and their gender – Factor 3.

Empirical frequencies			
Reasons	Male	Female	Total
"BIO" label does not guarantee that this is a Bio product.	0	1	1
Different taste	2	17	19
Shorter shelf life of products	9	14	23
I buy them, I have no reason to stop buying them	0	1	1
Unavailable in the stores	0	1	1
Does not belong to the assortment of my favourite brand	6	8	14
I do not see any difference	9	20	29
High price	25	90	115
Total	51	152	203
Theoretical frequencies			
Reasons	Male	Female	Total
"BIO" label does not guarantee that this is a Bio product.	0.251231527	0.748768473	1
Different taste	4.773399015	14.22660099	19
Shorter shelf life of products	5.778325123	17.22167488	23
I buy them, I have no reason to stop buying them	0.251231527	0.748768473	1
Unavailable in the stores	0.251231527	0.748768473	1
Does not belong to the assortment of my favourite brand	3.517241379	10.48275862	14
I do not see any difference	7.285714286	21.71428571	29
High price	28.89162562	86.10837438	115
Total	51	152	203
Statistics			
Reasons	Male	Female	Total
"BIO" label does not guarantee that this is a Bio product.	0.251231527	0.084294789	
Different taste	1.611376311	0.540659157	
Shorter shelf life of products	1.796227936	0.602681742	
I buy them, I have no reason to stop buying them	0.251231527	0.084294789	
Unavailable in the stores	0.251231527	0.084294789	
Does not belong to the assortment of my favourite brand	1.752535497	0.588021779	
I do not see any difference	0.403361345	0.135338346	
High price	0.524191686	0.175880105	
Test statistics	9.13685285		
Critical value	14.06714045		

Note: Source: Own processing.

It is very interesting that despite the "trust" was the biggest issue for people who do not buy organic dairy products, it was also one of the main reasons for people who buy them. We have identified the most common reasons for purchasing organic dairy products (question no. 10: For what reasons would you buy organic (BIO, ECO) dairy products?). In case of this question, 133 respondents chose Support of domestic milk producers (which is 65.5%), 132 respondents chose a Higher quality of these product and the third most important factor was the Food Safety Guarantee (almost 40%). About 30% of respondents chose better conditions for farm animals and support of organic milk farmers and 23.5% chose positive environmental impact.

Two respondents did not choose any of the options and two would not buy them under any circumstances (Figure 2).

Reasons of reduced interest in buying organic dairy products could be on the side of demand and on the side of supply as well. In connection with this we included question no. 11 (Do you consider the range of organic (BIO, ECO) dairy products in your region to be sufficient?) into analysis.

Surprisingly, 75% of respondents consider the range of organic dairy products insufficient in their region. Just the remaining quarter thinks that there is a sufficient range of organic dairy products in their region, and there is no need to improve it (Figure 3). But we find differences between people living in cities and in the countryside. While 35% of people living outside the city consider the product range to be sufficient and 7% rather sufficient, in cities these figures were lower – 25% sufficient and 5% rather sufficient. This means a total difference by 12%. The product range is considered to be insufficient in the countryside by 52% of respondents, which is a difference by 17% compared to a sufficient one. For people living in cities, it is 62% and the difference is 37%.

Considering fact, that price is a factor with strong impact on demand generally, we included it into analysis as well. Higher price of organic products can potentionally discourage price elasticity for consumers to the extent that they completely stop buying products or reduce their consumption considerably. In view of this, we included question no. 12 (How much are you willing to pay for organic (Bio, Eco) dairy products in addition to the price of the goods?). The results was expected, if respondents had a choice how much they would pay in addition to the price of dairy products for being organic, they would choose the lowest possible values. Up to 42% of them would only pay less than 20% and 45% of respondents identified 20% as their border. Just 12.5% of respondents would invest at least 40% extra for organic products, only one percent would invest 60% in addition to the price of the products and only one would invest more. Despite the fact that men and women tend to different price elasticity, we can not confirm this in our samle, but 60% or more than 60% in addition to the price of the products are willing to pay mainly men. Another fact is that most of these respondents are already buying organic products, although not all. For these products, the price is already 50% higher. Therefore, their willingness to pay for these products should be higher than they showed in the questionnaire. Besides, the level of income is likely important factor.

In connection with previous outcomes, the level of income was included as a second factor. The goal was to examined the dependency between the level of income of the respondents and their willingness to pay for organic products. The results showed in Table 2 clearly states that $\chi 2 = 37.64 > \chi$ таб. = 26.29. Therefore, we have accepted alternative hypothesis, and thus, we can confirm the dependency between the customers' average income per month and their willingness to pay for organic dairy products. Despite the undenaiably high impact of price elasticity, this is not the only important aspect. Out task was aslo to find another features and therefore we asked respondents (question no.13) What would discourage them from buying organic (BIO, ECO) dairy products. As shows Figure 4, more than a half of respondents (115 respondents), could be discouraged by high price of organic products. 11% of respondents would discourage shorter shelf life of such products, 9% different taste and 7% would not buy organic products that does no belong to the range of their favourite brand. There were also some minor opinions among answeres: organic dairy products are unavailable in the storesa and BIO label does not guarantee that it realy is organic product. Of course there are some loyal customers as well which buying these products ans have to reason to stop. But, there are still 29 respondents which does not see any difference between organic and non-organic products. Similar connection between consumers income and food consumption highlited also **Benda-Prokeinová and Hanová (2016)** as well as **Kozelová et al. (2018)**, who state that the main impact factors affecting food consumption in Slovakia are the consumers' income and food prices despite fact that The main criterion of food selection should not be connect with money, but health concerns. On the other side, according to **Nagyová et al. (2019)** Slovak consumers are gradually becoming more rational, especially in terms of information literacy. As there is a wide range of food products in store, many respondents try to choose the food according to their own mind. For these reasons, they seek to obtain the necessary information by studying, reading information on product labels, and in many cases also via Internet.

Except for any other differences, diverse gender approach is also visible in consumers' decision making. Therefore, we wanted to find out whether there is a dependency between the reasons that would discourage consumers from buying organic dairy products and their gender (Factor 3).

The results of analysis showed that $\chi 2 = 9{,}12 < \chi$ таб. = 14,07 (Table 3). Therefore, we have accepted null hypothesis, and thus, the gender of respondents and the reasons that would discourage consumers from buying organic dairy products are independent. This outcome does dot explain the differences in consumers' willingness to buy organic products, but it brings beneficial result that gender oriented approach in marketing of these products would not be effective.

Our outcomes aslo suggests that Slovak consumers become more focused on products quality and their range. This puts pressure on the domestic food industry. There is only one major producer of organic dairy products on the

market - Rajo. Similar products are already offered by market follower Tami. It added the Bio product line to its portfolio. Both offer Bio Milk, Bio Yoghurt and Bio Butter as well. Since companies are using organic milk produced in Slovakia and approaching high quality, these products also have a higher price to compensate higher costs of production. Enforcement in this market segment is still very difficult also because of necessity of extra investments, the creation of new contracts with producers of organic milk, the creation of new contracts with intermediaries who will buy organic products, staff training, and the need to obtain a certificate for the production of organic dairy products. Transporting milk from new suppliers and all additional ingredientsin BIO quality are also more expensive. Investments required for such project can be found in foreign capital, but it shifts the possibility of gaining (extra) profit to the distant future. Despite ther poor domestic competitive environment, our producers must face very strong foreign competition. There are not just producers from Austria (Biotrend), which is country with very strong organic rear, but also strong piece competitors from Czech Republic (Hollandia) and Germany (Milbona). But then again, those who can enforce in this environment will increase its market share focusing on the new target group and can gain and extra competitive advantage by demonstrating their social responsibility in the eyes of customers.

CONCLUSION

The idea of organic production based on the implementation of strict rules in the agricultural primarily production (crop and dairy) with the goal of production high quality products and subsequently high quality food, labeled as BIO or Organic by state control organization. Production under this strict control is more demanding and expensive in comparison with traditional conventional agricultural production. Products in this system have higher quality, but higher price as well. There are several organic producers in Slovakia, but just a few of them are involved in the dairy production, which is connected with the general situation of milk sector in Slovakia. Additonally, there is a lack of processing subjects in the Slovak food industry and recently just the two of them are involved in labeled production of organic milk products of BIO quality. This poor willingness of domestic processors is connected with the several factors and the fear of whether demand will be sufficient is one of them. Our analysis focused on the demand side of mentioned problem and examined dependency of age category, level of income and gender of consumers on their willingness to buy organic dairy products. Surprisingly, we found no dependency between the age category of the respondents and purchase of organic dairy products as well as between the reasons that would discourage consumers from buying organic dairy products and their gender. On the other side, analysis clearly shows the dependency between the customers' average income per month and their willingness to pay for organic dairy products. In addition, Slovak consumers are becoming increasingly demanding according to products quality. Naturally, the market supply of high quality products would be increased. But, this increase would be possible only in case of increase of investments in processing technologies. Overall objective of this investment is not to increase short-term profits, but other benefits, which will also generate higher profits in the long term.

Based on the survey, producers should focus mainly on three products: milk, butter and yogurt, which were most frequently bought and have the highest probability to be successful as BIO products. Most Slovak consumers prefer domestic dairy products, it can be also an advantage for company. 75% of respondents believe that there are not enough dairy products on the market, so producers can meet the needs of consumers looking for these products.

Respondents also consider prices to be reasonable, which means opportunity to sell new Bio products at a slightly higher price. Most consumers are willing to pay more if the product is of high quality, tasty, has the appropriate composition and origin of production. All of these factors suggest that organic dairy products can be accepted by consumers in the market. Marketing campaign should be focus on more educated people who are especially interested in the quality of products and their composition. These consumers would also belong in the main target group, which include individuals with higher income.

The survey also showed that production of organic products improves the perception of corporate social responsibility among consumers. The reasons why respondents would buy these products were for example: Support for farmers producing organic milk, Support for domestic producers of milk, Higher quality of products and others. New Bio production line therefore can help companies get better reputation, increase market share, attract new customers, but mainly, demonstrate the social responsibility of its business in the eyes of its customers.

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EXPRESSION PATTERN OF THAUMATIN IN THE SELECTED RED VARIETIES OF *VITIS VINIFERA*, L.

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ABSTRACT

Vitis vinifera L. is a specie that is adapted to a very variable range of climates, from cold up to the desert one, but especially it grows in the temperate Mediterranean regions and continental areas of Europe. Grape is a widespread consumed fruit as well as processed to musts, juices or wine. The health beneficial effects of grapes and wine are very well known due to their high nutritional value and unique phytochemical composition. Despite many health protective and beneficial effects of *Vitis vinifera*, a part of population suffer to allergic reactions to this fruit. Allergens of wine and grapes are: endochitinases, lipid-transfer protein and thaumatin. Thaumatin is a protein having a sweet taste belonging to the PR5-like proteins. These proteins are very difersified in their functions and were described to be involved in stress responses and fruit ripening, but are expressed in healthy grape fruits in a constitutive manner and needn´t to be expressed only as a answer to the stress. Thaumatin is a minor allergen in grape, but belonging to the suspected panallergens relevant to the food cross-allergy induction, its importance is quite high. Another importance of this protein is a technological one, as reported to aggregate in wine to form a visible haze unless removed prior to bottling. In this study, expression of thaumatin-like allergen was analysed in the grapes of selected varieties. Grapes of four red varieties of *Vitis vinifera*, L. were obtained in the season 2017 in the Sabo winery that belongs to the Malokarpatská wine region. Fresh maturated grapes of varieties Alibernet, Cabernet Sauvignon , Frankovka modrá and Dornfelder were analysed. Expression changes of thaumatin was calculated by delta delta Ct method. Dornfelder was found as to have the lowest activity in thaumatin-like gene activity, mainly when comparing to the Cabernet Sauvignon and Frankovka modrá. Alibernet, on the other side, has the expression level of thaumatin very similar when comparing to the Cabernet Sauvignon and Frankovka modrá.

Keywords: *Vitis vinifera*, L.; thaumatin; expression; red varieties

INTRODUCTION

Vitis vinifera (Family *Vitaceae*), commonly known as grapes, is one of the oldest cultivated plants all over the world. It adapted to a vast range of climates, from the cold areas of Russia to the desert regions of California, especially it grows from the temperate Mediterranean regions to the continental areas in Central Europe. This fruit is widespread consumed either directly or as wine. The Western Europe is the world's biggest producer of grapes, mainly the France, Italy and Spain are the major producers of wine (approximately more than 16 millions tons per year) **(Pastorello et al., 2003)**. Additionally, social involvement in grape harvesting and farming is very high in same areas of these states, with the participation of 20% to 30% of the local population **(Brito et al., 2008)**. The health beneficial effects of grapes and wine are very well known due to their high nutritional value and unique phytochemical composition. *Vitis vinifera* is a major source of polyphenols, flavonoids, anthocyanins, phenolic acids, stilbenes, vitamins (A and C), minerals (phospor, calcium) and carbohydrates **(Arora et al., 2016)**. It has recently been observed that moderate consumption of grapes or red wine has many health beneficial effects: anti-asthmatic, cardioprotective, cytotoxic, anti-aging, hepatoprotective, anti-inflammatory, antioxidant **(Cui et al., 2002; Bathomeuf et al., 2006; Ahmad and Khan, 2012; Masani et al., 2012)**. In addtition, some studies were demonstrated, that grapes and red wine has anticancer properties. Resveratrol, belonging to the class of stilbenes, secondary metabolites produced by this plant, has showed a promising role as multidrug anti-cancer agent in cancer chemoprevention and treatment and also in treatment of neurological diseases **(Varoni et al., 2016; Andrade et al., 2018)**.

Despite many health protective and beneficial effects of *Vitis vinifera*, a part of population suffer to allergic reactions to this fruit. **Pastorello et al. (2003)** characterized and identified the major allergens of wine and grapes: 30-kd endochitinase 4A and 4B, 9-kd lipid-transfer protein (LTP) and 24-kd thaumatin by mass spectrometry and amino acid sequencing. They also reported that LTP protein has a high rate of sequence

homology with peach and cherry LTPs proteins, which gives a molecular basic of allergen cross-reactivity **(Pastorello et al., 2003)**. The precise molecular mechanism of action of grape allergens has not been yet studied and therefore requires our attention and also need to develope novel diagnostics methods and improve treatment management in this field.

Thaumatin is a protein to be identified in tropical plant *Thaumatococcus danielii* Benth and having a sweet taste. A specific domain common to osmotin-like proteins and a kinase receptor of PR5-like proteins was decribed in the structure and grouped together they are the base of thaumatin-like protein family **(Wang et al., 2011)**. These proteins are very difersified in their functions and were described to be involved in stress responses **(Yan et al., 2017)**.

Pathogenesis-related proteins are expressed in healthy grape fruits in a constitutive manner and needn´t to be expressed only as an answer to the stress **(Charron, Giegé and Lorber, 2003)**. They aggregate in wine to form a visible haze unless removed prior to bottling **(Ferreira et al., 2001; Waters, Wallace and Williams, 1991)**. Thaumatin-like protein structure was defined in grape by **Marangon et al. (2014)** and a physico-chemical parameters relevant for the haze formation mechanism were determined by these authors.

Beside the relevance in winemaking, thaumatin is defined as a minor grape allergen together with the chitinases **(Vassilopoulou et al., 2007)**. Its relevance is important, because of grapes are consumed not only processed, but fresh, too, and some severe allergic reactions were reported in the case of grape consumption in the Mediterranean region before **(Kalogeromitros et al., 2006)**. Grape allergy is clinicaly manifestated by severe symptoms mainly in patients suffering of multiple allergies and preferencially to LTP (lipid transfer proteins) containing foods **(Vassilopoulou et al., 2007)** and an association with peach and cherry allergy was observed **(Pastorello et al., 2003)**. Actually, no specific information exist in the litetarure about the grape variety based differencies on the genomic or transcriptomic level for the allergens and their genes. In this study, expression of thaumatin-like allergen was analysed in the grapes of selected varieties.

Scientific hypothesis

Thaumatin-like protein is variety dependant in its gene expression in the red grape varieties.

MATERIAL AND METHODOLOGY

Biological material

Grapes of four red varieties of *Vitis vinifera*, L. were obtained in the season 2017 in the Sabo winery that belongs to the Malokarpatská wine region (all of the grapes were inspected to be without any infection marks). Fresh maturated grapes of varieties Alibernet (sy. Odesskij černyj), Cabernet Sauvignon (syn. Vidure Sauvignon, Petit Bouschet, Bouchet-Sauvignon, Burdeos Tintowere), Frankovka modrá (syn. Černé starosvětské, Černý muškatel, Čierny Zierfandler, Karmazín, Lampart, Limberger, Noir de France, Blaufränkisch, Limberger, Limberger schwarz, Schwarze Fränkische, Franconia nera) and Dornfelder were harvested and trasported immediately to the laboratory where were kept them in -50 °C until further processing.

RNA extraction and cDNA synthesis

Total RNA was extracted using the GeneJet Plant RNA Purification Mini Kit (ThermoFisher) following the manufacturer instruction with a minor modification of the weight of homogenized tissue used – a 80 ng was used. RNA concentration and A260/A280 nm ratios were determined by Implen Nanophotometer. 1% agarose gels were prepared to visualize the integrity otf the RNA. cDNA synthesis was performed from 72 ng of total RNA using the Tetro cDNA synthesis kit (BIOLINE) with the oligodT primer.

Thaumatin expression analysis

A two-step protocol was used for thaumatin expression analysis where the actin (GenBank accession AY847627) was used as the internal control during qPCR and the amplification was performed using 5x Hot FirePol® EvaGreen® qPCR Mix (Solis BioDyne) on a Biorad CFX qPCR thermal cycler. Following program was used: 95 °C for 10 minutes followed by 35 cycles of 95 °C for 10 seconds, 59 °C for 20 seconds and 72 °C for 20 seconds ended by dissociation curves analysis of amplified thaumatin products by heating the amplicon from 65 °C to the 95 °C. Thaumatin specific primers were designed on the basis of coding region of the genomic sequence stored in the GeneBank under accession AF227324.

Statistic analysis

A standart qPCR approach with the technical triplicates was used in the study and the relative expression values were calculated by the method described by **Livak and Schmittgen (2001)**. Expression levels were determined as the number of amplification cycles obtained in the reaching of the treshold in the exponential phase of the PCR. The calculations of data presented as means and standart errors as well as graphs were prepared and performed in Microsoft Excel for Windows.

A parametric two-tailored t-test was performed for obtained thaumatin Ct values **(Smyth, 2004)** using the online platform T-Test Calculator at the significance level 0.05 **(Social Science Statistics, 2019)**.

RESULTS AND DISCUSSION

Here, expression profiles of thaumatin-like protein were analysed in four red grape varieties – Alibernet, Cabernet Sauvignon, Frankovka modrá and Dornfelder. Dissociate curves of qRT-PCR amplified thaumatin products calculated during the melting procedure (from 65 °C to 95 °C) showed a single melting peak with melting temperature (Tm) of 88.5 °C, indicating specific product (Figure 1). Agarose gel electrophoresis of these products confirmed amplification of a single product and no primer-dimer formation were generated during the reactions (data not shown). Control reactions of NTC generate clearly differenciated products.

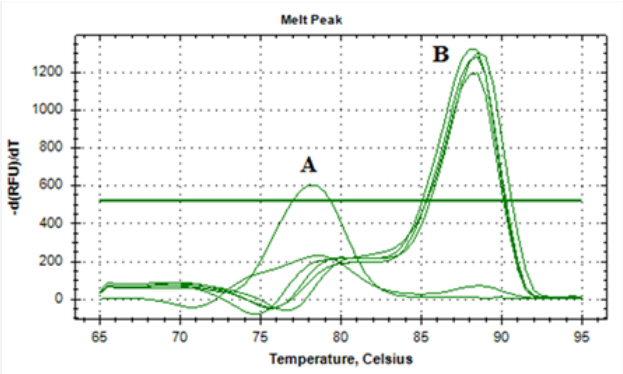

Figure 1 Melting temperatures of analyzed thaumatin products (B) and primer dimers (A) as visualized by dissociation curves.

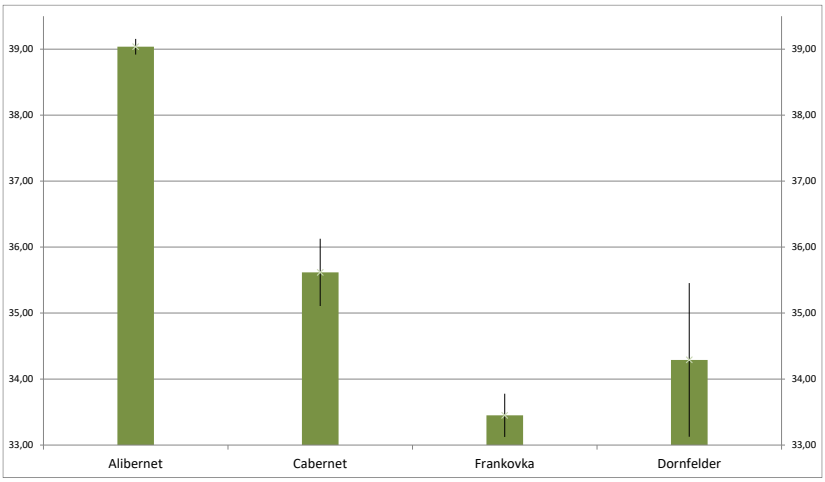

Figure 2 Characteristics of generated Cts for the triplicates of analysed red grape varieties.

For the correction of sample-to-sample variation, normalization against the actin gene was used and the generated Cts has ranged from 33.01 up to the 37.11 for the individual analysed grape varieties. Generated Cts for the thaumatin ranged from 33.1 up to the 39.11 with the variety average and standart deviations illustrated in the Figure 2.

Expression changes of thaumatin was calculated by delta delta Ct method **(Livak and Schmittgen, 2001)**. Dornfelder was found as to have the lowest activity in thaumatin-like gene activity, counted in 100 fold percentage change, mainly when comparing to the Cabernet Sauvignon and Frankovka modrá. Alibernet, on the other side, has the expression level of thaumatin very similar when comparing to the Cabernet Sauvignon and Frankovka modrá (Figure 3).

T-test was used for generated Cts to inspect the variety dependance in generated thaumatin amplicons level in the red grape varieties (Table 1) with the results that correspond to the quantified fold changes in the thaumatin expression for the analysed grape varieties.

Food allergies, defined as an immune response to food proteins, affects approximately 8% of young children and 2% of adults in western countries and their prevalence appears to be rising like all allergic diseases **(Cianferoni and Spergel, 2009)**. Few allergic reactions to grapes are described in literature as case reports and are ussually limited to respiratory tract mucosa (e.g. seasonal rhinoconjuctivities, asthma symptoms; anaphylactic reactions and oral allergy syndrome) **(Senna et al., 2001; Brito et al., 2008; Arora et al., 2016)**.

Other molecular studies reported a possible relationship between allergic reactions to grapes and other botanically unrelated fruits including peach and cherry **(Giannoccaro et al., 1998; Pastorelo et al., 2003)**. The ultimate cause of these human disorders are antifungal food allergens.

Grape allergy was reported to be specific to a certain grape variety for individual patients while tolerated others variety, or some patients may be allergic to grape but not wine **(Giannoccaro et al., 1998; Bircher, Bigliardi and Yilmaz, 1999)**.

Different allergens were identified in grapes, one of which is thaumatin. A total of thirtythree thaumatin-like genes were identified in the grape genome **(Yan et al., 2017)**. They are distributed in the chromosomes 1; 2; 3; 4; 6; 8; 12; 13; 14; 15; 16; 17 and 18 and varied from 573 bp up to the 24 345 bp in the length. Only a limited information exist about the natural genomic and transcriptomic variability of the thaumatin in grape genetic resources. Direct PCR was applied previously do detect the genomic sequences of thaumatin in the biological material such grapes, stormy wines and musts **(Žiarovská et al., 2018)**.

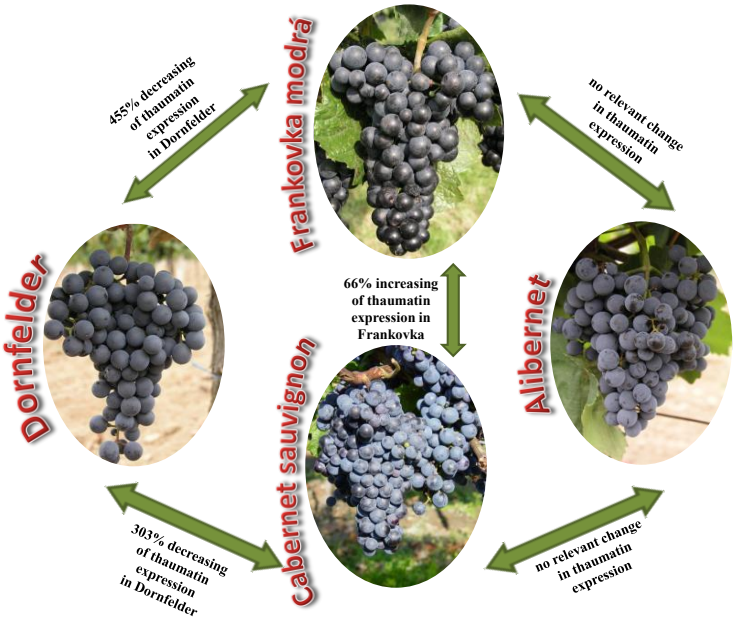

Figure 3 Expression profiles of thaumatin-like gene in the cross-comparison among analysed grape varieties.

Table 1 T-test values of thaumatin generated Cts of analysed grape varieties.

compared varieties	*p*-value	t-value	significant difference
A – C	0.420	-0.8	no
A – F	0.286	-1.13	no
A – D	0.002	7.02	yes
C – F	0.001	6.19	yes
C – D	0.002	6.05	yes
F – D	0.002	7.00	yes

Note: A – Alibernet; C – Cabernet; F – Frankovka; D – Dornfelder.

Expression profiles of different thaumatin-like proteins were analysed by **Yan et al. (2017)** in varieties Red Globe, Shang-24, Hunan-1 and Shuangyou under the inoculation of three different pathogens with the conclusion, that expression of this gene family is broadly influenced by *Botrytis cinerea*, *Elsinoe necator* and *Elsinoe ampelina*. Up to date, no specific information can be found for the expression differencies among different grape varieties.

Members of the thaumatin-like protein family are described as important allergens in peaches, too **(Palacín et al., 2010)** and they were described to be allergens in other fruit: cherry, apple, banana, kiwi, or olive and in pollens, too. This family is supposed to belonging to panallergens that are responsible for pollen-fruit cross-reactivity **(Breiteneder, 2004)**.

Expression of thaumatin in grape is reported up to now to be affected by different panthogens. Subsequences of inoculation by anthracnose, powdery mildew and Botrytis were analysed in the sense of thaumatin-like genes expression in three different grape varieties by **Yan et al. (2017)** with the conclusion, that different genes were increased in their expression following each of the inoculation pattern. The other knowledge is, that PR proteins are expressed constitutively even in healthy grape fruits **(Charron, Giegé and Lorber, 2003)** and that is why analysing the natural varietal differencies is relevant.

CONCLUSION

Thaumatin is a minor allergen in grape, but belonging to the suspected panallergens relevant to the food cross-allergy induction, its importance is quite high. Another importance of this protein is a technological one, as reported to aggregate in wine to form a visible haze unless removed prior to bottling. Expression differencies of thaumatin-like gene were analysed in four red grape varieties – Alibernet, Cabernet Sauvignon, Frankovka modrá and Dornfelder. Real-time PCR approach and normalization against actin was used. None fold change in thaumatin expression was found for comparing varieties Alibernet-Cabernet and Alibernet-Frankovka modrá. Dornfelder was found to have lowest level of thaumatin expression with the expression fold change of 4.5.

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USE OF SOME BIOSTIMULANTS TO IMPROVE THE GROWTH AND CHEMICAL CONSTITUENTS OF SWEET PEPPER

Rabab Maraei, Noha Eliwa, Amina Aly

ABSTRACT

The experiment was conducted during two successive seasons 2016 and 2017 on sweet pepper plants to study the effect of foliar application of some natural extracts (fulvic acid at 2, 4 and 6% or algae at 1, 2 and 4 $g.L^{-1}$) were applied three times along each season (after 2, 4 and 6 weeks of planting). The influence was evaluated through the response of vegetative growth, and some physical and chemical characteristics of sweet pepper fruits. The results obtained showed that the algae extract at 1 $g.L^{-1}$ in most cases was better than the other spray treatments investigated to improve most fruit characteristics (length, diameter and yield of fruits), vegetative growth, and chemical properties followed by 6% fulvic acid. With regard to organic acids, malic and citric acids are the main organic acids found in sweet pepper. Malic, succinic and glutaric acids were higher in 1 $g.L^{-1}$ algae extract treatment, but the concentration of citric acid was higher in 6% fulvic acid treatment. Therefore, algae extract and fulvic acid could be safely recommended as a natural biostimulants application for improving most desirable characteristics of sweet pepper grown under the same experimental condition.

Keywords: sweet pepper; fulvic acid; algae; vegetative growth; fruit characteristics

INTRODUCTION

Sweet pepper is one of the most important vegetable crops as well as important exportable crops in Egypt and it is considered an excellent source of bioactive nutrients. Vitamin C, carotenoids and phenolic compounds are the main antioxidant components **(Marín et al., 2004)**. The levels of these compounds in peppers and other vegetables depend on several factors, such as cultivar, type of agriculture (organic or non-organic), maturity and storage conditions **(Lee and Kader, 2000)**. Sweet pepper or its hot varieties (*Capsicum annuum* L.) are important vegetable crops that can be used to produce seasonings such as paprika spices. Green pepper is an important condiment containing phenolic compounds such as 3,4-dihydroxyphenyl ethanol glucoside, 3,4-dihydroxy-6-(N-ethylamino) benzamide and phenolic acid glycosides, suggesting a high radical scavenging activity of them. Addition of paprika spices (dried fruits of the pepper family) and pepper spices (mainly black pepper, white or green peppers) could change foods sensory quality and also, due to the antioxidant's presence, lower oxidation in the foods. Antioxidants help to control oxidation in foods effectively, therefore usage of spices such as paprika or pepper spices are also of great economical interest. Polyphenols are often responsible for the antioxidant capacity **(Škrovánková et al., 2017)**. Plant biostimulants, can be considered as substances that can be added to the environment around the plant and encourage growth and have positive effects on nutrition. They also increase plant tolerance and protective effects against various types of environmental stress such as water deficit, salinity and exposure to temperatures unsuitable for growth **(Du Jardin, 2015)**. Biostimulants are not nutrients but they facilitate nutrient uptake or contribute beneficially to promote growth and stress resistance **(Brown and Saa, 2015)**. The role of biostimulants, specifically in regard to growth promotion and nutrient availability, has been reviewed **(Du Jardin, 2015)**. Many categories of specific biostimulants have been extensively reviewed such as protein hydrolysates **(Colla et al., 2015)**, seaweed extracts **(Battacharyya et al., 2015)**, silicon **(Savvas and Ntatsi, 2015)**, chitosan **(Pichyangkura and Chadchawan, 2015)**, humic and fulvic acids **(Canellas et al., 2015)** plant growth-promoting rhizobacteria **(Ruzzi and Aroca, 2015)**. Seaweed extracts (SWE) as biostimulants are emerging as commercial formulations for use as plant growth promoting factors, and a method to improve tolerance to salinity, heat, and drought. Seaweeds are red, green, and brown macroalgae that represent 10% of marine productivity. Macroalgae have been used as organic fertilizers for thousands of years and are still in use **(Craigie, 2011)**. Currently, there are over 47 companies producing and marketing various algal extracts for agricultural use; the majority of the formulations are from

the brown algae, *Ascophyllum nodosum* **(Sharma et al., 2014)**. Fulvic acid is an important fraction of soil organic matter, an important portion of the dissolved organic C pool in soils **(van Hees, Lundström and Giesler, 2000)**. It is humic acids with a higher oxygen content and lower molecular weight **(Bulgari et al., 2015)**. Fulvic acid plays an important role in the acid – base buffering capacity of soil, and transfer of metal ions and organic compounds into soil **(Senesi and Miano, 1995)**. Foliar application of marine bioactive substances to grape plants (*Vitis vinifera* L.) increased leaf water potential and stomatal conductance under drought stress **(Mancuso et al., 2006)**. Humic and fulvic acids are produced by the biodegradation of organic matter resulting in a mixture of acids containing phenolate and carboxyl groups.

Scientific hypothesis

Evaluation of the effect of foliar application of some natural extracts (fulvic acid and algae) on vegetative growth, and some fruit physical and chemical characteristics of sweet pepper.

MATERIAL AND METHODOLOGY

The experiment was conducted during the two seasons 2016 and 2017 where the sweet pepper seeds (*Capsicum annuum* L.) were sown in trays in the mid-March in both growing seasons in the experimental farm of Natural products Department, National Center of Radiation Research and Technology. After 45 days from seeds sowing, transplants were transplanted into soil. They planted on both sides of lines 70 cm apart and 40 cm between plants. Plants were grown at 29 and 18 ºC day/night temperature conditions. All agricultural needs were done. Soil mechanical and chemical analysis of the experimental site is shown in Table 1. Fulvic acid (2, 4 and 6%) and algae extract (1, 2 and 4 $g.L^{-1}$) were sprayed after two, four and six weeks of transplanting. A complete randomized block design (CRBD) with three replicates was used.

Table 1 Physical and chemical properties of experimental soil in 2016 and 2017 seasons.

Soil properties	Experimental year	
	2016	2017
pH (1:1)	7.32	7.31
EC (1:1) $dS.m^{-1}$	2.2	1.8
Soluble anions ($meq.L^{-1}$)		
$CO_3^{=}$	1.5	1.3
HCO_3^{-}	4.5	3.6
Cl^{-}	8.0	5.9
$SO_4^{=}$	11.7	9.8
Soluble cations ($meq.L^{-1}$)		
Ca^{++}	10.5	9
Mg^{++}	3.5	2
K^{+}	0.50	0.4
Na^{+}	11.2	9.2
Sand (%)	57.3	58.4
Silt (%)	21.2	18.2
Clay (%)	21.5	23.4
Texture class	Sandy clay loam	Sandy clay loam

Measurements and analysis

The effect of the differential investigated spray treatments was evaluated through the response of the following measurements.

Vegetative growth parameters

After 90 days from transplanting, four plants per replicate were randomly chosen to measure, plant height (cm), root length (cm) and branches/plant.

Physical characteristics of fruit

Mature pepper fruits were harvested manually at full colour stage. Fruit length, fruit diameter and fruit yield/plant (kg) were determined.

Chemical characteristics

Photosynthetic Pigments

Chlorophyll a, chlorophyll b and carotenoids were determined in leaves **(Vernon and Seely, 1966)**. The results were calculated as $mg.g^{-1}$ fresh weight.

Ascorbic acid content

Ascorbic acid was measured as described by **(Jagota and Dani, 1982)**. Fresh weight (0.2 g) sample was homogenized in 1.5 mL 10% (w/v) trichloroacetic acid (TCA) at 4 °C. After centrifugation at 3000 × g for 5 min, 0.3 mL of the supernatant was made up to 2 mL volume with distilled water. A 0.2 mL 10% (v/v) Folin phenol reagent (Sigma Chemical Co.) was then added to the mixture, and vigorously shaken. After 10 min reaction time, maximum absorbance was measured at 760 nm. A result of the reaction between ascorbic acid and Folin phenol reagent, L (+) – ascorbic acid (Hamburg chemistry, Germany) was used as a standard.

Enzymes extraction

Sample (0.5 g) from each treatment were homogenized in 100 mM pre-chilled sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinyl pyrrolidone (PVP) (w/v) at 4 ºC. The extraction ratio was 4.0 mL buffer for each one gram of sample. The homogenate was centrifuged at 15.000 x g for 15 min at 4 ºC. Supernatant was used to estimate the activities of peroxidase and polyphenol oxidase. Proteins content was determined in the enzymes extract **(Bradford, 1976)**.

The activity of peroxidase (POD)

Peroxidase was assayed following method the method of **Hammerschmidt et al. (1982)**. The reaction mixture (2.9 mL) consisted of 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6 containing 10 mM hydrogen peroxide). Volume of 100 µL of the enzyme extract was added to launch the reaction which was determined spectrophotometrically (Jasco V-530, Jappan) at 470 nm per min. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 min^{-1} mL^{-1}. The POD activity expressed as unit min^{-1} mg^{-1} protein.

The activity of polyphenol oxidase (PPO)

Polyphenol oxidase was assayed following method the method of **Oktay et al. (1995)**. The reaction mixture

consisted of 600 µL catechol (0.1 M) and 100 µL enzyme extract was completed to 3.0 mL with 0.1 M phosphate buffer pH 7. The absorbance was registered at 420 nm by spectrophotometer. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 min^{-1} mL^{-1}. The enzyme activity was expressed as unit min^{-1} mg^{-1} protein.

Preparation of ethanolic extract

The fruits of sweet pepper were freeze dried (Virtis model 10-324) and ground. The ground samples were extracted using ethanol 80% and used in chemical analyses. Phenolic content was determined **(Shahidi and Naczk, 1995)** using the Folin-Denis reagent. The results were expressed as $mg.g^{-1}$ of gallic (GAE) equivalent of the dry weight.

Antioxidant activity by

Reducing power

Power reduction was determined by **Oyaizu (1986)** technique. Each 0.5 mL sample was mixed with 0.2 M sodium phosphate buffer 2.5 mL and 1% potassium ferricyanide 2.5 mL and incubated at 50 °C for 20 min. Following the addition of 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged for 10 min at 200 g. The upper layer (5.0 mL) was blended with 5.0 mL of deionized water and the test sample was read against the blank at 700 nm.

Metal chelating

Metal chelating activity was consequently evaluated by 0.1 mM $FeSO_4$ (0.2 mL) and 0.25 mM ferrozine (0.4 mL) in 0.2 mL of samples **(Chew et al., 2009)**. After 10 min of incubating at room temperature, absorbance of the blend was recorded against a void reagent was registered at 562 nm. The lower absorption of the test sample suggested greater chelating capacity of ferrous ion. The control contained all the reagents except test sample.

Organic acids analysis

Organic acids were separated by high performance liquid chromatography (HPLC) Knauer, Germany, flow rate was set at 0.6 $mL.min^{-1}$, UV detector set at $\lambda = 214$ nm, column oven temperature kept constant at 65 °C. The column used was Rezex@ column for organic acids analysis, mobile phase was 0.005 M H_2SO_4, data integration by clarity-chrom software.

Statistic analysis

All the statistical analyses were performed using an ANOVA, and Duncan's multiple range tests **(Duncan, 1955)** were applied to compare the results of the experiments ($p \leq 0.05$). A three replicates was used.

RESULTS AND DISCUSSION

Vegetative growth parameters

Table 2 shows the effect of spraying with fulvic acid and algae extracts on vegetative growth parameters (plant height, root length and branches/plant). All treatments have a positive effect on vegetative growth parameters and the best treatment is algae extract at 1 $g.L^{-1}$, resulting in improved growth parameters followed by fulvic acid 6% treatment in both seasons. It was clear that the growth parameters increased through the application of these extracts. The spraying of humic acid increased growth compared with control in *Thuja orientalis* plants because of its direct effect on solubility and transport of nutrients **(Zaghloul et al., 2009)**. Also, correspond, the increase in shoots characteristics may also be due to the auxins content in the seaweed extracts that have an effective role in cell division and enlargement, resulting in increased the shoot growth **(Arancon et al., 2004; Gollan and Wright, 2006)**. On the other hand, the least increase over control in all evaluated growth measurements was always associated with foliar application of algae extract at 4 $g.L^{-1}$.

Physical characteristics of fruit

Data presented in Table 3, shows that all spraying treatments led to increase the physical characteristics of fruits (length, diameter and yield of fruit) compared to control in both seasons. The results indicated that treatment of algae extract at 1 $g.L^{-1}$ gave the highest value of these characteristics, followed by the treatment of fulvic acid at 6% in both seasons. It is clear that fulvic acid and algae extract improved the quality of sweet pepper fruits. The use of humic substances gave the highest grain yield of winter wheat compared to control **(Bezuglova et al., 2017)**. Also, the application of humic acid and calcium nitrate alone caused a significant increase in growth parameters, photosynthetic pigments of pepper plants under normal and salt stress conditions **(Akladious and Mohamed, 2018)**. Fulvic acid as an organic fertilizer chelating the minerals (non-toxic) and water binder and thus maximizes uptake through the leaves, which increases the productivity of the plant **(Malan, 2015)**.

The stimulatory effects of fulvic acid are directly related to increase uptake of nutrient such as nitrogen, phosphorus, potassium and micronutrients **(Silva et al., 2016)**, vitamins, some growth regulators and polyamines as algae extract. Also, these extracts, such as fulvic acid, contain phenols that are antioxidants.

Therefore, their use on plants will improve the vegetative growth of plants in addition to the physical and chemical properties of fruits, which will be reflected in increased productivity. Fulvic acid and humic acid when used with a concentration of 40 $mg.kg^{-1}$ increased flowering and growth parameters such as shoots number, plant diameter, flowers number and root length compared to control plants in impatiens walleriana **(Esringu et al., 2016)**. Spraying mango trees with algae extracts alone or combined with natural or plant extracts was very effective in improving fruit set, fruit retention, yield and enhanced fruit quality **(Ahmed et al., 2014; Abed El Hamied, 2014)**. Also, the application of humic substances, especially at suitable concentrations, may alleviate the damages induced by the stress, probably via the enhanced nutrient uptakes and induced physiological changes **(Hamideh et al., 2013)**.

Chemical characteristics

Photosynthetic Pigments

Fulvic acids and algae extracts applications affected fruit chlorophyll content during both two seasons (Figure 1).

Table 2 Effect of fulvic acid and algae extracts spraying on growth parameters of sweet pepper plants during the first and second seasons.

Treatments		Plant height (cm ±*SD*)	Root length (cm ±*SD*)	Branches/plant ±*SD*
First season				
Fulvic acid	Cont (Zero)	101.0 ±0.954 ^{e}	11.67 ±0.268 ^{g}	26.00 ±0.306 ^{f}
	2%	105.0 ±0.814 ^{d}	18.47 ±0.277 ^{d}	30.33 ±0.493 ^{d}
	4%	113.7 ±1.26 ^{c}	20.00 ±0.178 ^{c}	34.33 ±0.794 ^{c}
	6%	124.7 ±0.252 ^{b}	22.07 ±0.378 ^{b}	38.00 ±0.517 ^{b}
Algae extract	1 g.L^{-1}	132.6 ±0.458 ^{a}	25.70 ±0.265 ^{a}	43.00 ±0.379 ^{a}
	2 g.L^{-1}	104.7 ±0.802 ^{d}	15.00 ±0.225 ^{e}	27.00 ±0.755 ^{e}
	4 g.L^{-1}	103.7 ±0.208 ^{d}	13.00 ±0.187 ^{f}	26.67 ±0.631 ^{ef}
Second season				
Fulvic acid	Cont (Zero)	102.0 ±2.08 ^{f}	11.67 ±0.764 ^{f}	25.33 ±1.02 ^{e}
	2%	107.3 ±1.80 ^{d}	18.67 ±0.697 ^{c}	29.67 ±0.862 ^{d}
	4%	115.4 ±1.16 ^{c}	21.67 ±0.289 ^{b}	33.33 ±0.603 ^{c}
	6%	126.5 ±1.89 ^{b}	22.70 ±0.289 ^{b}	36.67 ±0.751 ^{b}
Algae extract	1 g.L^{-1}	133.8 ±0.764 ^{a}	24.50 ±0.904 ^{a}	44.33 ±0.854 ^{a}
	2 g.L^{-1}	106.7 ±0.289 ^{d}	16.00 ±0.297 ^{d}	28.67 ±0.802 ^{d}
	4 g.L^{-1}	104.7 ±0.812 ^{e}	14.33 ±0.404 ^{e}	26.67 ±1.01 ^{e}

Note: Values are mean of three replications ±standard deviation. Different letters indicate statistically significant differences at $p \leq 0.05$.

Table 3 Effect of fulvic acid and algae extracts spraying on physical characteristics of sweet pepper fruits during the first and second seasons.

Treatments		Fruit length (cm ±*SD*)	Fruit diameter (cm ±*SD*)	Fruit yield/plant (kg ±*SD*)
First season				
Fulvic acid	Cont (Zero)	9.00 ±0.577 ^{b}	8.83 ±0.305 ^{b}	0.8950 ±0.021 ^{f}
	2%	10.17 ±0.631 ^{ab}	9.83 ±0.681 ^{ab}	1.700 ±0.011 ^{d}
	4%	10.50 ±0.584 ^{ab}	10.17 ±0.513 ^{ab}	1.900 ±0.01 ^{c}
	6%	11.67 ±0.543 ^{ab}	11.00 ±0.608 ^{a}	2.183 ±0.013 ^{b}
Algae extract	1 g.L^{-1}	12.17 ±0.601 ^{a}	11.50 ±0.304 ^{a}	2.550 ±0.21 ^{a}
	2 g.L^{-1}	9.67 ±0.325 ^{ab}	9.17 ±0.255 ^{b}	1.583 ±0.10 ^{d}
	4 g.L^{-1}	9.17 ±0.275 ^{b}	8.93 ±0.413 ^{b}	1.200 ±0.009 ^{e}
Second season				
Fulvic acid	Cont (Zero)	8.37 ±0.231 ^{d}	8.00 ±0.152 ^{e}	0.8467 ±0.031 ^{f}
	2%	10.33 ±0.0.312 ^{ab}	9.50 ±0.254 ^{bcd}	1.500 ±0.044 ^{d}
	4%	10.90 ±0.415 ^{ab}	9.87 ±0.274 ^{abc}	1.800 ±0.032 ^{c}
	6%	11.50 ±0.502 ^{a}	10.67 ±0.159 ^{ab}	2.000 ±0.0521 ^{b}
Algae extract	1 g.L^{-1}	11.67 ±0.344 ^{a}	11.00 ±0.312 ^{a}	2.467 ±0.0458 ^{a}
	2 g.L^{-1}	9.60 ±0.218 ^{bc}	9.33 ±0.321 ^{cd}	1.417 ±0.021 ^{d}
	4 g.L^{-1}	8.50 ±0.140 ^{cd}	8.33 ±0.173 ^{de}	1.167 ±0.0252 ^{e}

Note: Values are mean of three replications ±standard deviation. Different letters indicate statistically significant differences at $p \leq 0.05$

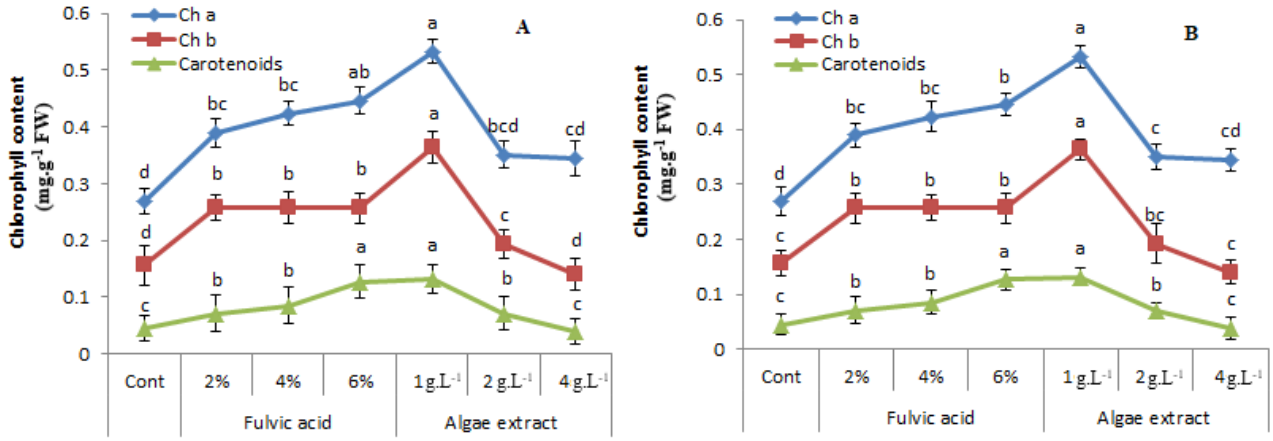

Figure 1 Effect of fulvic acid and algae extracts spraying on chlorophyll content (mg.g^{-1} FW) of sweet pepper fruits during the first (A) and second (B) seasons. Note: Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

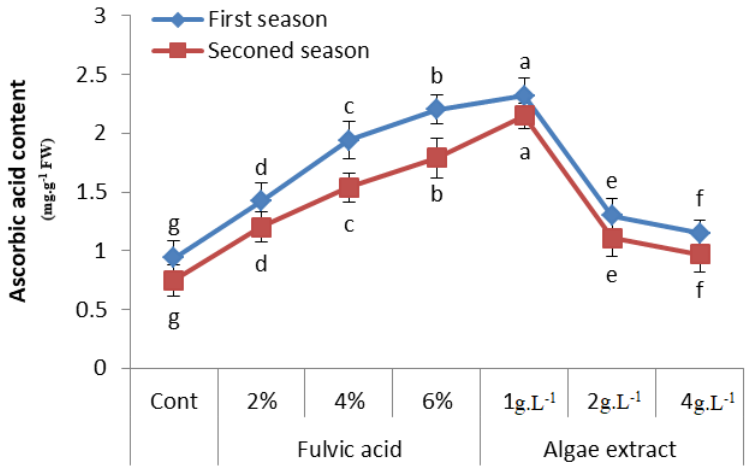

Figure 2 Effect of fulvic acid and algae extracts spraying on ascorbic acid content (mg.g^{-1} FW)) of sweet pepper fruits during the first and second seasons. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

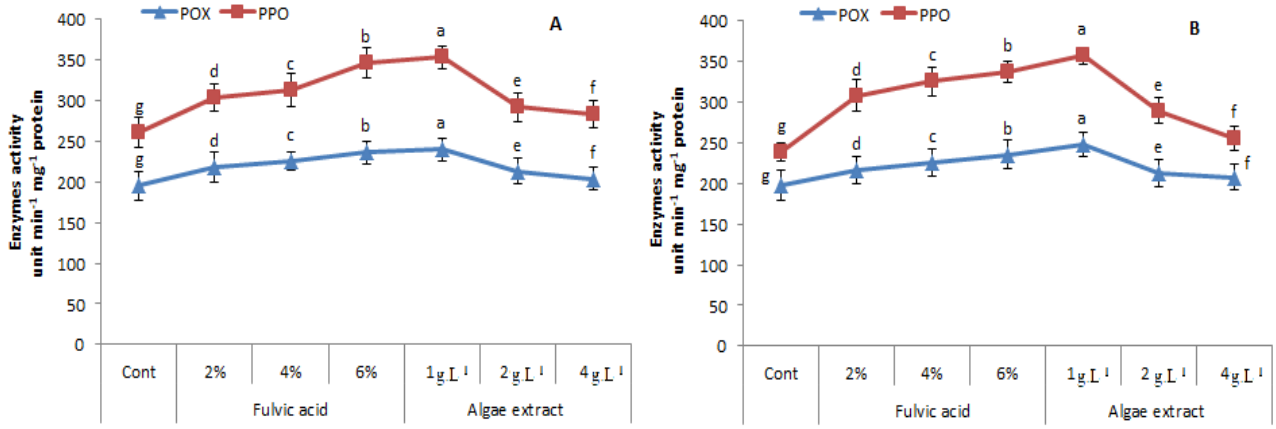

Figure 3 Effect of fulvic acid and algae extracts spraying on peroxidase (POX) and polyphenol oxidase (PPO) activities (unit min^{-1} mg^{-1} protein) of sweet pepper fruits during the first (A) and second (B) seasons. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

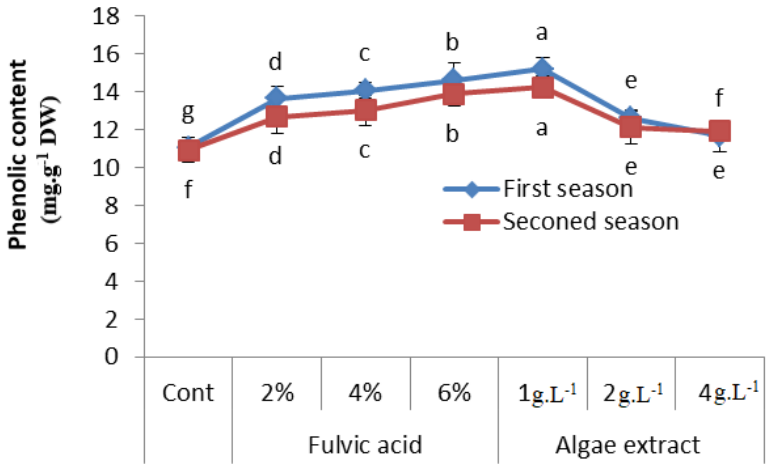

Figure 4 Effect of fulvic acid and algae extracts spraying on phenolic content (mg.g^{-1} DW) of sweet pepper fruits during the first and second seasons. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

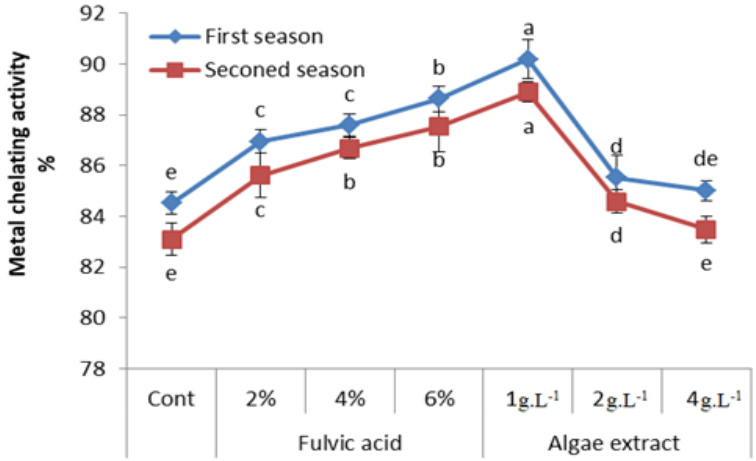

Figure 5 Effect of fulvic acid and algae extracts spraying on metal chelating activity % of sweet pepper fruits during the first and second seasons. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$

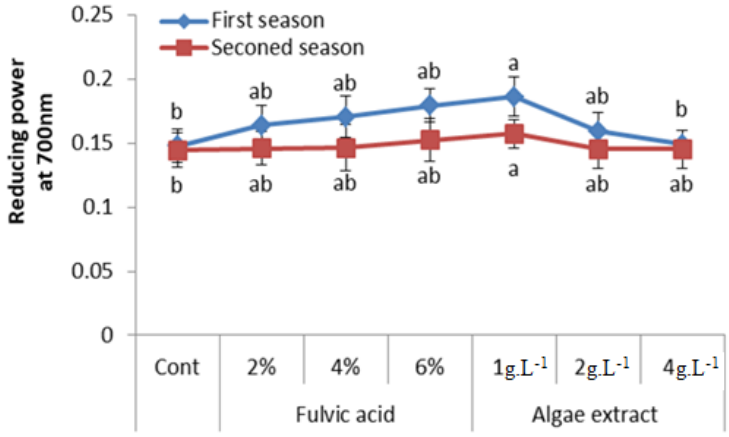

Figure 6 Effect of fulvic acid and algae extracts spraying on reducing power (at 700 nm) of sweet pepper fruits during the first and second seasons. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

Table 4 Effect of fulvic acid and algae extracts spraying on organic acids (µg.mg^{-1} DW) of sweet pepper fruits during the first and second seasons.

Organic acid (µg.mg^{-1} DW)	Control	Fulvic acid			Algae extract		
		2%	4%	6%	1 g.L^{-1}	2 g.L^{-1}	4 g.L^{-1}
First season							
Oxalic acid	7.15	8.61	8.66	6.75	6.66	7.02	5.32
Citric acid	15.64	20.64	24.33	29.54	28.97	27.64	22.30
Malic acid	50.31	4764	52.64	60.31	66.43	67.5	51.97
Succinic acid	13.23	18.34	20.33	18.88	21.70	18.42	17.02
Glutaric acid	2.35	2.13	2.50	2.11	3.65	3.89	2.75
Propionic acid	0.02	0.005	Nd	Nd	Nd	Nd	Nd
Second season							
Oxalic acid	6.01	7.03	7.27	5.50	5.03	5.66	3.27
Citric acid	13.67	19.55	26.75	38.05	36.04	30.31	23.23
Malic acid	45.60	41.20	45.83	52.94	73.70	52.82	45.49
Succinic acid	11.99	15.55	19.14	17.35	19.29	15.47	13.05
Glutaric acid	3.17	2.46	3.21	1.88	5.68	3.90	3.41
Propionic acid	0.013	0.02	0.014	0.014	0.013	0.01	Nd

Note: Nd = Not detected.

Results indicate that fruits chlorophyll content increased with most of treatments. The highest content of fruit chlorophyll was obtained from algae extract (1 $g.L^{-1}$), followed by fulvic acid (6%).

Seaweed extracts contain cytokinins that stimulate physiological activities (such as activation of some enzymes that involved in photosynthesis), increased total chlorophyll in the plant and photosynthesis activity that will be positively reflected on shoot characteristics **(Thomas, 1996)**.

Ascorbic acid content

The effect of spray treatments with fulvic acids and algae extracts on ascorbic acid content, depends on the concentration used (Figure 2). All extracts stimulated ascorbic acid over control. Spraying with the algae extract (1 $g.L^{-1}$) gave the highest content of ascorbic acid (5.38 and 4.75 $mg.g^{-1}$ FW in the first and second seasons, respectively) followed by spraying fulvic acid 6% (4.48 and 3.86 in both seasons, respectively). Humic substance application at different concentrations improved tomato vitamin C **(Dorais et al., 2008; Mauromicale, Longo and Lo Monaco, 2011)**.

Enzymes activity

The results in Figure 3 showed that spraying of algae extract at 1 $g.L^{-1}$ resulted in peroxidase and polyphenol oxidase activities 240.08 and 353.64 unit min^{-1} mg^{-1} protein, respectively while fulvic acid (6%) gave 236.00 and 346.44 unit min^{-1} mg^{-1} protein, respectively compared with control (195.22 and 261.70 unit min^{-1} mg^{-1} protein, respectively) in the first season. The same trend was observed in the second season. Treatment with algae extract (1 $g.L^{-1}$) gave the highest value for the activity of each enzyme. In general, the best results were obtained from algae extract (1 $g.L^{-1}$) followed by fulvic acid extract (6%).

Phenolic content

It is clear from Figure 4 that all sprayed concentrations of fulvic acid and algae extracts increased the phenolic content of sweet pepper fruits compared to the control. The highest values in this respect were recorded when plants were sprayed with algae extract at 1 $g.L^{-1}$ (15.21 and 14.24 $mg.g^{-1}$ DW in the first and second seasons, respectively) followed by fulvic acid at 6% (14.62 and 13.89 $mg.g^{-1}$ DW in the first and second seasons, respectively) compared to control (11.06 and 10.93 $mg.g^{-1}$ DW in the first and second seasons, respectively).

The organic fertilizer applications significantly affected total phenolic content. Plants cannot allocate resources for growth and defence at the same time, and there is competition between proteins and phenols in plants for common precursors involved in their biosynthesis **(Aminifard et al., 2012; Riipi et al., 2002)**. The use of humic acid and calcium nitrate individually improves the quality of fruits, total phenols and antioxidant activity in pepper fruits under normal and saline conditions **(Akladious and Mohamed, 2018)**. These results led us assume that pepper plants may benefit from fulvic acid fertilizer for protein synthesis and growth development. On the other hand, organic acids and amino acids act as precursors or stimulants of plant hormones and growth substances, as well as secondary compounds in plants **(Vernieri et al., 2006)**. Also, when the light is sufficient for regular photosynthetic rates, additional carbon (C) may be allocated for the synthesis of C-based secondary compounds such as phenols in plants treated with organic fertilizers **(Toor et al., 2006)**.

Antioxidant activity

Figures 5 and 6 show the effect of spray treatments with the extracts of fulvic acid and algae on the antioxidant activity. This indicates a significant difference between all treatments and control in metal chelating activity. The best results were obtained from algae extract at 1 $g.L^{-1}$ followed by 6% fulvic acid in both seasons. However, there were no significant differences between all treatments and the control in reducing power in both seasons.

Organic fertilizers increased the antioxidant activity **(Aminifard et al., 2012)**. There are a number of factors that can affect the total antioxidant capacity in plant tissues such as light intensity, temperature and cultivar as well as soil type and soil content of humic compounds (humic acid and fulvic acid) where the high content of humic compounds in the soil led to increase antioxidant activity **(Rimmer, 2006)**. Another view that explains the increase of antioxidant compounds in organic foods is that as the use of insecticide, fungicide and herbicide is limited in organic agriculture, the plants allocate more resources to fight pathogenic attacks, which include the generation of antioxidant compounds **(Winter and Davis, 2006)**. Thus, these findings reveal that fulvic acid had a positive influence on the antioxidant activity of sweet pepper fruits.

Organic acids

Organic acids have sour and fresh taste that impart unique flavour to the food **(Williams, 2001)**. Citric, malic, and succinic acids are the major organic acids in peppers **(Luning et al., 1995)**. The current study reports the determination of oxalic, citric, malic, succinic, glutaric and propionic acids in sweet pepper by HPLC. The results which summarized in Table 4, showed that the organic acids in the pepper fruits decreased in the order of malic >citric > succinic >oxalic >glutaric >propionic. Malic and citric acids are the principal organic acids found in peppers **(Jensen, 2007)**. While, the concentrations of glutaric and propionic acids in the sweet pepper were low. The concentrations of malic, succinic and glutaric acids were higher in 1 $g.L^{-1}$ algae extract treatment, but the concentration of citric acid was higher in 6% fulvic acid treatment **(Luning et al., 1995)**.

CONCLUSION

The use of plant biostimulants has positive effects on growth and bioactive compounds in sweet pepper plants, especially when used at appropriate concentrations. Fulvic acid and algae extracts improved most fruit characteristics (length and diameter of fruits), vegetative growth, and chemical properties. With regard to organic acid, malic and citric acids are the main organic acids found in sweet peppers malic, succinic and glutaric acids were higher in 1 $g.L^{-1}$ algae extract treatment, but the concentration of citric acid was higher in 6% fulvic acid treatment. The results of the current study showed that the best treatment is algae extract at 1 $g.L^{-1}$ which gave the best value in most

results followed by 6% fulvic acid so that it can be safely recommended as a natural biostimulants.

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The electrical conductivity of sheep's milk and the possibility of mastitis detection

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ABSTRACT

Measurement of electrical conductivity (EC) is a method frequently used in dairy cows during milking in milking parlours, but especially in robotic milking as a low-cost mastitis detection method. The aim of this study was to evaluate the relationship between somatic cell count (SCC) and EC of milk in sheep reared in Slovakia as factors for monitoring subclinical mastitis on the basis of a bacteriological examination of udder health. Samples were collected individually from both halves of the udder from 295 sheep of different breeds from eight farms during evening milking. Based on SCC, the samples (590) were divided into classes (SCC < 2×10^5, $2 \times 10^5 \leq$ SCC < 4×10^5, $4 \times 10^5 \leq$ SCC < 6×10^5, and SCC ≥ 6×10^5 cells.mL^{-1}), (SCC < 7×10^5 and SCC ≥ 7×10^5 cells.mL^{-1}) and (SCC < 1×10^5 and SCC ≥ 1×10^5 cells.mL^{-1}) respectively. Based on the presence of pathogens in the udder halve, they were classified as "major pathogens" (14), "minor pathogens" (161) and "without pathogens" (415). The presence of a pathogen had a significant effect on the increase in EC, SCC and protein content and decrease in content of lactose. We found a significant correlation between EV and SCC at first classification only in cases where all data was analysed jointly (r = 0.531), SCC ≥ 6×10^5 (r = 0.403) and SCC < 2×10^5 (r = 0.214). In the second and third classification, we found significant correlations in both cases, the SCC < 7×10^5 (r = 0.270) and the SCC ≥ 7×10^5 (r = 0.382) and SCC < 1×10^5 (r = 0.136) and the SCC ≥ 1×10^5 (r = 0.557). The electrical conductivity showed a stronger correlation with the lactose and protein content than LogSCC. We can argue that measuring the electrical conductivity of sheep milk may be a possible alternative for mastitis detection in sheep. EC can be useful in detecting animals with level of SSC greater than 6×10^5 cells.mL^{-1}.

Keywords: electric conductivity; somatic cell count; sheep milk; mastitis

INTRODUCTION

For sheep farmers it is very important to know the health status of the udder. Increasing SCC leads to a significant reduction in daily milk production, decrease in lactose and a moderate increase in fat and protein **(Caria et al., 2016; Tančin et al., 2017; Baranovič et al., 2018)** however, it significantly aggravates the coagulation properties of milk **(Abdelgawad et al., 2016)**. Measuring the electrical conductivity (EC) of milk during milking has been studied in cattle as a low-cost mastitis detection method that can be easily automated **(Romero et al., 2017)**. Milk normally has an EC of between 4.0 and 6.0 mS.cm^{-1} **(Ferrero, Valledor and Campo, 2014)**, but bacterial infection of the udder results in an increase in Na^+ and Cl^- and decreases in the K^+ levels **(Kitchen, 1981)**, which causes an increase in EC. This is widely used as a method of monitoring mastitis infections. When measured conductivity is in extreme values (6.5 – 13.00 mS.cm^{-1}) at 18 °C, this indicates mastitis **(Ferrero, Valledor and Campo, 2014)**. **Caria et al. (2016)** achieved a sensitivity of 73.08% and a specificity of 75.46% in their study, with an EC threshold of 4.84 mS.cm^{-1} for sheep milk. There are only a few reports that have been published about the effect of mastitis on the conductivity of sheep's milk. This led us to a decision to evaluate the relationship between SCC and EC of milk in sheep reared in Slovakia as factors for monitoring subclinical mastitis on the basis of a bacteriological examination of udder health.

Scientific hypothesis

The presence of pathogens in sheep milk significantly increases the electrical conductivity of milk.

The presence of pathogens in sheep's milk significantly increases SCC in milk.

Increasing the number of somatic cells increases the electrical conductivity in milk.

There is a moderate positive relationship between SCC and EC.

The presence of pathogens in sheep's milk significantly decreases lactose content in milk.

The presence of pathogens in sheep's milk significantly increases protein content in milk.

There is a moderate negative relationship between SCC and EC.

MATERIAL AND METHODOLOGY

Samples from 590 udder halves of 295 machine milking ewes of different breeds from eight farms were collected during evening milking. Milk samples were collected aseptically after cleaning the teats, especially teat-ends with antibacterial wipes (GAMA Healthcare Ltd, UK). Sampling always started with the right udder half, the first two strips were placed separately, next 10 mL were used for EC measurement with a handheld conductometer Milk Checker N-4L (Oriental Instruments Co., Ltd., Japan) with compensation the measured EC on a standard temperature of 25 °C, 1 mL was aseptically gathered into sterile test tube for cytobacteriological analysis and an additional sample of 50 mL was taken for somatic cell count and a basic components analysis. Immediately after removal, the milk sample was stored in a portable refrigerator at 5 – 15 ° C. The samples were transported to the laboratory and refrigerated at 4 °C. Milk samples (inoculum 10 µl) streaked onto selective culture medium PM test (LabMediaServis s.r.o., CZ) were incubated at 37 °C for 24 h. Isolated strains of pathogens were then verified by typing with BBL Crystal® (Becton, Dickinson & Co., New Jersey, USA). Somatic cell count was determined using a Somacount 150 (Bentley Instruments, Inc., Chaska, Minnesota, USA), milk composition was determined by MilkoScan FT120 (Foss, Hillerød, Denmark).

Statistic analysis

The correlation of EC with SCC was analysed (Proc Corr, SAS ver. 9.3; SAS Institute Inc., 2011) according to SCC intervals by **Romero et al. (2017)** ($SCC < 2 \times 10^5$, $2 \times 10^5 \leq SCC < 4 \times 10^5$, $4 \times 10^5 \leq SCC < 6 \times 10^5$, and $SCC \geq 6 \times 10^5$ cells.mL^{-1}), by **Caria et al. (2016)** ($SCC < 7 \times 10^5$ and $SCC \geq 7 \times 10^5$ cells.mL^{-1}) and **Barth, Burow and Knappstein (2008)** ($SCC < 1 \times 10^5$ and $SCC \geq 1 \times 10^5$ cells.mL^{-1}). EC and SCC variables were transformed into base 10 logarithms. The relationship of the EC and SCC variables with fixed effects was analysed by a one-way ANOVA (Proc GLM; SAS/STAT ver. 9.3; SAS Institute Inc., 2011), the mean differences were determined by the Scheffe's test.

RESULTS AND DISCUSSION

After the pathogen analysis, we found that 175 animals were free of the pathogen in the udder and in 120 animals the pathogen was present in at least one half of the udder. 76 animals (25.8%) from the "free of the pathogen" category had $SCC < 1 \times 10^5$ and EC ranging from 0.0 to 0.4. In total, 175 udder halves (29.7%) were infected, from that 55 animals were infected in both halves and

Table 1 Descriptive statistics of EC (mS.cm^{-1}) by type of pathogen.

category	N	Mean	SD	Median	Minimum	Maximum
without pathogens	415	4.6335^{B}	0.7579	4.5	3.1	10.3
major pathogens	14	5.8786^{A}	1.6912	5.3	2.9	9.6
minor pathogens	161	5.2919^{A}	1.2376	5.0	3.5	11.5

Note: A, B – means with different letters are significant (p <0.001); SD – standard deviation.

Table 2 Descriptive statistics of LogSCC (log cells.mL^{-1}) by type of pathogen.

category	N	Mean	SD	Median	Minimum	Maximum
without pathogens	415	4.8999^{C}	0.5836	4.8325	3.4772	7.0000
major pathogens	14	6.5047^{A}	0.9081	6.6447	4.3424	7.5887
minor pathogens	161	5.8489^{B}	0.7564	6.0418	3.9542	7.4532

Note: A, B – means with different letters are significant (p <0.001); SD – standard deviation.

Table 3 Spearman correlation coefficients and descriptive statistics of EC (mS.cm^{-1}) by SCC class (x 10^3 cells.mL^{-1}) according to **Romero et al. (2017)**; **Caria et al. (2016)** and **Barth, Burow and Knappstein (2008)**.

SCC class	r	N	Mean	SD	Median	Minimum	Maximum
Romero et al. 2017							
SCC < 200	0.214^{***}	392	4.4992^{A}	0.5443	4.5	2.9	7.9
200 ≤ SCC < 400	0.036^{NS}	34	4.7765^{A}	0.6282	4.8	3.5	6.7
400 ≤ SCC < 600	-0.138^{NS}	21	4.7810^{A}	0.6022	4.8	3.8	6.2
SCC ≥ 600	0.403^{***}	143	5.8091^{B}	1.3779	5.5	3.5	11.5
Caria et al. 2016							
SCC < 700	0.270^{***}	456	4.5432^{A}	RE0.5624	4.5	2.9	7.9
SCC ≥ 700	0.382^{***}	134	5.8619^{B}	1.4028	5.5	3.5	11.5
Barth, Burow and Knappstein 2008							
SCC < 100	0.136^{*}	301	4.4581^{A}	0.5424	4.5	2.9	7.9
SCC ≥ 100	0.557^{***}	289	5.2433^{B}	1.1882	5.0	3.1	11.5
all data	0.531^{***}	590					

Note: * p <0.05; *** p <0.001; A, B – means with different letters are significant (p <0.001); SD – standard deviation.

Table 4 Descriptive statistics of lactose (%) and protein (%) by type of pathogen.

category	N	lactose				protein			
		Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
without pathogens	415	4.97^{A}	0.48	1.15	6.13	5.66^{B}	0.68	3.94	7.74
major pathogens	14	4.10^{B}	0.80	2.80	5.70	6.55^{A}	1.43	4.82	9.97
minor pathogens	161	4.59^{B}	0.80	1.79	6.06	5.82^{AB}	0.97	3.87	9.95

Note: A, B – means with different letters are significant (p <0.001); SD – standard deviation; Min. - Minimum; Max. – Maximum.

Table 5 Descriptive statistics of lactose (%) and protein (%) by SCC (log x 10^3 mL^{-1}) classes with division according to **Romero et al. (2017)**; **Caria et al. (2016)** and **Barth, Burow and Knappstein (2008)**.

SCC class	N	lactose				protein			
Romero et al. 2017		Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
SCC < 200	392	5.06^{A}	0.35	3.67	6.13	5.68	0.77	3.94	9.97
200 ≤ SCC < 400	34	4.95^{A}	0.43	3.85	5.85	5.69	0.74	4.76	7.72
400 ≤ SCC < 600	21	4.88^{A}	0.42	3.54	5.47	5.64	0.95	3.87	8.67
SCC ≥ 600	143	4.25^{B}	0.85	1.15	5.89	5.86	0.86	4.41	9.47
Caria et al. 2016									
SCC < 700	456	5.03^{A}	0.37	3.54	6.13	5.67	0.79	3.87	9.97
SCC ≥ 700	134	4.22^{B}	0.85	1.15	5.89	5.85	0.84	4.41	9.47
Barth, Burow and Knappstein 2008									
SCC < 100	301	5.06^{A}	0.36	3.67	6.13	5.74	0.78	3.94	9.97
SCC ≥ 100	289	4.63^{B}	0.75	1.15	5.89	5.71	0.82	3.87	9.47

Note: A, B – means with different letters are significant (p <0.001); SD – standard deviation; Min. - Minimum; Max. – Maximum.

Table 6 Spearman correlation coefficients among milk variables (n = 590).

	LogSCC	lactose	protein	EC
LogSCC	1.000			
lactose	-0.373***	1.000		
protein	-0.022	-0.526***	1.000	
EC	0.531***	-0.393***	-0.152***	1.000

Note: *** p <0.001.

65 animals with only one half. In 14 samples (2.4%) major pathogens were detected (*Staphylococcus aureus* (5 samples), *Streptococcus agalactiae*).

The presence of the pathogen had an significant effect ($F_{(2;587)}$ = 37.06; p <0.001) on the increase in electrical conductivity (Table 1), no significant differences were found between the minor and major pathogens. EC of the infected glands (n = 175) without considering the type of pathogen was (Mean ± SD) 5.3389 ± 1.2836 $mS.cm^{-1}$.

Similarly as above, the presence of the pathogens had an significant effect ($F_{(2;587)}$ = 155.61; p <0.001) on the increase in LogSCC (Table 2), but the major pathogens increased the LogSCC level significantly higher than minor pathogens. This goes along with the results of other studies **(Linage et al., 2017; Gonzalo, 2018)**.

The correlation between SCC and EC for all animals (Table 3) was higher (a moderate relationship) than that found by **Caria et al. (2016)** (r = 0.306) or **Romero et al. (2017)** (r = 0.33), but corresponds to the data reported by **Peris et al. (1991)**. The strongest correlation was, similarly to **Romero et al. (2017)** (r = 0.25) in SCC ≥ 6 × 10^5 class. This correlation may indicate that EC may be used in this class for mastitis detection. Also, differences in the EC were statistically significant ($F_{(3;586)}$ = 86.67; p <0.001) only between the SCC ≥ 6 × 10^5 class and the other classes. When ordering EC according to **Caria et al. (2016)** significant differences between means ($F_{(1;588)}$ = 261.08; p <0.001) were found. Lower value of EC in class SCC < 7 × 10^5 as in classes 2 × 10^5 ≤ SCC < 4 × 10^5 or 4 × 10^5 ≤ SCC < 6 × 10^5 in classification above (Table 3) was caused by counting a greater number of cases from the SCC < 2 × 10^5 class to this class.

The lactose and protein content was significantly affected by the presence of pathogens (Table 4) but without significant differences between minor and major pathogens groups. In the SCC class classification (Table 5), we found significant differences in lactose content only between the SCC ≥ 6 × 10^5 class and the other classes. In the classifications according to **Caria et al. (2016)** and **Barth, Burow and Knappstein (2008)**, the differences between the classes were statistically significant. However, there are no differences between classes in protein content.

The negative correlation between LogSCC and lactose content (Table 6) corresponds to findings from other authors **(Scharch, Süß, and Fahr, 2000; Olechnowicz et al., 2009; Caria et al., 2016)**, but our values are lower than those reported by **Olechnowicz et al. (2009)** and **Scharch, Süß, and Fahr (2000)**. The electrical conductivity showed a stronger correlation with the LogSCC than lactose and protein content reported, although it is still only a weak relationship.

CONCLUSION

We can argue that measuring the electrical conductivity of sheep milk may be a possible alternative for mastitis detection in sheep. EC can be useful in detecting animals with level of SSC greater than 6×10^5 cells.mL^{-1}. But we can not estimate a threshold for healthy animals. Perhaps, if we obtain more data from animals in the $2 \times 10^5 \leq$ SCC $< 4 \times 10^5$ and $4 \times 10^5 \leq$ SCC $< 6 \times 10^5$ (cells.mL^{-1}) categories, it will be possible to specify the threshold in the future. However, since electrical conductivity is influenced by several factors **(Romero et al., 2017)**, it would be more appropriate to think about multiple individual assessments in milking parlours rather than using a portable device for mastitis detection.

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VARIETAL VARIABILITY OF LESS GROWN MINTS: INFLUENCE ON SELECTED ANTIOXIDANTS

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ABSTRACT

Genus *Mentha* belongs among the important part of spice, aromatic and medical plants. It includes a large amount of varieties and forms which are not spread generally because of low knowledgeability about possibilities of individual varieties using and also about their high content of bioactive substances. Five less-known varieties and forms (*Mentha* x *piperita* var. ´Danica´, *Mentha* x *piperita* var. ´Chocolate´, *Mentha arvensis* f. Banana, *Mentha sp*. f. Mojito, *Mentha sp*. f. White Grape) with different aroma and plant habitus were chosen to estimate the content of essential oils, chlorophyll *a*, chlorophyll *b* and carotenoids. From obtained results varietal variability of individual varieties and forms have been specified and compared with commonly used *Mentha* x *piperita*. The content of essential oils was estimated by steam distillation. The pigment content was determined by spectrophotometric measurement of absorbance at a wavelength of 649 nm (chlorophyll *a*), 665 nm (chlorophyll *b*) and 450 nm (carotenoids). The results showed that ´Danica´ and ´Chocolate´ varieties have reached the highest amount of essential oils. Most of the chlorophyll *a*, *b* and carotenoids content reached commonly used *Mentha* x *piperita*, but there wasn´t found significant difference when compared with ´Danica´ and ´Chocolate´. The effect of varietal variability on the content of qualitative characteristics in the case of essential oils, chlorophyll *a* and *b* was confirmed according to used statistical analyse (p <0.05). The varietal variability of tested mint forms and varieties on carotenoids content was not confirmed.

Keywords: mint; essential oils; carotenoids; chlorophyll

INTRODUCTION

Mentha x *piperita* L. and *Mentha arvensis* L. are perennial plants belonging to *Lamiaceae* family, originating from Europe but spread around the world and cultivated in many different climates **(Heydari et al., 2018)**. Mint essential oils have long been used in various forms such as in management of plant pathogens and insect pests, in traditional medicine as well as in culinary and cosmetics **(Singh and Pandey, 2018)**.

The essential oils are included in the group of terpenes; these are volatile substances soluble in ethanol. The quality and quantity of essential oils is influenced by the environment, the growing methods, the way of harvesting, the post-harvest processing of plants and the way of essential oils obtaining **(Šalamon, 2015)**. The use of essential oils is extremely diverse depending on the source, quality, extraction procedure, etc. Essential oils have proven industrial applications in the manufacture of perfumes, cosmetics, soaps, shampoos, or cleaning gels. Another interesting aspect of these oils is their potential as therapeutic agens in aromatherapy or as active principles or excipients of medicine **(Tančinová et al., 2018)**. Another significant application of essential oils is in the agrofood industry, both for producing beverages and for flavoring foods **(Ríos, 2016)**.

Chlorophyll as a photosynthetic pigment occurs in green plants in two forms: chlorophyll *a* and chlorophyll *b*, bound on a protein as chromoprotein. The ratio of chlorophyll *a* to chlorophyll *b* is usually 3:1 **(Hudec et al., 2002)**. The basic structure of the chlorophyll molecule is the porphyrin ring, which is formed by four heterocyclic nuclei with 10 double bonds and a centrally bonded magnesium atom **(Kleňová and Turianica, 2010)**.

Chlorophylls are fitted and accompanied by carotenoids in chloroplasts, on tylacoid membranes. They are naturally decomposed simultaneously with the breakdown of chloroplast membranes at the end of activity of green plant parts **(Ivanišová, 2014)**. Many studies support that chlorophylls and its derivatives have antioxidant properties **(İnanç, 2011)**. Chlorophyll as well as carotenoids, has the ability to react with singlet oxygen and it is acting as an

extinguisher of free radicals as an antioxidant **(Kleňová and Turianica, 2010)**.

The main compounds with the antioxidant properties in mints are phenols, ascorbic acid and carotenoids **(Capecka, Mareczek and Leja, 2005)**. Based on the results of a number of clinical and epidemiological studies there was confirmed the antioxidant effect of carotenoids in the prevention and treatment of certain types of cancer, cardiovascular and ocular diseases and photosensitivity disorders **(Fiedor and Burda, 2014)**.

At the present many experts have agreed on that the antioxidants taken in their natural state have greater efficacy and usability than their equivalent amounts taken in pure form as a food supplement **(Chrpová, 2010)**.

Scientific hypothesis

According to different aroma and plant habitus (colour of the leaves) there is prediction of different content of essential oils and pigments (chlorophyll *a*, *b* and carotenoids) in selected mint varieties and forms.

MATERIAL AND METHODOLOGY

Small plot field experiment with mint was conducted in area of the Department of Vegetable production, SUA Nitra, in 2016 and 2017. Before planting the plot preparing was done according to the cultivation technology of mint species, and on the basis of the agrochemical analysis of the soil, the land was fertilized with a dose of nitrogen fertilizer DASA in the amount of 0.44 kg 20 m^2 on 5th of May 2016. Nonwoven fabric was applied on 9th of May 2019. From each variety 10 plants in a spacing 0.4 x 0.4 m were planted. Selected range of *Mentha* spp. was purchased through the Lumigreen internet store, which has the widest spectrum of the mint genus at Slovak market. After the planting, nitrogen in the form of ammonia (LAD) was applied at 0.64 kg.20 m^{-2} in two dosages in 2016 and in 0.50 kg.20 m^{-2} in two dosages in 2017. Additional irrigation was used as required. Pesticides during the vegetation period (both years) were not applied because the plants were not infected with diseases and pests and also the content of selected antioxidants was monitored.

Soil – climatic conditions

The plot area is situated in a very warm agro climatic area based on climatic conditions, which is characterized by warm lowland climate with long to very long, warm and dry summer, mild, dry to very dry winter with duration of snow cover from 30 to 40 days a year. The average annual rainfall in the area is 500 – 600 mm **(Hreško, Pucherová and Baláž, 2006)**. The average annual temperature varies between 9 – 10 °C. Average July temperatures are 18 °C to 20.5 °C and average January temperatures are -1 °C to -3° C. The wind prevails in the northwest; less frequent winds are east, northeast and west. Southwest, south and southeast winds are the least common **(Špánik, Repa a Šiška, 2002)**.

Characteristics of selected less grown mint varieties and forms

Mentha arvensis f. Banana (Figure 1) – A fast growing low variety comes from France. Light green leaves are small, oblong-ovate to elliptic, with sparsely saw – leaves edge, covered with simple trichomes. It blooms from June to September with purple flowers. It grows to a height of 45cm (60cm). It requires humid, permeable soil, sunny position, well tolerates by also half-shade, it is also suitable for growing in containers. It has a characteristic banana menthol flavour.

Mentha x piperita var. 'Danica'(Figure 2) – The ´Danica´ variety is 80 cm tall. The leaves are oblong-elliptical to lanceolate, toothed, 4 – 8 cm long and 2.5 – 3.5 cm broad, green to red-green. The flowers are 3 – 5 mm long, lilac, or pale violet, produced in clusters on tall, branched, tapering spikes; flowering from July till September. It spreads via rhizomes to form clonal colonies.

Mentha x piperita var. ´Chocolate´ (Figure 3) – mint variety grows up to 30 – 60 cm high. The leaves are shaggy, opposed, elongated, egg-shaped, lanceolate, with a slightly arched edge, bronze-green. The stem is reddish-brown. The cup is a cylindrical purple pike on the top of the stem. It blooms from June to September. It requires moist, nutritious and permeable soil, sunny position.

Mentha sp. f. Mojito (Figure 4) – The variety comes from Cuba, brought to Europe in 2006. It creates upright stems 45 – 60 cm high. The leaves are opposed, seated or short stems, pale green, markedly wrinkled, egg-shaped, with a cut base, the edge of the leaf blade is sharply arched. The flowers are white in colour; they have a conical to cylindrical shape. It blooms from July to September. It requires light, aerated and humus soil, plenty of soil moisture. Full Sun to Partial Shade. It is the main ingredient of the alcoholic beverage 'Mojito'.

Mentha sp. f. White Grape (Figure 5) – It is a fast growing variety with size up to 50 – 90 cm. The stems are red in the lower part. The leaves are opposed, on short stalks, dark green, wide to oval, markedly wrinkled, with petiole edges, cheeks and ruby covered with trichomes. The flowers are conical of pale violet colour. It blooms from July to September. Plants grow in moist, permeable, nutritious soils, in direct sunlight or in half-shade.

Harvesting and post-harvest treatment

The harvest of the mint was carried out twice a season for estimation of quantitative characteristics (yields). For qualitative parameters were used plants from second harvest in 31th of August in 2016, and 10th of August in 2017. The harvest was carried out mechanically (by knife); the whole plants were cut 10 cm above the surface of soil. After harvest each variety was prepared for analysis according to the chosen methodology **Hegedűsová, Mezeyová and Andrejiová (2015)**. The process of determination of photosynthetically active pigments and essential oils was carried out in the laboratories of the Research Institute AgroBioTech SUA in Nitra and of Department of Vegetable production, FHLE, SUA in Nitra.

Determination of quantitative and qualitative parameters

Estimation of essential oils content

In the dried drug the essential oils content was estimated by the distillation method by using distilled apparatus according to **(Hegedűsová, Mezeyová and Andrejiová, 2015)**.

Estimation of chlorophyll a and chlorophyll b content

The chlorophyll *a* and chlorophyll *b* were determined spectrophotometrically (Spektralquant PHARO 100) laterally in the acetone extract on the wavelengths 649 nm and 665 nm in homogenisated fresh plant (150 – 200 g) **(Hegedűsová, Mezeyová and Andrejiová, 2015)**. Number of analysed samples for average content of chlorophyll *a* and *b* was 10 in case of each variety.

Estimation of total carotenoids content

The extraction of samples was done at the Laboratory of Beverages, AgroBioTech Research Center, Slovak University of Agriculture (SUA) in Nitra. The content of total carotenoids was estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract on spectrophotometer PHARO 100 at 450 nm wavelengths **(Hegedüsová, Mezeyová and Andrejiová, 2015)**.

Statistic analysis

A statistical analysis was performed by using of the Statgraphic Centurion XVII (StatPoint Inc. USA). Obtained results were evaluated by analysis of variance (ANOVA) and average values were tested by LSD test performed at the significance level of 95%.

RESULTS AND DISCUSSION

The content of essential oils was ranged from 0.67 ±0.30 (White Grape) to 3.10 ±0.39 mL.100 g^{-1} (´Danica´) in average from both tested years according to Table 1. The varietal variability was confirmed according to used statistical analyzes (p <0.05) between tested mint varieties, when F. Banana (0.86 ±0.05 mL.100 g^{-1}) and White Grape had the lowest content of the essential oils and ´Danica´ reached the highest value. Generally used *Mentha* x *piperita* reached higher value of essential oils in comparison with f. Banana and f. White Grape. The other varieties ´Chocolate´, ´Danica´ and f. Mojito were richer in essential oils, what was also statistically confirmed. Varietal variability was confirmed also by **Boukhebti et al. (2011)** where *Mentha spicata* had 1% off essential oils and *Mentha pulegium* 0.87%. Similarly the highest content of essential oils was found in *Mentha spicata* L. 'Moroccan'(0.82%) and *Mentha piperita* L. 'Glacialis' leaves, while the least in *Mentha suaveolens* Ehrh. 'Variegata' (0.07%) **Tarasevičienė et al. (2019)**. **Hussain et al. (2010)** determined that amount of essential oil depended on species and cultivation time of mint. The authors found that amount of essential oil in *M. arvensis* was 17.0 g kg^{-1}, *M. piperita* 12.2 g kg^{-1}, *M. longifolia* 10.8 g kg^{-1} and *M. spicata* 12.0 g kg^{-1} in summer grown plants, respectively 9.20, 10.5, 7.00 and 9.50 g kg^{-1} in the winter crops. The influence of climatic condition, cultivation and soil composition was obvious in our results, when significant difference was found between tested years in essential oils content in tested varieties at p <0.05 (Table 1).

According to Table 2 the chlorophyll *a* content estimated in fresh matter ranged in average from 84.74 ±4.49 mg.100 g^{-1} in case of f. Banana to 125.54 ±0.48 mg.100 g^{-1} (*Mentha* x *Piperita*). Second light coloured variety f. Mochito had also low level of chlorophyll *a* content with reached value 87.92 mg.100 g^{-1}. Generally used *Mentha* x *Piperita* reached highest values in comparison with all tested forms and varieties, the significant difference was confirmed (p <0.05) with f. Mojito, f. White Grape and f. Banana. Also **Straumite, Kruma and Galoburda (2015)** monitored the effect of varietal variability on the plant pigment content in leaves and stems of nine different species and mint varieties. The highest chlorophyll *a* content in leaves was reached by *M.* x *piperita* L. var. ´Bavarian´ (0.849 mg.g^{-1} of fresh matter) and the lowest *M.* x *piperita* L. var. ´Almira´ (0.321 mg.g^{-1}), with difference of 164%. The ´Chocolate´ mint variety contained 0.361 mg.g^{-1} (36.1 mg.100g^{-1}) of chl *a* in fresh matter. Because of differences in comparison with our results it is needed to say, that the chlorophyll content in fresh plant matter varies depending on the place of cultivation, its climatic conditions, the soil composition, the date of cutting, etc. **(Bohn et al., 2006)**. It is obvious f. e. on the statistically confirmed influence of the year on chlorophyll *a* according to values of our trial (Table 2). **Tewari et al. (2012)** tested the chlorophyll *a* content in case of *Ocimum kilimandscharicum*, where 96.57 mg.100 g^{-1} was measured what is comparable with the our results of light mints like f. Banana or f. Mochito.

The chlorophyll *b* content estimated in fresh matter moved in order 41.86 ±1.39 mg.100 g^{-1} (f. Banana) <47.63 ±2.25 mg.100 g^{-1} (f. Mojito) <53.32 ± 3.54 mg.100 g^{-1} (f. White Grape) <59.97 ± 15.95 mg.100 g^{-1} (´Danica´) <62.62 ±5.48 mg.100 g^{-1} (´Chocolate´) <to 67.40 ±10.51 mg.100 g^{-1} (*Mentha* x *Piperita*) according to Table 3. The difference in varietal variability of tested forms and varieties was statistically confirmed at p <0.05. **Grzeszczuk and Jadczak (2009)** estimated the biological value of fresh matter in five different mint varieties and species, with the highest chl *b* content in case of *M. aquatica* L. – 519.14 mg.kg^{-1} (51.914 mg.100g^{-1}) and the lowest in *M.* x *piperita* L. var. citrata Ehrh. with 325.55 mg.kg^{-1}, (32.56 mg.100g^{-1}). According to **Tarasevičienė et al. (2019)** the amount of chlorophyll *b* in *Mentha piperita* 'Glacialis' was 6.6-times higher than in *Mentha piperita* 'Swiss'. Total amount of chlorophylls in leaves was the highest in *Mentha piperita* 'Glacialis' (0.376 ±0.014 mg.g^{-1} FW) and *Mentha suaveolens* 'Variegata' (0.307 ±0.011 mg.g^{-1} FW), while in *Mentha piperita* 'Swiss' the lowest (0.057 ±0.002 mg.g^{-1} FW).

According to Table 4 total carotenoids content moved in 2 – years average from 5.80 ±5.54 mg.100 g^{-1} FM (f. Banana) to 10.38 ±5.16 mg.100 g^{-1} FM (f. Danica).

Table 1 The content of the essential oils in selected mint genus representatives [mL.100 g^{-1} DM]*.

variety/form	2016^{A}	2017^{B}	average
´Danica´	2.83 ±0.25	3.37 ±0.10	3.10 ±0.39^{d}
´Chocolate´	2.73 ±0.25	2.74 ±0.16	2.73 ±0.01^{cd}
f. Banana	0.83 ±0.11	0.90 ±0.01	0.86 ±0.05^{a}
f. White Grape	0.45 ±0.21	0.88 ±0.03	0.67 ±0.30^{a}
f. Mojito	1.68 ±0.04	3.14 ±0.03	2.41 ±1.04^{c}
Mentha* x *Piperita	1.75 ±0.14	1.27 ±0.06	1.51 ±0.34^{b}

Note: a, b, A, B – Different letters in the upper index represent a statistically proven difference (p <0.05, LSD test, ANOVA), Statgraphic XVII.
*average ± standard deviation, DM = dry matter.

Table 2 The content of the chlorophyll *a* in selected mint genus representatives [mg.100 g^{-1} FM]*.

variety/form	2016^{A}	2017^{B}	average
´Danica´	143.12 ±15.40	82.00 ±5.66	112.56 ±43.22^{bc}
´Chocolate´	128.14 ±9.74	103.29 ±2.59	115.71 ±17.57^{bc}
f. Banana	87.91 ±1.60	81.56 ±1.09	84.74 ±4.49^{a}
f. White Grape	112.32 ±1.04	92.00 ±4.28	102.16 ±14.37^{ab}
f. Mojito	91.71 ±4.44	84.14 ±4.93	87.92 ±5.36^{a}
Mentha* x *Piperita	125.88 ±19.46	125.21 ±5.41	125.54 ±0.48^{c}

Note: a, b, A, B – Different letters in the upper index represent a statistically proven difference (p <0.05, LSD test, ANOVA), Statgraphic XVII.
*average ± standard deviation, FM = fresh matter.

Table 3 The content of the chlorophyll *b* in selected mint genus representatives [mg.100 g^{-1} FM]*.

variety/form	2016^{A}	2017^{A}	average
´Danica´	71.25 ±19.38	48.70 ±3.88	59.97 ±15.95^{bcd}
´Chocolate´	66.49 ±3.34	58.74 ±1.47	62.62 ±5.48^{cd}
f. Banana	40.87 ±2.33	42.84 ±0.06	41.86 ±1.39^{a}
f. White Grape	55.82 ±0.87	50.82 ±2.34	53.32 ±3.54^{abc}
f. Mojito	46.04 ±3.95	49.22 ±3.46	47.63 ±2.25^{ab}
Mentha* x *Piperita	59.97 ±4.81	74.84 ±1.92	67.40 ±10.51^{d}

Note: a, b, A, B – Different letters in the upper index represent a statistically proven difference (p <0.05, LSD test, ANOVA), Statgraphic XVII.
*average ± standard deviation, FM = fresh matter.

Table 4 The content of the carotenoids in selected mint genus representatives [mg.100 g^{-1} FM]*.

variety/form	2016^{A}	2017^{A}	average
´Danica´	14.02 ±3.19	6.73 ±0.07	10.38 ±5.16^{a}
´Chocolate´	5.16 ±0.49	13.09 ±1.33	9.13 ±5.60^{a}
f. Banana	9.72 ±0.26	1.88 ±1.32	5.80 ±5.54^{a}
f. White Grape	13.61 ±1.23	3.16 ±1.29	8.38 ±7.39^{a}
f. Mojito	10.72 ±0.07	2.33 ±0.54	6.52 ±5.94^{a}
Mentha* x *Piperita	6.39 ±0.01	14.89 ±0.01	10.64 ±6.01^{a}

Note: a, b, A, B – Different letters in the upper index represent a statistically proven difference (p <0.05, LSD test, ANOVA), Statgraphic XVII.
*average ± standard deviation, FM = fresh matter.

Figure 1 *Mentha arvensis* f. Banana.

Figure 2 *Mentha x piperita* var. 'Danica'.

Figure 3 *Mentha* x *piperita* var. 'Chocolate'.

Figure 4 *Mentha sp*. f. Mojito.

Figure 5 *Mentha sp.* f. White Grape.

The values differed, but according to used statistical test (p <0.05) the significant varietal variability on carotenoids for tested varieties and forms wasn´t confirmed. Carotenoids content in the mint plants differed in relation to species according to **Tarasevičienė et al. (2019)** where for *Mentha piperita* 'Glacialis' leaves the amount of carotenoids was the highest (0.310 ±0.005 mg.g^{-1} FW) and in *Mentha piperita* 'Swiss' the lowest (0.053 ±0.002 mg.g^{-1} FW) with significant varietal variability at p <0.05. **Straumite, Kruma and Galoburda (2015)** on the basis of the measurements stated the highest value of carotenoids 16.9 mg.100g^{-1} in stems of *Mentha spicata* ´Marokko´ and the lowest in *Mentha* x *piperita* ´Granada´ leaves (3.8 mg.100g^{-1}). **Rubinskienė et al. (2015)** tested in addition to the content of carotenoids also the effect of different drying methods on the chemical composition and colour of leaves in two varieties of peppermint. For the ´Peppermint´ variety the total carotenoid content was 5.7 mg.100 g^{-1} of fresh matter and for the ´Krasnodarskaja´ variety 5.8 mg.100 g^{-1}. All mentioned results are similar to our estimations.

CONCLUSION

The new mint varieties are characterized by a different flavour and aroma, which is directly connected with qualitative bioactive substances, especially with the quality and quantity of essential oils. On the basis of obtained results the significant effect (p <0.05) of varietal variability was found on the essential oils content when the highest values reached *Mentha* x *piperita* var. ´Danica 'and *Mentha* x *piperita* var. ´Chocolate´. *Menta sp.* f Mojito has also reached a higher value than the commonly available *Mentha* x *piperita*, so it can be recommended for intense cultivation not only as an interesting mint in the popular drink, but also because of the high content of essential oils. In view of other the monitored parameters, *Mentha* x *piperita* reached the highest content in case of chlorophyll *a* (125.54 mg.100 g^{-1} FM) and chlorophyll *b* (67.40 mg.100 g^{-1} FM), as well as in the case of carotenoids (10.64 mg.100 g^{-1} FM). ´Danica´ and ´Chocolate´ varieties reached very similar values of chlorophyll *a*, *b* and carotenoids compared to *Mentha* x *piperita*, the differences were not statistically significant at p <0.05. In the case of f. Banana, f. White Grape and f. Mochito the significant differences have been found and they reached lower values in the contents of monitored qualitative characteristics.

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CAPITAL TAXATION EFFICIENCY OF AGRICULTURAL BUSINESSES IN THE SLOVAK REPUBLIC

Alena Andrejovská, Ján Buleca, Veronika Puliková

ABSTRACT
Effective tax rates are presented by indicators of the actual corporate tax burden, which take into account the impact of all the elements listed in the legislation. The submitted contribution explores the issue of effective taxation through effective average tax rates (EATRs) focusing on agricultural production enterprises. The analysis assessed the effect of changing the statutory tax rate (and other taxes and factors) on changing the effective average rate of capital. Taxation efficiency was monitored for selected intangible and tangible assets for 2004 and 2018. Analysis indicated a depreciation tax shield that tracked the amount of tax savings on capital investment as well as the economic rent of the project with taxation. The analysis showed that a 3% increase in the statutory rate over the reference period increased the effective average corporate rates for intangible assets by 13.35%, tangible assets by 14.25% and inventories by 16.63%. The highest annual tax saving was achieved in 2018 for tangible assets of € 4,647.50, with a four-year return.

Keywords: agriculture; burden; efficiency; tax; asset

INTRODUCTION
Market economy, capital mobility, and corporate tax efficiency are the concepts that are related to each other, and are currently putting strong pressure on investors as well as government officials in their decision making on investment placement. Major changes in the tax systems of the EU countries have resulted in the globalization and digitalization of the economy, which has substantially increased the geographical mobility of taxation. This has created a competitive environment between the tax systems that raised concerns about the level and fairness of the tax policies in a global perspective. From the point of view of economic efficiency, the tax systems should ideally be "neutral", particularly as regards the economic decisions. In fact, differences in corporate taxation in individual countries can only mean the differences in social security costs, producer costs, favouring one type of producer before another, and so on. Therefore, it is important to monitor the effective tax rates that examine the tax bases and provide sufficient information not only to investors about the volume and allocation of their investments, but also to government officials who create the tax legislation and modify the structure of tax systems.

Corporate tax rates are one of the decisive factors that influence the investors in deciding on the location of their investments and their business activities. The first and important dimension is the statutory tax rate (STR), given by the tax laws of each country. This is the easiest and the most accessible way to get information on the tax conditions in a country, which is certainly not sufficient. It is more important to monitor the total tax burden, which represents the level of the corporate tax, as the share of the taxes paid on the total income and the profit of the enterprise in the country **(Bird, 2000; Gupta, 2007; Bayer, 2012)**. Inappropriateness of using the statutory rates as an objective indicator in monitoring and subsequently comparison of the corporate tax rates has led to the deduction of the effective tax rates (ETR), which have significantly higher informative value **(Baker and McKenzie, 1999; Barrios, Nicodème, and Sanchez Fuentes, 2014; Delgado, Fernandez-Rodriguez, and Martinez-Arias, 2014)**.

The tax rate, which significantly influences the tax burden in the form of statutory, effective and average rates, is an important information not only for investors but also for politicians and economists **(Bánociová et al., 2014)**. In recent decades, the corporate tax system has undergone dramatic changes due to a fall in statutory but also effective tax rates, and a substantial widening of the tax base through the depreciations **(Devereux, Griffith, and Klemm, 2002; Liu and Cao, 2007; Egger and Raff, 2015)**. The countries have strategically responded to tax cuts in competing countries that have helped attract the foreign investors. There are many methods for calculating the effective rates used to determine the effective taxation, such as a macro-based backward-looking measures, a micro-based

backward-looking measures, and a micro-based perspective measure. As reported by **Devereux and Griffith (1998)**; **Sørensen (2004)**; **Devereux, Lockwood, and Redoano (2008)**, the use of these methods depends on the data used, from the time perspective (past/future), but also from the monitored area (micro/macro-level). All three methods are based on the assumption that the market of production factors is competitive and the production function has the usual characteristics. In such a case, the decision on where and how much to invest is affected not only by the rate of capital taxation but also by other production factors (wages, energy, and land).

A number of empirical studies **(McKenzie, Mintz and Scharf, 1997; Devereux and Griffith, 1998, Devereux and Griffith, 2003; Devereux, Griffith and Klemm, 2004; Kubátová and Říhová, 2012; Barrios, Nicodème and Sanchez Fuentes, 2014; Šimková, 2016)** investigated the impact of effective corporate tax rates on the company's economic behaviour, including their location, selection of investment opportunities, and spill-over income strategies. Others **(Gordon and Slemrod, 1998; Arnold and Schwellnuss, 2008; Vartia, 2008; Arnold et al., 2011; Vegh and Vuletin, 2015)** have used these rates to address the tax competition issues. **Suzuki (2014)** in his study in Asian countries, assessed the tax holidays as a means of attracting foreign capital and, in turn, the impact of tax holidays on the effective tax rates that varied according to the volume of capital contribution into individual schemes. Tax holidays for typical investments could be increased not only by the EATR, but also by EMTR (assuming 10% profit rate surplus), reflecting the extraordinary generous depreciation policies of some Asian countries.

The crucial indicator, apart from the tax holidays, is the size of the country and investment tax relief in form of the contributory capital that government can provide to individual investors. Small countries with almost zero effective tax rates can attract the most foreign capital. This finding is in line with a simple theoretical model of tax competition in which the optimal behaviour of small countries determines the reduction of the revenues at the source of taxation to the absolute minimum **(Gordon, 1986; Zodrow and Mieszkowski, 1986)**. Larger countries can maintain relatively high effective tax rates. This finding is based on the asymmetric tax competition **(Bucovetsky, 1991)**, as well as the "new trade theory" **(Haufler and Stahler, 2013; Baldwin and Krugman, 2004)**. The theory of asymmetric tax competition has determined the differences in capital elasticity between small and large countries, where higher tax rates settings are more balanced. According to the new trade theory the countries with a large domestic market can still maintain higher tax rates. It should be kept in mind that when analysing the tax competition, which is often influenced by the level of effective tax rates, we should take into account the volume and allocation of the investment. An analysis of such tax competition is a challenge for the future.

Scientific hypothesis

In the study, we calculate the effective tax rate for agriculture companies for conditions in the Slovak Republic. In the long run, the effective tax rate is lower than the statutory one. This trend is recorded worldwide. Basic hypothesis: Is the effective tax rate in Slovakia lower than the statutory rate in 2004 and 2018?

MATERIAL AND METHODOLOGY

The methodology for EATR calculation for capital was proposed by **King and Fullerton (1984)**, and extended by **Devereux and Griffith (1998)**. It represents the ratio of the actual rate of return before tax, required to reach a zero economic rent after tax (where the cost of capital is an initial investment), and the actual rate of return after tax for the shareholder.

The main source in calculating the effective average capital tax rate was the database of corporate taxation of the Centre for European Economic Research **(ZEW, 2018)**, which provides estimates of effective average tax rates (EATRs) for European countries classified according to the asset types and sources of their funding within the period 1998 to 2018.

The aim of the contribution was to analyse and evaluate the efficiency of taxation of selected types of intangible and tangible assets of agricultural holdings on the basis of accounting and tax legislation in the Slovak Republic (SR) through the construction of the EATR model in the year 2004 (entry of the Slovak Republic into the EU) and the year 2018.

The assets were classified into seven categories of intangible and tangible assets (intangible assets; agricultural buildings; machinery for agriculture and forestry; basic herd and the draught animals; permanent crops; land; and inventories). The design of the EATR model takes into account the discounted value of multiplying of the variability of tax discrimination, and the difference between the revenues and the costs of the project. The revenues were taxed at the required rate of return and accounting depreciations without the impact of inflation. The costs reflected the shareholder's discount rate, accounting depreciation, and inflation. They include the formula (1 – NPV tax depreciation shield), which expresses the tax savings from the depreciations. The sources for capital funding were divided into three groups, weighted by the **OECD (2011)** weights, and processed according to the OECD long-term statistics averages:

1. undistributed profit (55%);
2. new deposit (10%);
3. debt (35%).

The volume of corporate tax and the revenues from the interest deduction, that highlight the differences between the different ways of funding, are positively correlated.

Additional input data:

(r): real rate of return determined as 5% of the alternative investment,

(p): required rate of return before tax determined at the 20% level,

(π): inflation rate (of 2%),

(δ): accounting depreciation rate determined by **ZEW (2018)**,

(τ): effective statutory tax rate (22%),

(e): effective real estate tax rate determined from the statutory real estate rate (n) 0.25%, reduced by the corporate tax rate (22%). Since the **ZEW (2018)** model considers a market value that does not share in all countries

with a purchase price, it determines a uniform and optimal basis to capture the market value of 0.36%.

(v): Valuation of inventory loss may use:

- *FIFO method*: this method is used for valuation of inventory loss when the first inventory increase valuation price is used as the first price for inventory loss valuation (v = 1).
- *LIFO method*: is used for inventory valuation when the last inventory increase valuation price is used as first to evaluate the inventory loss. In the Slovak Republic (SR) this method is not allowed (v = 0). The weighted arithmetic mean is determined from actual purchase prices as the share of inventory in stock value, and the total inventory in stock state in the quantitative units, at least once per a month (v = 0.5).
- *Predetermined Inventory Price*: this is the price for fast-moving inventories (mostly in agriculture), in case of which we often do not yet know their price at the time of placing in storage (v = 2).

($\emptyset$): tax depreciation for tangible assets will be used in a straightforward or accelerated manner in accordance with the **Law no. 595/2003**. Intangible assets are depreciated in accordance with this Act for a maximum of 5 years up to their entry price.

(i): a nominal interest rate that would increase with the increase of inflation and an increase in the real interest rate.

(ρ): the shareholder's discount rate.

(γ): the variability of the shareholder's tax discrimination, which reflects the ratio of the funds from the investment to the alternative investment funds. If we eliminate the personal income tax at this value, a value of 1 is set, as the shareholder will not be discriminated when deciding for the investment, but for the possibility of depositing of his funds in the bank.

(A): the depreciation tax shield is determined by multiplication of the net present value by the tax coefficient.

(τ): tax savings, since the depreciations constitute a cost item that reduces the tax base of the company. In case of an increase of corporate tax rates, or a decrease in nominal interest rates, this saving will increase. Indicator in the form:

$$A = \tau\phi \quad \left\{\left(\frac{1}{(1+\rho)}\right) + \left(\frac{1}{1+p}\right)^2 + \cdots + \left(\frac{1}{1+\rho}\right)^T\right\} \qquad (1)$$

The effective average tax rate (EATR) is defined as the ratio of the current value of the taxes paid and the net present value of the revenue flows, excluding the initial investment costs. The method of the EATR determination consist of the proportional reduction of the economic rent generated by the investment due to the $EATR = (R^* - R)/R^*$ taxation. This method does not define the EATR for investment projects which are marginally without taxation $R^* = 0$. A different approach that follows the difference between R^* and R in relation to the net present value of the return on investments before tax $p/(1+r)$ was proposed by **Devereux and Griffith (1998)**. This relationship takes into account the impact of marginal personal effective tax rates on the capital gains accruing to the investors from this investment, which reduces the post-tax economic rent:

$$ATR = \frac{\frac{R^* - R}{p}}{\frac{}{(1+r)^t}}, \qquad (2)$$

where the R * is the economic rent flows from the project without tax and expresses the difference between the required rate of return before tax and the real interest rate from the next investment. To determine the present value of the project's profit, it is necessary to discount it with the real interest rate:

$$R^* = \frac{p-r}{1+r} \qquad (3)$$

The evaluation tracks the different assets and the equation is adjusted (reduced/increased) by the individual indicators.

Intangible assets, machinery for agriculture and forestry, basic herd, and the draught animals were calculated using the equation in the basic form:

$$R_{1,3,4} = \frac{\gamma}{1+\rho} * \{[(p+\delta) * (1+\pi) * (1-\tau)] - [\rho + \delta * (1+\pi) - \pi] * (1-A)\} \qquad (4)$$

For agricultural buildings and permanent crops, equation (4) was reduced by e − tax on real estate (from buildings: the tax rate + (number of floors * surcharge on the floor) * the size of the building) and (from land: tax rate * the size of the land * value of the land), this tax is the direct cost to this type of asset.

$$\mathrm{R}_{2,5} = \frac{\gamma}{1+\rho} * \{[(\mathrm{p}+\delta) * (1+\pi) * (1-\tau)] - [\rho + \delta * (1+\pi) - \pi] * (1-\mathrm{A})\} - \mathrm{e} \qquad (5)$$

In case of the land, accounting and tax depreciations are excluded from equation (5), i.e. δ = 0, (1 - A) = 0 (the land constitutes a specific group of undepreciated assets).

$$R_6 = \frac{\gamma}{1+\rho} * \{[p * (1+\pi) * (1-\tau)] - [\rho - \pi]\} - e \qquad (6)$$

For inventories, *e*: the tax on property is excluded from the equation (6), and the whole formula is reduced by the multiplication of the tax rate, the inflation rate, and the inventory valuation method. If the company decides for the *LIFO* method (the cost also includes the increase in the price level, we will insert 0 instead of v, that will reset the whole expression). In case of the *FIFO* method v = 1, while in the method of the weighted arithmetic mean v = 0.5, and in case of the method of predetermined inventory price we will use the v = 2 (which we have set as the basis, since it represents the agricultural fast-moving inventories).

The equation has the form:

$$R_6 = \frac{\gamma}{1+\rho} * \{[p * (1+\pi) * (1-\tau)] - [\rho - \pi]\} - v * \tau * \pi \qquad (7)$$

The investment was realized from the own funds (from undistributed profit and the new deposits) and from the external resources (out of debt). In the absence of personal taxes, γ = 1, the last indicator will always be zero, and capital costs for investments funded by new capital and investments funded by retained earnings will be equal. The difference is only in financing in the form of debt. To keep costs as low as possible, the companies try to optimize their capital structure. Corporate tax is the cost of equity financing, and often this cost is higher than other costs, such as this in form of interests, which are a tax-deductible item, thereby causing a reduction in the tax base, so called interest tax shield. Therefore, the economic rent of the project with taxation should be increased by the ratio of the discounted value of the difference between the discount rate of the shareholder and the nominal interest rate, and by the interest tax shield. It is necessary not to forget the effective

rate of real estate tax paid in the period of direct investment activity $(1 + e)$.

Equation for debt financing has the form:

$$F^{DE} = \frac{\gamma*(1+e)*(\rho-i+i*\tau)}{1+\rho} \quad (8)$$

Equation for financing through a new deposit has the form:

$$F^{NE} = -\frac{\rho(1-\gamma)(1+e)}{1+\rho} \quad (9)$$

Statistic analysis

For the calculation of ETR for agricultural companies in the Slovak Republic we use the method from **Devereux and Griffith (2003)**. Calculations were based on this methodology and modified for the conditions of Slovakia. Specifically, there were changes in the methodology for calculating tax deductions for all monitored assets, as well as determining the valuation of inventories and tax burden from the point of view of real estate tax. The calculation is extensive and the individual sub-calculations were given in the previous paragraph.

RESULTS AND DISCUSSION

The EATR for agricultural holdings was monitored within the years 2004 and 2018, with the entry year 2004, when a 19% flat tax was introduced. Since this period, the statutory rate has increased up to the current 22% (used in the analysis), (23% tax rate in 2013 represents the only change since 2004). Table 2 takes into account developments since 1991, which are connected to the formation of the Slovak Republic.

The tax depreciations that are necessary to determine the tax base have undergone a change that has increased the number of depreciation groups from 4 to 6 and prolonged the depreciation period for individual groups in 2015 (Table 3).

For the straight-line method of depreciation, the share of the entry price and depreciation period was used. This method takes only a fraction of the annual depreciation, depending on the number of months since the property was put into use. In the last year, the remaining months of the year are counted. The tax and accounting depreciation rates for the monitored assets are mentioned in the methodology of the work.

Real estate tax (land tax and building tax) is a local tax and is imposed by a city or municipality (Table 4).

The land tax was determined by multiplying the land area in m^2 and the corresponding value per $1m^2$. The building tax was determined by the area of the built-up area in m^2 and the tax rate determined in the generally binding regulations. The **ZEW (2018)** calculates the tax on invested capital (real estates) in buildings by an indirect method. Table 4 shows a four-fold increase in the level of the nominal real estate tax base since 2005. In the effective real estate tax, the amount has been distributed with the direct correlation since 1991, when it increased by 0.01% up to the year 2005. After this period, there was also a single four-fold increase.

The funding methods that were processed during the analysis were oriented to financing from undistributed profit, new deposits and debt. In analysing there is an absence of personal taxes (the dividend tax that will be applied in the Slovak Republic for the year 2017 (in 2018)). Capital costs for investments funded by a new deposit and investments funded by undistributed profits will be equal. The analysis revealed an effective average rate, a tax shield, expressing the tax savings and an economic rent of the project with taxation, which means the financial benefit of the related project (Table 5).

The differences occurred between the two monitored periods, 2004 with the rate of flat tax applied in that period at the level of 19%, and the current 22% tax rate. When assessing the depreciation tax shield, the highest annual tax saving for the 100,000 € model investment was achieved for tangible assets (machinery for agriculture and forestry, together with base herd and draught animals), where their four-year depreciation period in 2004 brought savings to taxes of € 16,050; and € 20,550 to € 20,590. On the other hand, the lowest tax savings were found for investments into agricultural buildings, as these assets have the longest return (20 years) and the lowest opportunity to reduce the tax base through tax depreciation. The tax savings amount for 2004 would be € 9,990 and an annual savings of just € 499.50. For 2018, it would increase up to € 11,590. Intangible assets for the five-year return period showed 15.54% (in 2004) and 17.99% (2018) savings from the purchase price.

Another monitored indicator, creating the part of the EATR, is the economic rent of the project with tax, which expresses the size of the project's financial benefit with the aspect of taxation. The lowest EATR rate belongs to the highest value of the indicator (R).

In an investor decision-making on investment placement into the agricultural assets in the Slovak Republic, the most favourable option would be the investments into the land and agricultural inventories, which showed the highest levels of the economic rent, but the lowest EATR rate. The economic rent on land was 0.1033 in 2004 and dropped to 0.0976 by 2018 when founding from own resources. Decrease in funding from 0.1159 to 0.1122 was found also when funding from foreign sources. Subsequently, the EATR rate showed the lowest taxation 20.79% in 2004, increasing to 23.78% in 2018 with funding from own resources, and also in funding from foreign resources the rate recorded increase from 22.05% to 25.25% within the monitored period. A similar, but slight 10% increase was shown fin case of the inventories. The EATR rate reached 22.73% in 2004 and 26.51% in 2018 in inventories when funding from own resources, and 24.00% and 27.98% when funded by foreign resources. The negative decision would be the investment into intangible assets (EATR average of 43.41%) and to tangible assets: machinery for agriculture and forestry, basic herds, and draught animals, where the EATR rate was 47.83% in 2018 when funded by own resources, and 49.29% when financed by foreign resources. This was the highest EATR rate for monitored tangible assets over the reference periods. The differences between the observed periods were due to the 3% increase in the statutory rate, reflected in the result of the calculations by increasing the effective average corporate rates for intangible assets by 13.35%, for tangible assets by 14.25%, and for inventories by 16.63%.

Table 1 Input data for analysis.

Asset	Accounting depreciation ZEW (δ)	Recalculated life	Tax depreciation (∅)
I. Intangible assets	15.35% = 0.1535	5 years	100/5 = 20%
II. Agricultural buildings	3.1% = 0.031	20 years	100/20 = 5%
III. Agricultural and forestry machinery	17.5% = 0.175	4 years	100/6 = 25%
IV. Basic herd and draft animals	17.5% = 0.175	4 years	100/6 = 25%
V. Growing units of permanent crops	4.5% = 0.045	12 years	100/12 =8,33%
VI. Estates	x	x	x
VII. Inventory	x	x	x

Note: Source: own processing according to (**ZEW, 2018**).

Table 2 Development of corporate tax rates in Slovakia.

Year	Statutory tax rate	Effective tax rate
1991 – 1999	40	40
2000 – 2001	29	29
2002 – 2003	25	25
2004 – 2012	19	19
2013	23	23
2014 – 2016	22	22
2017 – 2018	21	21

Note: Source: own processing according to (**ZEW, 2018**).

Table 3 Depreciation period for tangible assets.

Group	Years	Assets
1.	4	agricultural and forestry machinery, basic herd and draft animals
2.	6	-
3.	8	-
4.	12	basic herd and draft animals
5.	20	agricultural buildings
6.	40	-

Note: Source: own processing according to (**ZEW, 2018**).

Table 4 Development of real estate tax rates in Slovakia (%).

Year	Statutory tax rate	Effective tax rate
1991 – 1999	0.11	0.07
2000 – 2003	0.11	0.08
2004	0.11	0.09
2005 – 2012	0.44	0.36
2013 – 2016	0.44	0.34
2017 – 2018	0.44	0.35

Note: Source: own processing according to (**ZEW 2018**).

Table 5 EATR calculation values (2004 – 2018).

Title	Values			
	Tax depreciation rate	Accounting depreciation rates	Depreciation tax shield (A) 2004	2018
Intangible assets	20%	15.3%	0.1554	0.1799
Agricultural buildings	5%	3.1%	0.0999	0.1159
Agricultural and forestry machinery	25%	17.5%	0.1605	0.1859
Basic herd and draft animals	25%	17.5%	0.1605	0.1859
Growing units of permanent crops	8.33%	4.5%	0.1250	0.1448
Estates	-	-	-	-

Economic rent after tax	Retained earnings		New deposit		Debt	
	2004	2018	2004	2018	2004	2018
Intangible assets	0.0666	0.0564	0.0666	0.0564	0.0792	0.0710
Agricultural buildings	0.0874	0.0794	0.0874	0.0794	0.1000	0.0940
Agricultural and forestry machinery	0.0630	0.0518	0.0630	0.0518	0.0756	0.0664
Basic herd and draft animals	0.0630	0.0518	0.0630	0.0518	0.0756	0.0664
Growing units of permanent crops	0.0826	0.0741	0.0826	0.0741	0.0952	0.0887
Estates	0.1033	0.0976	0.1033	0.0976	0.1159	0.1122
Inventories	0.0996	0.0924	0.0996	0.0924	0.1122	0.1070

EATR (in %)	Retained earnings		New deposit		Debt	
	2004	2018	2004	2018	2004	2018
Intangible assets	40.06	45.41	40.06	45.41	41.32	46.87
Agricultural buildings	29.14	33.34	29.14	33.34	30.40	34.08
Agricultural and forestry machinery	41.95	47.83	41.95	47.83	43.21	49.29
Basic herd and draft animals	41.95	47.83	41.95	47.83	43.21	49.29
Growing units of permanent crops	31.66	36.12	31.66	36.12	32.92	37.58
Estates	20.79	23.78	20.79	23.78	22.05	25.25
Inventories	22.73	26.51	22.73	26.51	24.00	27.98

Note: Source: own processing.

We could state, that the effective rates that assessed the location of the investment used take into account the economic conditions associated with the cost of the capital, the amount of accounting and tax depreciations, the rate of inflation, and the nominal interest rate (the so-called discounted shareholder rate). With the existence of taxes, the return on investment is changing and ensuring the optimality requires the same return on different types of investment at a given margin. These rates will take into account the most optimal and the most effective conditions for investors to decide. **Cozmei (2015)** proved, that the effects of globalization have a significant impact on a wide range of national policies, including economic and tax policy. She stated that one of the manifestations is the competition of the countries in lowering the corporate tax rates in order to gain more foreign capital investment, which, on the other hand, endanger the collection of corporate income taxes. The author also stated that based on her findings it has not been confirmed, that over time the decrease in pressure on corporate tax rates has reflected in a decline of the corporate revenues. According to **Blechová (2015)**, the impact of the taxes on the return of planned investments (in case of their implementation in different countries) was negatively correlated, the higher was the indicator of effective average taxation, the less attractive were these countries for potential investors. In our case, the rate was based on the type of capital, and the land and inventories were the most attractive investments for prospective investors. **Devereux (2006)**; **Feld and Heckemeyer (2011)**; and **Devereux, Griffith, and Klemm (2004)** stated that the differences in the tax rates had a clear impact on the location of investments. The tax rate (effective average, but also marginal) and the legal tax base will be the decisive factors based on which the future investors will decide on the volume and allocation of their investments. In other words, investors do not control the tax revenues that differ endogenously with output fluctuations and changes in the tax base due to other factors, the rates are decisive. On the contrary, **Feldstein, Dicks-Mireaux, and Poterba (1983)**; **Dwenger, Rattenhuber, and Steiner (2017)**; and **Arulampalam, Devereux, and Maffini (2012)** confirmed that the increase in corporate tax rates resulted in an increase of negative impacts through lower investment and thus to a reduction in returns from other production factors, such as capital. The authors further stated that while small countries with a small share of domestic markets set their effective tax rates to almost zero values, large countries maintain much higher effective tax rates. In

developed countries with high capital incomes, various tax breaks, contributions and tax holidays can lead not only to increased EMTR but also to an increase in EATR **(Mendoza, Razin, and Tesar, 1994)**. **Šimková (2016)** in her analysis, following the EATR design for Slovak conditions stated that setting the tax rate is a rather complicated process of seeking a compromise. On the one hand, the countries want to maximize the taxes because they represent the income of the state budget, and on the other hand there are the interests of the business sphere and the consumers who take the taxes as a necessary evil.

The *Corporate Taxation Principle* means that the profit is immediately taxed at the shareholders' level (the tax rate of the shareholders is used as a tax rate for investment profits). Since the taxation of capital gains is limited to each asset, capital gains tax on shares cannot be considered. There are many empirical studies and research that deal with effective corporate taxation. **Arachi and Biagi (2005)**; and **Hanlon and Heitzman (2010)** investigated the impact of the differences in effective rates on investment decisions in European countries. **Alvarez and Koskela (2005)**; and **Gries, Prior and Sureth (2012)** followed in theoretical level the impact of taxation on investment under uncertainty conditions. **Devereux, Griffith, and Klemm (2002)**; and **Stickney and McGee (1982)** noted, that in various forms of EATR tracking, capital can be funded from different resources, including the use of debt. All these outcomes have highlighted the importance of monitoring effective taxation and its need for decision-making of the foreign investors.

CONCLUSION

An analysis of the structure and description of the construction of the effective average tax rate (EATR) model impact on capital, as well as the changes in statutory tax rate (and other taxes) and other factors reflection into the change in the effective rate were investigated. An important aspect was also the way of funding either by own or by the foreign resources. The analysis depicted a tax depreciation shield that determined the amount of tax savings on capital investment. The highest annual tax saving was achieved in 2018 for tangible assets (machinery for agriculture and forestry; and basic herd and the draught animals) and consisted of a yearly savings of € 4,647.50, with a return in four years, with the depreciation period having played an important role here. The lowest tax savings were obtained in the investments into agricultural buildings (€ 579.50), as these assets have the longest return and the smallest possibility to reduce the tax base through the tax depreciations. Intangible assets with the shortest time of return showed 15.54% (in 2004) and 17.99% (in 2018) savings from the purchase price. The EATR included an economic rent of the project with taxation, which reflected the size of the project's financial benefit with the aspect of taxation. In the analysis, the lowest value was found in case of the land and the inventories in both observed periods under both funding ways. A negative decision would be made by an investor if he would invest in an intangible asset; and in tangible assets in machinery, devices, and equipment.

Significant differences also occurred in the assessment of individual observed periods, as a 3% increase in the statutory rate over the period, increased the effective average corporate rates by an average of 14.74%.

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RESEARCHING OF THE CONCENTRATION DISTRIBUTION OF SOLUBLE LAYERS WHEN MIXED IN THE WEIGHT CONDITION

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ABSTRACT
The analytical and experimental analysis of the processes associated with the formation of structural systems, which includes adsorption, its main paths of formation, patterns of influence on the structure of the environment and its behavior at deformation influences is carried out. The ways of choosing the optimal variant of the adsorption diffusion process for providing the maximum or minimum value of parameters (criterion) are proposed. The physical essence of the relation of the length of the sorbent layer with the time of its protective action (number of bound substance) is considered, which allows to practically characterize the work of the sorbent layer under dynamic conditions. It is noted that the determination of dynamic combined power flow influences during the process of mixing of components plays an important role in the structure formation of the suspension and promotes the construction of calculations for the construction of mixing equipment. The obtained data give an answer a series of questions about the theory of adsorption and diffusion (adsorption actions of van der Walsh forces on surfaces) and the ability to regulate the effect of combined power flows directly affect these process transformations (concentrations). For a illustration and understanding of the general execution of research, depending on the method of applying force, the degree of its previous dispersion and its physical and mechanical properties, a scheme of causal relationships between components and parameters that determine the change in the structure of the components in mixing process on a new discrete machine. The principle of discrete-momentum mixing of components in the weight condition and mechanical influence of the formable working body is considered. Based on the process of mixing the components in the working chamber of the machine, a mathematical model is proposed.

Keywords: dough; injection; thermal conductivity; distribution of heat; heat flux; roll; phase; environment

INTRODUCTION
One of the main direction of rational technical support for the process of mixing components in different industries is machines with working organs of known designs. To date, there is not enough variety of designs of working bodies that perform a technological operation on mixing heterogeneous environment. Many of these machines need to be refined and explored to introduce new developments in manufacture. Particular attention is paid to small, efficient machines with controlled processes occurring in the working chamber. When creating or upgrading small machines for intensive mixing, special attention should be paid to substantiating the technological scheme and the appropriate constructive solution of the decisions taken **(Zvyagin and Vorotnikov, 2004)**. Since the design parameters are interrelated with the process, it significantly influences the possibility of manufacturing such a machine in the conditions of enterprises, technological possibilities of which are aimed at repair and restoration of equipment.

Machines intended to mix components should produce an effect on them in such a way that the raw materials and finished products loss are minimal and the quality of the semi-finished product is high. This leads to the need to ensure the full compliance of process conditions, geometric shapes and structural parameters of the working bodies and structural and mechanical properties of environment.

In his writings **(Zvyagin and Vorotnikov, 2004; Sekundov, 1977)** the author noted that the lag of scientific developments from the needs of practice is explained by an extremely wide range of properties of technological environments, the variety of materials used for their transportation and the difference in the conditions of their exploitation. In addition, the study of the mixing of the viscous medium is associated with a certain complexity due to the need to engage in solving the problems of modern knowledge and methods in various fields of science: physical and chemical mechanics of materials, rigid body physics, metallurgy, etc.

Implementation of the principles of rational mixing requires fundamentally new approaches to the creation of a new generation of technological equipment. The difference between a new machines should be based on a constructive solution of parameters at given physical and chemical properties of a mixture of components. Therefore, the rapid introduction into the industry of technological processes in a weighted condition, due to the fact that when

we give the method of conducting the process favorable conditions for its intensification are created. This is due to the increase of the phase contact area, the improvement of mass exchange and heat transfer, the reduction of energy consumption on the impact of hydraulic resistance of the system and the creation of conditions for the transition from the processes of periodic to semi-continuous and continuous.

Analysisoflatestresearches

In the study of the adsorption process in the stationary layer Shylov **(Keltsev, 1984)** introduced the concept of the dynamic activity of the sorbent. He, on the basis of a number of studies, proposed an equation that allows us to practically characterize the work of the sorbent layer under dynamic conditions. This equation links the length of the sorbent layer with the time of its protective action (the amount of bound substance) and has the form:

$$\tau = KL - t_0 \quad (1)$$

Where:
τ – time of protective layer property, min.;
K – protective action coefficient, the value associated with the components dosing rate (fluid) into the interaction system, after the formed region of the gradient and the velocity of its motion, has become constant, min.m^{-1};
L – length of the layer, m;
t_0 – time needed for the formation of the gradient region of theinteracting component concentration velocity in the weight state within the medium, min.

It follows from the equation that the values of K and t_0 are empirical values for the practical characteristics of the interacting systems. The coefficient of protective action of K depends on the sorbent properties, interaction conditions, aerodynamic conditions and physico-chemical parameters of the flour-air mixture. The presence in the equation τ0 is due to the fact that, owing to the high and constant concentration in the flour, the adsorption property of the initial layer forming the velocity gradient region, is used in a short time and the time of the protective action per unit length of the layer influences less than for the subsequent layer.

In the work of other researchers **(Braginsky, Begachev and Barabash, 1984)** it is shown that the presence in equation (1) t_0 is regular and is a consequence of the short velocity of the adsorption process in dynamic conditions. This leads to the fact that the concentration conditions of any layer and the protective action time are different from the operating conditions of the subsequent layers.

Several of researchers indicate that the formation of the front of mixture does not end when dosed at a sufficiently large length of the layer and that the equation (1) only formally shows the adsorption process in dynamic conditions. However, this equation is the only one that allows us to characterize the system components interaction in dynamic conditions. The adsorption process is heterogeneous and consists of:
- diffusion of droplet molecules between sorbent particles to its outer surface;
- diffusion of droplet molecules inside and active surface of the sorbent;
- sorption on the surface of sorbent pores.

The kinetics of the heterogeneous reaction is determined by the time course of its individual components, and the rate of the heterogeneous process coincides with the speed of the slowest process. Since the adsorption process itself is a purely superficial phenomenon, adsorption is practically instantaneous. Therefore, we can assume that the speed of the adsorption process is determined by the diffusion velocity, it means that it passes mainly in the diffusion region. In the diffusion region, all reactions have the first concentration of the reacting component at constant pressure. The constant of the reaction rate is the diffusion constant. So the expression of the external speed and internal diffusion can be represented by the equation:

$$\frac{da^1}{d\tau} = \beta^1 \left(c_0 - c^1 \right)$$

and

$$\frac{da^{11}}{d\tau} = \beta^{11} \left(c^1 - c^{11} \right)$$

in general, the adsorption rate

$$\frac{da}{d\tau} = \beta \left(c_0 - c \right)$$

Where:
β – the mass transfer coefficient of;
C – the mixture concentration, the equilibrium amount of the component that interacts with the unit volume of the adsorbent, kg.m^{-3}.

The value is reversed β, the diffusion resistance is present.

$$\frac{1}{\beta} = R = R_{internal} + R_{external}$$

To date, there is no complete theory describing the adsorption process in dynamic conditions. There are three points of view on the kinetics of the adsorption process. One author considers **(Levich, 1952)**, that the velocity of the adsorption process is determined by the speed of external diffusion, others are derived from representations **(Lykov, 1954)**, that the kinetics of adsorption is determined by the rate of internal diffusion. The third – to the corresponding period, the speed of the process is determined by external diffusion, and then – the speed of internal diffusion of the sorbent component. Consequently, the moment of these changes by different authors is determined differently.

Levich (1952) gave a general method for determining the diffusion flow from the surface. In his theory, the diffusion layer adopts an appropriate and quantitative definition: there is a convective and molecular transfer of matter on the outer boundary of the diffusion layer – one order of magnitude. The change in concentration c in the diffusion layer is determined by the differential equation:

$$D \frac{\partial^2 c}{\partial y^2} = v_x \frac{\partial c}{\partial x} + v_y \frac{\partial c}{\partial y} \quad (2)$$

Where:
v_x i v_y projections of fluid velocities.

On the basis of literature data and research analysis, the third point of view most correctly display the essence of the adsorption process in dynamic conditions. Despite the large number of works that display the essence of the adsorption process, to date, in the literature on the adsorption of flour in the liquid phase, there is no rational calculation methodology. Using the methodology it would be possible to use the design of new constructive and technological parameters of the machine, to establish ways to intensify the mixing of components and to select the optimal conditions for conducting the technological process.

Formation of the problem and the purpose of the researching

Modern food technologies are developing in the directions of technological processes intensification, therefore, it was necessary to obtain new experimental data for the qualitative design of modern types of mixing equipment, taking into account the filling of the working chamber, its length or contact area to ensure rapid concentration change.

In his writings **(Lykov, 1954; Strenk, 1971)**notes that the creation of a qualitative mixture is not equivalent to obtaining a uniform concentration of solids particles in the liquid throughout the volume of the apparatus. Often is hard to achieve this, but it is not necessary. The uniform concentration of the suspension in the entire volume of the mixer does not matter, but it is important that all solids are kept in a suspended liquid. Therefore, it is necessary to create a large turbulence of the liquid around the grains in order to reduce the thickness of the laminar layer on the boundary of the liquid – solid.

A typical technological process associated with the formation of structural systems is the process of adsorption. Therefore, new advanced technological methods of interaction of the solid phase with gas, liquid phases in the heavy (boiling) layer and adsorption in the fluid layer (hypersorption) appeared in the food and pharmaceutical industries. The rapid growth of the technique of adsorption requires further researching of the statics and kinetics of the adsorption process for its use in various conditions, especially when mixing the components **(Pawel et al., 2016)**.

The mixing process is considered as a purely mechanical process of mutual penetration (particles of a continuous medium with the same physical properties between particles of a continuous medium and other physical properties) in order to obtain a homogeneous continuous medium with new properties that differ from the properties of the mixed enviroments.

Mixing water and flour is a heterogeneous process. The speed of heterogeneous processes depends on the surface of the interphase contact and on diffusion. The increase in the reaction rate is mainly due to the renewal of matter on the contact surface of the phases, since the diffusion rate is negligible. The heterogeneous reaction consists of several successive stages. For example, the reaction between the liquid and the particles of flour passes through the following stages:

- supply of molecules of any reagent from the solution to the surface of the phase separation;
- adsorption on the surface;
- chemical reaction on the surface;
- desorption of reaction products;
- diffusion of reaction products in the surrounding volume.

The resulting velocity of the entire process is determined by the speed of the last stage – the volume mixing in the working chamber of all components. If this stage is a chemical reaction, mixing is used to align the concentration field. In this case, a large circulation is required. Therefore, the main attention must be paid to the researching of the heterogeneous system structure formation in a mass condition under the influence of mechanical, vibrational, gravitational, hydrodynamic factors. It is necessary to use a combined method of research: theoretical and experimental. On the basis of research, to obtain mathematical models that allow to substantiate the structural, technological parameters and the mechanism of the adsorption process for the design of new mixing machines.

The mechanism of structuring is of fundamental importance for mixing components with given properties. There are several mixing mechanisms. The first one, when the diameter of the formed droplet of the emulsion is up to 20% of the diameter of the center of structure formation. Drops as a heat-carrier, due to adhesive-sorption forces, are fixed on flour particles, and due to their interaction, an intense penetration of the liquid occurs and the formation of a new layer of solid components on the surface of the mixture.

The second option when the diameter of the formed drop is more than 20% of the diameter of the center of structure formation. The solution forms a pellicle on the surface of the flour in the zone of intense heat-mass transfer and, a new layer of the mixture is formed (Figure 1). In case of exceeding the critical value of moisture content, the mixture is destroyed due to the reduction of strength and drains over the walls of the working chamber, or due to the creation of liquid bridges appear agglomerates.

Both methods of mixing are determined by the method of introducing the liquid phase into the apparatus and the hydrodynamic regime of the fluidized bed. To ensure the layered growth of the forming mixture structure forming, the required diameter of the liquid droplets should not exceed 300 μm. So, the presence of a dispersing device that can distribute a heterogeneous liquid phase inside a fluidized bed, and providing a predetermined droplet size, is a prerequisite for mixing the components in the design of a new machine.

The use of a fluidized-bed technique with mechanical stirring allows you to obtain a suspension with a uniform distribution of components throughout the volume of the working chamber of the machine. This multifactorial process with stochastic nature requires complex mathematical modeling **(Neuvazhaev, 2000)**.

Scientific hypothesis

A significant number of transported substances in the environment, changes in their concentrations, the interaction between them and microorganisms, the presence of stimulants, etc., lead to relative instability of the system. Under these conditions, there is an understanding of which direction should assess the effects of individual factors. At first glance, it may seem that in best case should meet the maximum satisfaction or security at the upper levels of the factors of influence. However, the negative effects should

also be programmed, for example, by the values of osmotic pressures, double and triple effects of factors, deterioration of quality indicators, etc. Evaluating the effects of changes in concentration (composition of factors), is more difficult. The results of transformations of physical factors determined from this point of view are not enough. If the influence of temperature is carefully observed, then there is no complete point of view relative to the change in the concentration in the layers of the movable components.

The change in the concentration of dissolved flour in the liquid phase of the environment means decreasing of the osmotic pressure and increasing the mass transfer mechanism at the interface between the surfaces of microbial cells and the liquid phase of the environment.Dynamics of changes in the direction of concentration in the three-phase system is possible due to forced mixing. Such effect on the formed environment is one of the most rational, since its manifestations relate to the total volume of the formed medium with the complete exclusion of local zones without their features. The implementation of transient processes based on concentration in three-phase systems is possible due to the successive synthesized gas and solid phases, which have signs of energy-saving technology.

The presence of complete flour interaction at the level of the state of saturation in the liquid phase establishes on their surfaces the separation of the phases of the gas-liquid-solid body and in the liquid film, which causes the main change in concentration, the resistance of the mass transfer, the acceleration of dissolution. Therefore, the speed of the mixing process is not only external, but also corresponds to the essence of the physical and chemical phenomena that occurs when mixing the components. Indeed, the mixing process should be considered as adsorption of flour to moisture with the help of mechanical influences.

Such a conclusion deserves an experimental review and development of proposals for use. A special need for effective flour disintegration technology refers to technologies in which partially exist an intensive aeration process that impedes their productivity and efficiency. However, the peculiarities of phase synthesis in blending machines practically remain aside from these studies. The latter relates to the change in the concentration in the layers of the high-altitude unevenness of the interacting components in the working chamber, the holding capacity and high-altitude gradient of solubility of the flour. It is important that in both these directions should expect to increase the efficiency of technological processes.

The generated impulse energy impacts on the basis of accumulated energy potentials when mixing components in the baking industry should lead to a limitation of material losses. The realization of phenomena of change in concentration in the formed media in the mixing technologies should be considered at the level of the simulation method.

MATERIAL AND METHODOLOGY

The material for research was a liquid aqueous-flour mixture (pre-ferment) with a moisture content of 65 – 75%. The mixture was prepared from high quality wheat flour, and the moisture content of the flour was 13.9 ±0.2%. The tempering temperature was within the range of 24 – 350 °C. The volume of the pre-ferment in all cases was 0.008 m^3, and the density was $\rho = 1066$ $kg.m^{-3}$. The required amount of raw material was dosed by mass. The amount of water added in mixing Gv (in mL) was determined by the method **(Drobot, 2010)** (Table 1).

$$G = G_c(W_0 - W_c)/(100 - W_c)$$

Where:
Gc– total weight of flour, gr;
W_0 – moisture content of pre-ferment, %;
Wc – average weighted moisture content of raw materials, %.

The water temperature (t_0 C) of water consumed for mixing the pre-ferment, provided its temperature is 280 °C, was calculated by the formula:

$$t_o = t_n + c_b G_b (t_n - t_b)/c_b G_b + K$$

Where:
To – set temperature, °C.;
c_b – heat capacity of flour, $kJ.kg^{-1}$ K, (c_b =1.257);
G_b– heat capacity of water, 4.19;
t_b – flour temperature, °C.; Gm– amount of water, g;
K – correction factor (in summer 0 – 1, spring and autumn – 2, winter – 3.

Method of work execution

The research process included the mixing of ingredients at different modes: the rotational speed of the shaft, the geometric dimensions of the working body, the distance between the working body and the bottom of the working chamber.

To determine the existence of a functional dependence describing the kinetics of some features of the adsorption process in a weighted state, a researhing was conducted on the physical model (Figure 2) in the TNTU laboratory.

According to the design features of a new mixing machine designed, the free-fall flour is doped into a cylindrical chamber 1 and is wetted downward by a sloping jet of liquid components 6. The jet of liquid components is formed by a pressure device with nine holes in the form of nozzles. The components are supplied from the dispenser of liquid components 4, which is experimentally installed at a certain height so that the prescribed dosage amount is dosed within 1 – 1.5 minutes.

For the control of leakage velocity, duration and quality of liquid components sputtering, the researching of dynamic activity in a curved state on the dispenser of liquid components was performed. The speed was changed from 2 to 3 $L.min^{-1}$, it corresponded to the speed at which the absorbent layer passed to the suspended state. The supply pressure of the components was controlled by the installation height of the dispenser.

In our experiments, the way of studying the heterogeneity of the distribution of solid particles of flour in the stream was chosen by analyzing the photos obtained on the microscope. Technologies of automated image analysis in the technological process allow to shorten the time and increase the accuracy of product quality control. The processing of digital images is a rather vivid and obvious example of conversion and analysis of measurement data,

which are widely used in industrial systems of machine vision.

The light scattering phenomenon is at the basis of optical methods for estimating the size, concentration and structural and morphological features of agglomerates of the disperse phase. The main methods for the investigation of disperse systems using this phenomenon are electronic ultramicroscopy.

For data processing automation, a microscope, a digital camera, which provides image registration at predetermined intervals, image capture devices and digital camera control, is used. Also, software is used to interpret the information received in a convenient way.

Pre-ferment obtained for different mixing modes was examined. The main controlled parameter is the discrete-pulse input of components into the middle of the working chamber, which depends on the design parameters. The mixture was prepared by the developed method – mixing in a weighed condition with the influence of the container working bodies, which created the internal circulatory contour of the components in the chamber. After every 30 seconds of the process, by use the probe, five samples of a mixture of different parts of the volume of the working chamber were selected. Further samples were explored on a microscope.

Figure 3 shows an example of a 600x magnification shot using a biorex Konus-3 microscope and a Sigeta UCMOS 5100 digital camera with a resolution of 5 megapixels in the automatic shooting mode open in the ToupView program area.

The next step is to bring the digital image to the physical units of measurement using a scale based on the digital image of the measuring line with the same magnification factor.

Since the color of the sample is not interesting for further research, to facilitate further transformations, the photo was transformed into shades of gray.

Compared to the results of the visual analysis, while processing a part of the objects is lost. The main problem is the presence of unclosed areas that are not properly processed by the program. Therefore, various combinational lighting options were used to improve the "relief" of the image and the exclusion of "illumination", as well as the multiple use and combination of software filters. In Figure 4 are represented processed micrographs of samples.

The research results are mathematically processed using software packages: Excel, MathCAD.

Statistic analysis

Given the chaotic interaction of the components during their mixing, where the change in concentration occurs between the working plates, in the space of the working chamber and its surface, the task of planning the experiment with the use of a full factor experiment of the second order is drawn up. With two factors, the model of the experiment's function has the form: $y = f(x_1 . x_2)$

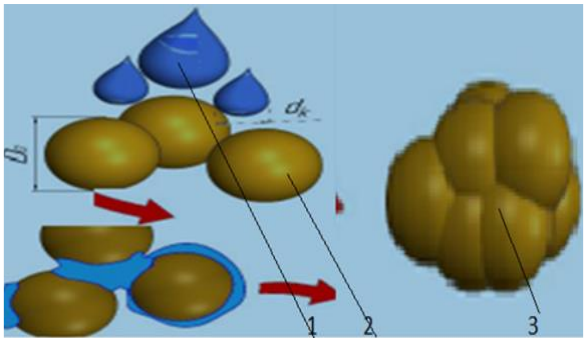

Figure 1 Fixing the drop 1 on the flour particles 2, their interaction, the formation of the mixture 3.

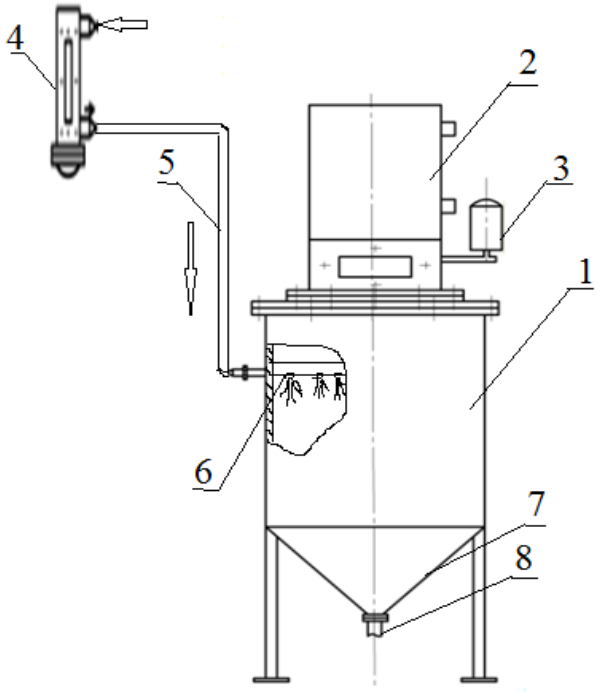

Figure 2 Structural diagram of the physical model of the experimental installation.
Note: cylindrical-conical working chamber – 1. On top mounted vibro-dispenser of loose components 2 with direct current actuator 3; the membrane dispenser 4 is installed to the left and is connected to the spray device 6 installed inside the chamber with mechanical nozzles with the supplied pipeline 5; the cone of the chamber 7 arranges the discharge of the mixture (emulsion) through the pipe 8.

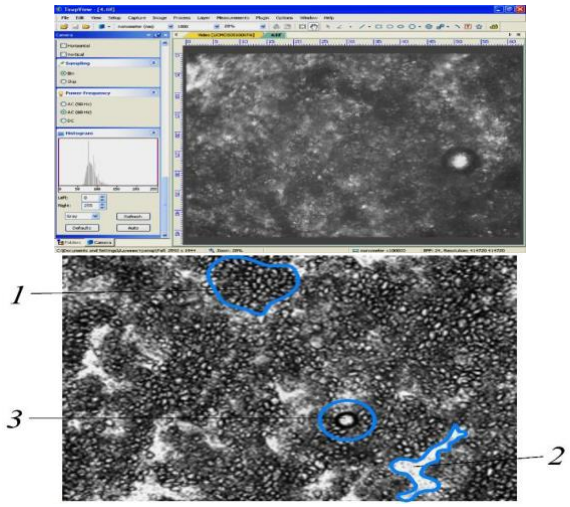

Figure 3 Example of a 600x magnification shot using a biorex Konus-3 microscope and a Sigeta UCMOS 5100 digital camera with a resolution of 5 megapixels.
Note: a) ToupView program work area; b) microscopic sample of pre-ferment: 1 – solid phase; 2 – liquid phase; 3 – air phase.

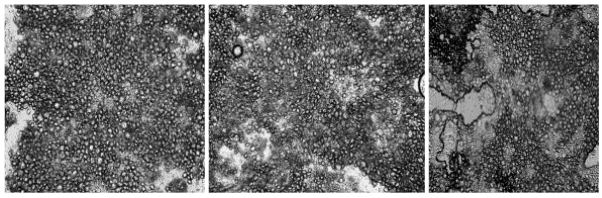

Figure 4 Microphotographs of pre-ferment in different modes.
Note: a) weighted condition; b) surface of cylindrical chamber; c) mixing by a working body.

Table 1 The ratio of the amount of flour and water to the boils of different humidity.

Raw	Amount of raw materials for pre-fermentinmoisture content		
	65%	70%	75%
Flour[g]	2600(40%)	2100(35%)	1800(30%)
Water [mL] (yeast emulsion)	4600 (60%)	3900 (65%)	4200 (30%)
Concentration, [kg.kg^{-1}]	0.666	0.538	0.429

According to the results of the experiment, we obtain a regression equation of the second order.

$$У = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$$

To conduct experiments a plan with corresponding matrices of experiment planning with the number of experiments and boundaries of factors change has been prepared. The matrix is a list of options taken in this series of experiments. Independent variables were chosen from the analysis of the nature of the effect on the change in concentration. As the optimization parameter, the frequency of rotation of the plate working body and their number on the shaft.Accordingly accepted: y_1 – number of turns of the working body; y_2 – n umber of working bodies. Experiments were conducted on the basis of mathematical planning (Table 3). Determining which factors influence on the change in concentration, determine their level variation and step of variation. The main factors and the equation of variation is given in the Table 2.

Output parameters were:
$У_1$ – change in the concentration of components in the height of their mixing in the gap between the plates. The height of mixing of the mass of components was recorded during the process by the method of visual control on a scale applied to the working camera.
$У_2$ – change in the concentration of components by mixing them in the working chamber. The mixed mass of components was selected by the sampler and recorded by the method (visual inspection according to the photographs).
$У_3$ – change in the concentration of components upon completion of their mixing in the working chamber.

On the basis of experimental data of mixing intensification, mathematical processing was performed using an application StatgraphicsPlus XV.1

According to the data of the dispersion analysis, the influence of the factors of the experiment is statistically significant at the value p = 5%((for example: p <0.05).

Influence of independent variables on output parameter $У_1$. The greatest influence on the change in the concentration in the gap between the plates has the effect of their amount on the shaft (X_2) (p-value for $У_1$ = 0.0017).

Table 4 shows the regression coefficients obtained during the mathematical processing of experimental data.

Using the obtained data of regression coefficients, we make a regression equation for $У_1$

$$У_1 = 0.125556 – 0.0166667X_1 – 0.0266667X_2 + 0.00666667X_1^2 + 0.0X_1X_2 – 0.00333333X_2^2$$

Experimentfailedfor $У_1$ = 0.0001111

Influence of independent variables on output parameter $У_2$. The greatest influence on the concentration change in volume has the influence of rotation frequency of plates on the shaft (X_1) (p-value for $У_2$ = 0.0338).

Table 5 shows the regression coefficients obtained during the mathematical processing of experimental data.

Using the obtained data of regression coefficients, we make a regression equation for $У_2$

$$У_1 = 70.8889 – 0.283333X_1 – 0.633333X_2 – 0.183333X_1^2 – 0.2X_1X_2 + 0.266667X_2^2$$

Experimentfailedfor $У_2$ = 0.104

Influence of independent variables on output parameter$У_3$. The greatest influence on the change of concentration in volume has the influence of rotation frequency of plates on the shaft (X_1) (p-value for $У_2$ = 0.0001). Table 6 shows the

regression coefficients obtained during the mathematical processing of experimental data.

Using the obtained data of regression coefficients, we make a regression equation for $У_3$

$У_3 = 83.5556 – 9.0X_1 – 9.83333X_2 + 1.66667X_1^2 + 2.25X_1 X_2 – 1.83333X_2^2$

Experimentfailedfor $У_3 = 1.52$

The results of the computational experiments allowed to study the kinetics of changes in the concentration of components in the volume of the working chamber. The rational constructive parameters, which contribute to the intensification of the process of mixing the components, are determined.

RESULTS AND DISCUSSION

Distribution of concentrations of soluble layers during mixing

Today there is information about the mechanism of mixing in laminar mode, which proves in the complex movement of the exchange process. In the presence of circular motion of a fluid in a cylindrical working chamber, on the turns of the flow at its transition from the central zone to the reverse, this sharp change in the direction of flow leads to a restructuring of the profile of velocity and the appearance of landslide deformation, which facilitate the mixing of the components. Therefore, the difference between the existing mechanisms of mixing and mixing in a closed profile channel (Figure 5) is considered.

The influence of the physical nature, dispersion, the content of the mixture on the section forming the circulating circuit and the design parameters of the machine at the stage of the mixing process is relevant.

It is also necessary to justify the basic laws of improving the technological requirements and parameters of this process.

Accelerated in the air, a stream of liquid components is found with spray particles of flour during its free fall, partially takes it, hits the walls of the cylindrical chamber and the rotating plate of the working body (Figure 6).

Liquid components, getting on TRO, are sprayed and get an extra speed, which contributes to a greater number of wetted particles of flour. The particles are pressed against each other, mixed, and their speed is lowered at the bottom of the camera. Due to the cylindrical configuration of the working chamber, the jets (emulsions) are automatically lowered, where the surface of the working body acts on them. In this case, the time from the moment of entering the flour and liquid components into the chamber and until they reach the bottom of the chamber is $\tau 1 = 5 – 10$ s.

In the first period of time, the adsorption process is heterogeneous, which determines the first and second stages of mixing. We will assume that the process of adsorption is a purely superficial phenomenon that occurs instantaneously. This allows us to assume that the velocity of the adsorption process is determined by the diffusion velocity at constant pressure (atmospheric). Therefore, a number of questions regarding the pre-ferment blending process of free fall in the first stage have not yet been resolved and require further consideration.

When adsorbed in a working chamber, where the kinetics of a heterogeneous reaction is determined by the concentration of components and process conditions, the rate of the adsorption process is determined by the diffusion velocity, that is, the entire process occurs predominantly in the diffusion region, which is divided into two periods. The first period of adsorption – a period characterized by the amount of liquid components that the adsorbent – flour – perceives during time t1 to the contact surface of the working body and the mixing chamber. This plot is shown in Figure 5.

The first period – from the moment of the delivery of ingredients and until the completion of one of them – flour. The liquid components are dosed for 20 – 30 seconds longer. This period can be combined with the submission and termination of the ingestion of doses; then the second period in our case will be very short, but without the necessary effect of adsorption. Structure of flour streams from flour vibrator (VDB), flows of liquid components from the membrane dispenser of liquid components (MDRCs) when rotation of the container's working body, where the speed of the wheel is significantly greater thanthe speed of the mixture of components, in the working chamber of the mixing machinebetween the layers there is mixing not only in the places rotation of the flow, in the longitudinal displacement, in the boundary layer, but also in a significant part of the volume.

The working chamber has consistently connected zones with different mechanisms and level of mixing (Figure 5). The level of deviation of the heterogeneity of the solution of the local concentration C from the average (boundary) is considered that the smaller the value of the heterogeneity, the better the components are mixed. Since mixing occurs at its constant motion (weigth condition, mechanical action of the working body, vibration, gravitation), then it is expedient to apply the mathematical model (2) with the following boundary value problem at the first and second stages with the ideal mixing:

$$\frac{\partial^2 c}{\partial x^2} \cdot D_L - \frac{\partial c}{\partial x} \cdot u = \frac{\partial c}{\partial t} \qquad (3)$$

With the initial conditions:

$$C(x,0) = 0, \ \left.\frac{\partial c}{\partial x}\right|_{x=L} = 0,$$

Where:

c – current concentration of the mixture components in the operating chamber;

t – time;

u – linear flow rate.

The left side of the equation reflects the change in mass concentration in the pseudo-layer over the period of component dosing. The second part reflects a change in the concentration, fixed in time. Consider the scheme of the flow structure in weight mixing (Figure 5).

Based on the obtained data, mathematical calculations were performed to select the optimal function, which most likely reflects the dependence of the change in

concentration on time from the mixing method at the first and second mixing stages.

Based on the concepts **(Braginsky, Begachevand Barabash, 1984;Pirogov,Leonovand Shilov, 2008)** of relative mass C and volumetric S concentrations of one of the two components in the solution and their interrelation, taking into account that the radial and longitudinal mixing is insignificant in straight lines of the initial and upstream streams, where the ideal mixing of the components takes place in a curved state and when they move into the working chamber and on the surface of the working body, and the ideal displacement in the process of centrifugal forces with longitudinal mixing, therefore, the process of perfect mixing of the first zone characterizes the dynamics of the concentration of concentrates which limited by time intervals up to 100 s.

The calculation of the change in the ideal mixing concentration in the working chamber volume is carried out by equation:

$$\frac{dS}{dt} = \frac{\upsilon}{V}\left(S' - S\right) \tag{4}$$

Where:

S and S' – current and equilibrium mixture concentration of components in working chamber,

t – time,

υ – volume flow rate,

V – volume of the working chamber.

The left side of the equation reflects the change in volumetric concentration in the pseudosilver for the period of component dosing. The second part reflects a change in the concentration fixed in space (volume) and speed when stirred in a stationary field. Then:

$$-\frac{d\left(S' - S\right)}{dt} = \frac{\upsilon}{V}\left(S' - S\right) \tag{5}$$

$$-\frac{dy}{dt} = w \cdot y; \quad -\int \frac{dy}{y} = \int w \cdot dt;$$

$$-\ln|y| = w \cdot t + C;$$

$$\ln|y| = C - w \cdot t;$$

$$y = e^{C - w \cdot t}, \; S' - S = C_0 \cdot e^{-\frac{\upsilon}{V}t};$$

at $t \to \infty$, $S \to S'$;

at $t = 0$, $S = 0$, $S' = C_0 \cdot 1$;

$$S(t) = S' - S' \cdot e^{-\frac{\upsilon}{V}t}, \; S(t) = S'(1 - e^{-\frac{\upsilon}{V}t}).$$

Initial conditions for the rate of flow of liquid components (ur) and flour (ut) to the mixing cylindrical chamber, kg.s^{-1}:

$ur := \frac{2.05}{90}$ ur = 0.023 $ut := \frac{3.55}{60}$ ut = 0.059 t := 0,0.1 .. 240

Conduct the construction of graphical dependence of the volume velocity of flux changes in a chamber at given parameters:

$$urc(t) = \begin{vmatrix} ur \cdot t \cdot if\ 0 \le t \le 95 \\ 0 \cdot if\ 0 \le t \prec 5 \\ 205 otherwise \end{vmatrix} utc(t) \begin{vmatrix} ut \cdot t \cdot if\ 0 \le t \prec 60 \\ 355 otherwise \end{vmatrix}$$

$$uc(t) = urc(t) + utc(t)$$

Accordingly, the change in the maximum mass concentration in the volume of the mixture under given conditions:

$v(t) := \frac{d}{dt}uc(t)$ vv := 10 $ss := \frac{ut}{ur + ut}$ ss = 0.722

Algorithm for calculating the change of current and equilibrium concentration underthe conditions of the process in the working chamber of the machine (Figure 10, Figure 11, Figure13).

A general analysis of mathematical modeling and refined mathematical calculations of the rate of change in concentration shows (Figure 7, FIgure 8, Figure 9, Figure 10, Figure 11, Figure 12, Figure 13 and Figure 15) that at the beginning of the process flour and liquid components interact actively, with time the activity decreases and, starting from 20 s. dosing it turns out differently. The results of the researching (Figure 14) also indicate an intensive acceleration of the process at the initial stage, which confirms the assumption of the effect of mixing activity in the suppressed state with the help of the vibrational action of the VDB. This changes the physico-chemical properties of the components, which is essential for kinetically-controlled processes.

The results of the researching and the analysis of the mathematical models of the process showed that the mass concentration is reached by 90 seconds of mixing. During this period, the first critical value is traced, where the curve has the first shift, which indicates that after 60 s the adsorption of the free surface of the flour ends and the absorption of free and bound flour begins.

Under different conditions of the process, it is observed that the volumetric consumption of raw materials (flour), which we can regulate with the help of a vibrating dispenser, significantly affect the change in concentration. It is also worth noting that the volume of the chamber is important, which can be changed during the research process. Such a change affects the creation of a larger contact, admired by the flow of liquid components.

From the scientific point of view, this is explained by the fact that at the beginning of the process, the adsorption bond is based on the molecular interaction of the flour with liquid components, which are the most strongly bound to the flour and are kept by the molecular force field on the external and internal active surfaces of the flour **(Dolomakin, 2015; Scram, 2003)**. Adsorption moisture on the surface of the flour accelerates the concentration of the first period with the help of vibration action, which after 20 s of the process practically does not affect the intensification, since the formed liquid component layer, which continuously increases with time, these vibrations absorb.

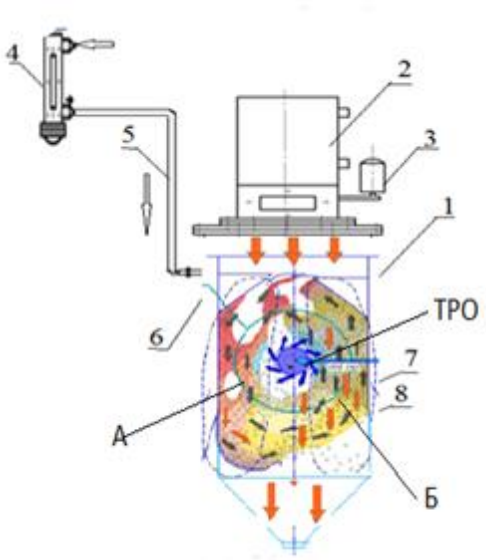

Figure 5 Motion scheme of the mixing components.
Note: A – circulation circuit component movement; B– force influence on the components; TPO–plate working body.

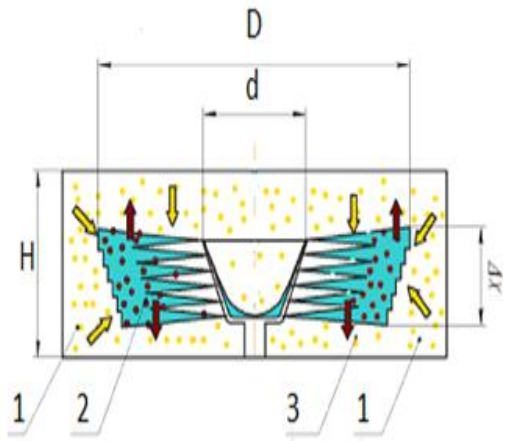

Figure 6 Distribution of components under the influence of the container's working organ.
Note: 1–particles of flour; 2–forming mixture; 3 – air bubbles (occlusion).

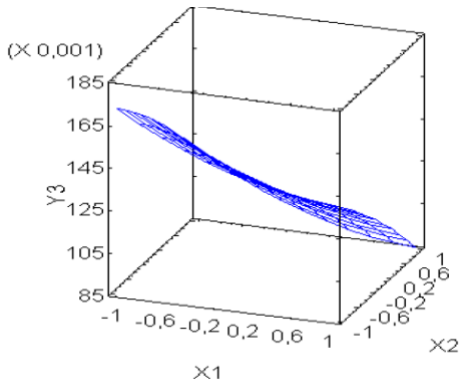

Figure 7 Influence on the change in the concentration of components between rotating plates.

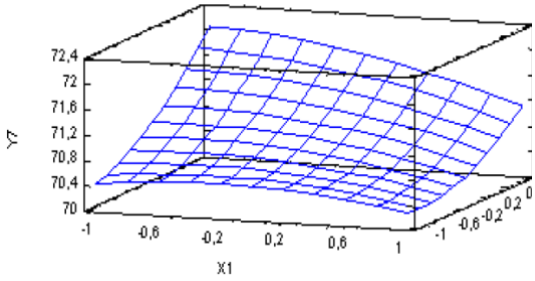

Figure8 Influence on the change in the concentration of components by the frequency of plates rotation.

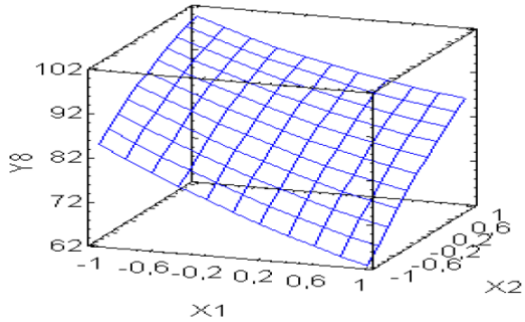

Figure 9 Influence on the change in the concentration of components by the frequency of plates rotation.

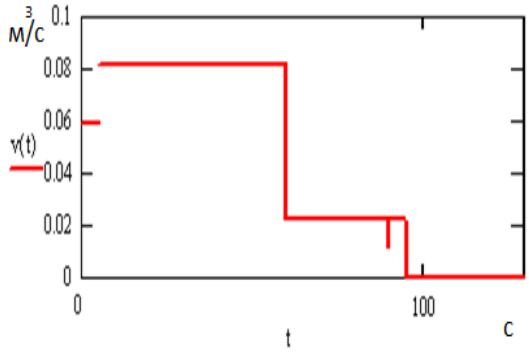

Figure 10 Volume change in flow velocity during its dosing period.

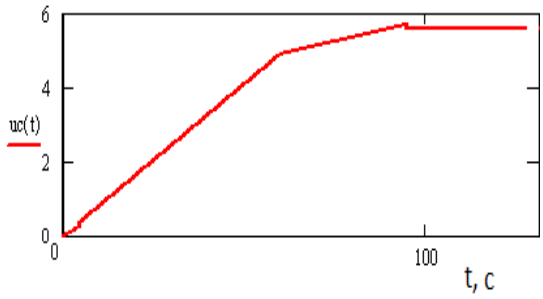

Figure 11 Change of the limiting volume concentration for the period of component dosing.

$$s1(t) := ss\cdot\left(1 - e^{\frac{-v(t)}{vv}\cdot t}\right)$$

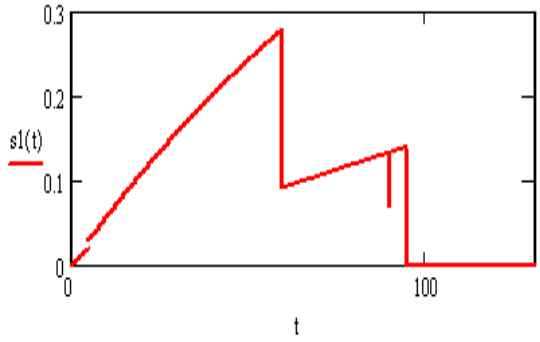

Figure 12 Concentration in the outer transition layer, which varies with time.

$$s2(t) := ss\cdot\left(1 - e^{\frac{-uc(t)}{vv}\cdot t}\right)$$

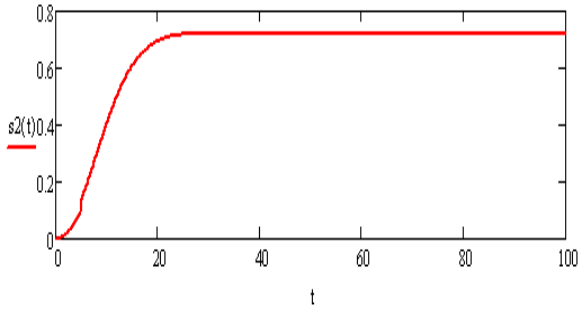

Figure13 Total concentration in the mixture of components.

$$s3(t) := s2(t) - s1(t)$$

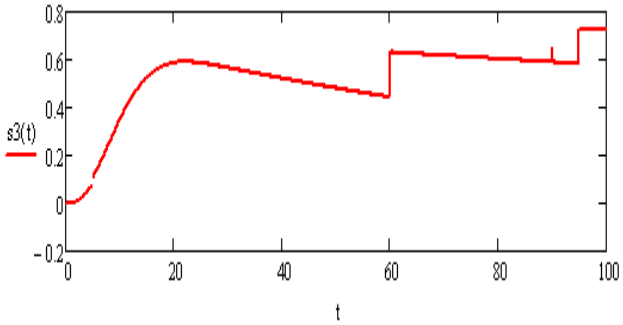

Figure 14 Concentration in the inner layer of components.

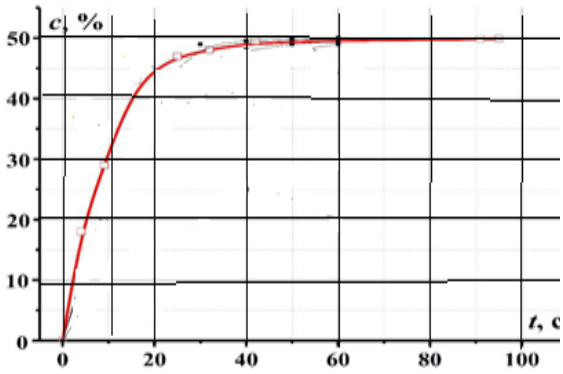

Figure 15 Change in concentration in the pre-ferment when it is formed in various parameters.

It has been experimentally established that the impulses formed by the fluctuations of the dispenser lattice practically do not reach the free surfaces of the container's working body and the working chamber, but fade in the components layers. In this condition, the formation mass concentration increases the contact with the surfaces, reduces periodically the displacement with partial amplitude, but it accelerates the process.

Table 2 Main factors and the equation of variation.

Plan characteristics	Variable factors	Variable factors
	Number of rotations of the working body X_1, min^{-1}	Number of working bodiesX_2,pcs
The main level, $X_1^{(0)}$	90	3
The step of variation	30	1
Lower level $X_1^{(-)}$(-1)	60	2
Upper level, $X_i^{(+)}$(+1)	120	4

Table 3 The experiment plan and its results.

Experiment number	X_1 min^{-1}	X_2 pcs	y_1 %	y_2 %	y_3 %
1	1	-1	0.17	709.5	85
2	0	-1	0.15	70.4	72.
3	-0	-10	0.14	70.3	62
4	1	0	0.15	70.1	94
5	0	0	0.13	70.8	83
6	-1	0	0.11	70.5	77
7	1	1	0.12	72	100
8	0	1	0.098	72	92
9	-1	1	0.09	71	86

Table 4 Regression coefficients.

Coefficients	value
constant	0.125556
A:X_1	-0.0166667
B:X_2	-0.0266667
AA	0.0066667
AB	0.0
BB	-0.00333333

Table 5 Regression coefficients.

Coefficients	value
constant	70.8889
A:X_1	-0.283333
B:X_2	0.633333
AA	-0.183333
AB	-0.2
BB	0.266667

Table 6 Regression coefficients.

Coefficients	value
constant	83.5556
A:X_1	-9.0
B:X_2	9.83333
AA	1.66667
AB	2.25
BB	-1.83333

CONCLUSION

Based on the results of exrching of sampling at certain time intervals and mathematical calculations, it was established that the maximum water absorption capacity of flour 72.2% is reached after 85 – 90 s while observing the operating modes: the frequency of rotation of the working organ 1.67 s^{-1}, the rate of dosing of liquid components of 0.023 $L.s^{-1}$, flour 0.059 $kg.s^{-1}$. The further process of mixing showed that the absorption of liquid components by surface-active flour slowed down over time due to the fact that the flour, which did not interact with the dosing process, leveled the moisture content.

The leveling of the moisture of various components occurs with the transition to a solution of soluble parts of the flour, which significantly affects the structure and further mechanical properties of the suspension. Therefore, up to 60s during hydration, the flour interacts with moving components, and this process continues with a simultaneous swelling, which does not occur immediately, but eventually.

The rate of formation of consistency is determined by the dosage of liquid components, since this is the main component of the solution and a slight difference in the time of the dosage of components contributes to a uniform, in a short time to ensure the stage of rational parameters of the mixing process. Accordingly, the first stage is performed qualitatively, and the second does not require vigorous mechanical processing, therefore, it is in a state of rest.

Without taking into account this fact, the mechanism of researching will not be complete. The importance and peculiarity of flour dissolution in the context of pseudo-mixing, taking into account vibration action, allows us to determine the size of thechamber, the speed of rotation of the body and the rate of dosing of flour and liquid components in a mass proportion.

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COMPARISON OF ANALYSIS AND THE NUTRITIONAL VALUE OF FRESH COMMON CARP, FROZEN AND SOUTHERN CANNED TUNA

Ali Aberoumand, Afsaneh Fazeli

ABSTRACT

Freezing and canning are suitable methods to delay the spoilage of marine products and improve their physico-chemical and organoleptic properties. The fish were transported to the ice in proportion to 1 to 3 (w/w) inside the boxes, and then moved wastes. The purpose of this project is to analyse and to compare the nutritional value of fresh, frozen the fish and canned tuna fish. Nutrient composition and pH of the fresh fish fillet and moisture and ash contents and other nutrient composition were measured by the standard AOAC method. The results showed that the percentage of frozen fish protein was 17.41 and the highest moisture percentage for frozen fish with 72.23. The level of energy (kcal) of canned fish with 393.36 kcal was the highest level. The pH of the canned fish with 7.28 was the highest pH. The percentage of drip and WHC in frozen fillet found 6.7% and 6% respectively. From the obtained results, it can be concluded that despite the low amounts of protein and ash in canned fish, the fat and energy content was the highest. The protein content of the fish is frozen, and its pH indicates that it was better than fresh fish from point of quality.

Keywords: fresh fish; frozen fish; canned tuna fish; nutritional value

INTRODUCTION

Today's economic considerations have led to the use of affordable, high-value food for the production of canned fish from affordably priced fish. On the other hand, the sharp reduction led to the use of some new types of low consumption in canning. In this study, the measurement of protein, fat, and ash in common carp was investigated. Tuna fishes include several large and red fish from the *Scombridae* fish family. Unlike other types of fish that have white meat, the tuna fish have pink or dark red meat. In their meat, the amount of myoglobin found in the tissues of the muscle. Tuna meat has a lot of protein and vitamins. Most tuna fish live in the warm sea; the blue wings are about 3 inches long. Albacore is another type of tuna fish. The purpose of this project is to analyse and comparison the nutritional value of fresh carp, frozen carp and canned tuna fish. Although frozen storage is a valuable method of preservation, but the quality of fish is still decreased, and some physic-chemical changes of protein occur such as the changes of muscle texture and flavour loss which is liable to cause consumer rejection. Fish quality depends on safety, nutritional quality, availability, convenience and integrity, freshness, eating quality and the obvious physical attributes of the species, size and product type **(Kharestan et al., 2006; Rezaee, 2004; Razai et al., 2004; Rezavi, 2007; Ghanbari et al., 1988; Nasrallah and Novirian, 2012; Yeganeh et al., 2011; Abbas et al., 2008; Nga, 2010)**. The purpose of this project is to analyse and compare the nutritional value of fresh, frozen and canned tuna fish.

Scientific hypothesis

Nutritive quality of fresh, frozen and canned fish is different.

MATERIAL AND METHODOLOGY

This research was conducted in the fall of 2018 at the Laboratory of Fisheries. The fish purchased from the fish market of Behbahan, known as the common carp, *Cyprinus carpio*. The fresh fish were transferred to the Fisheries Laboratory of Khatam-al-Anbia University of Technology in Behbahan, in the form of plastic in plastic. Part of fresh fish is placed in a freezer at -18 °C to freeze. The fresh and frozen fish are filleted after thawing for the tests, such as nutrient composition and pH of the fresh fish fillet. The moisture content and amount of ash and other compounds and nutritional and qualitative indices were measured by the standard **AOAC (2005)** methods.

Moisture measurement method

The moisture content of fresh fillet was determined by drying part of the sample for 24 h and expressed as percentage and was measured with constant weight.

Various types of moisture measurements were used, such as electric ovens, desiccators that contain a humidifier, laboratory scale, thin metallic dishes with a diameter of at least 60 mm, a height of 25 mm, and special containers for measuring moisture. We cooled the dictator in the laboratory temperature. 5 to 10 g of the sample was placed to the container. After 6 h, container was removed from the oven, then it was weighing exactly after cooling in the desiccators. And again, we put it back to the oven, after one day, so the weighing operation was repeated in the way mentioned, if the two obtained weight differ, then this must be repeated until we take a constant weight.

Determination of ash

We use dry method to measure ash. To this purpose, we place 5 g of the sample in a Chinese plant. Heat in a furnace was 500 – 550 °C until we obtained a bright grey colour. And in the end what remained as the ash is calculated by **AOAC (2005)**.

Fat Measurement

Total fat was measured by Soxhlet method. For this purpose, 5 g of homogenized sample was placed in a dish and heated in 35 CC HCl with distilled water. The fat sample obtained by the solvent ether was extracted using a Soxhlet apparatus. The remaining solvent was evaporated. After drying, the balloon weight was determined until it reached the constant weight. The difference between initial and final weight shows fat content as a percentage **AOAC (2005)**.

Raw Protein Measurement

Measurement protein was performed using the using the kjeldahl method. For this purpose, 5 g of homogeneous fillet with 20 mL of concentrated sulfuric acid and 8 g of catalysts were placed in a special dish, and then 30 min at 350 °C heated. After digestion, the sample was transferred automatically to NaOH solution, 32%. The distillation vapours were transferred in a container containing boric acid 2% and a few drops of the reagent. Then by HCl normal titrated **AOAC (2005)**.

PH measurement

The pH of the fresh fillet was measured using a digital pH meter of the Parta bell of the pcd650 model. Preparation of the samples was carried out to measure the pH, based on the standard method, and based on this method, 10 g of homogeneous fish fillet in 100 mL of water was mixed. The pH was measured using pH meter.

Tuna fish

Canned fish were purchased from the southern fish canning factory, its production date was 12.1.2008 and its expiry date is 12.1.2009.

Tuna fish moisture analysis

Moisture was carried out by drying. For this purpose, 5 g of homogeneous samples were placed in a petri dish, dried for 16 h in a temperature of 100 °C to 120 °C in reached a constant weight. The sample percentage of moisture content was considered **AOAC (2005)**.

The energy content of the canned fillet was calculated by Scholes method.

Methods of measuring the water drop and WHC

The water holding capacity was measured using a method by **Rørå et al. (2003)**, using centrifugation. 2 g of fish were weighed and placed in a centrifuge tube with Maidstone, Whatman England paper (V1). The centrifugation was carried out at 4000 rpm for 10 min at 10 °C and the wet filter paper (V2) was weighed to 50 °C before reaching constant weight (V3). The following formula were used to calculate the WHC:

$$\text{Water holding capacity} = (V2\text{-}V3) / S \times 100$$

Where: S represents the sample weight of the fish.

For determination of drop water, before and after thawing, sample weighted and after thawing water extracted from fillet. The following formula were used to calculate the water drop content:

$$\text{Water drop} = \text{before thawed sample weight - after thawed sample weight / before thawed sample weight} \times 100$$

Statistical analysis

Data analysis was done in three replications. The Duncan's mean comparison test was used at 5% level. The calculations were performed using SPSS 19 software (IBM, USA). Results were expressed as mean of triplicate trials. Data were analysed by one-way analysis of variance on the means of values ($p < 0.05$).

RESULTS AND DISCUSSION

The results showed that the percentage of protein in fresh fish was higher than the canned fish protein. Another study on lipid levels showed that fat content in fish increased with pass time. The pH of the canned fish was the same as the frozen. It decreased with increasing time after processing and does not follow a particular trend. The amount of ash in canned fish was less. Increasing the amounts of fat during the storage period in two types of canned fish compared to the fresh fat content in this study agreed with the results of **Greenfield and Kosulwat (1991)**.

In the present study, there was a positive and significant correlation between pH and fluid drop in most of the maintenance days and found that there was generally a reverse relationship between drop and muscle pH. Such a model might be able to observe for the initial hours of fishing, but during the maintenance period other than pH, other factors also have an impact on the amount of water checked. Hence, it seems that the difference in WHC can be attributed to the difference in the degradation of the autolysis or microbial for structure of the muscle. Increasing the maintenance days increased the distance between cells in muscle fibers, and muscle water holding capacity decreased. The results showed that the protein content of fresh fish was higher than the canned fish protein.

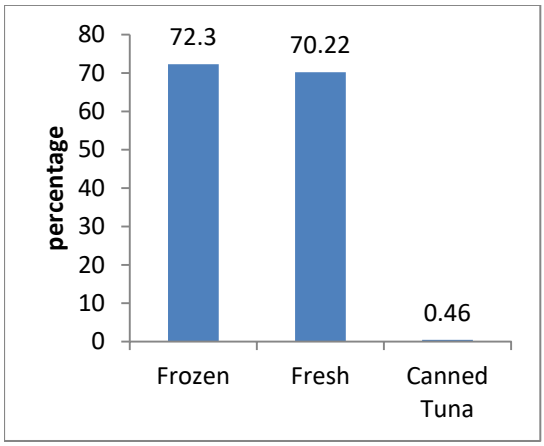

Figure 1 Comparison of moisture contents of fresh fish with frozen and canned fish tuna.

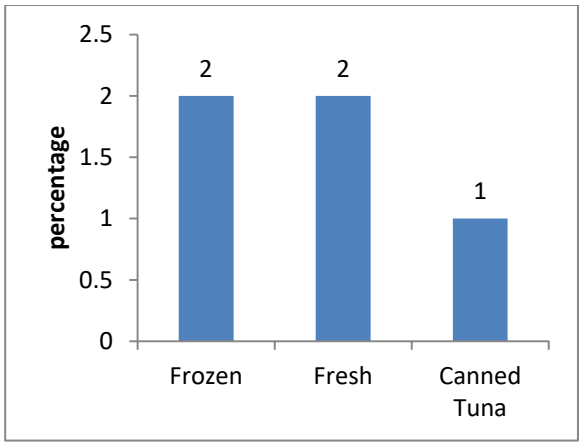

Figure 2 Comparison of ash contents in fresh fish with frozen and canned fish tuna.

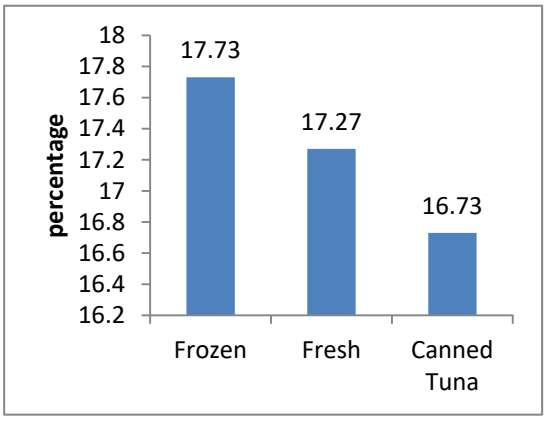

Figure 3 Comparison of protein contents of fresh fish with frozen canned fish tuna.

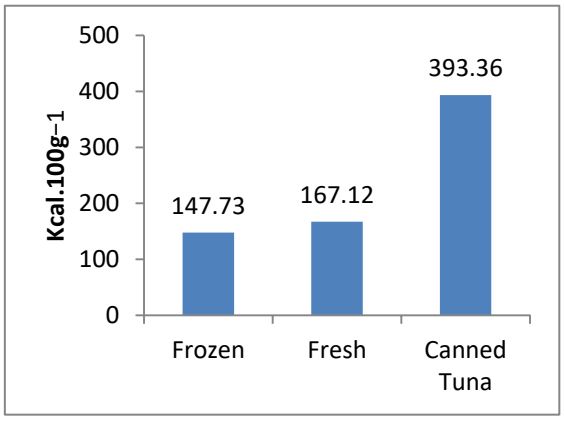

Figure 4 Comparison of energy value (kcal) in fresh and frozen fish fillet with canned fish tuna.

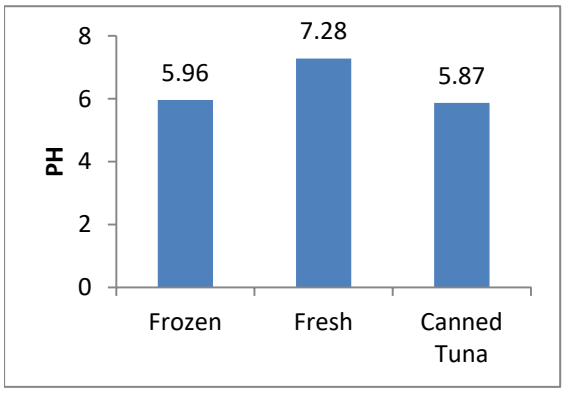

Figure 5 Comparison the pH of fresh fish with frozen frozen and canned tuna.

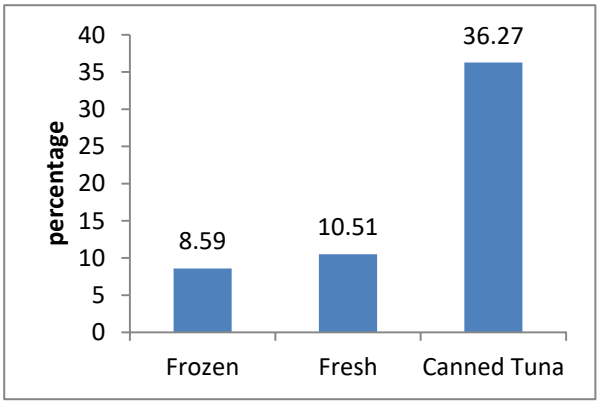

Figure 6 Comparison of fat percentage in fresh fish with frozen and canned tuna

The fat content of fresh fish was less than the canned fish. Because oil can be added to cans. The fat content of the fresh fillet was higher than the frozen fillet. The amount of ash of fresh fillet with frozen didn't differ. But it was more than canned fish ash. The moisture content of fresh fish was higher than that of frozen fish and was much higher than that of canned fish. The energy in kcal/g of canned fish was more than fresh fillets and frozen fillets, respectively. The reason was that the amount of fat in canned fish was much larger than the other two samples. The energy content of the frozen fillet was the smallest, since some water along fat was removed by thawing.

An efficient method for showing qualitative changes in fish meat, was calculating fish muscle water holding capacity that show the study of changes in its microstructures during storage. Ability to keep water was considered as one of the important parameters in the quality. Because WHC for the consumer and for industry was important. the reduction of muscle WHC was often as a result of a structural changes in the muscle, which occurs after fish death. Study of the trend of spoilage in tropical fish can provide useful information on the reduction of spoilage, handle and fish distribution in the area. There is little information about the changes in muscle fibers and its relation to the water holding capacity in the fishes of

Gulf of Persian Gulf and Oman Sea. The study was to investigate the changes in fish muscle tissue microstructures and their association with muscle water holding capacity of 14 days in a refrigerator **(Olsson, Seppola and Olsen, 2007)**.

The percentage of WHC in frozen fillets was 6% and the percentage of drop in frozen fillets was 4.7%. The percentage of fresh fillet fat in this study was 10.51%, which was lower than the fresh fat content in the fresh fillet in another fish (11.31%). The protein percentage of the fresh fillet was 17.27%, which high compared with the protein percentage of the other sample (3.96%). So, there is a lot of significant difference (p <0.05). The fresh fillet moisture content of this study was 70.22%, which was compared with the moisture content of the fresh fillet (81.9%) and the difference was not significant (p <0.05). The pH of the fresh fillet of this study was 5.87, which was compared with the pH of the freshly prepared fresh fillet (7.3) in another sample, which showed a significant difference between them (p <0.05). The percentage of fat in the frozen fillet of this study (8.39%), which was compared with the percentage of frozen fillet fat of the other sample, was 12.96%. different was significant (p <0.05). The percentage of moisture in the frozen fillet was 71.23%, which was compared to 73.3% of the frozen fillet in another sample. There was little difference (p <0.05). The percentage of protein in the frozen fillet was 17.41% but in frozen fillet protein in another sample was 67.74%. Tuna fat content in this study was 36.27%, compared to the other fish 39.3%, which is not significantly different (p <0.05). The percentage of ash of tuna was 1% compared to 2% of the other fish. The energetic value percentage of tuna in this study was 36.339 kcal, compared with a different sample of tuna fish (369.11) (p <0.05).

The major component of fish was moisture. From the maximum moisture content in were observed in *Cyprinus carpio* (78.51%), **Hantoush et al. (2015)**, that found more than present study result (70%), also according to results of **Hantoush et al. (2015)** the lipid content in the muscles of *Cyprinus carpio* was (3.16%), and protein value was in *Cyprinus carpio* (14.74%), but protein and fat values in present study found more than this. The low values of fat in the fillet of these species could be due to the high metabolism required for spawning **(Osibona, Kusemiju and Akande, 2006)**. The high muscles protein contents may be from the equally high protein content of their diets **(Osibona, Kusemiju and Akande, 2006)**. The moisture, protein, fat, ash content of fresh common carp mince was found to be 77.84%, 16.95%, 3.15% and 1.17%, respectively **Bavitha (2015)**, compared to present study showed that showed except moisture content, proximate composition contents in present study found more than **Bavitha (2015)**, results. Comparison between moisture and fat content in Bighead Carp showed a correlation between the results which correlate well with the expected inverse ratio between water and lipid matter as it had been previously described **(Aubourg et al., 2005)**. Increasing in water content may increase the rate of lipid hydrolysis, thus reduce the fat content **(Latip, Zzaman and Yang, 2013)**. **Siddique et al. (2011)** observed decreasing in total lipid content while assessing the effect of freezing time on nutritional value of *Puntius sophore, P. saranaand P. gonionotus* during the frozen storage at -5 °C of 20 days. This was due to both lipid oxidation and hydrolysis had taken place during ice and cold storage **Latip, Zzaman and Yang, 2013)**. The protein content in Bighead Carp showed a significant decrease (p <0.05) throughout chilled-frozen storage **(Latip, Zzaman and Yang, 2013)**. During investigation decreasing trend was found in fillet of *Mystus seenghala* in frozen samples **(Gandotra et al., 2012)**. This decrease was rapid and higher in fresh stored at -10 °C compared with those stored at -30 °C. The decrease in protein solubility could be due to protein denatured and protein aggregation induced by frozen storage. The stabilization of myofibrillar proteins is directly related to better fish quality **(Martinez, Friis and Careche, 2001)**. Thus, the decreasing in protein content showed that there was a decreasing in fish quality during frozen storage **(Latip, Zzaman and Yang, 2013)**, that not agreed with present study. During chilled-frozen storage, the ash content in Bighead Carp showed a significant decrease **(Latip, Zzaman and Yang, 2013)**, which not agreed with present study results, this different duo to the location and environmental conditions of the fish and the method of analysis and type of apparatus. Canning process, including the addition of filling materials, that to affect the macronutrient contents of the canned product compared with that of the raw ones **(Herawati, Susanto and Kurniadi, 2016)**. This effect was found in this study in terms of moisture (decreased) and fat (increased) contents **(Bahurmiz, Al-Sa'ady and Adzitey, 2018)**, this is agreed with present study.

CONCLUSION

This research was conducted on the nutrients of fresh fish of common carp and frozen fish and canned tuna fish in the southern Iran. The results obtained showed the percentage of canned fat (36.27%) that was the highest percentage. The ash amounts of frozen and fresh with 2% found higher than ash in canned fish with 1%. The percentage of protein in frozen fish was the highest and the highest percentage for moisture found for frozen fish. The except moisture content, proximate composition contents of carp fresh fish in present study found more than **Bavitha (2015)** results. The level of energy (kcal) of canned fish was the highest. The pH of canned fish was the highest pH. The percentage of drop in frozen fillets was 6.7%. WHC level was 6%. This effect canning was found in this study for moisture content decreased and for fat value increased that compared with frozen and fresh fishes, was agreed with other authors. It can be concluded that despite the low level of protein and ash in canned fish, the highest fat and energy was obtained. The protein content of the frozen and its pH indicates that it was better than fresh fish in terms of quality.

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EVALUATION OF SELECTED PARAMETERS OF EDAM TYPE CHEESE PACKED UNDER FOIL WITH NATURAL ANTIMICROBIAL AGENTS

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ABSTRACT

The aim of this study was to evaluate the properties of essential oils packed in foils derived from different plant sources used in Edam type cheese on selected parameters (total viable count of microorganism, coliform bacteria, micromycetes, sensory parameters and instrumental colour). Essential oils have antibacterial and antifungal activities against microorganisms. However, the concentration of these substances applied in cheeses should be considered carefully because of their possible negative influences on sensory parameters. Mixture of the essential oils (clove/cinnamon/thymol in a 1:2:1 ratio), three concentrations (3.9 %, 6.6 %, 9.0 %), respectively mixture of the essential oils (eugenol/thymol/cinnamon in a 1:1:1 ratio), three concentrations (0.10 %, 0.19 %, 0.24 % as a 5% solution in limonene in a dry coating) were used. Samples wrapped in polystyrene dishes were stored in the refrigerator at 3 – 6 °C. Analyses were made after 48 h, 168 h (144 h), 216 h (240 h) respectively. Taste is the most affected by presence of essential oils. The effectiveness of the film with the mixture A seems to be more effective in eliminating microorganisms. Negative sensory changes were observed at higher concentration. Based on the results, the tested foils seem to be promising materials suitable for packaging of cheese.

Keywords: packaging; sensory evaluation; colour; microbiological quality

INTRODUCTION

Active packaging becomes more and more important, rapid progress and growth of applications have been observed because of consumers food preferences **(Khaneghah, Hashemi and Limbo, 2018)**.

Active packaging has appeared as strategy for the control of microbial growth, thereby increasing food quality and safety of the packaged foodstuffs **(Wen et al., 2016)**.

Plants contain many substances that show antimicrobial effects. The most well-known and long-term studied are the essential oils that are carriers of the aromatic properties of many spices and medicinal plants such as cumin, anise, fennel, cinnamon or cloves. Substances as carvone, eugenol, thymol, anethol, phenchon and others are often occurring **(Conner and Beuchat, 1984; Doyle, Beuchat and Montville, 2001)**.

The application of essential oils in the film has been proven to be advantageous for avoiding direct application on the food, thus reducing undesirable interference in sensory parameters. It also allows for a gradual release of antimicrobial compounds on the surface of the packed food, prolonging the time of action/protection, and acting exactly where the risk of contamination is greatest **(Coma, 2008)**.

Active compounds from plants show a different effect on microorganisms. Gram-negative bacteria are more resistant to antimicrobial agents than gram-positive bacteria due to their cellular structure walls, as reported by **Gyawali and Ibrahim (2012)**. Their effectiveness depends on several factors, as pH, temperature, oxygen, concentration and variability of the components **(Tajkarimi, Ibrahim and Cliver, 2010; Burt, 2004)**.

Antimicrobial agents derived from spices are used to reduce the incidence of pathogenic bacteria and improve overall quality, can also be used to prolong the shelf life of food products **(Tajkarimi, Ibrahim and Cliver 2010; Jay, Loessner and Golden, 2005)**.

Milk and dairy products are an important part of the diet. Dairy products cover from 20 to 30% of protein, 15% of lipids and about 80% of calcium from food and positive aspects of milk are enhanced by the used cultural microflora **(Vorlová et al., 2017)**.

Scientific hypothesis

The aim of this work was to test the impact of the spray film mixtures of plant extracts without direct contact with the packaged food on microbiological parameters (Total viable count of microorganism, coliform bacteria, micromycetes), sensory characteristics (colour, odour, consistency, taste) and instrumental colours (L* lightness, a* redness, b* yellowness) of packaged Edam type cheese during storage.

MATERIAL AND METHODOLOGY

Experimental design

Two mixtures of natural antimicrobial agents were prepared for spreyed on foils.

Mixture A: a mixture of the essential oils (clove/cinnamon/thymol in a 1:2:1 ratio), three concentrations (3.9%; 6.6%; 9.0%).

Mixture B: a mixture of the essential oils (eugenol/thymol/cinnamon in a 1:1:1 ratio), three concentrations (0.10%; 0.19%; 0.24% as a 5% solution in limonene in a dry coating).

A layer of 27.5 x 17 cm lacquer coating, corresponding to the area of disposable polystyrene plates for food packaging in which antimicrobial tests were performed, was applied to the film. To the cleaned dishes (alcohol 60%) were added 100 g Edam type cheese, the active foil was glued to the dishes by means of a hot-pistol, the plates were incubated in the refrigerator at 3 – 6 °C for 48 h, 168 h (144 h) and 216 h (240 h).

Microbiological analysis

Microbiological analysis was performed by smear of the surface area of 5 x 5 cm. The following groups of microorganisms were determined for control and specimens taken at the time of establishment of the experiment. Total viable count (TVC) on PCA agar with skimmed milk on cheese samples (Biokar Diagnostics, France) at 30 °C for 72 hours, coliform bacteria on VRBL (Biokar Diagnostics, France) at 37 °C for 24 hours, micromycetes (mould and yeast) to Chloramphenicol glucose agar ((Biokar Diagnostics, France) at 25 °C in 120 hours. The results are shown in CFU x 25 cm^{-2}.

Sensory analysis

Sensory evaluation of cheeses packaged in active foil was performed in the tests of mixtures A and B. The sensory parameters evaluated colour on cut, odour, consistency and taste during the storage time. A sensory evaluation was carried out by 8 evaluators (3 men, 5 women) in special room under **ISO 6658:2010** condition in the sensory laboratory of the Department of Food Technology, Mendel university in Brno, Czech Republic. All sensory evaluators buy and consume cheeses regularly. 100 mm line scale ranging from 0 at the left to 100 at the right were used. Descriptors expressed as the hedonic scores, where 0 is unpleasant and 100 is pleasant.

Instrumental colour analysis

The surface colour of cheese was measured on the Konica Minolta Spectrophotometer CM-3500d (Konica Minolta, Japan). The SCE (specular component excluded) mode and 8 mm slot were used. The L* (lightness), a* (redness) and b* (yellowness) were evaluated. Colour variation was determined as total colour difference ΔE^*_{ab} **(Saláková, 2012)**.

Statistic analysis

Data collected from experiments were statistically rated nonparametric Kruskal-Wallis multi-choice test for comparing the treatment by programme STATISTICA 12.

RESULTS AND DISCUSSION

Two mixtures od natural anitimicrobial agents (essential oils) were used in these experiments.

Total viable count of microorganisms

The results of microbiological analysis of mixture A (clove/cinnamon/thymol in a 1:2:1 ratio) in three concentrations (3.9%; 6.6%; 9.0%) are shown in Table 1. The data was compared with control packaging without essential oils.

The microbiological analysis was done during storage time 48 h, 168 h and 216 h. There were found no statistically significant differences (p >0.05) among treatment with different concentration of essential oils in mixture A. The TVC of fresh cheese (before packaging) was 2.1 x 10^4 CFU.cm^{-2}. After packaging, the TVC decreased in all concentration of essential oils.

The result of microbioogical analysis of mixture B (eugenol/thymol/cinnamon in a 1:1:1 ratio) in three concentrations (0.10%; 0.19%; 0.24% as a 5% solution in limonene in a dry coating) are shown in Table 2. The microbiological analysis was done during storage time 44 h, 144 h and 240 h. The TVC of fresh cheese (before packaging) was 4.2 x 10^4 CFU.cm^{-2}. There were found no statistically significant differences (p >0.05) among treatment with different concentration of essential oils in mixture B.

Coliform bacteria

Coliform bacteria were not detected or were detected in minimal CFU in both mixtures. Essential oils containing carvacrol and thymol can decompose the membrane of *E. coli*, facilitating the entrance of strong essential oils components such as eugenol into the *E. coli* and allowing them to interact with the relevant proteins **(Pei et al., 2009)**. **Khaneghah, Hashemi and Limbo (2018)** reported, that antimicrobial packaging technology reduces health risks and improves the safety and quality of food products by reducing or inhibiting microbial growth.

Micromycetes

There were found minimum CFU of micromycetes in mixture A with different concentrations of essential oils during storage time. Higher incidents of micromycetes was detected in cheese packed under foils with mixture B in all concetrations of essential oils. It seems that mixture B is less effective against growth of yeast and moulds. There were not found statistically significant differences (p >0.05) among concentrations or days of storage, respectively. According **Garnier, Valence and Mounier (2017)** more than 60 species of yeast have been identified as spoilage agents of dairy products and about 100 mould species have been identified so far as being responsible for dairy product spoilage. Mould spoilage can also lead to the formation of off-flavors and can also contaminate heat-treated dairy product.

Table 1 Microbiological analysis of Edam type cheese – mixture A (clove/cinnamon/thymol in a 1:2:1 ratio).

		Mixture A			
parameters	**time**	**0% A**	**3.9% A**	**6.6% A**	**9.0% A**
TVC CFU x 25 cm^{-2}	48 h	1.3 x 10^4	8.7 x 10^2	1.3 x 10^4	1.6 x 10^4
	168 h	8.5 x 10^3	1.2 x 10^4	9.3 x 10^3	4.0 x 10^3
	216 h	6.6 x 10^3	5.6 x 10^3	4.6 x 10^3	1.5 x 10^4
coliform bacteria CFU x 25 cm^{-2}	48 h	ND	ND	ND	ND
	168 h	ND	ND	ND	ND
	216 h	ND	ND	ND	ND
micromycetes CFU x 25 cm^{-2}	48 h	3	ND	ND	ND
	168 h	5	ND	1.7 x 10^2	2.8 x 10^3
	216 h	3	1.1 x 10^2	7.3 x 10^2	3

Note: TVC – total viable counts of microorganism, CFU – colony forming units, ND – not detected.

Table 2 Microbiological analysis of Edam type cheese – mixture B (eugenol/thymol/cinnamon in a 1:1:1 ratio).

		Mixture B			
parameters	**time**	**0% B**	**0.11% B**	**0.19% B**	**0.24% B**
TVC CFU x 25 cm^{-2}	48 h	2.1 x 10^5	3.6 x 10^5	1.8 x 10^5	2.1 x 10^5
	144 h	7.7 x 10^4	5.8 x 10^4	1.7 x 10^5	1.5 x 10^5
	240 h	1.5 x 10^6	6.4 x 10^5	9.5 x 10^5	7.9 x 10^5
coliform bacteria CFU x 25 cm^{-2}	48 h	ND	4.5 x 10^1	ND	ND
	144 h	ND	ND	ND	18
	240 h	3	3	ND	ND
micromycetes CFU x 25 cm^{-2}	48 h	1.9 x 10^3	1.1 x 10^2	1.1 x 10^3	6.3 x 10^2
	144 h	6.9 x 10^3	9.2 x 10^3	5.8 x 10^4	3.6 x 10^4
	240 h	6.4 x 10^5	8.5 x 10^5	6.2 x 10^5	1.5 x 10^6

Note: TVC – total viable counts of microorganism, CFU – colony forming units, ND – not detected).

Table 3 Sensory evaluation of Edam type cheese – mixture A (clove/cinnamon/thymol in a 1:2:1 ratio).

		Mixture A			
parameters	**time**	**0% A**	**3.9% A**	**6.6% A**	**9.0% A**
colour	48 h	54 ±13	57 ±10	53 ±15	55 ±14
	168 h	74 ±22	69 ±20	81 ±20	70 ±18
	216 h	81 ±15	71 ±20	85 ±17	75 ±21
odour	48 h	33 ±22	31 ±15	19 ±22	26 ±17
	168 h	81 ±19	30 ±18	47 ±18	21 ±16
	216 h	78 ±16	46 ±16	45 ±11	28 ±11
consistency	48 h	60 ±17	66 ±14	58 ±17	63 ±12
	168 h	66 ±17	46 ±22	65 ±13	61 ±20
	216 h	61 ±15	63 ±9	67 ±8	39 ±20
taste	48 h	35 ±18	41 ±21	21 ±25	30 ±21
	168 h	69 ±22	25 ±26	33 ±19	12 ±14
	216 h	71 ±11	56 ±12	64 ±15	35 ±12

Note: mean ± S. D.

Table 4 Sensory evaluation of Edam type cheese – mixture B (eugenol/thymol/cinnamon in a 1:1:1 ratio).

		Mixture B			
parameters	**time**	**0% B**	**0.11% B**	**0.19% B**	**0.24% B**
colour	48 h	81 ±14	81 ±14	80 ±14	80 ±14
	144 h	79 ±19	75 ±20	78 ±18	75 ±21
odour	48 h	83 ±16	65 ±+á	65 ±18	51 ±25
	144 h	76 ±21	68 ±23	65 ±19	61 ±17
consistency	48 h	81 ±13	79 ±14	76 ±14	66 ±15
	144 h	80 ±17	77 ±16	78 ±15	80 ±12
taste	48 h	84 ±11	70 ±10	53 ±18	29 ±23
	144 h	74 ±16	69 ±13	70 ±14	59 ±15

Note: mean ± S. D.

Table 5 Instrumental colour evaluation of Edam type cheese – mixture A (clove/cinnamon/thymol in a 1:2:1 ratio).

		Mixture A			
parameters	**time**	**0% A**	**3.9% A**	**6.6% A**	**9.0% A**
L*	48 h	83.60 ±0.13^{a}	82.76 ±0.36^{a}	80.38 ±1.18^{a}	81.37 ±1.29^{a}
	168 h	76.86 ±1.83^{b}	79.00 ±1.41^{b,c}	79.05 ±0.93^{a}	79.62 ±0.73^{a}
	216 h	80.56 ±0.48^{c}	80.00 ±0.89^{c}	80.23 ±0.30^{a}	79.08 ±0.62^{a}
a*	48 h	2.16 ±0.40^{a}	1.86 ±0.31^{a}	1.46 ±0.50^{a}	1.26 ±0.43^{a}
	168 h	0.62 ±0.48^{b,c}	1.56 ±0.31^{a}	1.67 ±0.15^{a}	1.05 ±0.10^{a}
	216 h	1.32 ±0.22^{c}	1.49 ±0.24^{a}	1.91 ±0.04^{a}	1.98 ±0.19^{a}
b*	48 h	28.06 ±0.41^{a}	28.09 ±0.80^{a}	29.17 ±1.02^{a}	28.86 ±0.59^{a}
	168 h	29.90 ±0.62^{b}	31.35 ±1.23^{b}	31.17 ±0.84^{b}	29.01 ±0.45^{a}
	216 h	29.42 ±0.24^{a}	29.75 ±0.96^{a}	31.09 ±0.31^{a}	33.19 ±0.64^{b}

Note: L* – lightness, a* – redness, b* – yellowness, mean ± S.D., different superscripts in the same columns show significant differences (p <0.05).

Table 6 Instrumental colour evaluation of Edam type cheese – mixture B (eugenol/thymol/cinnamon in a 1:1:1 ratio).

		Mixture B			
parameters	**time**	**0% B**	**0.11% B**	**0.19% B**	**0.24% B**
L*	48 h	82.67 ±0.17^{a}	82.13 ±0.41^{a}	83.97 ±0.04^{a}	81.96 ±0.15^{a}
	144 h	83.04 ±0.30^{b}	82.50 ±0.79^{b}	83.57 ±0.54^{a}	83.97 ±0.23^{b,c}
	240 h	83.46 ±0.47^{b}	83.99 ±0.77^{c}	83.43 ±0.27^{a}	83.69 ±0.44^{c}
a*	48 h	2.58 ±0.46^{a}	3.03 ±0.06^{a}	2.56 ±0.11^{a}	2.58 ±0.35^{a}
	144 h	3.24 ±0.27^{a}	3.23 ±0.23^{a}	2.74 ±0.16^{a}	2.76 ±0.41^{a}
	240 h	2.75 ±0.12^{a}	2.74 ±0.35^{a}	2.67 ±0.35^{a}	2.69 ±0.14^{a}
b*	48 h	29.92 ±0.42^{a}	29.87 ±0.38^{a}	29.25 ±0.40^{a}	30.24 ±0.36^{a}
	144 h	30.64 ±0.37^{a,b}	30.53 ±1.05^{a,b}	28.79 ±0.68^{a}	29.77 ±0.85^{a}
	240 h	28.55 ±0.04^{a,c}	28.78 ±0.57^{a,c}	28.79 ±0.26^{a}	28.48 ±1.00^{a}

Note: L* – lightness, a* – redness, b* – yellowness, mean±S.D., different superscripts in the same columns show significant differences (p <0.05).

Sensory analysis

The data collected during sensory evaluation of cheese packed under foil with mixture A of essential oils are shown in Table 3.

Colour on cut of cheese before packaging was according sensory evaluators 64 ±15. Sensory assessors judged cheese colour after 48 h worse than cheese before packaging in all essential oil concentration. The best colour evaluation had cheese with concentration 6.6% of essential oils during time of storage. Odour of cheese before packaging was 77 ±14. The best odour evaluation was for cheese packed without essential oils and the worst wit concentration of essential oils 9.9%. The use of essential oils had negative effect on odour, it corresponded with **Garnier, Valence and Mounier (2017)**.

No statistically significant effect (p >0.05) on consistency was found for all concentratin of essential oil. Consistency of cheese before packaging was 67 ±17, after 216 h of storage time the worst evaluation had 9.9% of essential oils on foil. Taste is the most affected by presence of essential oils. Before packaging was 70 ±13 and the worst evaluation was for concentration 9.9% of essential oils during storage time. Essential oils have intensive aroma, application at high concentrations could result in sensory defects **(Khorshidian et al., 2018)**.

The data collected during sensory evaluation of cheese packed under foil with mixture B of essential oils are shown in Table 4. Sensory analysis was performed only after 48 h and 144 h due to deteriorative microbiological parameters.

No statistically significant effect (p >0.05) on colour and consistency was found for every concentration of essential oils. The worst evaluation was for concentration 0.24% of essential oils during storage time.

Characteristic of sensory properties of cheese, i.e. appearance, colour, taste and aroma and texture, are the result of running biochemical process primarily during ripening of cheeses **(Vítová et al., 2011)** and during packaging.

Instrumental colour measurement

The results of instrumental colour are presented in Table 5 and Table 6. Colours of cheese may scope from pale yellow to deep red-orange, depending upon the application and consumer preference **(El-Nimr et al., 2010)**.

There were found statistically significant differences (p >0.05) during storage time. Minor differences were noted in use mixture B. Colour at the beginning of experiment range from L* 81.69, a* 2.13, b* 28.98 (mixture A) to L* 83.53, a* 2.70, b* 27.50 (mixture B). After 168 h were found colour difference ΔE^*_{ab} from 2.33 to 5.14, the highest colour difference was found in concentration 9.9% (mixture A) after 216 hours of storage. Statistically significant differences (p <0.05) in L* value between concentrations of mixture A at time of storage 168 h. After 144 h were found colour differences ΔE^*_{ab} from 0.55 to 1.36.

It is clear that the lightness decreased during storage and the yellowness b* increased during storage for use of mixture A. When using mixture B, the opposite trend was found.

CONCLUSION

These films appear to be promising materials for packaging cheese. However, it is necessary to confirm this other test focusing primarily on the choice of the appropriate concentration of the active substance and possible negative effects on the sensory properties of the food. The effectiveness of the film with the mixture A (clove/cinnamon/thymol in a 1:2:1 ratio), seems to be more effective in eliminating microorganisms. Negative sensory changes were observed at higher concentration.

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EFFECT OF ESSENTIAL OILS OF *MYRTACEAE* PLANTS ON THE *PENICILLIUM COMMUNE*

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ABSTRACT

The aim of this research was to determine the inhibitory effect of vapor phase of five essential oils (EOs) on the growth of seven strains of *Penicillium commune* isolated from moldy milk products. Another objective was to determine the minimum inhibitory doses (*in vitro* and probit analyses) of EOs, which at concentration 625 µL.L^{-1} of air completely inhibited the growth of all strains. The antifungal activity was evaluated by the micro-atmosphere method. The essential oils used in this study were extract of plants from family *Myrtaceae*. Only one essential oil – clove (from *Syzygium aromaticum* L.; leaves) completely inhibited the growth of all strains during cultivation at 25 °C and 5 °C. Eucalyptus essential oil (from *Eucaliptus globulus*; leaves), tea tree essential oil (from *Melaleuca alternifolia* Cheel; leaves), cajeput essential oil (from *Melaleuca leucadendra* L.; leaves and twigs), niaouli essential oil (from *Melaleuca quinquenervia* (Cav.) S. T. Blake; leaves) have different effects on the growth of *P. commune* strains. The order of tested essential oils according to the inhibition effect on the growth of the strains of *P. commune* (from the strongest to the weakest effect) was: clove > tea tree > cajeput > niaouli > eucalyptus. Clove EO that completely inhibited the growth of all strains was used to determine minimum inhibitory doses (MIDs). The MIDs were 125 µL.L^{-1} of air for two strains of *P. commune* and 250 µL.L^{-1} of air for five strains of *P. commune* on the 7th and 14th day of cultivation, also. Using probit analysis, predicted MIDs90 and MIDs50 were calculated. The MIDs90 were determined from 104.93 to 301.37 µL.L^{-1} of air.

Keywords: *Penicillium commune*; essential oils; antifungal activity; vapor phase

INTRODUCTION

Today's consumers demand food that is minimally technologically processed and without synthetic preservatives or additives, because of the possible adverse health effects. Therefore, the food industry is now focused on finding solutions that fully satisfy the criteria of consumers while retaining the food safety. The application of natural antimicrobial agents such as extracts, essential oils, components of spices, and other aromatic plants could be significant in resolving these problems. These agents may be useful as additives in limiting or preventing the development of harmful fungi in food, as food surface protectants, or in modified atmosphere packaging of food **(Kocic-Tanackov et al., 2014)**. Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural, and food industries. Because of the mode of extraction, mostly by distillation from aromatic plants, they contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components, and aliphatic components **(Bakkali et al., 2008)**. Natural preservatives could also constitute a viable alternative to address the critical problem of microbial resistance, and to hamper the negative side effects of some synthetic compounds, while meeting the requirements for food safety, and exerting no negative impact on nutritional and sensory attributes of foodstuffs **(Pisoschi et al., 2018)**.

Molds are the most common cheese spoilage organisms which can lead to economic loss as well as raising public health concerns due to the production of mycotoxins **(Cheong et al., 2014)**. *Penicillium commune* is a microscopic filamentous fungus that very often causes molding of cheeses **(Lund, Filtenborg and Frisvad, 1995; Kure and Skaar, 2000; Kure et al., 2001; Lund, Nielsen and Skouboe, 2003; Garnier et al., 2017)**.

The aim of the present research was to determine the inhibitory effect of vapor phase of five essential oils on the growth of seven different strains of *Penicillium commune* isolated from moldy milk products. Another objective was to determine the minimum inhibitory doses of essential oils, which at a concentration 625 µL.L^{-1} of air completely inhibited the growth of all the strains.

Scientific hypothesis

Growth of *Penicillium commune* is affected by essential oils of family *Myrthaceae*.

MATERIAL AND METHODOLOGY

Plant essential oils

The essential oils used in this study were extracts of plants from family *Myrtaceae*. Specifically, we used clove essential oil (from *Syzygium aromaticum* L.; leaves), eucalyptus essential oil (from *Eucaliptus globulus* leaves), Tea Tree essential oil (from *Melaleuca alternifolia* Cheel; leaves) cajeput essential oil (from *Melaleuca leucadendra* L.; leaves and twigs), niaouli essential oil (from *Melaleuca quinquenervia* (Cav.) S. T. Blake; leaves). Essential oils were commercially produced.

Chemical composition of essential oils

Semi-quantitative composition of the essential oil samples was determined by gas chromatography coupled with mass spectrometry (GC-MS) using an Agilent 7890B oven coupled with Agilent 5977A mass detector (Agilent Technologies Inc., Palo Alto, CA, USA) and CombiPal autosampler 120 (CTC Analytics AG, Zwingen, Switzerland). Prior to the analysis, essential oil samples were diluted in hexan (HPLC ≥97%, Sigma Aldrich GmbH, Germany) to a concentration of 10 $\mu L.mL^{-1}$. One microliter of diluted sample was injected in inlet operated in split mode (1:10; 250 °C). Separation was achieved using a ZB-WAXplusTM capillary column (10 m × 0.1 mm × 0.10 μm) (Phenomenex Inc., Torrance, CA, USA) and the following oven temperature programme: 50 °C for the first 5 minutes, increased to 240 °C at the rate of 3 °C min^{-1}, where it was kept constant for 2 minutes. Helium was used as carrier gas at the constant flow (1.2 $mL.min^{-1}$ – constant flow). The mass detector parameters were as follows: ionization energy of filament: 70 eV, transfer line temperature: 250 °C, MS source temperature: 230 °C, quadrupole temperature: 150 °C. The mass spectrometer was programmed under electron impact (EI) in a full scan mode at m/z 40 – 400. The identification of compounds was carried out by comparing of mass spectra (over 80% match) with a commercial database NIST® 2014 and retention times of reference standards (nerol, linalool, geraniol, citral, α-pinene, and β-pinene). The semi-quantitative content of determined compounds was calculated by dividing individual peak area (excluded by solvent peak area) by total area of all peaks. Peaks under 0.10% were not counted.

Fungal strains using in research

Seven strains from different moldy milk products were used. Specifically: *Penicillium commune* KMi 177 (from moldy cheese flavored with pepper); *P. commune* KMi 270 (smoked cheese – block); *P. commune* KMi 276 (smoked cheese – slices); *P. commune* KMi 277 (smoked cheese – slices); *P. commune* KMi 370 (sour cream); *P. commune* KMi 402 (sour cream); *P. commune* KMi 403 (parenica – pasta filata). These strains belong to the Collection of Fungi of Department of Microbiology; Faculty of Biotechnology and Food Sciences SUA in Nitra, Slovakia. Five-day old cultures cultivated on Czapek yeast extract agar (CYA) at 25 ±1 °C were used for each experiment (CYA) **(Pitt and Hocking, 2009)**.

Antifungal activity of essential oils

The antifungal activity of selected essential oils was evaluated by the micro-atmosphere method. The test was performed in sterile plastic Petri dishes (Ø 90 mm) containing 15 mL of CYA. The evaluation by filter paper was made by the adapted method from **Guynot et al. (2003)**. Essential oils were tested in concentration 625 $\mu L.L^{-1}$ of air. A round sterile filter paper (Ø 40 mm) was placed in the lid of Petri dish, and 50 μL of essential oil was pipetted by micropipette on the paper. Dishes were kept in inverted position. Filter paper discs impregnated with sterilized distilled water were used as a control. Each strain was inoculated in the center of Petri dishes with sterilized needle. Dishes were tightly sealed with parafilm and incubated for 14 days at 25 ±1 °C and for 35 days at 5 ±1 °C (four replicates were used for each treatment). The diameters (Ø mm) of the growing colonies (from the reverse side) were measured with a digital caliper on the 3rd, 7th, 11th, and 14th day – the strains cultivated at 25 ±1 °C and on 3rd, 7th, 11th, 14th, 21st, 28th, and 35th day – the strains cultivated at 5 ±1 °C.

Inhibition of mycelial growth

According to **Cakir et al. (2005)** and **Kordali et al. (2008)**, growth inhibition of treated samples (T) against control (C) was calculated by the percentage of growth inhibition using the following equation:

$$\% \text{ of inhibition} = (C - T)/C \times 100$$

where, C is the mean of eight replicates of the hyphal extension (mm) of controls and T is the mean of eight replicates of the hyphal extension (mm) of plates treated with either essential oil.

Minimum inhibitory doses (MIDs)

Essential oils that completely inhibit the growth of all strains at both temperatures were used to determine their minimum inhibitory doses (MIDs). EOs dissolved in dimethyl lsulfoxid (DMSO) were prepared at different concentrations (625; 500; 250; 125; 63; 31.25; and 15.63 $\mu L.L^{-1}$ of air). For each fungal strain, a conidial spore suspension of 10^6 spore's ml^{-1} was prepared. The EVE™ Automatic cell counter (NanoEnTek, Korea) was used to determine the number of spores. Petri dishes (Ø 90 mm, two-sector, three replicates) containing 15 mL of CYA were inoculated by 5 μl spores suspension. Cultivation was carried out at the 25 ±1 °C and measured after 7 and 14 days. The MID (expressed as microliters of EOs per volume unit of atmosphere above the organism growing on the agar surface) was defined as the lowest concentration of the oil which did not permit any visible growth after 7 or 14 days in comparison with control sets.

Probit analyses

The ability of the strains to grow in the presence of EO was coded to binomial scale (1 – growth observed, 0 – without growth). Such data were processed by probit analysis in Statgraphics Centurion XV (Statgraphics) software. Doses that inhibit the growth in 50% respectively 90% of cases (MID50 and MID90) were reversely predicted from regression equation.

Statistic analysis

The statistical data evaluation was performed using SAS 9.3 package. The glimmix was used to test the effect of strain, essential oil, day, and the interaction of the strain, essential oil, and day on the mold growth with repeating random-residual-option and unstructured covariate structure. Ranked data were analysed. Holm-Tukey post hoc test was applied to performe for multiple comparison. Results were considered as significant at p value ≤0.05.

RESULTS AND DISCUSSION

In this study, the antifungal properties of five essential oils (clove, eucalyptus, Tea Tree, cajeput, niaouli) from family *Myrtaceae* were evaluated. Essential oils are complex mixtures of low molecular weight compounds extracted from plants by steam distillation and various solvents. Terpenoids and phenylpropanoids are the major constituents which provide characteristic aroma and biological properties to essential oils **(Raut and Karuppayil, 2014)**. According to authors **(Ben Farhat, et al., 2016; Méndez-Tovar et al., 2016; Dušková et al., 2016)** the effect of the growing seasons, different growth stage of plants, and climatic conditions of each year in terms of the essential oil content and composition were proven. Based on the above, we also focused on the composition of the essential oils used. Essential oils are very complex natural mixtures which can contain about 20 – 60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20 – 70 %) compared to other components present in trace amounts **(Bakkali et al., 2008)**. The GC-MS analyses of the essential oils led to identification of 78 compounds, 31 from them are presented in amount of ≥1 percentage minimally in one essential oil. The identified compounds (31) are listed in Table 1. The major components according to the specific essential oil were: clove – Eugenol (73.3%); eucaliptus – Eucalyptol (78.7%); Tee tree – 4-terpinenol (39.6%), gamma – Terpinene (20.0%) and (+)-4-Carene (9.38%); niaouli Eugenol (50.2%), alfa-pinene (9.78%), Viridiflorol (8%).

Antifungal activity of essential oils

All strains of *P. commune* without essential oil in the atmosphere – controls (without essential oil at the atmosphere), were grown on the first measurement (3rd day of incubation) at 25 ±1 °C. The intensity of the growth of the strains at 25 ±1 °C is shown in Figure 1. Dairy products are stored at low temperatures, so an additional cultivation temperature of 5 ±1 °C was used. At 5 ±1 °C (controls), the growth of two strains (KMi 270 and KMi 276) was recorded on the 3rd day and of other five strains on the 7th day. The intensity of the growth of the strains at 5 ±1 °C is shown in Figure 2.

Plant oils obtained from plants of three genera of family *Myrthaceae* were used in the research: *Syzygium*, *Eucaliptus,* and *Melaleuca.* The growth of the strains of *P. commune* was affected by all the essential oils used (Table 2 and Table 3). Only clove (from *Syzygium aromaticum* L.) essential oil, as the only one, completely inhibited the growth of the strains of *P. commune* at 5 ±1 °C, respectively 25 ±1 °C, throughout the experiment. **Guynot et al. (2003)** demonstrated the potential of clove essential oil aganist species belonging to *Eurotium, Aspergillus* and *Penicillium* genera. According to **Císarová, Tančinová and Medo (2016)** clove essential oil (500 µL.L^{-1} of air) completely inhibited growth of isolates of *Aspergillus flavus* and *Aspergillus parasiticus*.

Eucaliptus (from *Eucaliptus globulus*) essential oil only partially inhibited the growth of strains of *P. commune* at 25 ±1 °C and 5 ±1 °C. Even the growth stimulation of the strain *P. commune* KMi 277 was observed (at 25 ±1 °C). The influence of the eucalitptus esseniatial oil is shown in Figure 3 and Figure 4. Unlike our results using another type of volatile essential oil, **Ramezani et al. (2002)** showed a strong fungicidal activity of the volatile oil of *Eucaliptus citriodora*.

Three essential oils were used from genus *Melaleuca:* tea tree, cajeput, and niaouli. Tea tree (from *Melaleuca alternifolia* Cheel) essential oil showed a strong inhibitory effect on the strains of *P. commune*. The growth of six strains at 25 ±1 °C, respectively five at 5 ±1 °C was completely inhibited throughout all cultivations. Only one strain (*P. commune* KMi 403) in the presence of tea tree oil grew at 25 ±1 °C, but its growth was small (84.8 – 99.5% of inhibition). The influence of tea tree essential oil on the growth of the strains *P. commune* at 25 ±1 ºC is shown in Figure 5. Two strains of *P. commune* (KMi 276 and KMi 403) were able to grow in the presence of tea tree essentail oil at 5 ±1 °C from the 28th day of the experiment, but growth inhibition was significant (Figure 6). A weaker inhibitory effect was found in cajeput (from *Melaleuca leucadendra*) essential oil (Figure 7 and Figure 8) and the weakest in niaouli (from *Melaleuca quinquenervia* (Cav.) S. T. Blake) essential oil (Figure 9 and Figure 10). **Stević et al. (2014)** also reported excellent antifungal activity of tea tree essential oil against the most of tested fungi (species of genera *Fusarium, Aspergillus, Alternaria, Penicillium* and others).

Minimum inhibitory doses (MIDs)

In this study clove essential oil (625 µL.L^{-1} of air) was able to inhibit the growth of all strains at all days of cultivation on the Czapek yeast extract agar at 5 ±1 °C and 25 ±1 °C also. Clove essential oil was used for the determination of MIDs. The results are shown in Table 4. The MIDs were 125 µL.L^{-1} of air for the strains *P. commune* KMi 177 and KMi 277 and 250 µL.L^{-1} of air for the strains *P. commune* KMi 270, KMi 276, KMi 370, KMi 402 and KMi 403 on the 7th day of cultivation. On the 14th day of incubation, the same values of MIDs were determined. In some strains, the number of growing colonies increased, but this did not affect the MIDs. **Císarová et al. (2016)** determined lower MIDs of clove essential oil in vapor phase against the tested aspergilly strain 62.5 µL.L^{-1}.

Using probit analysis, predicted MIDs90 and MIDs50 were calculated. The results are shown in Table 5. The highest MIDs90 were determined for strain KMi 403 – 301.37 µL.L^{-1} of air on 7th day and 292.56 µL.L^{-1} of air on 14th day of cultivation. The lowest MIDs90 were determined for the strains KMi 177 and KMi 277 – 104.93 µL.L^{-1} of air on the 7th day and on the 14th day of cultivation.

Table 1 Essential oils tested for the fungicidal effect and their compounds (%)* determined by gas chromatography coupled with mass spectrometry (GC-MS).

	Compaund	Essential oils				
		Clove	Eucaliptus	Tea Tree	Cajeput	Niaouli
1	alfa-pinene		2.71	3.63	1.81	9.78
2	beta-pinene		0.19		1.14	
3	p-Cymene		6.37	5.95		
4	beta.-M+E5:G25yrcene				1.54	
5	gamma.-Terpinene		2.36	20.0		
6	gamma-terpineol			6.74		
7	D-Limonene	0.08	8.03	0.92	5.17	7.11
8	Eucalyptol	0.35	78.7		60.76	50.2
9	gamma-Terpinene				1.21	0.56
10	Caryophyllene	9.23		0.54	1.52	1.56
11	o-Cymene				1.22	1.66
12	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	2.68				
13	alpha-Terpinyl acetate					1.44
14	Linalool				3.49	
15	Nerolidol					3.59
16	Caryophyllene oxide	2.19				0.55
17	Humulene				1.09	0.36
18	Eugenol	73.3				
19	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	10.1				
20	alfa-selinene				0.87	
21	beta-selinene				1.04	
22	Aromandendrene			1.02		
23	alpha-guaiol				1.00	
24	beta-gurjunene			1.06		
25	(+)-4-Carene			9.38	0.33	
26	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-			0.71		2.27
27	4-terpinenol			39.6		
28	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene			1.35		
29	Cerd-8-ene			0.23		6.88
30	alpha-Terpineol			3.24	12.9	
31	Viridiflorol			0.12		8.00

Note: *listed are the components that represented min. 1% in at least one essential oil.

Table 2 The effect of essential oil on the growth of different strains during cultivation at 25 ±1 °C.

Strain of *P. commune*	Essential oil	Day of cultivation			
		3rd	7th	11th	14th
KMi 177	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	5.13 ± 1.17^{aB}	20.75 ± 1.09^{bB}	34.63 ± 0.99^{aB}	43.00 ± 0.71^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{aA}	0^{aA}	0^{aA}	4.63 ± 1.49^{bC}
	Niaouli	0^{aA}	2.75 ± 0.66^{bC}	6.88 ± 0.78^{cC}	9.75 ± 1.20^{dD}
	Control	16.00 ± 1.80^{aC}	30.63 ± 0.70^{bD}	41.63 ± 1.49^{cD}	45.88 ± 3.62^{cB}
KMi 270	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	1.16 ± 1.80^{aB}	21.25 ± 1.39^{bB}	29.63 ± 1.11^{cB}	35.00 ± 0.71^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	2.13 ± 1.62^{aC}	5.25 ± 2.63^{bC}	12.00 ± 3.00^{cC}	23.50 ± 2.78^{dC}
	Niaouli	3.13 ± 0.93^{aD}	7.75 ± 0.97^{bD}	15.50 ± 1.22^{cC}	25.25 ± 1.79^{dC}
	Control	20.00 ± 1.50^{aE}	27.25 ± 0.66^{bE}	32.88 ± 1.62^{cB}	39.00 ± 1.58^{dB}
KMi 276	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	8.50 ± 0.71^{aB}	22.63 ± 0.86^{bB}	27.38 ± 0.99^{cB}	35.63 ± 1.49^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{A}	0^{A}	0^{A}	0^{A}
	Niaouli	0^{aA}	1.88 ± 0.60^{bC}	4.63 ± 0.48^{cC}	9.88 ± 0.60^{dC}
	Control	16.38 ± 0.48^{aC}	25.00 ± 1.12^{bB}	31.25 ± 1.09^{cB}	38.38 ± 0.86^{dB}
KMi 277	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	7.38 ± 0.86^{aB}	31.38 ± 0.86^{bB}	36.75 ± 0.97^{cB}	41.25 ± 3.80^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{aA}	0^{aA}	0.38 ± 0.70^{bC}	3.88 ± 1.54^{cC}
	Niaouli	0^{aA}	4.38 ± 0.99^{bC}	9.38 ± 3.94^{cD}	14.88 ± 5.01^{dD}
	Control	14.75 ± 1.09^{aC}	31.88 ± 2.52^{bB}	36.13 ± 3.10^{cB}	39.25 ± 1.09^{dB}
KMi 370	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	3.00 ± 0.00^{aB}	16.50 ± 0.50^{bB}	27.00 ± 1.00^{cB}	35.00 ± 0.50^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{aA}	0^{aA}	2.50 ± 1.73^{bC}	4.88 ± 2.20^{cC}
	Niaouli	0^{aA}	3.50 ± 1.00^{bC}	9.25 ± 2.38^{cD}	13.75 ± 2.86^{dD}
	Control	10.63 ± 0.70^{aC}	25.75 ± 0.83^{bD}	35.13 ± 0.60^{cE}	40.25 ± 0.92^{dE}
KMi 402	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	1.00 ± 0.00^{aB}	14.63 ± 0.86^{bB}	27.50 ± 1.32^{cB}	34.38 ± 1.58^{dB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{aA}	0^{aA}	0.50 ± 0.5^{bC}	2.37 ± 0.99^{cC}
	Niaouli	0^{aA}	4.75 ± 0.83^{bC}	12.25 ± 1.79^{cD}	17.50 ± 1.50^{dD}
	Control	9.50 ± 0.50^{aC}	24.63 ± 0.48^{bD}	36.25 ± 1.20^{cE}	44.13 ± 3.10^{dE}
KMi 403	Clove	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	5.88 ± 1.69^{aB}	34.25 ± 1.71^{bB}	57.00 ± 1.22^{cBE}	66.13 ± 0.78^{cBE}
	Tea Tree	0^{aA}	0.25 ± 0.43^{bC}	4.88 ± 0.78^{cC}	11.12 ± 1.17^{dC}
	Cajeput	0.50 ± 0.87^{aC}	4.63 ± 1.31^{bD}	21.13 ± 2.37^{cD}	31.50 ± 1.50^{dB}
	Niaouli	2.75 ± 0.43^{aD}	21.75 ± 1.09^{bF}	40.25 ± 1.85^{cB}	59.88 ± 1.17^{dE}
	Control	19.75 ± 0.43^{aE}	46.25 ± 0.83^{bF}	67.13 ± 0.78^{bcE}	73.13 ± 0.78^{cBF}

Note: The numbers represent mean of colony size. a, b, c, d shows significant differences within row. A, B, C, D, E, F shows significant differences within column and within strain. Data presented as mean ± stdev in mm.

Table 3 The effect of essential oil on the growth of different strains during cultivation at 5 ±1 °C.

Strain of *P. commune*	Essential oil	Day of cultivation						
		3rd	7th	11th	14th	21st	28th	35th
KMi 177	Clove	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{a}	0^{aA}	2.00 ± 0.72^{bB}	3.88 ± 0.78^{cB}	8.25 ± 1.20^{dB}	13.25 ± 1.20^{eB}	17.75 ± 1.39^{fB}
	Tea Tree	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Niaouli	0^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.88 ± 0.60^{bC}	3.63 ± 0.99^{cC}
	Control	0^{a}	5.88 ± 1.05^{bB}	13.38 ± 0.70^{cC}	17.13 ± 1.45^{cC}	27.5 ± 1.22^{dD}	34.25 ± 1.79^{eD}	43.38 ± 2.96^{fD}
KMi 270	Clove	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{aA}	0^{aA}	1.88 ± 1.77^{bB}	2.25 ± 1.48^{bB}	6.88 ± 1.90^{cB}	9.50 ± 1.50^{dB}	15.63 ± 2.06^{eB}
	Tea Tree	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.88 ± 0.60^{bC}	2.00 ± 0.71^{bC}	5.00 ± 1.00^{cC}
	Niaouli	0^{aA}	0^{aA}	1.13 ± 1.17^{bC}	2.13 ± 0.33^{cC}	2.50 ± 0.71^{cD}	4.13 ± 0.60^{dD}	6.88 ± 1.27^{eD}
	Control	1 ± 1^{aB}	2.38 ± 1.49^{bB}	7.38 ± 2.45^{cD}	9.88 ± 3.02^{cD}	24.50 ± 1.58^{dE}	34.38 ± 1.32^{eE}	43.00 ± 2.00^{fE}
KMi 276	Clove	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{aA}	0^{aA}	0^{aA}	1.88 ± 0.60^{bB}	5.75 ± 1.09^{cB}	12.25 ± 1.39^{dD}	18.00 ± 2.18^{eB}
	Tea Tree	0^{aA}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.50 ± 0.50^{bC}	2.88 ± 0.60^{cC}
	Cajeput	0^{aA}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	2.75 ± 0.66^{bD}	4.38 ± 0.70^{cD}
	Niaouli	0^{aA}	0^{aA}	0^{aA}	0^{aA}	2.13 ± 0.60^{bC}	3.75 ± 0.43^{cE}	6.63 ± 1.32^{dE}
	Control	1.88 ± 0.33^{aB}	6.38 ± 1.22^{bB}	10.75 ± 0.66^{bB}	18.75 ± 0.97^{cC}	24.63 ± 0.70^{D}	36.00 ± 0.71^{dF}	41.50 ± 1.80^{dF}
KMi 277	Clove	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{a}	0^{aA}	1.38 ± 0.86^{bB}	2.50 ± 0.87^{cB}	6.00 ± 2.43^{dB}	10.13 ± 3.18^{eB}	16.88 ± 1.62^{gB}
	Tea Tree	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.38 ± 0.48^{bC}	3.63 ± 0.48^{cC}
	Niaouli	0^{a}	0^{aA}	0^{aA}	2.50 ± 0.71^{bB}	4.00 ± 0.87^{cC}	5.50 ± 0.71^{dD}	8.25 ± 1.20^{eD}
	Control	0^{a}	7.00 ± 1.22^{bB}	$11.25^{b} \pm 0.83^{C}$	18.75 ± 1.30^{cC}	32.25 ± 0.97^{dD}	35.00 ± 1.66^{eE}	39.50 ± 1.80^{eF}
KMi 370	Clove	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{a}	0^{aA}	2.00 ± 0.00^{bB}	3.75 ± 0.66^{cB}	8.00 ± 0.71^{dB}	13.00 ± 0.71^{eB}	15.13 ± 0.93^{fB}
	Tea Tree	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Niaouli	0^{a}	0^{aA}	0^{aA}	0^{aA}	1.00 ± 0.00^{bC}	2.13 ± 0.33^{cC}	4.13 ± 0.78^{dC}
	Control	0^{a}	4.63 ± 0.48^{bB}	9.38 ± 0.48^{cC}	12.63 ± 0.48^{cC}	18.00 ± 1.00^{dD}	22.63 ± 0.48^{eD}	28.50 ± 0.71^{fD}
KMi 402	Clove	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{a}	0^{aA}	1.00 ± 0.00^{bB}	2.75 ± 0.83^{cB}	7.75 ± 1.09^{dB}	12.63 ± 1.22^{eB}	14.38 ± 0.70^{fB}
	Tea Tree	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Cajeput	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Niaouli	0^{a}	0^{aA}	0^{aA}	1.13 ± 0.33^{bC}	3.00 ± 0.71^{cC}	4.00 ± 0.71^{eC}	5.38 ± 0.70^{fC}
	Control	0^{a}	3.25 ± 0.43^{bB}	9.25 ± 0.83^{cC}	12.50 ± 0.50^{cD}	20.50 ± 0.50^{dD}	23.75 ± 0.66^{dD}	30.50 ± 0.50^{eD}
KMi 403	Clove	0	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	Eucaliptus	0^{a}	0^{aA}	3.13 ± 0.78^{bB}	4.88 ± 0.60^{cB}	11.13 ± 0.78^{dB}	15.38 ± 0.70^{eB}	20.13 ± 1.17^{fB}
	Tea Tree	0^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.00 ± 0.00^{bC}	2.75 ± 0.43^{cC}
	Cajeput	0^{a}	0^{aA}	0^{aA}	0^{aA}	0^{aA}	1.00 ± 0.00^{bC}	3.25 ± 0.43^{cD}
	Niaouli	0^{a}	0^{aA}	0^{aA}	2.50 ± 1.12^{bC}	5.63 ± 0.86^{cC}	8.75 ± 0.66^{dD}	13.38 ± 1.66^{eE}
	Control	0^{a}	4.00 ± 0.71^{bB}	9.75 ± 0.66^{cC}	15.00 ± 0.50^{dD}	28.00 ± 0.71^{eD}	33.00 ± 0.87^{fE}	39.00 ± 0.71^{gF}

Note: The numbers represent mean of colony size. a, b, c, d, e, f, g shows significant differences within row. A, B, C, D, E, F shows significant differences within column and within strain. Data presented as mean ± stdev in mm.

Table 4 The inhibitory effect (in %) of clove essential oils on the growth of colonies (n = 6) of *Penicillium commune* on CYA at 25 °C after 7 and, respectively, 14 days of cultivation.

µL of EO.L^{-1} of air	Strain of *Penicillium commune*													
	KMi 177		KMi 270		KMi 276		KMi 277		KMi 370		KMi 402		KMi 403	
	7th	14th	7th	14th	7th	14th	7th	14th	7th	14th	7th	14th	7th	14th
625	100	100	100	100	100	100	100	100	100	100	100	100	100	100
500	100	100	100	100	100	100	100	100	100	100	100	100	100	100
250	100	100	100	100	100	100	100	100	100	100	100	100	100	100
125	100	100	83.33	83.33	50	33.33	100	100	83.33	50	50	50	66.66	66.66
62.50	0	0	0	0	50	50	0	0	33.33	0	33.33	0	0	50
21.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15.625	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: EO – essential oil, CYA – Czapek yeast extract agar.

Table 5 Minimal inhibition doses estimated by probit analyses.

Strain	7th day		14th day	
	MID_{50}	MID90	MID50	MID90
KMi 177	94.16	104.93	94.16	104.93
KMi 270	113.34	128.79	113.34	128.79
KMi 276	106.15	173.27	119.56	197.12
KMi 277	94.16	104.93	94.16	104.93
KMi 370	81.45	124.43	125.00	143.92
KMi 402	113.33	176.51	125.00	143.92
KMi 403	200.13	301.37	187.07	292.56
All strains	116.77	182.97	126.27	189.49

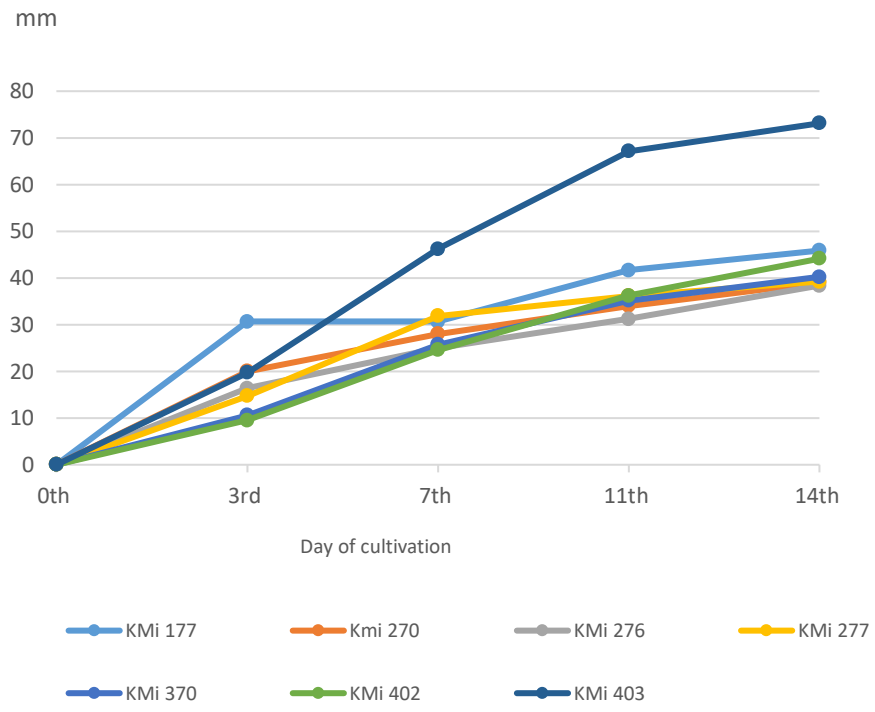

Figure 1 The growth of the strains of *Penicillium commune* on the Czapek yeast extract agar at 25 ±1 °C.

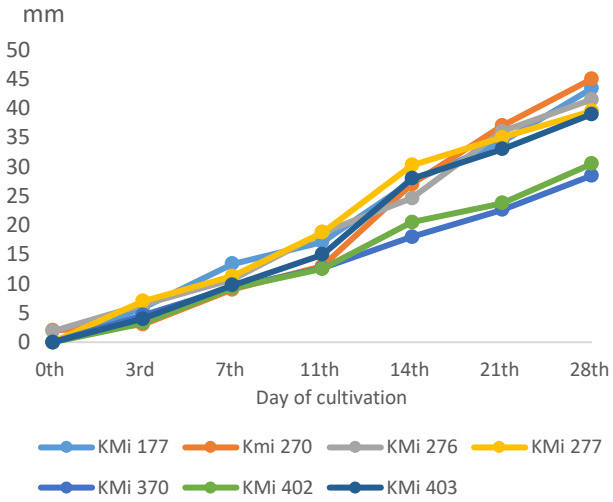

Figure 2 The growth of the strains of *Penicillium commune* on the Czapek yeast extract agar at 5 ±1 °C.

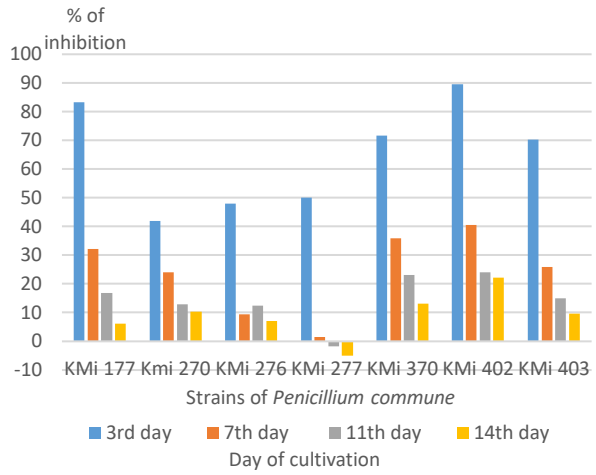

Figure 3 Influence of eucalyptus essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 25 ±1 °C.

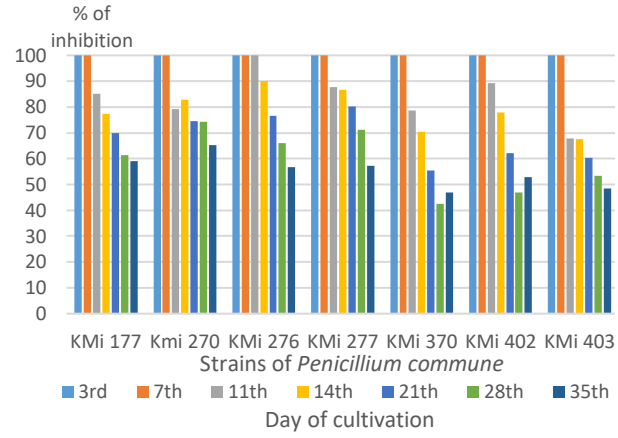

Figure 4 Influence of eucalyptus essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 5 ±1 °C.

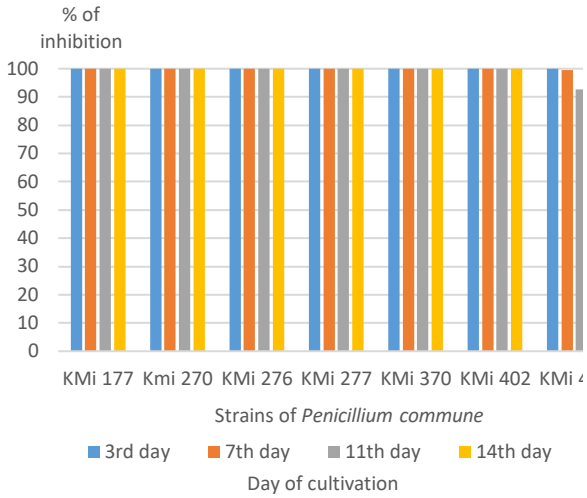

Figure 5 Influence of tee tree essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 25 ±1 °C.

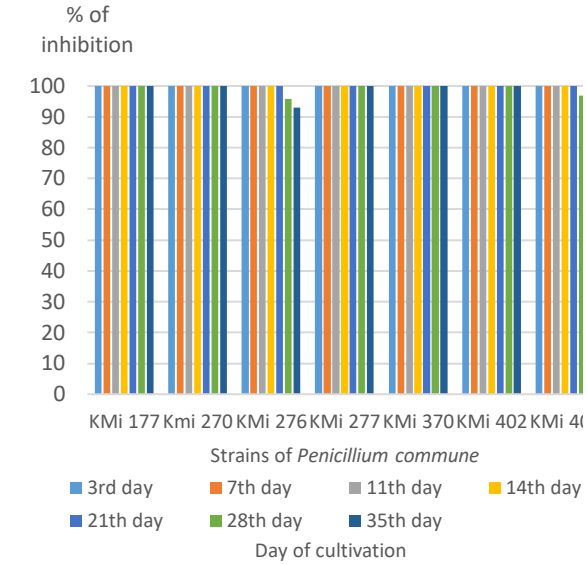

Figure 6 Influence of tee tree essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 5 ±1 °C.

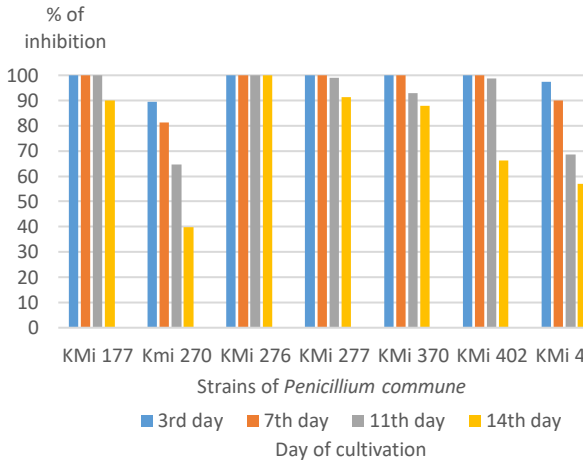

Figure 7 Influence of cajeput essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 25 ±1 °C.

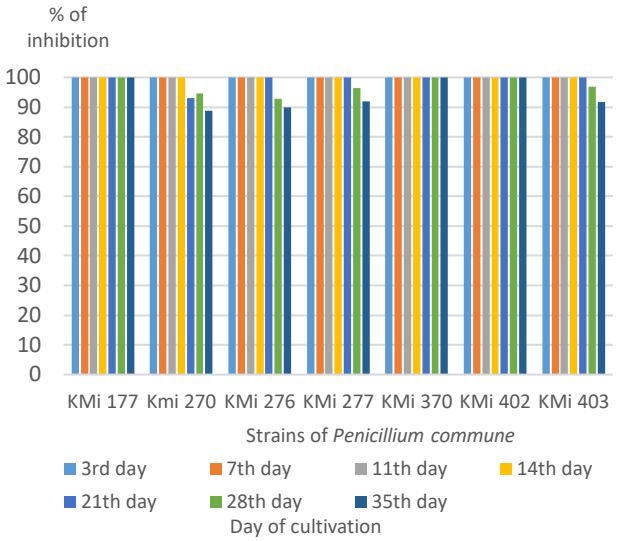

Figure 8 Influence of cajeput essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 5 ±1 °C.

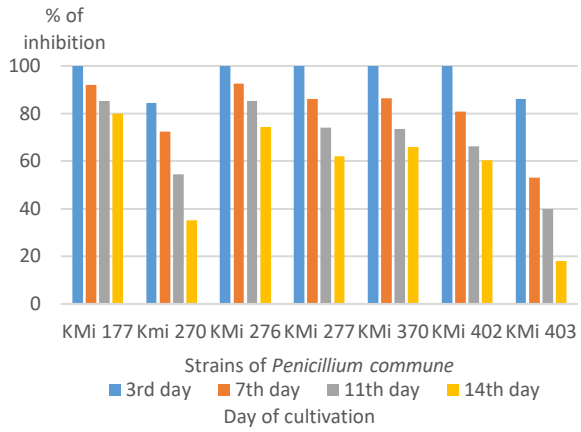

Figure 9 Influence of niaouli essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 25 ±1 °C.

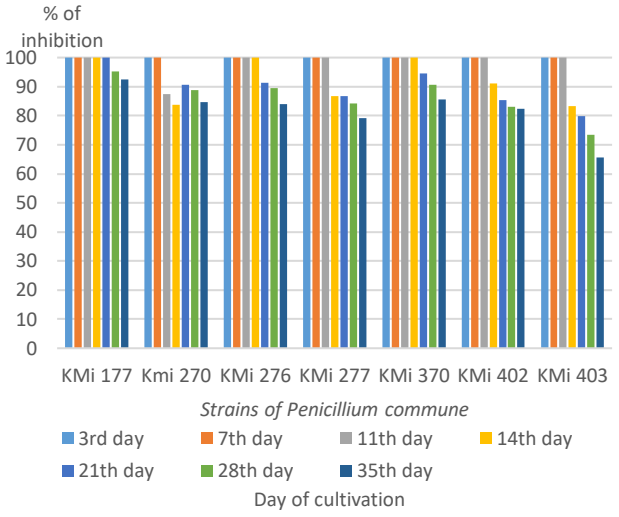

Figure 10 Influence of niaouli essential oil (625 µL.L^{-1} of air) on the growth of *Penicillium commune* on the Czapek yeast extract agar at 5 ±1 °C.

In our research, we have confirmed the ability of five essential oils from plants family *Myrtaceae* to inhibit (partially or completely) the growth of the strains of *P. commune*. Testing should be supplemented by testing the effects of essential oils on the sensory properties of foods. Our tested strains were obtained from the moldy dairy products whose sensory properties could be affected by essential oils.

CONCLUSION

In this study, we evaluated the antifungal properties of clove essential oil (from *Syzygium aromaticum* L.; leaves), eucalyptus essential oil (from *Eucaliptus globulus* leaves), tea tree essential oil (from *Melaleuca alternifolia* Cheel; leaves) cajeput essential oil (from *Melaleuca leucadendra* L.; leaves and twigs), niaouli essential oil (from *Melaleuca quinquenervia* (Cav.) S. T. Blake; leaves). The growth of the strains of *P. commune* was affected by all the essential oils used. But, only clove (from *Syzygium aromaticum* L.) essential oil completely inhibited the growth of the strains of *P. commune* at 5 ±1 °C and 25 ±1 °C respectively, throughout the experiment. The order of tested essential oils according to the inhibition effect on the growth of the strains of *P. commune* was (from the strongest to the weakest effect): clove > tee tree > cajeput > niaouli > eucalyptus. Clove EO that completely inhibit the growth of all strains was used to determine minimum inhibitory doses (MIDs). The MIDs were 125 µL.L^{-1} of air for two strains of *P. commune* and 250 µL.L^{-1} of air for five strains of *P. commune* on the 7th and 14th day of cultivation, also. According to probit analyses, the highest MIDs90 were determined for the strain KMi 403 – 301.37 µL.L^{-1} of air on the 7th day and 292.56 µL.L^{-1} of air on the 14th day of cultivation. The lowest MIDs90 were determined for the strains KMi 177 and KMi277 104.93 µL.L^{-1} of air on the 7th day and on the 14th day of cultivation, also.

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ANTIOXIDANT CAPACITY OF PLANT RAW MATERIAL OF *SCUTELLARIA BAICALENSIS* GEORGI

Olena Vergun, Liudmyla Svydenko, Olga Grygorieva, Oksana Shymanska, Dzhamal Rakhmetov, Ján Brindza, Eva Ivanišová

The aim of this study was to evaluate antioxidant capacity of *Scutellaria baicalensis* Georgi from two regions of Ukraine: Kyiv city (M. M. Gryshko National Botanical Garden of NAS of Ukraine (NBG)) and Kherson region (Experimental Facility "Novokakhovska" of Rice Research Institute of Ukrainian Academy of Agrarian Sciences (EFN of RRI)). Observation of plants and biochemical analyses conducted with plants collected in the stage of flowering. In study investigated and compared above-ground part of plants and separated organs: inflorescences, stems, leaves. Measured morphometric parameters (height of plants, length, and width of leaves, length, and diameter of inflorescence, the diameter of the stem) showed that the most variable was the length of inflorescence (12.79%) for NBG sample and diameter of the stem (33.33%) for EFN of RRI sample. Ethanolic extracts were screened for the antioxidant capacity. As standards were used gallic acid for polyphenol content (GAE), quercetin for flavonoids (QE), caffeic acid for phenolic acids (CAE), Trolox for antioxidant capacity (TE). The total content of polyphenol compounds was 42.43 – 86.13 mg GAE.g^{-1} DW (dry weight) (NBG sample) and 28.06 – 96.76 mg GAE.g^{-1} DW (EFN of RRI sample). The content of flavonoids was 9.39 – 62.97 mg QE.g^{-1} DW (NBG sample) and 10.64 – 66.07 mg QE.g^{-1} DW (EFN of RRI sample). The concentration of phenolic acids was 2.60 – 16.13 mg CA.g^{-1} DW (NBG sample) and 12.02 – 30.12 CA.g^{-1} DW (EFN of RRI sample). Antioxidant activity of plant extracts was measured by DPPH assay and reducing power method. The first method indicated an antioxidant ability 8.24 – 8.56 mg TE.g^{-1} DW (NBG sample) and 7.63 – 8.83 mg TE.g^{-1} DW (EFN of RRI sample). Reducing power of extracts was 51.48 – 306.09 mg TE.g^{-1} DW (NBG sample) and 63.33 – 260.24 mg TE.g^{-1} DW (EFN of RRI sample). Very strong positive correlation identified between total polyphenol content, total flavonoid content and reducing power. *Scutellaria baicalensis* is a rich source of antioxidants and potential raw of further pharmacological study in Ukraine as well as in other regions for improving and enrichment of relevant production.

Keywords: *Scutellaria baicalensis*; antioxidant activity; polyphenols; flavonoids; phenolic acids

INTRODUCTION

Species of genus *Scutellaria* L. belong to *Lamiaceae* Martinov., plants of which are known as aromatic, medicinal and food **(Bazzaz et al., 2011)**. Among species of this genus, the most known is *Scutellaria baikalensis* Georgi (Huangqin) that is an important medicinal species with a rich biochemical composition that contains, also, a metabolite previously thought to be unique for *Hypericum perforatum* L. **(Murch et al., 2004)**. Raw (dry roots) material of this plant widely used in Chinese herbal medicine as treatments of some diseases such as inflammation, hypertension, cardiovascular diseases, and tumor. Root extracts exhibited high antioxidant activity that appeared also due to the content of baicalin and baicalein, and other biologically active compounds **(Chen et al., 2000; Liu et al., 2017; Wang et al., 2017; Cheng et al., 2018)**. It is described that flavonoid wogonin from *Scutellaria baicalensis* roots appears anti-tumor and anti-metastatic action **(Kimura and Sumiyoshi, 2013)**. According to **Cheng et al. (2018)**, extracts and main flavonoids of *Scutellaria baicalensis* have an anticancer effect and this species is promising for anticancer therapy. As reported by **Cole et al. (2008)**, plant extracts can be effective also in the inhibition of liver fibrosis, insomnia, neuralgia etc. Besides of main flavonoids, in the plant raw material of this plant indicated flavones, diterpenes, phenylethanoid glycosides, amino acids, essential oils.

Extracts of *Scutellaria baicalensis* possess high potency to reduce lipid peroxidation **(Gabrielska et al., 1997)**. Study of **Gaire Prasad et al. (2014)** resulted that raw of *Scutellaria baicalensis* and its individual components showed a neuroprotective effect that combined from some pharmacological effects. The physiological effect of baicalein is regulation of apoptosis in damaged roots **(Hirunuma et al., 2011)**. Significant antioxidant activity was found in the extract of this plant **(Li et al., 2015)**.

The aim of this study was a comparing study of the antioxidant activity, total polyphenols, flavonoids, phenolic acid content of plant raw material of selected species of the

genus of *Scutellaria baicalensis* Georgi from two areas of Ukraine.

Scientific hypothesis

Comparative assessment of morphometric and antioxidant parameters of *Scutellaria baicalensis* plants from two regions of Ukraine.

MATERIAL AND METHODOLOGY

Plant materials

The plants material of *Scutellaria baicalensis* Georgi was collected from experimental collection of Cultural Flora Department of M. M. Gryshko National Botanical Garden of the NAS of Ukraine (NBS) (Kyiv; 50°24′55″N, 30°33′45″E) and collection of aromatic and medicinal plants of Experimental Facility "Novokakhovska" of Rice Research Institute of Ukrainian Academy of Agrarian Sciences (EFN of RRI) (v. Plodove; 46°45′16.2″N 33°20′55.1″E). The following morphometric parameters of investigated plants were conducted at the conditions of NBG (Figure 1) and EFN of RRI (Figure 2) in the period of flowering: height of plants in cm, length of leaf in cm, the width of leaf in cm, length of inflorescence in cm, the diameter of inflorescence in cm, the diameter of the stem in cm.

Figure 1 *Scutellaria baicalensis* Georgi in the period of flowering in the M. M. Gryshko National Botanical Garden of NAS, Kyiv.

Figure 2 *Scutellaria baicalensis* Georgi in the period of flowering-frutage in the Experimental Facility "Novokakhovska" of Rice Research Institute of Ukrainian Academy of Agrarian Sciences.

In this study investigated parameters of antioxidant activity of ethanol extracts of *S. baicalensis* from two regions of Ukraine in the stage of flowering. Plant samples were dried at 35 °C for four days. All biochemical analyses were conducted at the Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Resources, Department of Plant Storage and Processing.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

0.2 g of dried plant raw material was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for the next measurements: antioxidant activity, polyphenols, and flavonoids. All data expressed in mg of standard compound per gram of dry weight (DW).

Total polyphenol content (TPC)

Total polyphenol content extracts were measured by the method of **Singleton and Rossi (1965)** using Folin-Chiocalteu reagent. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Chiocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 250 $mg.L^{-1}$; $R^2 = 0.996$) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Total flavonoid content (TFC)

Determination of total flavonoids content was conducted using the procedure described by **Shafii et al. (2017)**. 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminum chloride, 0.1 mL of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.01 – 0.5 $mg.L^{-1}$; $R^2 = 0.997$) was used as the standard and the results were expressed in $\mu g.g^{-1}$ quercetin equivalents.

Total phenolic acid content (TPAC)

Determination total phenolic acids content of extracts was carried out using the method of **Farmakopea Polska (1999)**. 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent, 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of distilled water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1 – 200 $mg.L^{-1}$, $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ caffeic acid equivalents.

Antioxidant activity

Radical scavenging assay (DPPH)

The radical scavenging activity of samples was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH) **(Sánchéz-Moreno, Larrauri and Saura-Calixto, 1998)**. The extracts (0.5 mL) were mixed with 3.6 mL of radical solution (0.025 g of DPPH in 100 mL ethanol). The absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $mg.L^{-1}$; $R^2 = 0.988$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Reducing power (RP)

Reducing the power of extracts was determined by the phosphomolybdenum method of **Prieto, Pineda and Aguilar (1999)** with slight modifications. The mixture of sample (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) incubated at 90 °C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10 – 1000 $mg.L^{-1}$; $R^2 = 0.998$) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Statistical analysis

The statistically treated data are given in tables as the arithmetical mean values and their standard errors. Data were submitted ANOVA and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). We used the Dixon's Q test (DQn) at the significance level of $p < 0.05$. Correlation analysis was performed using Pearson's criterion. Variability of all these parameters was evaluated using descriptive statistics. Level of variability determined by **Stehlíková (1998)**.

RESULTS AND DISCUSSION

The morphometric parameters of plants of *Scutellaria baicalensis* in conditions of M. M. Gryshko National Botanical Garden represented in Table 1. Variability of measured parameters was from 2.45 to 12.79%, the most variable parameter was the length of inflorescence and the least variable the width of the leaf. The morphometric parameters of plants of *Scutellaria baicalensis* in conditions of Experimental Facility "Novokakhovska" of Rice Research Institute of Ukrainian Academy of Agrarian Sciences represented in Table 2. The most variable parameter was the diameter of the stem and the least height of plants. Based on obtained results, it's should be noted that significant differences found when comparing the diameter of inflorescences. Investigation of biological activities of plant raw material one of the most discussed areas of plant raw material study **(Cocan et al., 2018; Ohigashi et al., 1991)**. One of the most known biological activity of plant raw material is antioxidant capacity **(Kavalcová et al., 2014)**. In addition, plant raw material of *Lamiaceae* herb is a rich source of antioxidant compounds **(Matkowski, Tasarz and Szypuła, 2008)**. Antioxidant properties of herb from *Lamiaceae* studied in the context of the condition of raw. The higher values obtained by studying the dried material than fresh and frozen **(Adámková, Kouřimská and Kadlecová, 2015; Kouřimská, Ešlerová and Khatri, 2016)**.

According to **Liu et al. (2011)**, 80% ethanol extract of *Scutellaria baicalensis* demonstrated higher antioxidant capacity than other concentration of alcohol solution. Also, flavonoid, phenolic acids content and antimicrobial activity were higher at the 80% ethanol extraction.

The antioxidant capacity of plant extracts has been attributed to their phenolic contents that were determined by Folin-Ciocalteu reagent. The amount of total polyphenol content of *Scutellaria baicalensis* extracts is shown in Table 3 and Table 4. The content of polyphenols for plants of NBG was in the range from 42.43 to 86.13 mg GAE per g of DW (dried weight) depending on part of the plant. Plants collected from EFN of RRI accumulated polyphenols from 28.06 to 96.76 mg GAE per g DW. It should be noted the most content of polyphenol compounds indicated in the leaves.

Study of **Seo et al. (2013)** showed that content of different polyphenol compounds using liquid chromatography (HPLC-UV) depends on the organ of *Scutellaria baicalensis* and was 1715.7 $mg.kg^{-1}$ fresh weight of fruits (FW) for roots, 885.0 $mg.kg^{-1}$ FW for leaves, 622.4 $mg.kg^{-1}$ FW for flowers and 307.4 $mg.kg^{-1}$ FW for stems.

Previous data about the content of flavonoids concerning mainly of their concentration in the roots of *S. baicalensis* **(Kimura and Sumiyoshi, 2013; Kosakowska, 2017)**. According to **Kosakowska (2017)**, the mean value of the content of flavonoids in the roots was 0.33%. *Scutellaria baicalensis* flavonoids are active compounds of anti-inflammatory herbal medicine in China **(Gao et al., 1999)**. The most known active ingredients of genus *Scutellaria* are flavonoids such as baicalin, baicalein, and wogonin, which play important role in biological activities of these plants: antimicrobial, antifungal, antiviral **(Bazzaz et al., 2011)**.

According to **Cheng et al. (2018)**, more than 40 flavonoids identified from the raw of this plant. **Cole et al. (2008)** reported that baicalin and baicalein the most common flavonoids from the *Scutellaria baicalensis*. As resulted in **Grzegorczyk-Karolak, Wysokińska and Olas (2015)**, the content of some flavonoids was much lower than in roots and shoots. In the stems and leaves 21 flavonoids were detected **(Liu et al., 2011)**. The main flavonoids of the aboveground part of this plant are scutellarin, dihydroscitellarin, and glucuronides of apigenin and luteolin **(Olennikov, Chirikova and Tankhaeva, 2010)**.

In our study content of flavonoids compared with quercetin content. As noticed **Gao et al. (1999)**, flavonoids such as quercetin, luteolin, and catechin are better antioxidants than for example ascorbic acid or vitamin E. Total flavonoid content in ethanol extracts of *Scutellaria baicalensis* from NBG was from 9.39 to 62.97 mg QE per g DW and for it plants from EFN of RRI from 10.64 to 66.07 mg QE per g (DW) (Table 3 and Table 4). The least content of flavonoids was found in the stems of both samples. The high concentration of flavonoids indicated in all plants of the NBG sample and in the leaves of EFN of RRI sample.

Table 1 Morphometric parameters of *Scutellaria baicalensis* Georgi plants in conditions of M. M. Gryshko National Botanical Garden.

Parameter	min	max	mean	*SD*	*V* (%)
Height of plant, cm	60.02	70.00	64.30	1.17	5.68
Length of leaf, cm	4.00	4.40	4.24	0.04	2.98
Width of leaf, cm	0.22	0.51	0.35	0.03	2.45
Length of inflorescence, cm	12.00	17.00	14.30	0.58	12.79
Diameter of inflorescence, cm	7.11	9.00	8.21	0.26	10.21
Diameter of stem, cm	0.21	0.52	0.31	0.03	8.01

Note: min, max – minimal and maximal measured values; mean – arithmetic mean; *SD* – standard error of mean; V – coefficient of variation (%).

Table 2 Morphometric parameters of *Scutellaria baicalensis* Georgi plants in conditions of Experimental Facility "Novokakhovska" of Rice Research Institute of Ukrainian Academy of Agrarian Sciences.

Parameter	min	max	mean	*SD*	*V* (%)
Height of plant, cm	60.11	70.02	66.30	2.14	4.39
Length of leaf, cm	3.50	4.00	3.77	0.19	5.80
Width of leaf, cm	0.53	0.90	0.66	0.09	18.18
Length of inflorescence, cm	11.00	16.00	13.80	1.64	13.62
Diameter of inflorescence, cm	4.90	7.10	5.80	0.74	15.13
Diameter of stem, cm	0.20	0.45	0.33	0.09	33.33

Note: min, max – minimal and maximal measured values; mean – arithmetic mean; *SD* – standard error of mean; V – coefficient of variation (%).

Table 3 Total polyphenol, flavonoid and phenolic acid content of plants of *Scutellaria baicalensis* Georgi (NBS).

Part of plant	Total polyphenols (mg GAE.g^{-1})	Total flavonoids (mg QE.g^{-1})	Total phenolic acid (mg CAE.g^{-1})
Inflorescences	42.43 ±1.20	26.43 ±2.36	10.68 ±0.93
Leaves	86.13 ±2.06	61.53 ±1.91	13.98 ±0.25
Stems	45.36 ±0.56	9.39 ±0.52	2.60 ±0.26
All plant	81.24 ±3.91	62.97 ±0.63	16.13 ±0.53

Note: GAE (gallic acid equivalent); QE (quercetin equivalent); ± (standard deviation of the mean).

Table 4 Total polyphenol, flavonoid and phenolic acid content of plants of *Scutellaria baicalensis* Georgi (EFN of RRI).

Part of plant	Total polyphenols (mg GAE.g^{-1})	Total flavonoids (mg QE.g^{-1})	Total phenolic acid (mg CAE.g^{-1})
Inflorescences	67.50 ±0.89	45.89 ±0.98	15.40 ±0.32
Leaves	96.76 ±2.18	72.66 ±2.69	23.40 ±1.13
Stems	28.06 ±1.91	10.64 ±0.29	12.02 ±0.17
All plant	96.54 ±0.68	66.07 ±0.67	30.12 ±2.66

Note: GAE (gallic acid equivalent); QE (quercetin equivalent); ± (standard deviation of the mean).

Table 5 The correlation coefficients of a linear relationship between the different parameters of antioxidant activity of investigated plants of *Scutellaria baicalensis* Georgi from Kyiv region of Ukraine.

Parameter	TPC	TFC	TPAC	DPPH
TFC	0.943*			
TPAC	0.772*	0.939*		
DPPH	-0.065*	-0.358	-0.625	
RP	0.942	0.969*	0.878*	-0.177

Note: *Significant according to the *t*-test (p <0.05).

Table 6 The correlation coefficients of a linear relationship between the different parameters of antioxidant activity of investigated plants of *Scutellaria baicalensis* Georgi from Kherson region of Ukraine.

Parameter	TPC	TFC	TPAC	DPPH
TFC	0.995*			
TPAC	0.887	0.843*		
DPPH	0.519*	0.481*	0.447*	
RP	0.933*	0.906	0.980	0.356*

Note: *Significant according to the *t*-test (p <0.05).

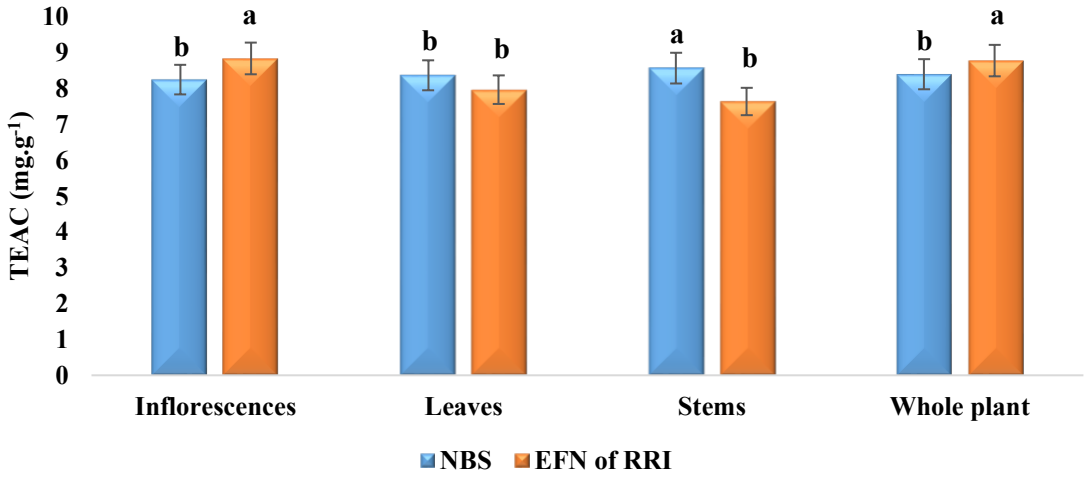

Figure 3 Antioxidant activity of extracts of plants of *Scutellaria baicalensis* Georgi depending on the region of growing (means in columns followed by different letters are different at p = 0.05. Each value represents the mean of three independent experiments (±*SD*)).

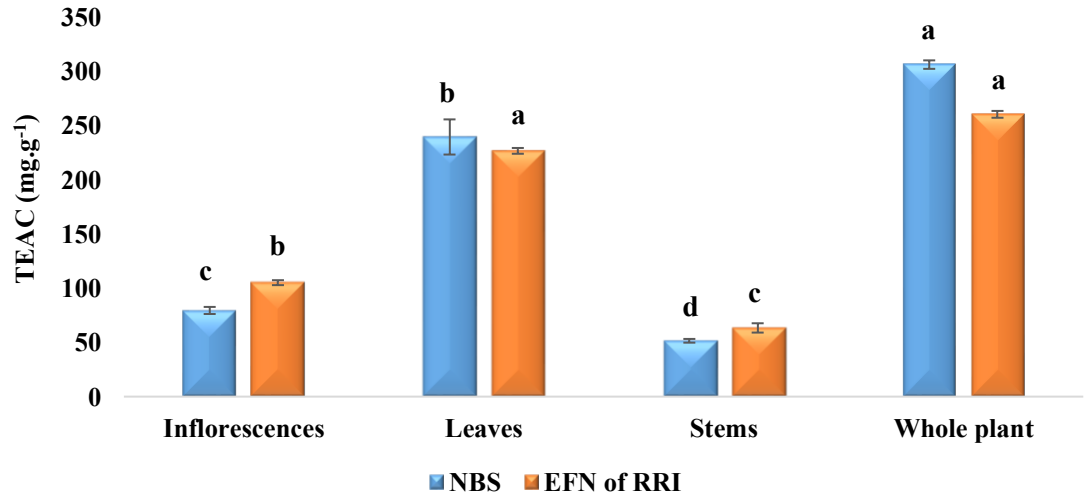

Figure 4 Reducing power of extracts of plants of *Scutellaria baicalensis* Georgi depending on the region of growing (means in columns followed by different letters are different at p = 0.05. Each value represents the mean of three independent experiments (±*SD*)).

Phenolic acids are derivates of benzoic and cinnamic acid that can exist in free and bound forms **(Leváková and Lacko-Bartošová, 2017)**. As flavonoids, phenolic acids play an important role as antioxidant protectors against diseases such as cardiovascular, cancer, inflammatory bowel syndrome etc. **(Saxena, Saxena and Pradhan, 2012)**. Also, this group of phenolic compounds connected with diverse functions in plant organism such as nutrient uptake, protein synthesis, enzyme activity, photosynthesis etc. The concentration of phenolic acids depends on conditions of growth, among which is temperature **(Robbins, 2003)**.

In this study total phenolic acid content was found in the range from 2.60 to 16.13 mg CAE per g (DW) for NBG sample and from 12.02 to 30.12 mg CAE per g DW for EFN of RRI sample. In addition, stems of both samples

accumulated the least content of phenolic acids and all plant raw had the higher content of it.

Radical scavenging activity of the ethanol extracts of investigated samples of *Scutellaria baicalensis* was screened against DPPH radical which is the most frequently used to determine the antiradical activity of several natural compounds **(Marinova and Batchvarov, 2011; Alam, Bristi and Rafiguzzaman, 2013)**. Also, in a review of **Alam, Bristi and Rafiguzzaman (2013)** described that ethanol the most frequently used for extraction of plant samples to detect the antioxidant capacity. In Figure 3 represented comparable results of radical scavenging assay of ethanolic extracts of two samples of *Scutellaria baicalensis*. The difference in this parameter wasn't significant and for sample growing in the condition of NBG, it was of 8.24 – 8.56 mg Trolox per g (DW). In another sample (EFN of RRI) this parameter was 7.63 – 8.83 mg Trolox per g.

According to **Seo et al. (2013)**, antioxidant activity by DPPH method was maximal in flowers and stems of investigated plants. In our study, the maximal value of this parameter was in the stem for plant sample from NBG and in inflorescences for EFN of RRI plant samples. Measuring of reducing the power of plant extracts one of the methods to determine the antioxidant capacity of plants. Determination of this parameter by phosphomolybdenum method based on reduction Mo (VI) to Mo (V) **(Alam, Bristi and Rafiguzzaman, 2013)**.

Results of reducing power measuring of ethanolic extracts investigated species of *Scutellaria baicalensis* showed in Figure 4. In total, the value of this parameter for NBG sample was of 51.48 – 306.09 mg Trolox per g, for EFN of RRI sample of 63.33 – 260.24 mg Trolox per g. The fewer results were obtained for stems of investigated plants but stems of EFN of RRI demonstrated higher reducing power than other parts of the plant. The highest reducing power exhibited extracts of all plant. As studied by **Seo et al. (2013)**, reducing power value was maximal for flowers of *Scutellaria baicalensis*, however, measured by another method than us.

Pearson's correlation analyses conducted for investigated plants (Table 5, Table 6). According to **Li, Wu and Huang (2009)**, between antioxidant activity and phenolic compounds exhibited significant correlation. This fact depends on investigated extract and structure of phenolic compounds **(Tatiya et al., 2011)**. In the study of **Vamanu et al. (2011)** wasn't found a correlation between phenolic compounds and antioxidant activity by DPPH method. Moreover, this research showed that existed a direct relationship between reducing power and antioxidant activity of investigated extracts, while in our study we didn't find a significant correlation between these parameters (very weak) for both samples.

We found a moderate positive correlation between total phenolic acids content, flavonoid content and total phenolic content and antioxidant activity by DPPH method for EFN of RRI sample, although NBS sample didn't show this relationship. Strong positive correlation identified between all investigated phenolic compounds and reducing power of extracts for both samples. Thus, in our case, we identified a significant relationship between the content of investigated polyphenol compounds and reducing power of extracts of two tested samples.

CONCLUSION

Investigated plant extracts of different parts of *Scutellaria baicalensis* Georgi from two Ukrainian regions exhibited high antioxidant activity in the stage of flowering. The high content of polyphenol compounds was identified in the leaves of both investigated samples. The present study demonstrates that the highest concentration of phenolic acids detected in all parts of plants. Some difference noticed in the accumulation of flavonoids: the highest values found in all parts of plants of NBG sample, whereas, for EFN of RRI sample maximum found in the leaves. Radical scavenging activity hasn't significant values that can say that this parameter does not depend on the area of growth for this species. Extracts of all plant demonstrated higher antioxidant activity by DPPH method and by phosphomolybdenum method than extracts of separated organs. The lowest values of these parameters found for stems. Correlation analyses found significant values in the relationship between investigated phenolic compounds and reducing the power of extracts. Moderate positive correlation noticed between phenolic compounds content and antioxidant activity by DPPH method for a sample from the Kherson region. Thus, two samples of *Scutellaria baicalensis* from two Ukrainian regions appears high potency of antioxidant activity that can be used for further study especially pharmacological.

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EFFECT OF SOUS-VIDE HEAT TREATMENT ON THE PHTHALIC ACID ESTERS CONTENT IN MEAT

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ABSTRACT

The aim of the study was to monitor the migration of the phthalic acid esters dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) from packaging to meat wrapped in plastic when heat treated by sous-vide method. A heat treatment temperature of 80 °C was used for 4 and 8 hours with reheating at 80 °C for 1 hour. The average DBP and DEHP concentrations in meat ranged from 2.24 to 4.66 mg.kg^{-1} and 2.29 to 10.35 mg.kg^{-1} of the original sample, respectively. The average DBP and DEHP concentrations found in plastic packaging ranged from 3.06 to 6.37 µg.g^{-1} and 5.70 to 7.83 µg.g^{-1} of plastic, respectively. The average concentrations of DBP in water bath range from 16.25 to 23.38 µg.l^{-1}, while the average concentrations of DEHP in water bath were determined between 0.24 and 1.82 µg.l^{-1}. The above results were compared with the results measured at sous-vide treatment temperature of 70 °C for 4 hours, 8 hours, and with 1 hour of reheating. The average concentrations of DBP and DEHP in all meat samples exceeded the specific migration limits of both phthalates (0.3 mg.kg^{-1} for DBP and 1.5 mg.kg^{-1} for DEHP).

Keywords: meat; plastic; water; phthalate; heating

INTRODUCTION

Phthalic acid esters are persistent pollutants, which serve as plasticizers in the industry. The most common are two esters of phthalic acid dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) **(Gao and Wen, 2016)**. DEHP as a plasticizer has been widely used in materials for both food and non-food use, which has led to its expansion into the environment. The plastic components can migrate from the contact materials into food. Migration does not only depend on the type of contact material but also on the composition of the food (pH, fat content, etc.) and the conditions of its processing and storing (time, temperature) **(Bradley, Castle and Driffild, 2019)**. Phthalic acid esters, such as plasticizers, can migrate because they are not firmly bound chemically in plastics **(Piotrowska, 2005)**. In addition, increasing temperature increases the release of plasticizers **(Nerín, Acosta and Rubio, 2002)**. Plastics, many times exposed to heating, in the case of microwave heating of microwave dishes, released more phthalate plasticizers into food than dishes previously unused **(Moreira, André and Cardeal, 2014)**.

The European Union legislation in the **Commission Regulation (EU) 10/2011**, which deals with food contact materials, sets specific migration limits also for DBP (0.3 mg.kg^{-1}) and DEHP (1.5 mg.kg^{-1}), where a specific migration limit indicates for a given substance the maximum permitted amount that may pass from the material to the food **(Commission Regulation (EU) No. 10/2011)**.

Esters of phthalic acid have become ubiquitous contaminants because of their wide use and subsequent leaching. They are also problematic because of their high stability and accumulation in the body **(Cao, 2010; Chen et al., 2017)**. They pose a danger to the food chain when irrigating with wastewater **(Tan et al., 2016)**. Their carcinogenic and teratogenic effects have been demonstrated and influence the reproductive capacity of the organism **(Velíšek and Hajšlová, 2009)**.

The sous vide technology is that the food is vacuum packaged in a plastic wrap and heated in a water bath at a combination of temperature and length of heat treatment. Sous vide heat treatment uses temperatures below 100 °C. There are more vitamins preserved in sous vide heat-treated products than with conventional heat treatment **(Schellekens, 1996)**. Vacuum packaged foods are thus heat-treated, cooled, then stored in a cold place and reheated before consumption **(Rhodehamel, 1992)**.

Scientific hypothesis

We anticipate that plastic packaging intended for wrapping meat for sous vide cooking releases phthalates into meat when different temperature and time modes are used (80 °C for 4 and 8 hours, 70 °C for 4 and 8 hours) and then 1 hour of reheating that simulates reheating before eating meat.

MATERIAL AND METHODOLOGY

Samples for analysis were prepared in collaboration with the Institute of Gastronomy of the University of Veterinary and Pharmaceutical Sciences Brno. Samples of meat (fresh chilled pork roast. pH 5.44 – 5.58) were purchased in the market network of Brno. The meat was divided into pieces of 150 g, and the pieces were vacuum packed in a packaging designed for heat treatment by sous-vide method. The meat temperature by packaging was at 6 °C. Cryovac® CN300 (Sealed Air Polska Sp. z o.o., Poland) multi-layer cooking bags, thickness 60 μm and with OTR (oxygen transmission rate) 13 cm^3/m^2/24 hr/bar at 23 °C and 0 % RH were used for packaging. Subsequently, the samples (n = 6) were heat-treated in a water bath at 80 °C per 4 hours and additional samples (n = 6) at 80 °C per 8 hours. After the heat treatment, half of the samples from both variants of heat treatment were immediately analysed, while the other half of the samples from both variants of heating were stored in the refrigerator for 24 hours and then heat treated in a water bath for 1 hour at 80 °C. Water samples were taken from the water bath before heat treatment and after one hour of heat treatment of the samples from both variants of the heating experiment. Similarly, samples of water from the water bath were taken before and after one hour heat treatment of vacuum-packed samples of meats treated at 70 °C per 4 hours and 70 °C per 8 hours.

In addition, samples of sous vide technologies were prepared for comparison in a similar manner at 70 °C per 4 hours and 70 °C per 8 hours, with repeated heating at 70 °C per 1 hour **(Jandlová, Jarošová and Kameník, 2018)**.

The determination of phthalic acid esters di-2-ethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) was carried out at the Department of Food Technology of the Mendel University in Brno.

Plastic wraps for meat packaging were first washed in distilled water and dried. For analysis, 10 x 10 cm wraps were taken and then weighed and crushed. Subsequently, they were extracted for 3 days with hexane: dichloromethane (1:1) solvents. The resulting extract was evaporated using a vacuum rotary evaporator, transferred to hexane and subsequently, in the absence of turbidity, transferred to acetonitrile for HPLC determination. If turbidity was present, the sample was centrifuged and the hexane portion was then transferred to acetonitrile **(Gajdůšková, Jarošová and Ulrich, 1996)**.

Meat samples were homogenized and about 50 g of the sample was weighed for analysis; the sample was frozen and lyophilized. Fat was extracted from the lyophilized sample with hexane: acetone (1: 1), the solvents were then evaporated on a vacuum rotary evaporator. Gel permeation chromatography separated the phthalate fraction which was clarified with sulphuric acid and transferred to vials with acetonitrile for HPLC determination **(Jarošová et al., 1999)**.

Water from the water bath was extracted three times with dichloromethane in a separatory funnel. The combined extracts were evaporated on a rotary evaporator and transferred to the vials with acetonitrile for HPLC determination **(Jarošová et al., 1999)**. Measurement was performed on HPLC with UV detection at a wavelength of 224 nm, acetonitrile was used as the mobile phase. Measurement was performed with HPLC system under UV detection at 224 nm, with acetonitrile as the mobile phase, and Zorbax Eclipse C8 column. The evaluation was done using the Data Analysis program (Agilent Technologie).

Statistic analysis

The data was processed using Microsoft Excel (Microsoft Corporation, USA) and Statistica 12 (StatSoft, USA) software. The Shapiro-Wilk's test was used for testing the normality of data and the Grubbs test was used to detect outliers ($\alpha = 0.05$). Furthermore, the independent samples t-test was run to determine the conformity of the means ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Table 1, Table 2 and Table 3 show concentrations of phthalic acid esters (DBP a DEHP) in meat, plastic packaging, and water from the water bath.

The average concentration of DBP and DEHP (Table 1) was 2.30 ±0.60 and 6.17 ±2.91 $mg.kg^{-1}$ respectively in meat samples heat treated at 70 °C per 4 hours, and 2.36 ±1.58 and 14.78 ±10.19 $mg.kg^{-1}$ respectively when heat treated at 70 °C per 8 hours. The results show that a longer period (8 hours) of heat treatment resulted in a higher migration of both phthalates from the packaging to meat.

The average concentration of DBP and DEHP was 2.89 ±1.94 and 6.95 ±2.95 $mg.kg^{-1}$ respectively in meat samples heat treated at 70 °C per 4 hours + 70 °C per 1 hour and 3.14 ±0.90 and 5.04 ±2.18 $mg.kg^{-1}$ respectively when heat treated at 70 °C per 8 hours + 70 °C per 1 hour. The results show that for DBP a longer period (8 hours) of heat treatment resulted in a higher concentration in meat.

The average concentration of DBP and DEHP was 4.60 ±1.06 and 10.35 ±2.32 $mg.kg^{-1}$ respectively in meat samples heat treated at 80 °C per 4 hours and 3.43 ±0.69 and 3.82 ±0.95 $mg.kg^{-1}$ respectively when heat treated at 80 °C per 8 hours. The results show that over a longer period of time the two phthalates decreased.

The average concentration of DBP and DEHP was 2.24 ±0.24 and 2.91 ±0.59 $mg.kg^{-1}$ respectively in meat samples heat treated at 80 °C per 4 hours + 80 °C per 1 hour and 4.66 ±1.87 and 5.08 ±0.96 $mg.kg^{-1}$ respectively when heat treated at 80 °C per 8 hours + 80 °C per 1 hour. The results show that for both phthalates a longer (8 hours) heat treatment resulted in higher concentrations in meat.

The average concentration of DBP and DEHP was 2.30 ±0.60 and 6.17 ±2.91 $mg.kg^{-1}$ respectively in meat samples heat treated at 70 °C per 4 hours and 4.60 ±1.06 and 10.35 ±2.32 $mg.kg^{-1}$ respectively when heat treated at 80 °C per 4 hours. The results show that at higher temperature (80 °C) of heat treatment there was a higher migration of both phthalates from the packaging into meat.

The average concentration of DBP and DEHP was 2.89 ±1.94 and 6.95 ±2.95 $mg.kg^{-1}$ respectively in meat samples heat treated at 70 °C per 4 hours + 70 °C per 1 hour and 2.24 ±0.24 and 2.91 ±0.59 $mg.kg^{-1}$ respectively when heat treated at 80 °C per 4 hours + 80 °C per 1 hour. The results show that the higher temperature of heat treatment resulted in a decrease in the concentration of both phthalates in meat.

Table 1 The average concentrations and standard deviation ($\bar{x} \pm SD$) of dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) in $mg.kg^{-1}$ of original mass in the samples of meat heat treated at 70 °C **(Jandlová, Jarošová and Kameník, 2018)** or 80 °C for 4 or 8 hours with the sous vide technology and then heat treated for 1 hour at 70 °C or 80 °C.

Heat-treated samples of meat	DBP ($mg.kg^{-1} \pm SD$)	DEHP ($mg.kg^{-1} \pm SD$)
70 °C for 4 hours	2.30 ±0.60	6.17 ±2.91
80 °C for 4 hours	4.60 ±1.06	10.35 ±2.32
70 °C for 4 hours + 70 °C for 1 hour	2.89 ±1.94	6.95 ±2.95
80 °C for 4 hours + 80 °C for 1 hour	2.24 ±0.24	2.91 ±0.59
70 °C for 8 hours	2.36 ±1.58	14.78 ±10.19
80 °C for 8 hours	3.43 ±0.69	3.82 ±0.95
70 °C for 8 hours + 70 °C for 1 hour	3.14 ±0.90	5.04 ±2.18
80 °C for 8 hours + 80 °C for 1 hours	4.66 ±1.87	5.08 ±0.96

Note: Each value represents the average of three determinations of meat samples.

Table 2 The average concentrations and standard deviation ($\bar{x} \pm SD$) of dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) in $\mu g.dm^{-2}$ and $\mu g.g^{-1}$ of packaging in the samples of plastic packaging used for vacuum packaging of meat heat treated at 70 °C **(Jandlová, Jarošová and Kameník, 2018)** or 80 °C for 4 or 8 hours with the sous vide technology and then heat treated for 1 hour at 70 °C or 80 °C.

Samples of meat plastic packaging – heat treated	DBP ($\mu g.dm^{-2} \pm SD$)	DEHP ($\mu g.dm^{-2} \pm SD$)	DBP ($\mu g.g^{-1} \pm SD$)	DEHP ($\mu g.g^{-1} \pm SD$)
70 °C for 4 hours	4.01 ±0.32	3.40 ±1.11	4.75 ±0.40	4.03 ±1.35
80 °C for 4 hours	3.73 ±0.79	7.99 ±0.64	3.06 ±0.65	6.56 ±0.54
70 °C for 4 hours + 70 °C for 1 hour	4.35 ±0.81	5.81 ±0.99	4.87 ±0.91	6.50 ±1.12
80 °C for 4 hours + 80 °C for 1 hour	4.42 ±0.15	6.70 ±0.16	3.77 ±0.21	5.70 ±0.14
70 °C for 8 hours	2.18 ±0.24	1.70 ±0.30	2.43 ±0.42	1.91 ±0.45
80 °C for 8 hours	7.28 ±4.15	8.34 ±0.77	6.37 ±4.04	7.02 ±0.29
70 °C for 8 hours + 70 °C for 1 hour	2.42 ±0.14	4.51 ±0.60	2.65 ±0.16	4.95 ±0.78
80 °C for 8 hours + 80 °C for 1 hour	6.27 ±1.10	9.48 ±0.17	5.18 ±0.96	7.83 ±0.20

Note: Each value represents the average of three determinations of packaging samples.

Table 3 The average concentrations and standard deviation ($\bar{x} \pm SD$) of dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) in $\mu g.l^{-1}$ in the samples of water from water bathes used for reheating the meat samples (previously treated with the sous vide technology) for 1 hour at 70 °C or 80 °C, taken before and after the heat treatment.

Samples of water from water baths used for meat samples reheating	Sampling – reheating	DBP ($\mu g.L^{-1} \pm SD$)	DEHP ($\mu g.L^{-1} \pm SD$)
70 °C for 1 hour (meat samples heat treated at 70 °C for 4 hours)	**Before**	16.25 ±0.84	1.82 ±0.07
	After	23.38 ±2.10	0.24 ±0.20
70 °C for 1 hour (meat samples heat treated at 70 °C for 8 hours)	**Before**	23.38 ±2.10	0.24 ±0.20
	After	18.78 ±4.71	0.83 ±0.55
80 °C for 1 hour (meat samples heat treated at 80 °C for 4 hours)	**Before**	18.78 ±4.71	0.83 ±0.55
	After	20.64 ±2.29	0.90 ±0.57
80 °C for 1 hour (meat samples heat treated at 80 °C for 8 hours)	**Before**	20.64 ±2.29	0.90 ±0.57
	After	22.49 ±8.25	1.16 ±1.51

Note: Each value represents the average of three determinations of water samples.

The average concentration of DBP and DEHP was 2.36 ±1.58 and 14.78 ±10.19 mg.kg^{-1} respectively in meat samples heat treated at 70 °C/8 hours and 3.43 ±0.69 and 3.82 ±0.95 mg.kg^{-1} respectively when heat treated at 80 °C/8 hours. The results show that over a longer period (8 hours) of heat treatment there was a decrease of DEHP, while DBP slightly increased.

The average concentration of DBP and DEHP was 3.14 ±0.90 and 5.04 ±2.18 mg.kg^{-1} respectively in meat samples heat treated at 70 °C per 8 hours + 70 °C per 1 hour and 4.66 ±1.87 and 5.08 ±0.96 mg.kg^{-1} respectively when heat treated at 80 °C per 8 hours + 80 °C per 1 hour. The results show that the higher temperature of heat treatment resulted in a higher concentration of both phthalates in meat.

At the temperature variant of 70 °C, with increasing time (from 4 to 8 hours) the phthalate concentration in meat increased and decreased at 80 °C. Additional heating for 1 hour resulted in a fall of phthalates in samples heat treated over 4 hours at a higher temperature (80 °C). After comparing mean values for independent samples using a t-test, for DBP and DEHP concentrations in meat products the mean values were equal for all samples (p >0.05) – always variants of different temperatures but same heating times were compared.

Even studies of other authors **(Zhang and Guo, 2009; Xue et al., 2010)** have shown the presence of phthalates in packaging materials and their migration from packaging to food. **Zhang and Guo (2009)** found that the migration of DEHP from PVC foil into meat increased with the increasing temperature and time. Maximum DEHP migration was at 90 °C and 30 minutes of action (1961.92 mg.kg^{-1}; 75.12 mg.dm^{-2}). The overall migration limit (60 mg.kg^{-1}; 10 mg.dm^{-2}) was exceeded in all the time and temperature combinations monitored, except for the combination of 10 °C and <41 hours where migration was not observed. In the light of the **Commission Regulation (EU) 10/2011** dealing with plastic food contact materials, given the specific migration limits for DBP (0.3 mg.kg^{-1}) and DEHP (1.5 mg.kg^{-1}), which set a maximum allowable quantity that may pass from materials to food, all samples exceeded the values indicated in legislation.

In our study, the average DBP and DEHP concentrations in all meat samples exceeded the specific migration limits.

The average DBP concentrations for plastic packaging at the temperature variant of 70 °C and 80 °C ranged from 2.43 to 4.87 and from 3.06 to 6.37 µg.g^{-1} respectively of the packaging.

The average DEHP concentrations for plastic packaging at the temperature variant of 70 °C and 80 °C ranged from 1.91 to 6.50 and from 5.70 to 7.83 µg.g^{-1} respectively of the plastic packaging.

At the temperature variant of 70 °C, with increasing time of heat treatment from 4 to 8 hours the phthalate concentration in packaging decreased and increased at 80 °C. Additional heating of the packaged meat samples in water bath for 1 hour resulted in a growth of phthalates in samples of packaging heat treated at 70 °C.

After comparing mean values for independent samples using a t-test, for DBP concentrations in µg.dm^{-2} for plastic packaging the mean values were equal for all samples (p >0.05), except for the variant of 70 °C per 8 hours + 1 hour versus 80 °C per 8 hours + 1 hour (p <0.05). After comparing mean values for independent samples using a t-test, for DEHP concentrations in µg.dm^{-2} for plastic packaging, only mean values for the variant 70 °C per 4 hours + 1 hour versus 80 °C per 4 hours + 1 hour were equal (p >0.05).

After comparing mean values for independent samples using a t-test, for DBP concentrations in µg.g^{-1} for plastic packaging the mean values were equal for all samples (p >0.05), except for the variants of 70 °C per 4 hours versus 80 °C per 4 hours (p <0.05) and 70 °C per 8 hours + 1 hour versus 80 °C per 8 hours + 1 hour (p <0.05). After comparing mean values for independent samples using a t-test, for DEHP concentrations in µg.g^{-1} for plastic packaging, the mean values were equal for all samples (p >0.05), except for the variants of 70 °C per 8 hours versus 80 °C per 8 hours (p >0.05) and 70 °C per 8 hours + 1 hour versus 80 °C per 8 hours + 1 hour (p >0.05).

In the t-test always variants of different temperatures but same heating times were compared.

Nerín, Acosta and Rubio (2002) states that the decomposition of polymer chain and additives occurs with increasing temperature, and additives, including phthalic acid esters, can be released from plastics.

Rastkari et al. (2017) studied the effect of storage time, temperature and bottle type on the migration of phthalates from packaging materials to fruit drinks. Drinks were filled into polyethylene terephthalate (PET) and high-density polyethylene (HDPE) bottles. The analysis performed before and after 2, 4 and 6 months of storage showed that the migration of DEP (diethyl phthalate), DEHP and DBP from packaging materials into aqueous solutions increased with the increasing acidity.

The average concentration of DBP and DEHP in samples of water from water baths before the heat treatment ranged from 16.25 to 23.38 and 0.24 to 1.82 µg.l^{-1} respectively. After reheating, the concentration of DBP and DEHP was from 18.78 to 23.38 and 0.24 to 1.16 µg.l^{-1} respectively. In most cases, the concentration of both phthalates after heating in water bath samples increased, except for DBP at 70 °C per 1 hour of heat treatment (meat sample heat treated at 70 °C per 8 hours) and for DEHP at 70 °C per 1 hour of water heating (meat sample heat treated at 70 °C per 4 hours). Evidently, the phthalic acid esters from plastic packaging were released into water.

After the t-test of the mean values for independent samples, where the same samples of water were compared before and after the heating, mean values of both DBP and DEHP concentrations were equal (p >0.05), except for the variant before and after the heating at 70 °C per 1 hour of samples heat treated at 70 °C per 4 hours (p <0.05).

Study by **Xu et al. (2010)** has dealt with PAE migration from plastic packaging into mineral water and kitchen oil. Oil was a better medium for the migration of phthalates, due to its lipophilic nature. It was also found that, with increasing temperature and contact time with the plastic material, the migration of phthalic acid esters was more significant.

Simoneau, Van den Eede and Valzacchi (2012) examined the migration of phthalate from baby bottles (n = 277) under the conditions of hot filling for 2 hours at 70 °C and found migration levels of diisobutyl phthalate (DIBP) and dibutyl phthalate (DBP) from 50 to

150 μg.kg^{-1}, while DEHP exhibited relatively lower levels of migration (25 to 50 μg.kg^{-1}).

The migration of phthalates from plastic bottles of four bottled water labels was monitored by **Vazquez et al. (2017)**. Samples were stored for 70 days at three different temperatures (8 +/-2 °C, 22 - 25 °C, 35 +/-1 °C). Dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and di-2-ethylhexyl phthalate (DEHP) have been identified in all four bottled water labels. The most common phthalate in all the samples studied was DEHP.

In a previous study by **Jandlová, Jarošová and Kameník (2017)**, the average concentrations of phthalic acid esters in the original raw meat mass and in the original plastic non-heated packaging have been reported, which we have also used for this study. In raw unpackaged meat the average concentration of DBP and DEHP was determined to be 7.56 and 13.82 mg.kg^{-1} respectively of the original mass. In the original plastic non-heated packaging the average concentration of DBP and DEHP was 22.47 and 11.76 μg.g^{-1} respectively of the plastic. If we compare the average concentrations of both phthalic acid esters found in the meat products heat treated with the sous vide technology at 70 °C and 80 °C with the average concentrations found in raw meat, the average concentrations of DBP and DEHP in heat treated meats were lower than in the raw meat, except for the variant of 70 °C per 8 hours, where the average DEHP concentration was higher in heat treated meat than in raw meat. If we compare the average concentrations of both phthalic acid esters in the original plastic packaging with the plastic packaging exposed to heating in water bath at 70 °C and 80 °C, the average concentrations of both phthalic acid esters were lower in the plastic packaging exposed to heating in water bath against the original non-heated plastic packaging.

CONCLUSION

The average concentrations of DBP and DEHP in sous-vide-treated meat are higher than the specific migration limits (0.3 mg.kg^{-1} for DBP and 1.5 mg.kg^{-1} for DEHP) laid down in the Commission Regulation (EU) No 10/2011.

The assumed hypothesis that the concentrations of phthalic acid esters will decrease with the heat treatment time and higher temperature has not been definitely confirmed.

In our study, we compared the mean values of concentrations by t-test ($\alpha = 0.05$).

Under sous-vide treatment at 70 °C per 4 hours, average concentrations of both phthalates in meat were lower than at 70 °C per 8 hours. When comparing the heat treatment at 80 °C per 4 hours and 80 °C per 8 hours, the average concentrations of both phthalates in meat decreased with a longer heat treatment time. Additional heating for 1 hour resulted in a decrease of both phthalates in samples of meat heat treated for 4 hours at higher temperature (80 °C). Our examined temperature and time variants of meat heating with sous vide technology suggest that the most suitable variant of meat sous-vide treatment is at 80 °C per 4 hours + 80 °C per 1 hour, even if this variant also constitutes exceeding the specific migration limits.

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RECOMBINANT METALLOPROTEASE AS A PERSPECTIVE ENZYME FOR MEAT TENDERIZATION

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ABSTRACT

Peptidase family M9 (MEROPS database) is true collagenases and contains bacterial collagenases from *Vibrio* and *Clostridium*. One of the producers of M9A subfamily peptidase is *Aeromonas salmonicida* (locus - ASA_3723). The aim of the study was production of recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* for further meat tenderization. Laboratory amounts of recombinant peptidase were obtained and test evaluation of enzyme activity was performed. Recombinant peptidase broke the peptide bond «Pro-Leu-Gly-Met-Trp-Ser-Arg» (one of the collagen chains, (Mw = 846.06)). The concentration of the substrate (peptide) after 180 min was 2 – fold decrease as compared with control. The maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. As a result of histological studies of beef shank samples, the specific effect of the supernatant on the structure of connective tissue was established. Muscle fibers have not changed. The recombinant enzyme could be used for the meat tenderization.

Keywords: meat tenderization; recombinant peptidase; M9 family peptidase

INTRODUCTION

Meat tenderness is a problem in the meat industry with important economic repercussion. The connective tissue contribution (termed a background effect) influence on tenderness. The main component of connective tissue is collagen, its content in the muscles of beef can reach 15% of dry weight **(Bailey and Light, 1989)**. Epimysium and perimysium contain type I collagen as the major component and type III collagen as a minor component; endomysium contain both type I and type III collagen as major components **(Light and Champion, 1984)**. In addition, such type of by-products can be used in the food industry **(Kotenkova and Polishchuk, 2019)**. The use of exogenous proteases to improve meat tenderness has attracted much interest recently **(Bekhit et al., 2014; Chanalia et al., 2018)**. Of particular interest for studies of collagenase activity is the M9 metallopeptidase family (MEROPS database), from microorganisms *Clostridium histolyticum* and *Vibrio alginolyticus* **(Eckhard, Schönauer and Brandstetter, 2013; Miyoshi et al., 2008; Nezafat et al., 2015)**.

Peptidase family M9 (MEROPS database) are true collagenases and contains bacterial collagenases from *Vibrio* and *Clostridium*. Active site represents of two histidine zinc ligands and the catalytic glutamate occur in the «HEXXH» motif. Based on structure and function, *Vibrio* proteases are combined into three classes. The *Vibrio* proteases in classes II and III are all members of the subfamily M9A in the MEROPS database and have collagenolytic activity. But they have significant differences in their structure and function. So, the Vibrio class II proteases have a zinc-binding motif «HEYTH», contain no C-terminal domain and cannot hydrolyse casein, while the *Vibrio* class III proteases have a zinc-binding motif «HEYVH», contain PKD-like domain and PPC domain and can hydrolyse casein. *Vibrio* collagenases hydrolyses the Pz peptide (Pz-Pro-Leu-Gly-Pro-D-Arg) by cleaving the same peptide bond Leu-Gly **(Zhang et al., 2015)**.

Peptidase M9 *Aeromonas salmonicida* is unassigned metalloprotease according to the MEROPS database (http://merops.sanger.ac.uk). It has zinc-binding motif «HEYVH» like a *Vibrio* class III peptidase, contain no C-terminal domain like *Vibrio* class II peptidase and presumable cannot hydrolyse casein. The methylotrophic yeast *Pichia pastoris* has become a fundamental tool for food biotechnology, especially for recombinant enzymes production.

Many proteases have been used in meat industry **(Angelovičová et al., 2018)**. A variety of proteases have been successfully expressed in *P. pastoris* **(Rabert et al., 2013; Queiroz Brito Cunha et al., 2018; Silva, Peres and Gattas, 2009; Macauley-Patrick et al., 2005)**, for example alkaline protease from *Aspergillus oryzae* **(Guo and Ma, 2008)**, neutral protease from *Aspergillus oryzae* **(Ke et al., 2012)**, aspartic protease from *Mucor mucedo* **(Yegin and Fernandez-Lahore, 2013)**, chymosin from *Rhizopus microsporus* **(Qian et al., 2017)**. For use in the meat industry, in particular, for meat tenderization, extracellular

aspartate protease has been expressed in *Pichia pastoris* from *Rhizomucor miehei* **(Tyagi et al., 2017)**.

The aim of the study was production of recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* for further meat tenderization.

Scientific hypothesis

We are expecting the influence of the recombinant metallopeptidase *Aeromonas salmonicida* on the connective tissue of meat and meat tenderness consequently.

MATERIAL AND METHODOLOGY

The subjects were M9 peptidase gene (ASA_3723) *Aeromonas salmonicida* (laboratory collection strain, isolated from the meat surface), vector plasmid pPic9K (Invitrogen, USA), competent *E. coli* DH5α cells (Invitrogen, USA), competent *Pichia pastoris* GS115 cells (Invitrogen, USA), super-natant from recombinant clones of *Pichia pastoris*, synthetic peptide «Pro-Leu-Gly-Met-Trp-Ser-Arg» («Almabion», Voronezh, Russia), beef shank samples.

Nucleotide sequence analysis

Analysis of the nucleotide sequence encoding the peptide gene *Aeromonas salmonicida* was performed using the NCBI database (https://www.ncbi.nlm.nih.gov). For analysis and comparison of nucleotide sequences, the BLAST program was used, the search for homologous sequences was performed in the GenBank database (http://www.ncbi.nlm.nih.gov/). Bioinformatic analysis of the sequences of the *Aeromonas salmonicida* coding peptidases of genes, the design of the primers was performed using the OligoAnalyzer Tool program (https://eu.idtdna.com/calc/analyzer).

Cloning of a metalloprotease gene

PCR was performed on an ANK-32 (Sintol, Russia) in a reaction mixture containing HS-Fuzz buffer, dNTP, 5'- and 3'-terminal primers, DNA, and HS-Fuzz DNA polymerase (NEB, England). Primers used to obtain the protease gene *Aeromonas salmonicida*: AACAATCTGGGTACAAGGT-forward primer; TCAGTGGGAGGAGTCGTTG-reverse primer. Screening of recombinant clones was performed using the PCR method with standard primers for the pPic9K vector. To isolate and purify the target PCR products, a kit was used to extract DNA from an agarose gel and Cleanup Standard reaction mixtures (Evrogen, Russia). Ligase-free cloning of the obtained PCR fragments into the pPic9K vector plasmid was performed using a FusionTM CF Dry-Down PCR Cloning Kit (Clontech Laboratories Inc, USA) non-gas cloning system. Electroporation *Pichia pastoris* was carried out on an electropor with the following parameters: voltage = 1500, capacitance = 25, resistance = 200.

Electrophoresis

Analysis of recombinant *Pichia pastoris* clones for the production of target proteins. One-dimensional electrophoresis was performed according to the method of Laemmli **(Laemmli, 1970)** in 12% polyacrylamide gel with the presence of SDS.

HPLC-MS/MS

Sample preparing: the substrate «Pro-Leu-Gly-Met-Trp-Ser-Arg» synthetic peptide (one of the collagen chains, (Mw = 846.06) was treated with the supernatant from recombinant *Pichia pastoris* clones in different ratios: sample A – 0.1 mg substrate/300 μL supernatant without inhibitor Protease Inhibitor Cocktail (Roche, Switzerland); sample B – 0.1 mg substrate/300 μL supernatant with inhibitor Protease Inhibitor Cocktail; sample C – 0.02 mg substrate/300 μL supernatant with inhibitor Protease Inhibitor Cocktail; sample D – 0.02 mg substrate/150 μL supernatant with inhibitor Protease Inhibitor Cocktail.

Mass spectral analysis was performed after 1 h, 2 h, 3 h. The reaction was inhibited with EDTA and EGTA solutions – metalloproteases inhibitors. The analysis of peptidase activity was carried out on chromatograph 7890A (Agilent Technologies, USA) with a 5975C VL MSD mass spectrometer (Agilent Technologies, USA). The following parameters of mass spectrometric detection were selected for analysis: source temperature – 100 °C; desolvation gas temperature – 320 °C; desolvation gas flow rate is 8 $dm^3.min^{-1}$; nebulizer needle pressure – 30 psi. The parameters of exposure to ions in the SIM and MRM mode and the conditions of ionization by spraying in an electric field (ESI) with the registration of positive ions: range, m/z 50 – 2000; voltage on fragmenter (Frag), B 70 – 150; dissociation energy (CE), B 0 – 40.

This technique makes it possible to determine the activity of peptidases in reaction with peptides of different sequences. The extent of its degradation was determined by the area of the daughter ions of the desired peptide. The protonated ions were used to determine the molar weight of the peptide degradation products and identify their amino acid sequence.

Histological analyse

Sample preparing: beef shank samples were treated with supernatant by injection (sample E) and immersion (sample F), the exposure time was 24 hours at 18 °C. The control was treated with saline. Further, all samples were fixed in 10% buffered formalin solution for 72 hours. Sections 16 μm thick were made on a MIKROM-HM525 cryostat (Thermo Scientific, USA), mounted on Menzel-Glaser glasses (Thermo Scientific, USA) and stained with Ehrlich haematoxylin and 1% aqueous-eosin solution, then concluded glycerin-gelatin. The study of histological preparations was carried out on an AxioImaiger A1 light microscope (Carl Zeiss, Germany) using the AxioVision 4.7.1.0 computer image analysis system. Magnification on all photos 400x (lens 40x and eyepiece 10x).

Shear force analyse

Sample preparing: beef shank samples were treated with supernatant by injection (sample E) and immersion (sample F), exposure time – 24 hours at 18 °C. Control sample of meat was treated with saline. Shear force measurement of samples was performed using a Shimadzu AGS-X (Shimadzu corporation, Japan) instrument according to the standard method of Warner and Brazler **(Goodson et al.,**

2002). After exposure, some samples were cooked at 70 °C for 3 hours. The shear force measurements were performed in 4 replicates.

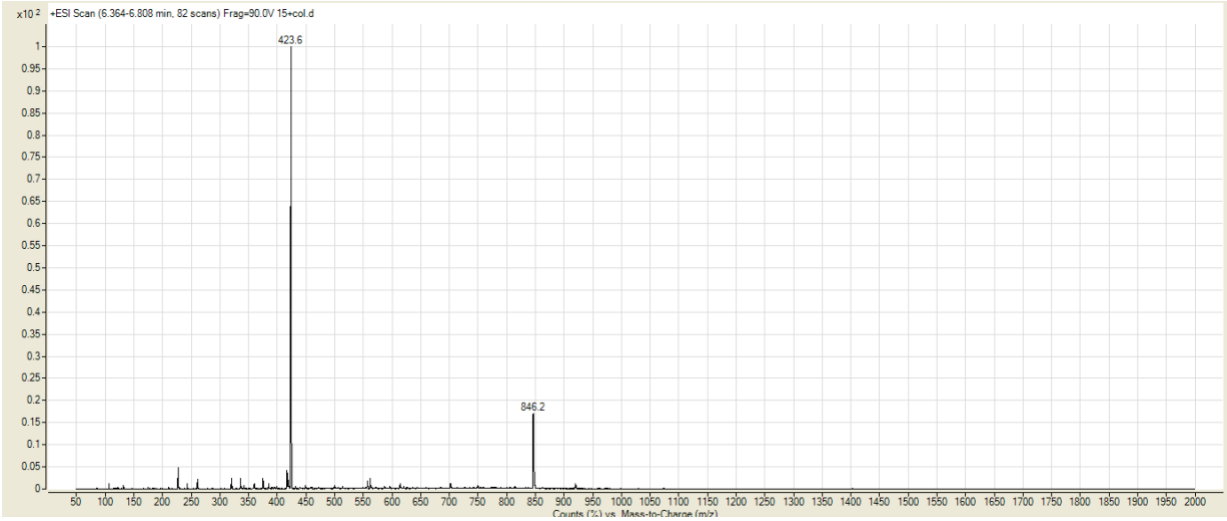

Figure 1 The mass spectrum of the peptide in the mode of selective ion monitoring (SIM) with electrospray ionization (ESI) with registration of positive measurements.

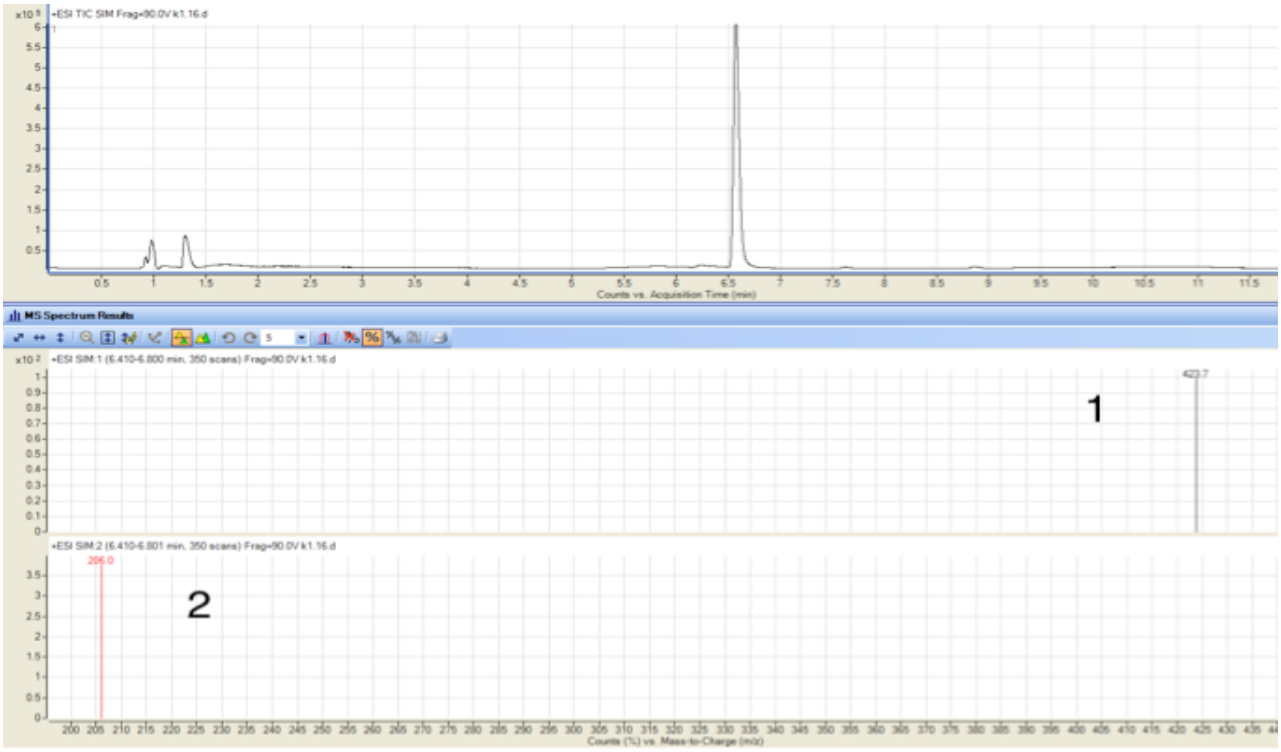

Figure 2 Chromatogram after enzymatic hydrolysis (SIM: 1 – 423.7; 2 – 206.0).

Table 1 Substrate degradation after enzymatic hydrolysis.

Time, min	Final substrate concentration, mol ±*SD*)				
	Control	sample A	sample B	sample C	sample D
60	13455.89 ±0.135*	11817.95 ±0.3531	12974.22 ±0.1641	2962.26 ±0.3602	2441.81 ±0.1302
120		8614.31 ±0.2471	9791.85 ±0.3570	2171.63 ±0.3189	2055.97 ±0.142
180	3547.54 ±0.135**	6310.73 ±0.1123	7195.99 ±0.2794	1759.57 ±0.192	1642.48 ±0.1511

Note: *- Control for A. B samples; **- Control for C. D samples.

Table 2 Result of shear force measurement of supernatant treated beef shank samples.

Cooking degree	Maximum shear force, $N.m^{-2}$ ±*SD*		
	Control	sample E	sample F
Raw beef shank	690242.6^{x} ±2.31671^{b}	413544.9 ±1.18845	639077.86 ±2.53349
Cooking beef shank	184.97 ±2.9245	149.355 ±4.6533	145.175 ±2.06103

Note: ^{X} – average value; ^{b} – standard deviation .

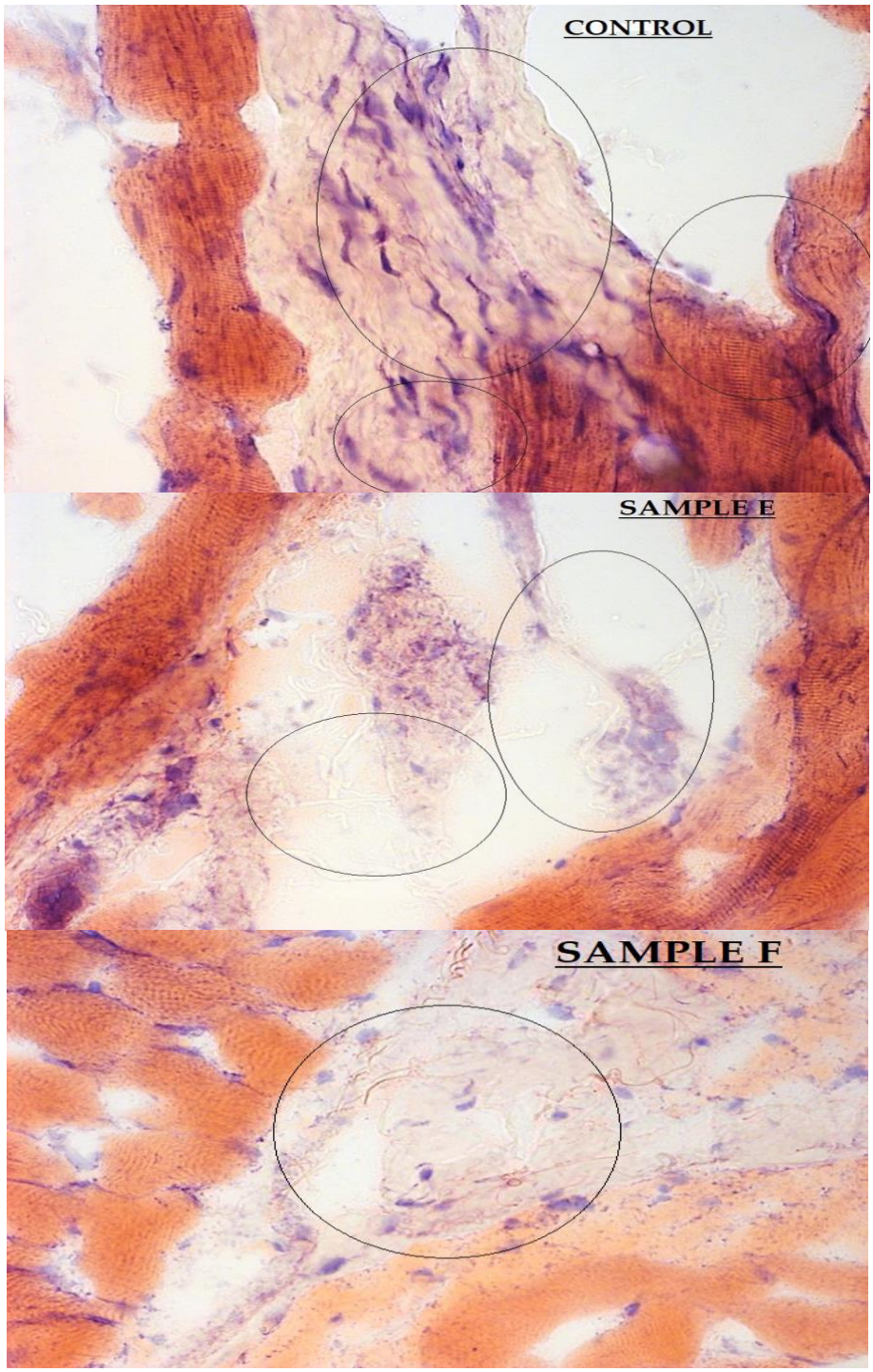

Figure 3 Result of histological analysis of supernatant treated beef shank samples. Note: *Magnification 400x.*

Statistic analysis

StatPlus 6.2.2.0 Software (AnalystSoft) was used. Average value and standard deviation (*SD*) values of shear force index and final peptide concentration was calculated. Statistical significance was determined by the examining the basic differences between values by Z-score. The differences with $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The active domain of the M9 *Aeromonas salmonicida* peptidase gene was cloned, transformation of *Pichia pastoris* cells was carried out, and supernatant from recombinant clones was accumulated. A 40 kDa band was observed on electrophoresis. Figure 1 shows the mass spectrum of the pure peptide "Pro-Leu-Gly-Met-Trp-Ser-Arg" in the selective ion monitoring mode (SIM) with an electrospray ionization (ESI) with registration of positive reactions. To build the calibration characteristics and the quantitative content of the peptide, m/z 423.6 was chosen as the molecular ion, and m/z 846.2 as the protonated daughter ion. After enzyme hydrolysis, products were obtained from which a peak was found with m/z 206.0. This corresponds to the sum of the mass's leucine-glycine dipeptide (Leu-Gly). The chromatogram of the sample after enzymatic hydrolysis is shown in Figure 2. Such activity can be compared with *Vibrio* collagenases hydrolyses the Pz peptide (Pz-Pro-Leu-Gly-Pro-D-Arg) by cleaving the same peptide bond Leu-Gly **(Zhang et al., 2015)**. For the quantitative determination of the dipeptide (Leu-Gly), the conditions of ionization in the SIM mode were selected. The rate of hydrolysis of the peptide "Pro-Leu-Gly-Met-Trp-Ser-Arg" was studied by reducing the area of the main peak with m/z 423.7 (Table 1). Table 1 shows the comparing the final concentration (mol) of the experimental and control samples, it was found that the concentration of the substrate (peptide) after 180 min significantly decreased by 2.13 times (sample A) and by 1.87 times (sample B). Samples A and B contained the same amount of substrate and were distinguished by the presence of an inhibitor of common proteases in the sample B. A 5-fold decrease in the substrate concentration (sample C) and a 2-fold decrease in the amount of enzyme with 5-fold decrease in the substrate concentration (sample D) did not affect the degree of substrate degradation. The final concentration of the substrate in C and D experimental samples after 180 min decreased by half (Sample C) and by 2.16 times (sample D) as compared with control.

In every case the statistical differences between peptide concentration into group were $p < 0.05$. In this, Z-score was in the range of 1.8 to 2.

Results of the shear force measurement are presented in the Table 2. In all cases, sample E and sample F were softer than the control sample. Sample E in raw form was noticeably softer than the control sample and Sample F. However, after heat treatment, the average values of the shear force of the experimental samples were approximately the same (149.355 $N.m^{-2}$ and 145.175 $N.m^{-2}$). As a result of the shear force measurement, it was established that recombinant collagenase had a significant effect on meat tenderness. On average, the maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. The authors **(Qian et al., 2017)** described a method for producing recombinant aspartate protease from *Rhizomucor miehei*, expressed in Pichia pastoris, able to find use in softening raw meat. The resulting enzyme had a high peptidase activity (3480.4 $U.mL^{-1}$). The effective of recombinant aspartate protease used in tenderizing pork. But pork had a low connective tissue content as compared with beef. It can be assumed that the tenderization of meat in this case is achieved by the degradation of muscle proteins, rather than connective tissue proteins. The authors **(Antipova and Glotova, 2006)** showed that the enzymes used to improve the quality of meat should have little effect on muscle tissue.

The histological analysis revealed differences between control and experimental samples. In the control the muscle fibers were characterized by a straightened shape, well-defined transverse striation and a fairly dense arrangement in the bundle. The oval-shaped nuclei were located directly under the sarcolemma of the fiber. Wavy connective tissue interimization layer tightly adhered to the bundles of muscle fibers. The nuclei in connective tissue layers were clearly detected on the preparation (Figure 3).

In the experiment sample E, marked detachment of the perimetry from the muscle bundles, its loosening. Also, disintegration of collagen fibrils, their thinning and partial fragmentation were revealed. Muscle fibers have not changed. Changes in the experiment sample F are similar to changes in the sample E, but more pronounced in the surface layers, where some homogenization of fibrillar structures was noted. In the deep layers, the loosening of connective tissue layers was mainly observed. Muscle fibers have not changed (Figure 3).

CONCLUSION

Recombinant metallopeptidase *Aeromonas salmonicida* by transformation *Pichia pastoris* was produced. Laboratory amounts of recombinant peptidase were obtained and test evaluation of enzyme activity was performed. Recombinant peptidase broke the peptide bond «Pro-Leu-Gly-Met-Trp-Ser-Arg» (one of the collagen chains, (Mw = 846.06)). The concentration of the substrate (peptide) after 180 min was 2 – fold decrease as compared with control. The maximum shear force of heat-treated samples had a 1.27 – fold decrease as compared with the control. As a result of histological studies of beef shank samples, the specific effect of the supernatant on the structure of connective tissue was established. Muscle fibers have not changed. The recombinant enzyme could be used for the meat tenderization.

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CHARACTERIZATION OF FRUIT TREES POLLEN

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ABSTRACT
One of the options to determine botanical origin of trees or honey is the analysis of pollen grains. The characteristics of pollen grains in Czech flora has not been sufficiently described yet. Within this work, fruit trees pollen of Czech origin was characterized on the basis of morphological and spectral description of pollen grains produced by fruit species of *M. domestica, P. armenica, P. persica, P. domestica, P. avium* and *P. cerasus*. The morphological characterization results of the studied fruit species are consistent with results by other authors, but certain differences between the pollen grains of some fruit trees were confirmed. Most morphological differences were confirmed among the *Malus* and *Prunus genera*. Results of morphological and spectral analyzes further confirmed the differences between some types of fruit trees, but homogeneity remained for individual species even in mixed samples. Morphological and spectral analysis can therefore be used for botanical identification of pollen. If this knowledge is applied to pollen analysis in honey, these methods can also be used to verify the botanical origin of honey.

Keywords: spectral characteristic; image analysis; palynology; honey; authenticity; melissopalynology

INTRODUCTION
Pollen grains have their typical characteristics according to botanical plant species. Differences are described between plant species **(Evrenosoğlu and Misirli, 2009)** as well as between individual varieties **(Hebda and Chinnappa, 1994; Geraci et al., 2012)**. In their study, **Radice et al. (2004)** demonstrated morphological differences in the size of pollen grains in one Forastero variety of *P. persica* (L.) Batsch sp. grown on different rootstocks. Although there are certain differences between plant species and varieties, application of their morphological characteristics is significant in terms of research on plant pollination **(Kermani et al., 2003; Asma, 2008)** as well as in forensic diagnostics **(Milne et al., 2004; Morgan et al., 2014; Arguelles, Reinhard and Shin, 2015; Bell et al., 2016)**. In addition to suitability of palynology for forensic diagnostics, the above authors describe some factors (climatic, time period, temperature effects) which alter the morphological characterization of pollen grains to a certain extent; but even with regard to the morphological change, such pollen grains can still be identified and characterized. Pollen grains are also used for authentic honey demonstration – this scientific discipline is called melissopalynology. One parameter is the presence of pollen grains itself. Another option is qualitative or quantitative palynology **(Louveaux, Maurizio and Vorwohl, 1978)**. Morphology of pollen grains is also used to prove the geographical origin of honey **(Rodopoulou et al., 2017; Carreck, 2018)**.

Scientific hypothesis
This article presents fundamental morphological and spectral characteristics of fruit trees pollen and verifies suitability of the obtained characteristics for the creation of mathematical models to discriminate fruit species pollens from each other.

MATERIAL AND METHODOLOGY
For pollen analysis, fruit trees pollen from southern Moravia was collected in 2018. Pollen collection and processing was performed in compliance with methods for preparation of comparative preparations under the Czech standard norm **(ČSN 570190:1974)**. Pollen was collected from fruit trees, namely from apple trees, apricot trees, peach trees, cherry trees, plumb trees, and sour cherry trees (*M. domestica, P. armenica, P. persica, P. domestica, P. avium,* and *P. cerasus*). Mixed samples of all examined varieties were produced for each species. Description of fruit species included in the collection is shown in Table 1.

Morphological and spectral mesurement
Morphological and spectral parameters were evaluated for pollen grains. A specified number of pollen grains (n = 10) was examined. Scanning for both analyzes was performed using the Eclipse Ci microscope (Nikon, JPN). 60x/0.80 lens (Nikon, JPN) was used, real magnification for morphometry was 600x. The pollen grains were captured by DFK 23U274 camera (The Imaging Source, GB). Morphometry was performed using NIS Elements ver. 6.5 (Laboratory Imaging, CZE). The measured morphological

parameters included Area, EqDiameter, VolumeEqSphere, VolumeEqCylinder, Perimeter, Perimeter Contour, MeanChord, Length, Width, MaxFeret, MinFeret, MaxFeret90, Circularity, Elongation, Orientation, LineLength, ShapeFactor, Convexity, Roughness, RoughnessInf.

Spectral analysis was performed using the USB4000-UV-VIS-ES spectrophotometer (Ocean Optics Inc., USA). The spectrum within the range of 420-750 nm was analyzed.

Statistic analysis

Correlation analysis, regression analysis, ANOVA, factor analysis, Cochrane-Orcutt estimation, and normal homogeneity test were carried out using the XLSTAT software ver. 2014.5.03 (Addinsoft SARL, New York, NY, USA).

RESULTS AND DISCUSSION

Melissopalynology is a scientific area that is demanding even on experienced evaluators who are able to interpret the origin of honey through characterization of pollen grains. The analysis principle lies in counting the pollen grains and their categorization into species. Pollen analysis allows for both, the geographical and botanical origin determination of honey **(Von Der Ohe, 2004)**. Only a few organizations in the world usage melissopalynology for botanical and geographical honey identification. This is mainly due to the high demands of the method on human resources. Using new imaging techniques and primarily correlation microscopic techniques, however, it is possible to better characterize the pollen grains and then use the characteristic parameters for automatic or semi-automatic systems to determine the origin of honey. In order to determine the origin and the botanical source of honey, characterization of pollen grains in the given regions must be available. Nonetheless, characterization of pollen from Czech honey-producing plants is not available and subsequent diagnosis of the authenticity of honey is therefore difficult. Table 2 shows the obtained morphological characteristics of pollen grains of fruit trees in southern Moravia.

The morphological characteristics demonstrate that the length and width of *M. domestica* pollen grains is scattered in harmony with **Nazeri (2008) and Evrenosoğlu and Misirli (2009)**. These parameters for *P. armeniaca, P. domestica*, are consistent with the results published by **Evrenosoğlu and Misirli (2009)** as well as **Geraci et al., (2012)**. Length of *P. persica* pollen grains is in line with **Evrenosoğlu and Misirli (2009)** and with the Spaccareli variety described by **Geraci et al. (2012)**, but the width is out of their reported range. On the other hand, our results are also consistent with the study **(Radice et al., 2004)** comparing diamether of pollen grains of forastero on various rootstocks. The diameter in this study was compared to the max Feret diameter which is defined as the distance between two parallel tangential lines rather than planes. Therefore, differences in pollen grains of Turkish varieties compared to Czech varieties can be expected. This finding can also be used to determine the geographical origin of honey. *P. Avium* described in an Italian study **(Geraci et al., 2012)** is in line with the Czech varieties. Length of *P. cerasus* pollen grains was in agreement with the study by **Sótonyi et al. (2000)**, in contrast to the width and diameter that did not overlap within the variance of the measured values in most of the studied varieties, except for the Germersdorfi óriás variety. Besides the different size of pollen grains of individual botanical species of fruit trees, certain differences between individual varieties were also confirmed. Pollen grain differences can therefore be utilized to identify pollen origin used in both forensic diagnostics **(Milne et al., 2004; Morgan et al., 2014; Arguelles, Reinhard and Shin, 2015; Bell et al., 2016)** as well as in melissopalynology **(Von Der Ohe, 2004)**. The characteristic microimages of fruit tree pollen grains are shown in Figure 1.

According to our measured results, some botanical species of fruit trees can be distinguished from each other based on the measured parameters (Table 2). The most diverse morphological parameters were found among the *Malus* genus (*M. domestica*) compared to *Prunus (P. domestica, persica, and cerasus*). The Malus genus was statistically significantly different from some prunus specimens in the parameters of length, circularity, convexity, perimeter, line length, roughness, max. feret, and perimeter contour (p <0.05). Cylindrical and tricolporate shape of *M. domestica* was in agreement with **Evrenosoğlu and Misirli (2009)** and **Nazeri (2008)**. Even with regard to identical shape of *P. persica* pollen grains **(Evrenosoğlu and Misirli, 2009)** described by several authors, a statistically significant difference in the circularity parameter was found between *M. domestica* and *M. Persica*. This result may be due to the high shape variability of *P. Persica* pollen **(Sótonyi et al., 2000)** or due to the transverse pollen grains position during image capturing. The most common morphological differences were reported between *M. domestica* and *P. domestica*. Differences can be attributed in particular to the shape characteristics that are described for *P. domestica* as triangular-obtuse-convex in the polar view and elliptic-acuminate-acute in the equatorial view **(Ćalić et al., 2013)**. The possibility to distinguish between *P. domestica and M. domestica* is an important finding especially of the great morphological variability of *P. domestica* **(Geraci et al., 2012)**.

A statistically significant difference was also demonstrated between *M. Domestica and P. cerasus* in the convexity parameter (p <0.05). A statistically significant difference in only one parameter can be attributed to the low homogeneity of *P. cerasus* pollen grains **(Anvari, 1977)** which is in morphological characteristic in ranges between 78 – 91% **(Bolat and Pirlak, 1999)**. Morphological differences, however, were also demonstrated within the *Prunus* genus. Primarily between *P. persica and P. domestica* in the parameters of area, perimeter, line length, max feret, min feret, max feret 90, eq diameter, perimeter contour. and volumetric parameters of volume eq sphere and volume eq cylinder. This result is in line with **Evrenosoğlu and Misirli (2009)** who described the shape of *P. persica* and *P. domestica* as cylindrical and tricolporate in our study best characterized by the parameter of circularity and convexity where no statistically significant difference was found.

Table 1 Characteristics of the analyzed samples of fruit trees.

Species	***Malus domestica* (Borkh.)**	***Prunus armeniaca* (L.)**	***Prunus persica* (L. Batsch)**	***Prunus domestica* (L.)**	***Prunus avium* (L.)**	***Prunus cerasus* (L.)**
Variety	Biogolden (Czech breed)	Leskora (Czech breed)	Red Haven	Wangenheim (GER import)	Hurlat (Czech breed)	Érdi Bötermö (Hungary breed)
Rootstock	A-2, reg. no. 584	M-VA-2, reg. no. 584	B-SB-1, reg. no.:1142	Mirobalan, reg. no.:1142	Mahaleb, reg. no.: 584	Mahaleb, reg. no.: 584
Variety	Goldstar	Harogem (Canada import)	Suncrest (Czech breed)	Althanova	Kordia (Czech breed)	Újfehértoi FürtösS (Hungary breed)
Rootstock	M25, reg. no.: 1216-09/17	B-VA-2 reg. no.: 1142	BM-VA-1, reg. no.: 584	Myrobalan, reg. no.: 584	Mahaleb, reg. no.: 584	MAN25, reg. no.: 1061
Variety	Rubin	Lebela (Czech breed)	Flamingo (Slovak breed)	Tuleu gras (RO import)	Horka (Czech breed)	Moravia (Czech breed)
Rootstock	B-VA-2 reg. no.: 1142	M-VA-2, reg. no 584	B-SB-1, reg. no.:1142	Myrobalan, reg. no.: 584	Mahaleb, reg. no.: 584	Mahaleb, reg. no.: 584
Amount of nectar mg	1.12	1.19	1.65	3.4	1.9	N/A
Nectar sugar content %	41	27	38	13	29.9	N/A
Nectar sugar amount mg / 24hr	0.45	0.32	0.63	0.44	0.57	N/A

Table 2 Morphological characteristics of fruit trees pollen.

	Length	SD	Width	SD	Circularity	SD	Elongation	SD	ShapeFactor	SD	Convexity	SD	Area	SD	Perimeter	SD	LineLength	SD	Roughness	SD
M. domestica	32.17[a]	8.88	22.78	7.20	0.92[a]	0.08	1.12	0.09	0.97	0.03	0.99[a]	0.01	783.36	453.52	101.05[b]	34.05	50.91[a]	17.20	0.97[a]	0.04
P. armeniaca	40.53	11.78	21.87	4.33	0.76	0.09	1.14	0.05	0.93	0.04	0.97	0.01	922.48	366.36	122.25	33.23	60.95	16.35	0.90	0.05
P. persica	36.00	14.38	17.11	3.30	0.73[b]	0.17	1.08	0.03	0.93	0.05	0.95	0.03	594.28[a]	177.69	103.53[b]	27.41	51.46[a]	12.64	0.87	0.09
P. domestica	55.15[b]	17.00	22.45	6.94	0.64[b]	0.17	1.14	0.07	0.91	0.05	0.95[b]	0.02	1170.39[b]	331.58	154.08[a]	28.02	77.55[b]	14.18	0.83[b]	0.11
P. avium	44.11	20.66	20.78	5.03	0.73	0.19	1.12	0.06	0.95	0.05	0.97	0.01	879.60	300.81	126.58	40.69	63.56	21.00	0.87	0.12
P. cerasus	48.89	12.15	19.97	3.00	0.67	0.14	1.15	0.08	0.91	0.06	0.95[b]	0.03	952.69	131.62	136.09	21.86	68.38	11.70	0.85	0.07

	MaxFeret	SD	MinFeret	SD	MaxFeret90	SD	Orientation	SD	RoughnessInf	SD	EqDiameter	SD	VolumeEq Sphere	SD	VolumeEq Cylinder	SD	Perimeter Contour	SD	MeanChord	SD
M. domestica	32.67[a]	9.71	29.12	8.65	29.82	8.86	77.00	52.91	1.03	0.04	30.51	8.82	18210.5	15414.1	12733.7	11625.1	101.05[a]	34.05	22.78	5.59
P. armeniaca	37.32	9.75	32.66	7.82	33.21	8.04	53.80	60.11	1.11	0.06	33.51	8.05	22174.6	11083.0	13820.5	6805.7	120.43	32.08	22.71	4.88
P. persica	29.37[a]	5.05	27.16[a]	4.58	28.10[a]	4.82	94.75	47.26	1.16	0.13	27.21[a]	4.31	11226.4[a]	4742.0	6572.5[a]	2437.2	101.93[a]	24.80	17.94	2.73
P. domestica	42.77[b]	5.64	37.75[b]	5.63	38.69[b]	6.26	102.20	56.49	1.23	0.17	38.26[b]	5.45	30929.9[b]	13076.4	18029.6[b]	8940.3	154.08[b]	28.02	23.90	5.21
P. avium	35.81	6.21	32.19	6.17	32.92	6.34	69.80	54.24	1.17	0.20	32.99	5.91	20407.7	10091.0	12073.0	5122.2	126.58	40.69	21.71	3.57
P. cerasus	39.25	4.79	34.22	2.95	35.53	2.94	85.83	56.40	1.18	0.09	34.76	2.46	22254.2	4490.4	12684.1	2097.5	134.97	23.19	22.11	1.99

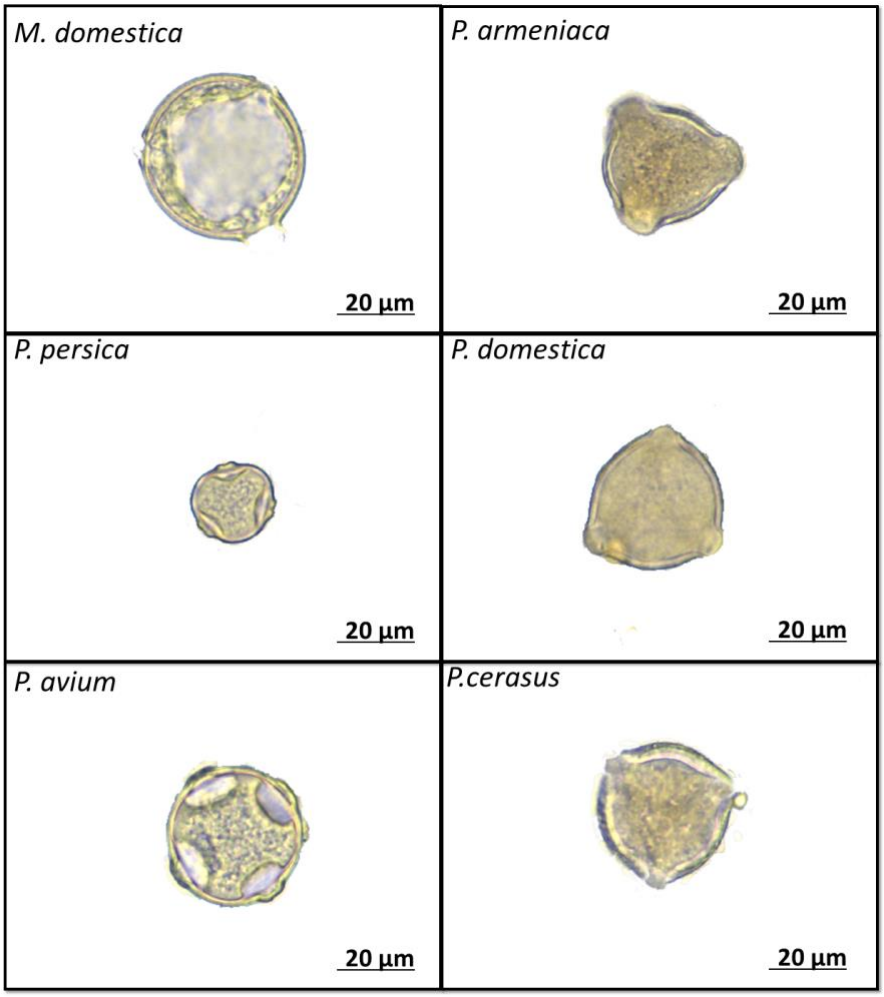

Figure 1 Pollen shape in parallel view.

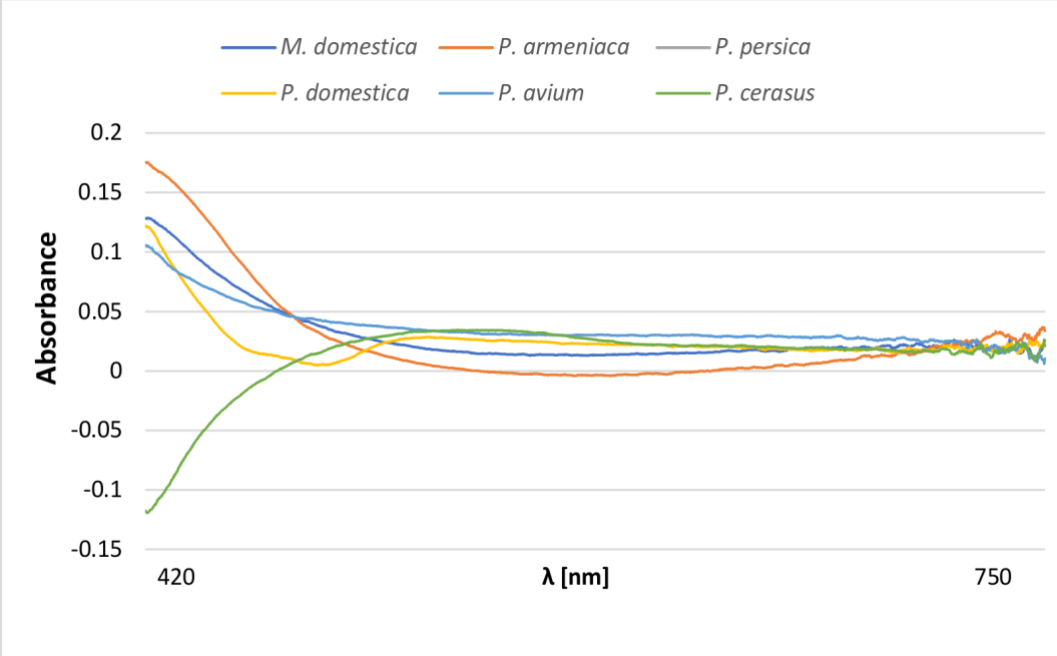

Figure 2 UV-VIS spectra plot of fruit tree species.

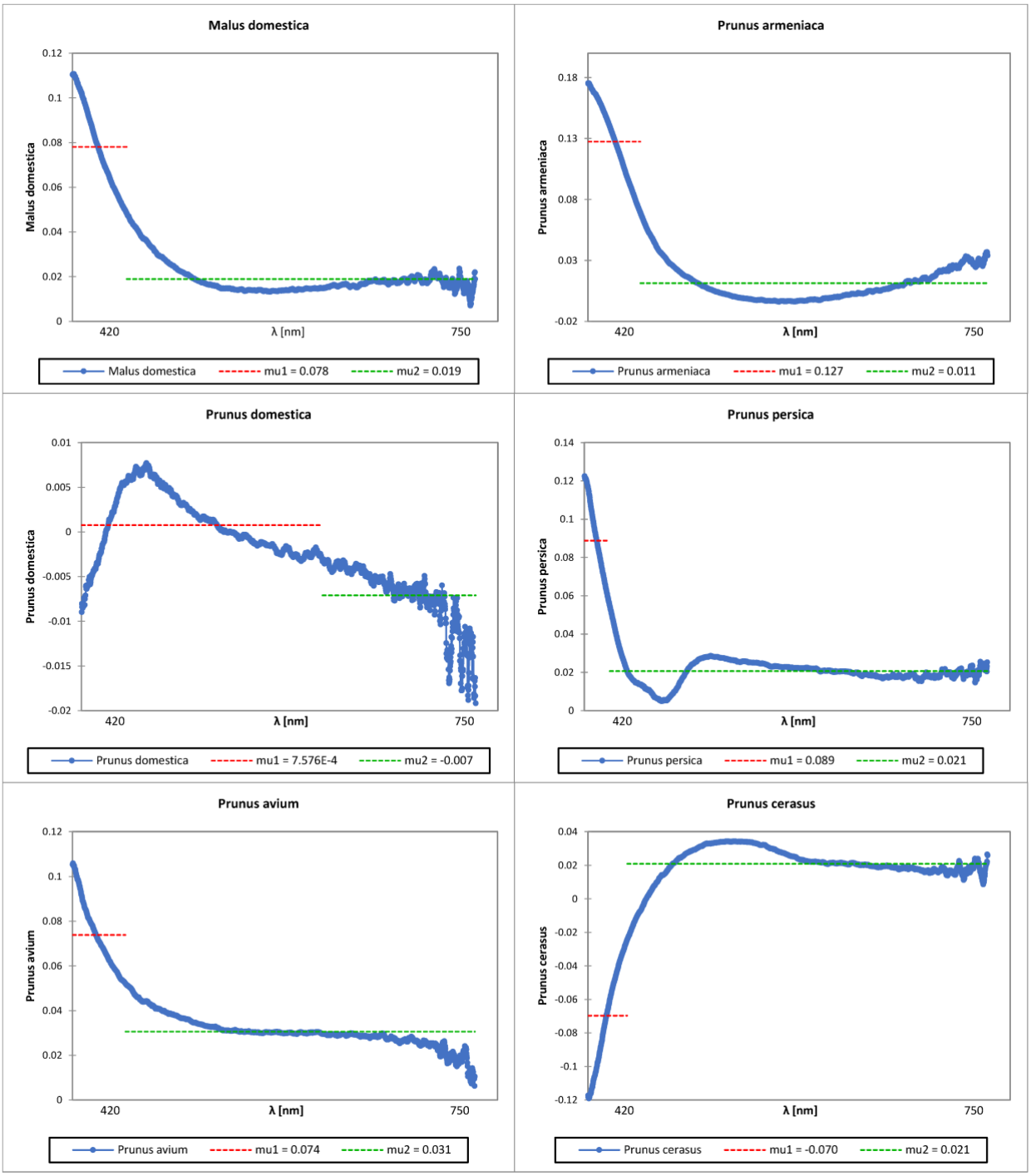

Figure 3 Standard normal homogeneity test (p <0.05).

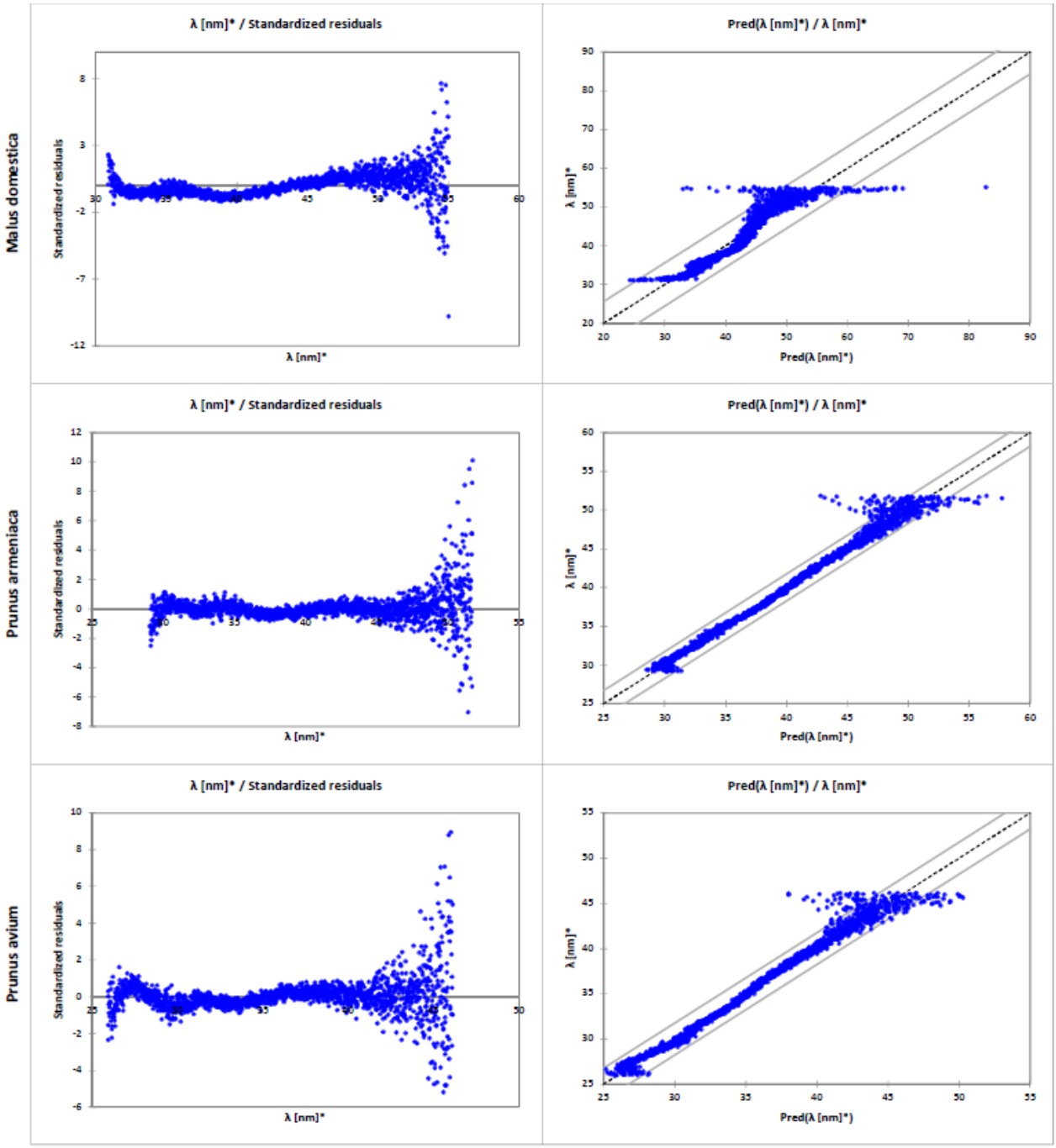

Figure 4 Cochrane-Orcutt prediction model of fruit tree pollen.

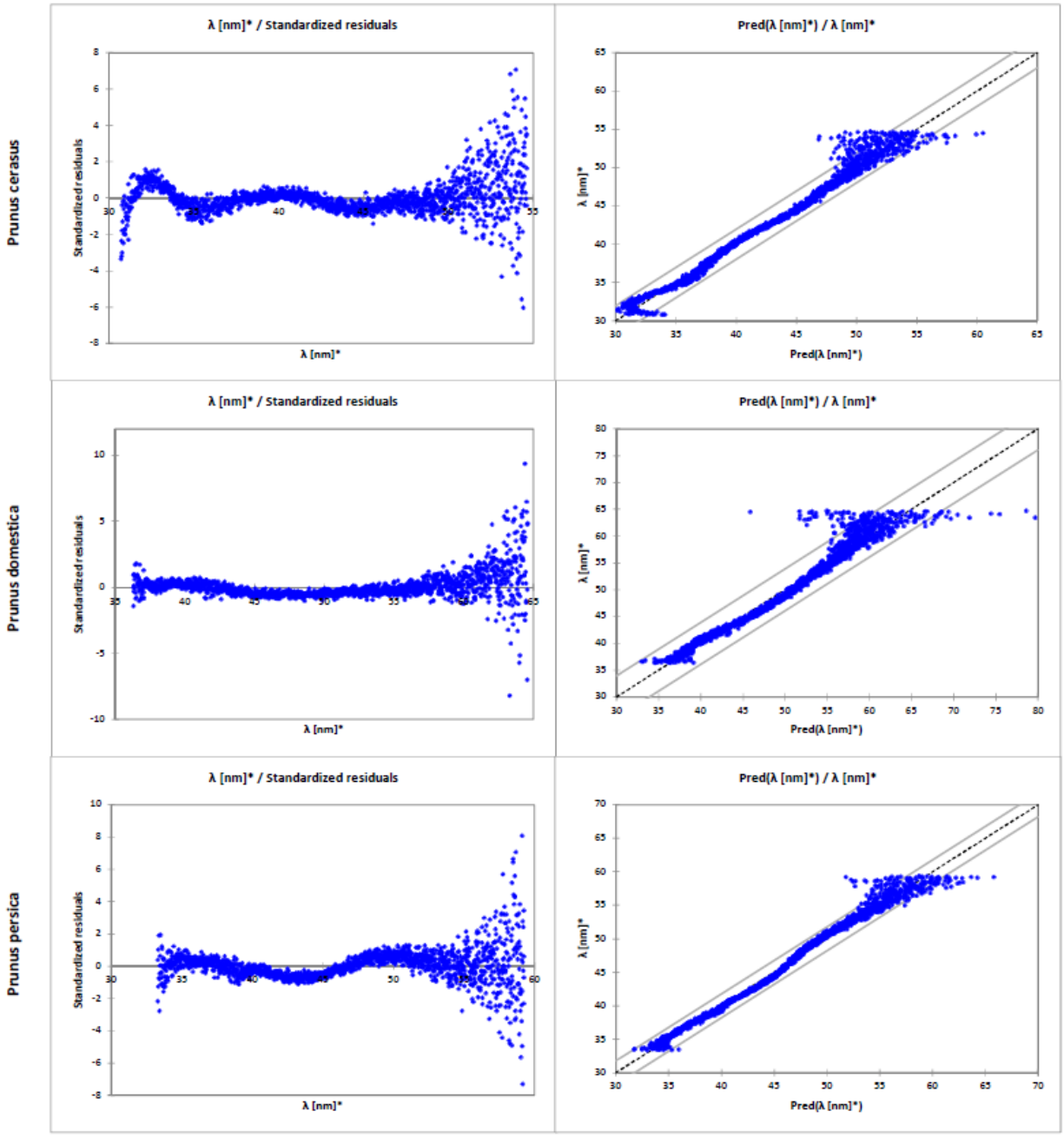

Figure 5 Cochrane-Orcutt prediction model of fruit tree pollen.

Although there was a statistically significant difference (p <0.05) in the min, max feret and max feret 90 parameters, which indicates that there are shape differences between the species, the pollen grains are rather irregular bodies and therefore they are better described by feret parameters that are more suitable for the description of irregular bodies than simple circularity or convexity. Circularity and convexity are values calculated of the object circumference and any roughness skews the results. Further, **Evrenosoğlu and Misirli (2009)** report differences in pollen grain surface which could also be reflected in the parameters we measured, namely in the perimeter and perimeter contour, indicating that the *P. domestica* surface (154.08 and 154.08) is more wrinkled than that of *P. persica* (103.53 and 101.93).

The statistical differences found within the *Prunus* genus are in agreement with **Wrońska-Pilarek and Jagodziński (2011)** who confirmed that the morphology of pollen grains is an important feature for distinguishing species within the Rosaceae family where the *Prunus* genus belongs. Taking into account the large number of morphological parameters described in the work, factor analysis of morphological parameters was also made, which outlined the most different morphological criteria of max feret 90 and circularity (Factor 1). Volumetric factors are preferably excluded with respect to the unequal longitudinal and transverse shape of the pollen grains, which could interpret the result with great error. As a secondary factor (Factor 2), roughness (convex hull perimeter/perimeter) and roughness inf (1/roughness) were selected. Given the reversed value of these two factors, in case of reducing the number of the

measured factors, it would be preferable to take a factor with lower correlation into account, namely the shape factor (R = -0.06 for Factor 1 and R = -0,21 for Factor 2, respectively). Factor analysis further showed that Factor 1 represents 58.9% and Factor 2 represents 24.7% of the decision criterion.

On the other hand, shape characteristics are not the only parameter that can be used to discriminate pollen of fruit species from each other. Another option is to evaluate the spectral characteristics of pollen grains. With conventional spectroscopic techniques, color evaluation within the visible spectrum of light can be considered. However, with regard to the availability of spectrophotometric techniques, it is not a problem to analyze also regions outside of visible spectrum of light. An important fact is that spectral analysis, especially in UV and NIR, also provides information about natural substances contained. In the UV-VIS region, spectral characteristics are often used to evaluate the content and amount of polyphenols **(Paradiso et al., 2016)**. Polyphenols represent a large group of natural photoactive substances presented throughout the plant kingdom. Their color ranges from red to purple and is determined by the number of OH-phenol groups reaching from 100 to 3000 daltons **(Anouar et al., 2012)**. Polyphenols achieve maximum absorption primarily in the UV region, because they are designed specifically to protect against UV light. In addition to OH groups, OCH_3 and glycoside groups are likewise responsible for UV absorption **(Harborne and Mabry, 2013)**. Though, the content of polyphenols is not represented only in vegetative plant forms. Polyphenols are also present in generative parts including pollen **(Serra Bonvehi et al., 2001; Almaraz-Abarca et al., 2007; Tian et al., 2007; Rzepecka-Stojko et al., 2015)**. In view of the yet unknown presence of polyphenols in pollen from Czech plants, the spectral analysis was used as a fingerprint methods. UV-VIS fingerprinting has been successfully used, for example, to compare grapes of red varieties in the spectral range of 160 – 600 nm **(Pop, Babeş and Bunea 2008; Casale et al., 2010)**. Average spectrum of fruit trees pollen is shown in Figure 2.

Figure 2 shows that individual UV-VIS spectra of fruit tree pollen differ from each other. Differences are mainly due to the different content of polyphenols **(Almaraz-Abarca et al., 2004)**. Differences were not observed only among the different types of fruit trees. Relatively large variability was also demonstrated between individual pollen grains. The variability is given both by differences in the pollen grains of individual flowers, as well as between cultivar variability **(Daoud et al., 2015)**. The UV-VIS absorbance variability of individual pollen grains is shown in Figure 4 and Figure 5. The Cochrane-Orcutt model was used to predict variability of the spectrogram, predicting the probability of occurrence of values in the 95% interval for further measurements. The chart illustrates that abnormal number is outside the interval only in certain wavelengths. Even considering a rather small group of pollen used for the analysis, it can be assumed that the whole spectrum analysis is a suitable method to determine botanical origin of pollen. Conformance of spectrograms in individual fruit species was also demonstrated by linear regression *M. domestica* (R^2 = 0.84), *P. armeniaca* (R^2 = 0.98), *P. persica* (R^2 = 0.99), *P. domestica* (R^2 = 0.94), *P. avium* (R^2 = 0.98), *P. cerasus* (R^2 = 0.98).

Abnormal numbers outside the interval were observed only in low and the highest wavelengths. It can be assumed that interference with glass optics used in the microscopes occurs particularly in the upper wavelength range. The measured absorbance was also tested for homogeneity within wavelengths. The test results showed that the absorbance across the wavelength spectrum is not homogeneous (p <0.05). Differences between individual varieties of fruit tree pollen were demonstrated (Figure 3).

The homogeneity test showed similarity between species of *M. domestica* (λ = 466), *P. armeniaca* (λ = 465), P. *persica* (λ = 441),), *P. avium* (λ = 465), *P. cerasus* (λ = 453). It can be assumed that the reason is a high content of polyphenols having a maximum absorbance between 250 – 410 nm. In the 270 – 280 range, phenolic acid and flavanones are involved **(Pop, Babeş and Bunea 2008; Anouar et al., 2012)**, in the 300 – 410 nm range, flavones and flavonols are involved **(Anouar et al., 2012)**. For *P. domestica*, the absorbance of the homogeneity test was different from the wavelength (λ = 620) which indicates a different representation of polyphenols. It can be assumed that *P. domestica* pollen contains a rather large amount of anthocyanins. Anthocyanins have absorbance between 520 – 546 **(Pop, Babeş and Bunea, 2008; Anouar et al., 2012)**. The above data show spectral variability between the pollen grains of the individual fruit trees, with variations within cultivars, and individual pollen grains are within the framework of predictive models. The spectral analysis can therefore be applied for fingerprinting of pollen grains similarly to studies performed on other analytes within the UV-VIS spectroscopy **(Pons, Le Bonté and Potier, 2004; Van Den Broeke, Langergraber and Weingartner, 2006; Pop, Babeş and Bunea, 2008; Casale et al., 2010; Zavoi et al., 2011)**.

CONCLUSION

Based on the morphological criteria of pollen grains, individual species of fruit trees can be discriminated from each other. Morphological differences of pollen grains within the *Prunus* genus are smaller. Comparison to the literature reveals that the morphological characteristics of pollen grains of fruit trees in the Czech Republic do not differ significantly from other countries. Exceptions are *P. persica, P. avium, and P. cerasus*, where Czech varieties can be assumedly distinguished from foreign varieties of these fruit trees based on the morphological characteristics. The Cochrane-Orcutt prediction model shows that variability in microspectrophotometric measurements of individual species within the wavelength range of 230 – 780 nm is low, but variations were found between individual spectrograms. It can therefore be assumed that the microspectrophotometric method can be used as a fingerprinting method to identification of pollen grains. It was also confirmed in the work that the pollen of individual fruit trees has a different content of photoactive substances, most likely polyphenols. Based on these results, it was confirmed that microspectrophotometric analysis is a suitable complementary method for determining botanical origin of pollen and, together with the morphological criteria, it can also be used as a fingerprinting method to determine botanical origin of honey based on pollen identification.

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POTENCY OF OKRA FLOUR (*ABELMOSCHUS ESCULENTUS*) IN IMPROVING ADIPONECTIN LEVEL AND TOTAL ANTIOXIDANT CAPACITY OF HIGH FAT DIET STREPTOZOTOCIN RAT MODEL

Ahdiyatul Fauza, Ahmad Ni'matullah Al-Baarri, Kis Djamiatun

ABSTRACT

T2DM has increase in global-morbidity and mortality. Oxidative stress and adiponectin-levels are important for insulin-resistance and pancreatic-β-cell-dysfunction in T2DM. Okra fruit is rich of quercetin and phytosterol which have positive-effect for T2DM. Research aimed was to study the effect of okra-flour to adiponectin-levels and total-antioxidant-capacity (TAC) in T2DM. Thirty Wistar-rats were divided randomly in five groups. K1 and (X1, X2 and X3)-treated-groups were in T2DM-condition-induced by high-fat-diet-(HFD)-Streptozotochin-(STZ)-nicotinamid-(NA). Healthy-controls-(K2)-group was also used. Okra-flour was given orally for 28 days at doses of 0.1; 0.2 and 0.3 g/Kg-body-weight/d to X1, X2 and X3-groups, respectively. Statistical program was used to analyse the different between pre-post-intervention, and between groups. Correlations between variables were also analysed. The serum-adiponectin and TAC-levels were measured by ELISA and ABTS-methods, respectively. By comparing pre and post-intervention, adiponectin levels of all-intervention-(X1, X2, X3)-group were increase ($p = 0.027$ for X1 and X2; $p = 0.028$ for X3), while in the same period the decrease were found in group K1 ($p = 0.026$) and K2 ($p = 0.028$). Increase-TAC-levels pre-post-intervention was observed in group all-intervention-groups ($p = 0.027$), while no change in K1 ($p = 0.66$) and the decrease in group K2 ($p = 0.039$). Reduce-fasting-blood-glucose-levels pre-post-intervention were shown in the all-intervention-groups ($p = 0.028$), while for the K1 groups was increase ($p = 0.028$). There were significant differences between the five-groups on fasting-blood-glucose-levels, adiponectin and TAC-levels, and X3-group showed the highest adiponectin and TAC-levels. Very-strong-correlations were found between glucose-adiponectin-TAC-levels-post-intervention. Okra-flour make better glucose-adiponectin and TAC-levels in T2DM-conditions. Okra dose of 0.30 g/Kg-body-weight/day is the best in increasing adiponectin and TAC-levels.

Keywords: Okra; T2DM; adiponectin; TAC

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is metabolic disease (metabolic syndrome) characterized by high blood glucose levels. Mechanisms of T2DM include first, reduce ability of pancreatic-β-cells in producing sufficient amounts of insulin, and second, insulin resistance when the body cannot use it effectively so that excess sugar occurs in the blood **(Melton, 2017)**. DM is a global problem due to the increase incidence particularly those caused by obesity. T2DM caused 1.5 million deaths in 2012 in which high blood glucose from normal was responsible for 2.2 million additional deaths as a result of an increased risk of cardiovascular disease and others, with a total of 3.7 million deaths associated with blood glucose levels in 2012. Many of the deaths (43%) occurred under the age of 70. In 2014 as many as 422 million people in the world suffered from DM with a prevalence of 8.5% among the population of adults suffering from T2DM **(WHO, 2016)**.

The relationship between obesity and T2DM occur through 2 mechanisms, namely insulin resistance that occurs due to obesity and failure of pancreatic β cell function. Abnormalities of the body's metabolism where there is a decrease in response or sensitivity from the action of insulin is called insulin resistance. The HFD-inducing-insulin resistance causes an increase in fat oxidation and subsequently increase reactive oxygen species (ROS) levels **(Styskal, Van Remmen and Richardson, 2012; Asghar, 2017)**. The high ROS level can reduce total antioxidant status (TAS) **(Moheildein et al., 2015)**. Insulin resistance is a result of positive energy balance, because positive energy balance increases adipose tissue, and reduces adiponectin production. Adiponectin is part of adipocytokine which

functions to increase insulin sensitivity by increasing GLUT4 translocation and plays a role in suppressing glucose production by inhibiting gluconeogenic enzymes **(Cheng et al., 2014)**. Adiponectin levels can be increased by consuming polyphenols from food **(Kim and Koegh, 2016)**.

Antioxidants bind to free radicals and afterward prevent the adverse effects of free radicals on the body **(Maritim et al., 2002)**. Plants are considered food that is beneficial in many diseases including diabetes in controlling blood glucose levels and preventing long-term complications **(Gallagher, Flatt and Duffy, 2003)**. Phytochemical studies show that polysaccharides, polyphenols, flavonoids, tannins, sterols and triterpenes are the main components of Okra (*Abelmoschus esculentus*) and those components have a variety of biological activities **(Sheu and Lai, 2012)**. Other study show that Okra fruit is rich in bioactive components, such as flavonoids, especially quercetin and phytosterols **(Sa'eed Halilu Bawa, 2016)**. Fresh Okra has flavonoids namely quercetin in amounts 60 – 75% **(Zhang, 2014)**. Okra fruit has phenols and flavonoids that have antioxidant effects and anti-diabetic effects, namely quercetin-3-O-ß-D-glucopyranosyl-(1→6)-ß-D-glucopyranoside and quercetin-3-O-ß-D-4'-O-methyl-ß-D-glucopyranoside **(Zhang, 2014)**.

This study aimed to determine whether intervention of okra fruit flour increase both serum-adiponectin and total antioxidant capacity (TAC)-levels in T2DM-rats induced by high fat diet (HFD) followed by Streptozotochin-(STZ)-nicotinamid-(NA)-injection.

The dosage variations of okra flour given in this study were 0.1; 0.2 and 0.3 g/Kg-body-weight-(BW)/day(d) **(Naeem, Mohammad and Ali, 2018; Sabitha, Ramachandran and Naveen, 2012)**. The intervention of okra fruit flour was carried out for 28 days.

Scientific hypothesis

Okra flour has various dosages which affect adiponectin levels, total antioxidant levels (KAT) and blood sugar levels in DMT2 wistar rats.

MATERIAL AND METHODOLOGY

Okra-flour preparation and Intervention

Okra was bought in the traditional market of Semarang. Choosed okra was those in good condition as indicated by green-colour, about 10 to 20 cm long, notched at the end and had fine feather. The fruit (seeds and skin) was then wash and blend by using a blender machine, then it was dry at low temperature 40 °C in drying cabinet. The dried ingredients are then mashed with mortar or blender. The powder is weighed and put in a plastic bottle and kept away from light and moisture for further use **(Naeem, Mohammad and Ali, 2018)**. The Okra-flour-suspension is given orally using a feeding tube given to experimental –Wistar-rats. The moderate doses were 0.10 and 0.20 $g.Kg^{-1}$-BW/d. These doses reduced blood glucose levels of T2DM-rat. The high dose was 0.3 $g.Kg^{-1}$-BW/d, twice the moderate dose **(Naeem, Mohammad and Ali, 2018; Sabitha, Ramachandran and Naveen, 2012)**.

Antioxidant activity test of okra flour

Antioxidant activity test of okra flour was carried out at the Diponegoro University Integrated Laboratory. Analysis of antioxidant activity with DPPH method was referring to the

Figure 1 Okra Fruit (*Abelmoschus esculentus*).

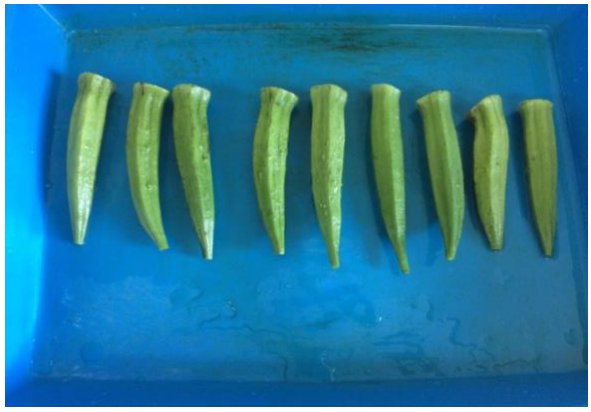

Figure 2 Preparation of Okra flour.

Figure 3 Preparation of Okra flour.

Figure 4 Okra flour.

previous method **(Suica-Bunghez et al., 2016)**. Preparation of DPPH solution was carried out using methanol (0.02 mg DPPH.mL^{-1} MeOH) with violet colour. A total of 0.045 g of okra flour samples were mixed with 1 mL of DPPH solution which was then incubated in a dark place for 1 hour. After that, the mixture is read the absorbance at $\lambda = 517$ nm with a spectrophotometer. The control was made from 0.5 mL of MeOH with 1 mL of DPPH solution. Antioxidant activity (AA%) was calculated by the absorbance control formula minus the absorbance of the sample then divided by the absorbance of the control then multiplied by 100.

Research design and Experimental-Animals

This research was a true-experiment study with randomized pre-post test with control group design. Animal used was male Wistar rats, aged 8 – 12 weeks, weighing 150 – 200 g, were acclimatized at the laboratory of the Center for Food and Nutrition Studies at Gajah Mada University, Yogyakarta. The rats were placed in individual stainless-steel cages at regulated temperatures (21 °C). They are housed at cleaned and germ-free place. The rats were fed with 20 g.d^{-1} of the Comfeed II standard-diet (7% fat) during the non-HFD period. They received ad-libitum water during the experiment. Animal care in the laboratory was carried out accordance with the Animal Laboratory Guidelines from the Central Laboratory for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta.

Thirty rats were divided into five groups which were T2DM-control-(K1), T2DM-intervention-(X1, X2 and X3) and healthy-control-(K2)-groups. The T2DM-condition was induced by HFD-STZ-NA. After a week of acclimatization, rats were conditioned on T2DM with oral HFD of 20 g.d^{-1} for 14 days then injected intraperitoneally with STZ (45 mg.Kg^{-1}-rat-BW) and NA (110 mg.Kg^{-1}-rat-BW). Injection of NA was carried out 15 minutes after STZ with the aim of preventing pancreatic cell-apoptosis β **(Ghasemi and Khalifi, 2014)**. T2DM-condition was indicated by fasting blood glucose serum >200 mg.dL^{-1} **(Bao et al., 2015)**. The X1, X2 and X3-groups were then treated with Okra-flour-suspension in doses of 0.1; 0.2 and 0.3 g.Kg^{-1}-rat-BW/d every day for total period of 28 days. The blood sample again was taken at the end of the trial (end of intervention), fasting blood glucose was taken through the retroorbital plexus. Blood samples were collected in a centrifugation tube and centrifuged 4000 rpm in 15 minutes. Adiponectin and TAC levels were analysed by ELISA and ABTS methods respectively. This study was approved by the Health Research Ethics Commission (KEPK) of the Faculty of Medicine Diponegoro University Semarang through ethical clearance No. 06/EC/H/FK-UNDIP/I/2019.

Statistic analysis

Results were expressed as mean ±*SD* (for normally distributed data) otherwise it expressed as median (min-max). Statistical difference was analysed by using one-way analysis of variance (ANOVA) followed by post hoc Bonferroni for normally distributed data, otherwise Kruskal-Wallis test followed by Mann-Whitney-U-test was used (SPSS 21). Spearman's correlative test was used to analyse the relation between variables. Statistical analyses were done by computer. The differences and correlations were considered significant at *p*-value <0.05 and 95% confidence intervals. The strength of correlations was determined by *r*-value.

RESULTS AND DISCUSSION

The data processed in this study was obtained from 30 Wistar-rats, which divided in each group consisting of 6 rats. The experimental animals used before T2DM had an average body weight of 173.25 g and blood glucose levels of 73.11 mg.dL^{-1}, while the T2DM animals after induction had an average body weight of 194.92 g and blood glucose levels of 259.18 mg.dL^{-1}.

Wilcoxon test between body weight before and after intervention showed that the intervention of okra flour increased body weight in the all-treatment-groups (the groups had $p = 0.027$; Table1), while K1-group experienced significant weight loss ($p = 0.027$). This suggested that Okra-flour increased body T2DM-rat-BW. Healthy-control-K2-group was experienced a significant increase in body weight ($p = 0.027$). Statistically difference was also found on the pre-post-intervention change (Δ) of rat-BW among all-groups (Kruskal Wallis test; $p < 0.001$), and then Mann-Whitney U-test between two-groups was performed. The Δ-rat-BW of all-intervention-T2DM-group was higher than T2DM-control-K1-group and this was significant (X1, X2 and X3 had same *p*-value; $p = 0.004$). This demonstrated that Okra-flour recover BW of T2DM-rat. The Δ-rat-BW of X1 and X2-groups were significantly lower than healthy-control-K2-group (X1 and X2 had same *p*-value; $p = 0.004$), while those of X3-group was not different than K2-group ($p = 0.411$). This demonstrated that Okra-flour in dose of 0.3 g/Kg-rat-BW/d normalized BW of T2DM-rat. All of these indicated that Okra-flour increased T2DM-rat-BW in dose dependent manner.

The blood glucose levels were decrease in the end of intervention, and these was significantly difference (X1, X2 and X3 groups had same *p*-value; Wilcoxon test, $p = 0.028$; Table 2), meanwhile those of T2DM-control-K1-group increased significantly ($p = 0.028$). This indicated that Okra-flour reduced glucose-levels of T2DM-rat. The blood glucose level of healthy-control-K2-group was also significantly increase in the end of study ($p = 0.028$), however this change was remained in the normal value. The pre-post-intervention-change (Δ) of blood-glucose-levels was significantly different among five-groups (Kruskal Wallis test; $p < 0.001$). The Δ-glucose-levels of all-intervention-T2DM-group was more apparent than T2DM-control-K1-group and this was significant (X1, X2 and X3 had same *p*-value; Mann-Whitney U-test, $p = 0.004$). This again indicated that Okra-flour reduced glucose-levels of T2DM-rat. The Δ-blood-glucose-levels were significantly different between intervention groups. The Δ-blood-glucose-level of X1 was significantly lower than X2 and X3-groups (X2 and X3 had same *p*-value; Mann-Whitney U-test, $p = 0.004$). Further analysis showed that the Δ-blood-glucose-level of X2 was significantly lower than X3-group ($p = 0.004$). All together these demonstrated that the smallest Δ-blood-glucose-level was found in Okra-flour-intervention-dose of 0.1 g.Kg^{-1}-rat-BW/d, while the biggest change was found in the dose of 0.3 g.Kg^{-1}-rat-BW/d. This indicated that Okra-flour reduced T2DM-rat-glucose-levels in dose dependent manner.

The adiponectin levels were significantly increase at the end of intervention in all treatment-group (Wilcoxon test of

X1 and X2-group had same *p*-value, $p = 0.027$; while X3-groups showed $p = 0.028$; Table 2). This indicated that Okra-flour intervention increased adiponectin levels of T2DM-rats. The adiponectin-level of T2DM-control-K1-group was significantly decrease ($p = 0.026$) at the end of study. Although this was also observed in healthy-control-K2-group, the adiponectin level in K2-group was obviously higher than all T2DM-groups. Kruskal Wallis test showed a significant difference in the pre-post-intervention-change (Δ) of adiponectin-levels among the five groups ($p < 0.001$) (Table 2). Mann-Whitney U-test showed the Δ-adiponectin-levels of all-T2DM-intervention-groups were significantly higher than K1-group ($p = 0.004$). This suggested that Okra-flour increased adiponectin-levels of T2DM-rats. The Δ-adiponectin-levels of X3-group was the higher than X1 and X2 (X1 and X2 had same *p*-vale; $p = 0.004$). The Δ-adiponectin-levels of X2-group was the higher than X1 ($p = 0.004$). These showed that the biggest Δ-adiponectin-levels was in those treated with Okra-flour in dose of 0.3 $g.Kg^{-1}$-rat-BW/d, the middle was those receive 0.2 $g.Kg^{-1}$-rat-BW/d, and the lowest was those treated with 0.1 $g.Kg^{-1}$-rat-BW/d. This indicated that Okra-flour increased T2DM-rat-adiponectin-levels in dose dependent manner.

The pre-post-intervention-TAC-levels were significantly increase in all T2DM-intervention groups (X1, X2 and X3-groups; Wilcoxon test, $p = 0.027$; Table 2), while K1-group showed no significant change of the pre-post-intervention-TAC-levels ($p = 0.066$). This suggested that Okra-flour increase T2DM-rat-TAC-levels. Kruskal Wallis test demonstrated was a significant difference in the pre-post-intervention-change (Δ) of TAC-levels among the five groups ($p < 0.001$) (Table 2). Mann-Whitney U-test showed the Δ-TAC-levels of all-T2DM-intervention-groups were significantly higher than K1-group ($p = 0.004$). This again indicated that Okra-flour increase T2DM-rat-TAC-levels. The Δ-TAC-levels of X1 was significantly lower than X2 and X3 ($p = 0.004$), while no significant different was found between those of X2 and X3-group ($p = 0.091$). This indicated that Okra-flour increased T2DM-rat-adiponectin-levels partly in dose dependent manner.

The Spearman test on all data from all rats at the end of study showed that very strong correlation was found between variables. A very strong positive-correlation was observed between TAC and adiponectin-levels (Table 3), while very strong negative correlation was found between blood-glucose-levels either with adiponectin-levels or TAC-levels. Those correlations were only weak before Okra-flour-intervention began.

Okra flour is recovered T2DM-rat-BW. This was based on a significantly higher T2DM-rat BW of Okra-flour-intervention-group of at the end of study than those of before the intervention. This was based on a significantly higher Δ-T2DM-rat-BW in those of Okra-flour-intervention-group than T2DM-control-K1-group, and this effect was dose dependent. The effect of Okra-flour on T2DM-rat-BW was as expected. Reduce BW occurred after STZ-injection during T2DM induction (data not shown).

This is also observed in previous studies observing the effect of STZ injection on BW **(Bermúdez-Pirela et al., 2007; Roopchanda et al., 2015; Rodrigues et al., 2015)**. Other mechanism involve in the reduce BW of T2DM is insulin resistance which increases lipolysis **(Zhou et al., 2013)**.

All together indicates that Okra-flour overcome any mechanism involve in the reduce T2DM-rat-BW.

Okra flour reduced glucose-level of T2DM-rat and increase both serum-adiponectin and TAC-levels. The effect of Okra-flour on blood-glucose, adiponectin-levels and TAC-levels were dose dependent. T2DM-control-K1-group showed increase blood-glucose-levels and reduce both serum-adiponectin and TAC-levels. Healthy-control-K2-group also showed increase blood-glucose-levels. Stress may be a factor for increasing blood-glucose levels. Blood collection in condition which activates stress response in rats through increased adrenaline and non-adrenaline **(Bowe et al., 2014)**.

Table 1 Intake and body weight.

Rat Group	Intake (Rerata ±*SD*) g			
	Pre Intervention	**Post Intervention**	***p***	**Δ**
X1	12.6 ±0.49	13.1 ±0.34	0.051	0.5 ±0.50
X2	12.9 ±0.25	12.7 ±0.69	0.686	-0.1 ±0.82
X3	12.8 ±0.31	12.4 ±0.33	0.153	-0.3 ±0.51
K1	13.9 ±0.16	14.0 ±0.28	0.647	0.07 ±0.36
K2	12.4 ±0.44	13.0 ±0.45	0.136	0.5 ±0.79
p^1	0.002	0.001		0.064
Rat Group	**Body Weight (Median (Min-Max)) g**			
	Pre Intervention	**Post Intervention**	***p***	**Δ**
X1	196.0 (183.0 – 203.0)	211.0 (199.0 – 216.0)	0.027	14.00 (11.00 – 16.00)
X2	199.5 (188.0 – 205.0)	219.5 (208.0 – 226.0)	0.027	20.00 (17.00 – 2 1.00)
X3	185.5 (178.0 – 210.0)	213.5 (205.0 – 236.0)	0.027	27.00 (25.00 – 29.00)
K1	197.0 (185.0 – 210.0)	181.0 (170.0 – 197.0)	0.027	-14.50 (-17.00 – -12.00)
K2	187.5 (175.0 – 193.0)	214.5 (205.0 – 220.0)	0.027	27.50 (26.00 – 30.00)
p^1	0.058	0.002		0.000

Note: Δ = change of body weight before and after treatment.

Table 2 Blood serum test.

Rat Group	Blood Glucose Level (Median (Min-Max)) (mg.dL^{-1})			
	Pre Intervention	Post Intervention	*p*	Δ
X1	253.8 (251.09 – 262.04)	144.9 (138.55 – 152.61)	0.028	-109.34 (-115.83 – -105.89)
X2	262.2 (258.03 – 264.60)	128.1 (124.50 – 129.32)	0.028	-134.29 (-135.36 – -132.33)
X3	262.4 (258.76 – 268.25)	108.4 (100.40 – 116.47)	0.028	-155.71 (-160.62 – -149.88)
K1	257.3 (250.73 – 262.04)	258.8 (253.01 – 263.05)	0.028	1.96 (0.68 – 2.39)
K2	73.1 (68.98 – 76.64)	74.7 (71.08 – 77.11)	0.028	1.52 (0.39 – 2.10)
p^1	0.000	0.000		0.000
Rat Group	**Adiponektin Level (Median (Min-Max)) (mg.L^{-1})**			
	Pre Intervention	**Post Intervention**	***p***	**Δ**
X1	3.1 (2.49 – 4.19)	9.4 (8.72 – 9.94)	0.027	6.23 (5.47 – 6.42)
X2	3.2 (2.21 – 3.72)	12.3 (11.50 – 12.90)	0.027	9.12 (9.04 – 9.31)
X3	3.2 (2.02 – 4.38)	13.8 (13.40 – 14.60)	0.028	10.58 (9.69 – 11.90)
K1	3.3 (2.68 – 3.91)	3.1 (2.30 – 3.62)	0.026	-0.23 (-0.48 – -0.19)
K2	16.1 (15.30 – 17.40)	15.6 (15.00 – 16.50)	0.028	-0.45 (-0.90 – -0.10)
p^1	0.006	0.000		0.000
Rat Group	**TAC Level (Median (Min-Max)) (mmol.L^{-1})**			
	Pre Intervention	**Post Intervention**	***p***	**Δ**
X1	0.2 (0.15 – 0.44)	0.9 (0.74 – 1.18)	0.027	0.59 (0.44 – 1.03)
X2	0.3 (0.15 – 0.44)	1.6 (1.62 – 1.91)	0.027	1.33 (1.18 – 1.61)
X3	0.2 (0.15 – 0.59)	1.9 (1.76 – 2.21)	0.027	1.69 (1.17 – 2.06)
K1	0.4 (0.15 – 0.59)	0.2 (0.15 – 0.29)	0.066	-0.15 (-0.44 – 0.00)
K2	2.2 (2.06 – 2.35)	2.0 (1.91 – 2.21)	0.039	-0.15 (-0.29 – 0.00)
p^1	0.005	0.000		0.000

Table 3 Spearman correlative test before and after intervention.

Variable	Pre Intervention		Post Intervention	
	r	*p*	*r*	*p*
Kadar adiponektin dan KAT	0.424	0.019*	0.919	0.000*
Kadar adiponektin dan glukosa darah	-0.582	0.001*	-0.980	0.000*
KAT dan kadar glukosa darah	-0.405	0.026*	-0.930	0.000*

Note: *p-value <0.05 = significant.

This then contributes in the increase of blood-glucose-levels **(Bowe et al., 2014)**. The stress effects on blood-glucose-levels, therefore may occur in the present study. The very strong correlations between blood-glucose, adiponectin, and TAC-levels after Okra flour intervention in T2DM-rats, strengthen the notion that Okra flour influence mechanisms contribute in regulating blood-glucose, adiponectin, and TAC-levels. Mechanisms which may explain these present finding have been studied. Serum adiponectin levels correlate with insulin resistance in T2DM. Adiponectin plays a role in maintaining insulin sensitivity through direct and indirect mechanisms. Adiponectin decreases glucose production in the liver by inhibiting gluconeogenic enzymes in the AMPK pathway and adiponectin also upregulates IRS-2 expression to strengthen insulin in the liver. Adiponectin increases GLUT 4 translocation to the skeletal muscle plasma membrane, so that glucose uptake increases **(Cheng et al., 2014)**. Conditions of hyperglycemia in T2DM can interfere with antioxidant status because the condition of hyperglycemia can produce excessive ROS and can disrupt the body's defence system. Low antioxidant status and high production of ROS can trigger oxidative stress **(Zhou et al., 2013; Dornellas et al., 2015)**. ROS is toxic because it has high reactivity to enzymes, so it can damage tissue **(Skovso,**

2014). In uncontrolled T2DM conditions, there is an increase in NADPH activity. Increased NADPH activity can increase the production of superoxide radical anions which indicates that there is an increase in oxidative stress **(Lim et al., 2018)**. The antioxidants contained in food contribute to giving hydrogen atoms to radical compounds taken from hydroxyl groups, thereby forming stable phenoxyl hydroxyls **(Nagarchi et al., 2015)**. Total phenol content in food directly correlated with KAT in mice tested **(Nagarchi et al., 2015)**. Further Okra flour study in more detail mechanisms involve will complete the present finding.

CONCLUSION

The administration of Okra flour with various doses tested, proved to significantly reduce blood glucose levels, increase adiponectin and TAC levels in T2DM wistar rats. The administration of Okra flour with a dose of 0.30 $g.kg^{-1}$ BB per day is most effective in reducing blood glucose levels, increasing adiponectin and TAC levels in DMT2 wistar rats.

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PHYSICAL FACTORS RELEVANT FOR EFFICIENT HAWTHORN FRUIT EXTRACTION

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ABSTRACT

Today, the healthy nutrition market is one of the most promising market niches; modern consumers increasingly claim fortified products for their diets. One of the ways to increase the biological and physiological value of products is their enrichment with extracts of plant origin. The aim of the work was to study the influence of various factors on the process of hawthorn fruit extraction and determine the optimal parameters of the technological process. The study objects were hawthorn fruit extracts produced by the statistical method of maceration (with stirring). In the extracts obtained, there were determined the quantitative content of tannins and pectin substances, dietary fiber and vitamins. Currently, the global health and wellness food market is steadily growing due to changes in consumer behaviour patterns and developing of healthy self-consciousness. The studies conducted by the authors have shown that extracting of plant materials and the efficiency of biologically active substances extraction are influenced by the following factors: the extractant pH, grinding type and size of raw materials and process parameters. The optimal technological regimes have been established. They are the extraction temperature of 60 °C and extraction time of 30 minutes. The appropriate grinding size of dry hawthorn fruit has been determined to be up to 2.8 mm of a particle. In case of milk being an extractant, a high extraction dynamic of pectic substances and dietary fibers was observed. So, the hawthorn milk extract has been revealed to have higher organoleptic characteristics.

Keywords: dietary fiber; extraction; fermented milk product; hawthorn fruit; pectic substances; tannins

INTRODUCTION

Currently, the global health and wellness food market is steadily growing due to changes in consumer behaviour patterns and developing of healthy self-consciousness **(Nikolaev, 2012)**. Food products, combining a fairly complete set of vitamins and minerals with other biologically active substances, such as dietary fiber, pectic substances and phospholipids, are appearing on the market. Sometimes the content of some enriching additives in one product is undesirable or impossible due to their taste incompatibility, instability or undesirable interactions with each other **(Kukharenko et al., 2008)**. One solution to this problem is to use food fortification with plant extracts. Extraction is the most acceptable way to obtain biologically active substances from plant materials with respect to efficiency and rationality and provides the maximum yield of biologically active substances that increase the physiological value of the product enriched **(Lyapin et al., 2009)**.

The extraction process is caused by a complex of interacting factors that make it difficult to establish general principles for intensifying this process **(Khalanskaya, Lodygin and Kurchenko, 2017)**.

To obtain various types of extracts, fresh and dried raw materials are applied. Fresh raw material is usually used in small quantities and on rare occasions because of its storage and transportation being complicated, as well as rapid decomposition of biologically active substances in it **(Aliyev and Stepanov, 2006)**. The raw materials subjected to drying undergo considerable changes, i.e., dry residue is obtained from cellular fluid; the inside of the cell is filled with air; after drying, the cell wall and membranes of the cell organelles acquire the properties of porous membranes. Therefore, depending on the raw materials used and the cell wall's physiological state, there are some features of the extraction of biologically active substances **(Tikhonov and Yarnykh, 2002)**. A number of physicochemical processes take place inside the cell of the dried raw material and on its surface under the action of the extractant, i.e., diffusion, desorption, dissolution, dialysis and leaching **(Leonova and Klimochkin, 2012)**.

Diffusion is the main physical and chemical process. The presence of a porous membrane of dry plant material during

extraction leads to the movement of the substance in both directions and the nature of diffusion through it constitutes a dialysis process. Biologically active substances whose molecules do not exceed the pore size can diffuse through the porous membranes of the cell wall, so, the vast majority of extraction preparations are obtained from dried plant materials.

In terms of the nature of diffusion, the main stages of extraction are distinguished **(Tretyakova, 2014)**. They are diffusion of extractable substances from the inside of the cells to their surface; diffusion of substances through the laminar sublayer, surrounding the particle and arising due to viscous forces of the extractant during the flow of raw materials through the layer; and convective diffusion of the extracted substances from the outer surface of the laminar sublayer into total extractant flow. Convective diffusion is the more effective, the more intense the hydrodynamic regime (mixing) is. The thickness of the laminar sublayer depends on the hydrodynamic regime.

Desorption is a part of the extraction process and takes place in the cells when the extractant penetrates them, which results in a concentrated solution ("primary juice") in cells. Due to the difference in osmotic pressures, soluble substances leave the cells, and the extractant penetrates into them; "colliding" the processes of osmosis and dialysis leads to swelling of plant material **(Zyubr and Vasiliev, 2008)**. Diffusion processes are mass transfer processes, occur spontaneously and proceed until true dynamic phase equilibrium is established between the phases under certain conditions. Equalizing the concentration on both sides of the cell membrane to achieve a state of mobile diffusion equilibrium means the completion of the process at this extraction mode **(Ishanhodzhaeva, 2012)**.

The phytocomponents extraction dynamics is influenced by a number of factors, i.e., the pH of the extractant, types of the extractant and raw material, its grinding size, ratio between the weight of the raw material and volume of the extractant, the temperature regime of extraction and duration of extraction.

So, when selecting the technological parameters of the extraction process, the following factors affecting the released biologically active substances should be taken into account: the acidic pH of the extractant helps to transfer difficultly soluble alkaloid compounds (complexes with tannins) into easily soluble alkaloid salts and dynamics of tannins extraction; temperature drop causes decrease in the solubility of tannins; while drying raw material, muciform substances destruct, which contributes to the increased extraction of pectin; in the extraction process, the difference between concentrations of the extracted substance in the liquid and solid phases gradually decreases, so it is necessary to determine the dynamic equilibrium of the extraction process at the appropriate time **(Terletskaya, Planer and Zinchenko, 2013)**.

An important intensifying factor in extracting biologically active substances is continuous mixing of raw material and extractants, which ensures the best hydrodynamic conditions of the process, i.e., the particles of the raw material do not become compressed and are constantly and intensively washed with extractants. So, the conditions of mass transfer improve, the driving force of the extraction process increases, and greater yield of extracting substances is reached **(Oorzhak, Ushanova and Repyakh, 2003; Sorokopud, Mustafina and Fedyaev, 2012)**.

Scientific hypothesis

Study the influence of various factors on the process of hawthorn fruit extraction and determine the optimal parameters of the technological process.

MATERIAL AND METHODOLOGY

The objects of the study are hawthorn fruit (*Crataégus*) extracts produced by the maceration (with mixing). To obtain extracts of hawthorn fruit (*Crataégus*), the following equipment was used: a laboratory mill LZM-1 (LAB-AGRO, Barnaul), laboratory scales VLT-1500-P (SARTOGOSM, St. Petersburg), infusing device AI-3 (Kiev production association MEDPARATURA, Kiev).

Hawthorn fruit (*Crataégus*) was extracted by the method of maceration with stirring. To study the factors influencing the efficiency of the extraction of tannins, pectic substances and dietary fiber, the following parameters of the extraction process were chosen:

- types of extractants, i.e., distilled water, whey, milk and cream;
- extraction temperature of 50 ±2 °C; 60 ±2 °C; and 70 ±2 °C;
- duration of extraction of 20 min, 30 min, 40 min and 50 min; and
- grinding size: up to 1.0; up to 2.8; and up to 5.6 mm.

The experiment was repeated three times; 185 prototypes were studied.

Figure 1 Photo of hawthorn fruit (*Crataégus*): fresh and dried.

Quantitative determination of tanning substances in the objects was carried out by the spectrophotometric method, pectic substances by the titrimetric method and dietary fiber by the enzymatic-gravimetric method **(Fedoseeva, 2005)**. Quantitative determination of vitamins in extracts was performed by the high-performance liquid chromatography.

Statistic analysis

Statistical data were processed using the Statistical program (StatSoft, version 9.0 (Dell, USA). The data are presented as averages. The differences between the samples were assessed using unpaired *t*-test. Correlation analysis with calculation of pair correlation coefficient, for establish the dependence of parameters was used. The significance of differences was determined by the Student's criterion (t). The level was considered significant at $p \leq 0.05$. The study was repeated three times.

RESULTS AND DISCUSSION

Under laboratory conditions, hawthorn fruit extracts were produced according to the technology that included the following steps: dried hawthorn fruit was ground in a cutting mill; the ground vegetable raw material (5%) was placed in a pre-warmed porcelain mug and filled with extractant; distilled water (pH of 6.46), whey (pH of 6.12), milk (pH of 6.54 and weight fraction of fat content of 2.3%) and cream (pH of 6.68 and weight fraction of fat content 10%) were used as extractants; the mug was capped, heated in a water bath to a temperature of 50 – 70 °C and kept for 30 minutes, periodically stirred; the resulting mass was filtered and squeezed; and the quantitative contents of extractable substances were determined with respect to the extractant used. The research results are presented in Figure 3.

Viscosity of the extractant had a great influence on the solubility and the substances' diffusion rate. The extraction efficiency depends on a large number of parameters, i.e., extraction method, extractant and technological parameters of extraction. In this regard, it is very important to find the optimal extraction parameters for obtaining extracts with the highest content of biologically active compounds **(Lincheva et al., 2017)**.

The experiments have shown that the nature of the extractant, temperature regime of the extraction process have a considerable impact on the yield of extractable substances (Figure 3). According to the Fik-Shchukarev's law, the amount of dissolved substance, diffusing through an extractant layer was directly proportional to the difference in concentrations of this layer, time and surface area of the layer and inversely proportional to the thickness of the diffusion layer. So, less viscous extractants had greater diffusion capacity. Increasing temperature of the extractant reduced the kinematic viscosity. Therefore, heating was used to reduce the viscosity during extraction **(Kňazovická et al., 2015)**. In cream, high viscosity was caused by milk fat and particularly by the formation of clusters of fat globules; structural viscosity was due to the interaction between protein and water. In cream, it was not casein that interacted, but shell protein that had larger contact surface with water, which reduced the diffusion coefficient; and mixing the extracting raw materials lead to mechanical destruction of hawthorn fruit tissue and mixing colloidal particles, which made the process of separation of the mixture after extraction more difficult. Therefore, the extract on the basis of cream had low values of extractable substances. Thus, whey and milk were established to be the most suitable extractants for the extraction of biologically active substances.

Some researchers extract hawthorn fruit using acidic methods for efficient pectin extraction **(Yenipinar and Yildirim, 2014)**.

A number of scientists also emphasize the influence of plant materials grinding and choice of temperature parameters on the extraction process **(Ivanova et al., 2008; Titova and Aleksanyan, 2013; Terletskaya et al., 2013)**.

The conducted experiments showed that the type of extractant and temperature regime of the extraction process had a significant impact on the yield of extractable substances. Compared with other extracts obtained, the whey extracts had an excess of tannins due to the low value of its active acidity, contributing to the intensification of the extraction process by converting low soluble compounds into highly soluble ones. High extraction dynamics of pectic substances was observed when milk being used as an extractant due to the interaction of pectin with casein and free calcium ions. The use of milk as an extractant also made it possible to obtain a high quantitative content of dietary fiber.

The study showed a direct relationship between the changes in the temperature regime of the extraction process and yield of extracted substances. The smallest quantitative content of extractable biologically active substances was observed at a temperature of 50 ºC. An increase in temperature caused their content increasing in direct proportion until the onset of dynamic equilibrium. The temperature of 60 ºC contributed to better separation of plant tissues and tearing of the cellular walls of hawthorn fruit, which led to the highest yield of extractable substances. So, in milk extract, the content of pectic substances was 62.98 $mg.100g^{-1}$, dietary fiber 176.08 $mg.100g^{-1}$. In whey extract contained tannins of 63.91 $mg.100g^{-1}$. These exceeded the weight fractions of tannins by 4.17%, pectic substances by 3.22% and cellulose by 3.82% compared with the extracts produced at a temperature of 50 °C.

The 70 °C temperature regime applied in the hawthorn fruit extraction led to peptization of substances, the extracts became slimy, and the extraction dynamics of biologically active substances decreased, the extractable substances in the obtained extracts had lower values than in extracts produced at 60 °C. The conducted studies showed that the most appropriate temperature regime for extracting hawthorn fruit was 60 °C.

Next, we identified the organoleptic characteristics of whey and milk 5% hawthorn fruit extracts. Tannins were characterized by a special tart, astringent taste, so the high content of these substances was unlikely to have the best effect on the organoleptic characteristics of the extracts, and further deteriorate the quality indicators of the product; therefore, it was important to conduct these studies. Organoleptic characteristics of the extracts are shown in Table 1.

The analysis of the indicators presented in Table 1 showed that the milk extract had higher organoleptic characteristics that allowed milk to be chosen as an extractant for further

research. Despite the high content of biologically active substances, whey extract was inferior to milk extract with respect to its organoleptic characteristics because of its inharmonious, sour, astringent taste of whey and tannins in it.

The influence of the duration of the extraction process on the intensity of hawthorn fruit extraction was studied. For this purpose, 5% hawthorn fruit plant material was extracted with milk at a temperature of 60 ºC for 20, 30, 40 and 50 minutes. The obtained extracts were examined to determine the quantitative content of biologically active substances; the results are presented in Figure 3. Figure 3 shows that the increase dynamics of the extractable substances content in the milk extract was observed in the period of 20 – 30 minutes; during this period, biologically active substances had time to dissolve and diffuse before they coagulated or swelled. A longer extraction process within 40 – 50 minutes decreased their concentration; the extracts became too viscous; the dissolved substances diffused into the extractant more slowly; and the yield of substances decreased. Thus, during the 30 minutes extraction, the diffusion and osmosis of biologically active substances proceed at the greatest speed; their maximum extraction occurred, so, this time mode of extraction was established to be the most acceptable.

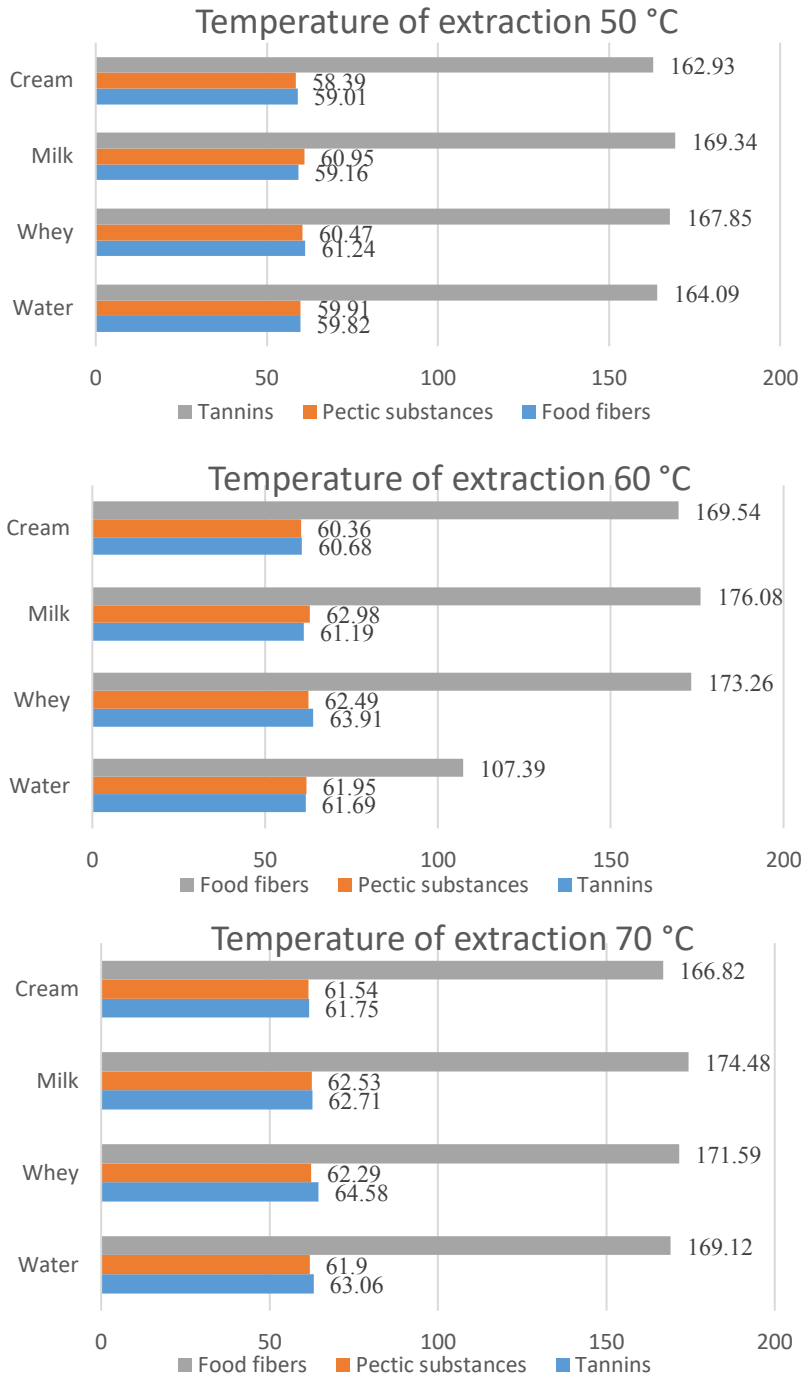

Figure 2 Weight fraction of extractable substances in the extract with respect to the extractant.

Table 1 Analysis of the indicators.

Indicator	Whey extract	Milk extract
Appearance and Consistency	Homogeneous consistency	Homogeneous consistency
Taste and smell	Inharmonic, sour, astringent taste of the extract with light aroma of hawthorn fruit	Milky taste and smell with pleasant aroma and sweetish taste of hawthorn fruit
Color	Brown, uniform throughout the mass	Cream, uniform throughout the mass

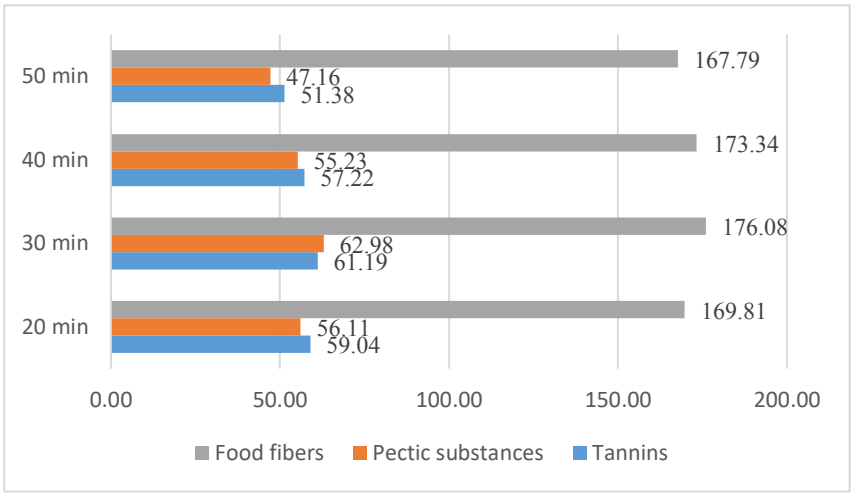

Figure 3 Weight fraction of extractable substances in the extractant (milk) with respect to the duration of extraction, mg.100g^{-1}.

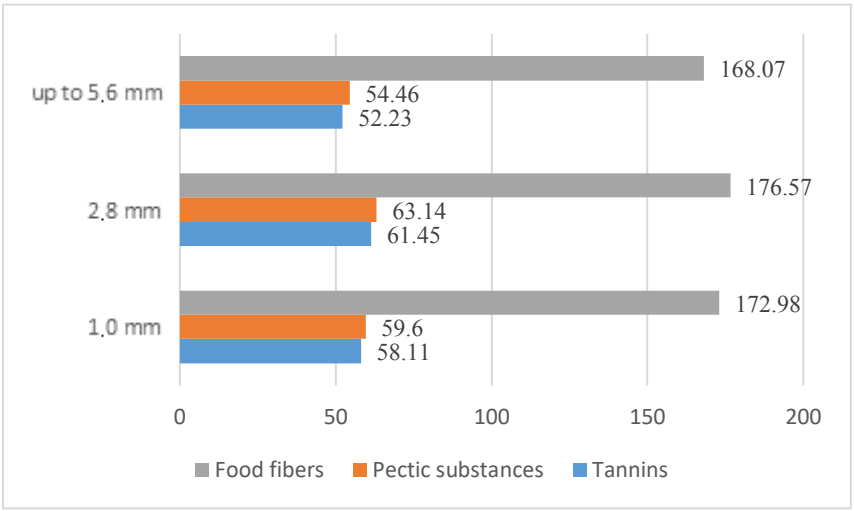

Figure 4 Weight fraction of extractable substances in the extractant (milk) with respect to the duration of extractionmg.100g^{-1}.

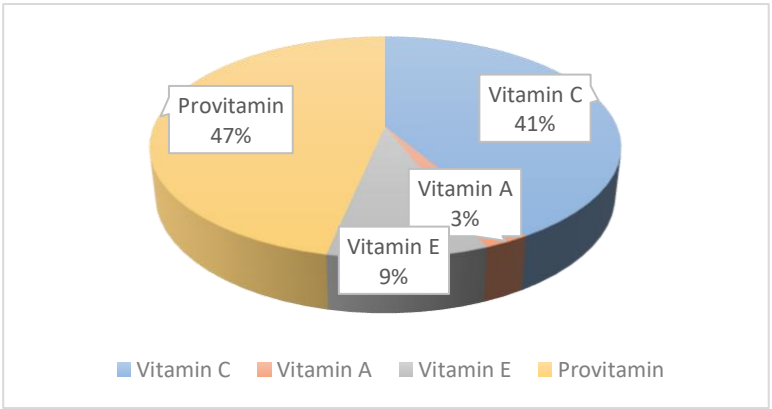

Figure 5 Vitamin composition of hawthorn fruit extract, mg.100g^{-1}.

To study the influence of the grinding size of dry hawthorn fruit on the yield of substances extracted, the raw material was ground before particles passed through a sieve with a hole diameter of 1.0, 2.8 and 5.6 mm and extraction with milk at a temperature of 60 °C for 30 minutes (Figure 4). The influence of the particle size of plant materials on the effectiveness of extraction was confirmed by the studies of some scientists; so, a decrease in the particle size of crushed raw materials (up to a certain minimum size) causes an increase in internal and external diffusion as well as mass exchanging, but the minimum size of raw materials must be accurately determined **(Iudina, 2015; Okhrimenko et al., 2011)**.

The data presented indicates that the appropriate grinding size for this type of raw material was a particle size of up to 2.8 mm, this grinding size contributed to the enlargement of the contact surface of the raw material with the extractor, its rapid penetration into the cells and hence the acceleration of the extraction process. The yield of extractable substances turned out to be maximum; the extract contained 61.45 mg.100g^{-1} of tannins, 63.14 mg.100g^{-1} of pectic substances and 176.57 mg.100g^{-1} of fiber.

Experimental studies indicated that a strong mechanical effect on the phytocomponent, i.e., grinding up to 1.0 mm in particle size was impractical and contributed to excessive cell destruction. An increase in grinding size of plant material enhanced the number of cells with a destroyed membrane and accelerated the leaching process. Fine powder of plant materials got easier compressed. In extracting, substances rapidly dissolved and swelled, and lumps were formed due to the adhesion of the slimy cells, settling to the bottom of the mug. All this greatly slowed down the extraction process. When grinding up to the size of 5.6 mm, active substances were incompletely extracted; in this extract, the pectic substances concentrated less and decreased by 8.68 mg.100g^{-1}, tannins by 9.22 mg.100g^{-1} and dietary fiber by 8.56 mg.100g^{-1} compared to the milk extract with raw material of a particle size being up to 2.8 mm. The vitamin composition of hawthorn fruit milk extract was determined. The results are presented in Figure 5.

The studies of the vitamin composition showed that the 5% hawthorn fruit milk extract contained the highest concentration of provitamin A (0.575 mg.100g^{-1}) that had antioxidant, adaptogenic and immunomodulatory properties and vitamin C (0.511 mg.100g^{-1}). The need for vitamin C especially increases in diseases of the gastrointestinal tract; ascorbic acid helps to strengthen the immune system and restore intestinal cells; it accelerates the elimination of toxins and toxins and improves digestion.

The optimal parameters of the extraction process are the extraction temperature of 60 °C and extraction time of 30 minutes. The decrease in these parameters was directly proportional to the yield of extracted biologically active substances; the increase in the temperature and duration of extraction also had a negative tendency, namely, the extracts became slimy and viscous, solutes diffused more slowly, and the dynamics of their extraction decreased.

The grinding size of dry hawthorn fruit was also important for the extraction; with respect to the high content of biologically active substances in the extract, the appropriate grinding size of the phytocomponent was the particle size up to 2.8 mm. The resulting extract contained vitamins C and E, provitamin A and vitamin A that increase the physiological value of the fermented milk product developed.

CONCLUSION

The experimental studies showed that the extraction is a complex process, combining diffusion, desorption, dissolution, dialysis and leaching. Plant material extracting is influenced by a number of factors that must be taken into account, i.e., the pH of the extractant, the types of extractant and raw material, grinding size of raw material and parameters of the technological process. The studies of the influence of various factors on the extraction efficiency of tannins, pectic substances and dietary fibers showed that milk was the optimal extractant with a high extraction dynamics of pectic substances and dietary fiber; the milk extract had high sensory characteristics, i.e., a homogeneous consistency, milky taste and smell with pleasant aroma and sweetish taste of hawthorn fruit, cream colour and uniform throughout the mass. Thus, the identified factors influencing the extraction process made it possible to establish that the statistical method of maceration (with stirring) with grinded hawthorn fruit being extracted allows obtaining an extract not only with a high content of pectin, tannins, dietary fibers and some vitamins, but with great sensory parameters. The introduction of the extract in food production will improve the quality indices and nutritional and biological values.

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PHYSICO-CHEMICAL STUDY OF STEROIDS FROM DIFFERENT MATURENESS CORN SILK MATERIAL

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ABSTRACT

This study shows an ultrasonic assisted extraction procedure of steroids from corn silk (CS). The total steroids contents were positively correlated with the ultrasonic assisted extraction time. The extracted steroids contents varied according to the different maturity stages of CS. There were three tested CS maturity stages: silking stage (CS-S), milky stage (CS-M) and mature stage (CS-MS). The β – sitosterol standardization method with 530 nm wavelength colorimetric measurements were applied to determine the content of extracted steroids. Measured steroids concentrations range were from 38.3×10^{-3} mg.mL^{-1} to 368.9×10^{-3} mg.mL^{-1} in different extraction time and CS maturity stages. The highest concentration of steroids, 368.9×10^{-3} mg.mL^{-1} was found in CS-MS sample with the 75 minutes ultrasonic extraction time. The fluorescence mapping techniques were used to confirm the existence of steroids. The thermal analysis illustrated a typical multistep decomposition process for the CS-S, CS-M and CS-MS samples. Two endothermic peaks were found: The first one was 54.3 °C for CS-S and CS-MS, 60.2 °C for CS-M, the second one, 397 °C (CS-MS), 415.1 °C (CS-M) and 419.7 °C (CS-S) attributed to the total thermal decomposition. The observed exotheric process found at 524 °C corresponded to CS-MS sample decomposition. The optimal ultrasonic-assisted extraction time for all samples under study CS-S, CS-M and CS-MS was about 75 minutes and the optimal steroids extraction contents obtained were 92.8×10^{-3} mg.mL^{-1} (CS-S), 124.2×10^{-3} mg.mL^{-1} (CS-M) and 368.9×10^{-3} mg.mL^{-1} (CS-MS) respectively.

Keywords: corn silk; ultrasonic extraction; maturity stages; steroids; UV-VIS

INTRODUCTION

Corn silk (CS) can be defined as the dried thrum and stigma of *Zea mays* L. (corn) which is cheap and high yielding. The by-product of agriculture is the common use of CS to be abandoned, burned or applied as fodder. Corn silk is remarkablely functional to the clinical diseases as diabetes, nephritis and hypertension etc **(Jin, 1980)**. Additionally, morden researches also revealed the healing functions of anti-fatigue **(Hu et al., 2010)**, anti-depression **(Mahmoudi and Ehteshami, 2010)**, anti-free radical, anti-cancer **(Ebrahimzadeh, Pourmorad and Hafezi, 2008; Maksimović and Kovačević, 2003)** and anti-radiation. Corn silk has been used as a cure to urinary tract infection, malaria and heart disease by aboriginal American Indians. Furthermore, the properties of cooling blood, purging heat and removing the damp and heat from human body of CS were also used as weight-losing products in many countries. It was found by the acute toxic test on rats, that cosrn silk extracts were not causing any mortality and was non-toxic at the dose of up to 5 g.kg^{-1} body weight. However at the doses about 1000 mg.kg^{-1} in long term treatments, toxic effects in liver were observed **(Ikpeazu et al., 2018)**.

The previous studies of the improvement of chicken meat nutrition quality by the fermentated corn fodder have made a success **(Angelovičová and Semivanová, 2013; Mačanga et al., 2017; Štenclová et al., 2016)**. Aside from that, corn products were also contributed to the improvement of the sensory quality of crackers **(Kuchtová et al., 2016)**. The alchohol from the fermentation technology of CS has been used in food and chemical industry **(Krejzová et al., 2017; Süli, Hamarová and Sobeková, 2017)**.

Corn silk steroids (CSS) are significantly valuable in the research of its nutrients, which is the cause of the functions of anti-cancer, anti-oxidant, cholesterol – reducing of the corn silk extracts **(Li and Lapcik, 2018)**. The total steroids extractive technology includes solvent crystallization, complexometry, saponification, distillation (simple distillation, molecular distillation), adsorption (column adsorption, high pressure fluid adsorption), supercritical carbon dioxide extraction, enzymic method etc. **(Hossain et al., 2014; Ren et al., 2015)**. **Ren et al. (2015)** extracted total steroid saponins from *Dioscorea zingiberensis* by means of microwave-assisted technique, the optimal extracting conditions were established as 75% ethanol as solvent, ratio of solid/liquid 1:20 (g.mL^{-1}),

temperature 75 °C, irradiation power 600 W and three extraction cycles of 6 minutes each **(Hossain et al., 2014; Ren et al., 2015)**. **Hossain et al. (2014)** applied ultrasonic-assisted technology on the extraction of steroidal alkaloids from potato peel waste, used response surface methodology to optimize the extracting conditions. The optimal ultrasonic-assisted extraction conditions were identified as an amplitude of 61 μm and an extraction time of 17 min which resulted the recovery of 1102 μg steroidal alkaloids per g dried potato peel. In contrast, solid liquid extraction yielded 710.51 glycoalkaloid $\mu g.g^{-1}$ dried potato peel. Recoveries of individual glycoalkoids using ultrasonic-assisted extraction yielded 273, 542.7, 231 and 55.3 $\mu g.g^{-1}$ dried potato peel for α-solanine, α-chaconine, solanidine and demissidine respectively. Whereas for solid liquid extraction yields were 180.3, 337.6, 160.2 and 32.4 $\mu g.g^{-1}$ dried potato peel for α-solanine, α-chaconine, solanidine and demissidine respectively **(Hossain et al., 2014; Ren et al., 2015)**.

UV-VIS method is a common methodology to determine the content and kinds of steroids **(Antonisamy and Eahamban, 2012)**. However, the optimization of extraction conditions, steroids properties comparison and the determination of the steroids kind according to the various maturity stages of corn silk are rarely reported. In this study, we expect the maturity stage will be a vital influential factor in the extracted total steroids content. This study focuses on the extraction procedures evaluation, kinetics and the maturity stage effects on the CS extracts, allowing in more detailed knowledgement for the developing novel nutrition or health care products to contribute to the human and veterinary applications.

Scientific hypothesis

UV-VIS and fluorescence techniques have been applied for the content and sorts of steroids in plants in modern research. However, the extraction conditions optimization, steroids properties comparison and analyzation and the determination of the steroids kind in different maturity stages of CS are rarely reported. We expect the maturity stage will be a significant effective factor for the total extracted steroids content. That is why, this research is focused on the quantification of the total steroids content extracted from different maturity stages of CS at defined ultrasonic-assisted extraction times.

MATERIAL AND METHODOLOGY

Corn silk samples were collected from the corn kernels type dent produced in a field in Southern Moravia agricultural region (Uherské Hradiště County, Czech Republic). Fresh corn silk fibers were first 14 days dried on air in the shade and then final drying was done in a thermostatic hot air drying oven (Hot air sterillizator Stericell 55 Standard, BMT Medical Technology, Czech Republic), pulverized and sifted through a 80 mesh sieve (Analysette 3, Fritsch, Germany) to obtain the final product powder samples. There were collected three types of corn silk materials, dependent on the growth stage. The first one was silking stage (assigned as CS-S), the second one was the milky stage (assigned as CS-M), the third one was mature stage (assigned as CS-MS) **(Rahman and Wan Rosli, 2014; Sarepoua et al., 2015)**.

All reagents and chemicals used in this research such as β-sitosterol, ethanol, phosphoric acid, sulfuric acid and ferric chloride were purchased from Sigma-Aldrich (USA) in an analytical reagents purity grade. As a solvent distilled water was used. Distilled water conductivity was about 0.6 $\mu S.cm^{-1}$).

UV/VIS spectrophotometer used was Lambda 25 (Perkin Elmer, MA, USA). Measurements were performed in the wavelength range from 200 to 700 nm in 1 cm quartz cells **(Marques et al., 2013)**. Thermogravimetry (TG) and differential thermal analysis (DTA) experiments were performed on simultaneous DTA-TG apparatus (Shimadzu DTG 60, Japan). Measurements were performed at heat flow rate of 5 $°C.min^{-1}$ in the static nitrogen atmosphere (gas flow of 50 $mL.min^{-1}$) at the temperature range from 30 °C to 550 °C. The apparat was calibrated using Indium as a standard **(Liu et al., 2005; Wu et al., 2008)**.

Fluorescence excitation-emission maps of the different maturity stages corn silk extracts were measured on a FLS980 fluorescence spectrometer (Edinburgh Instruments, UK). Each experiment was repeated 10 times.

Samples were pulverized in a table top blender (Philips HR2170/40, The Netherlands).

β-sitosterol standard curve determination procedure **(Hossain et al., 2014)**: Precisely weigh 10 mg β-sitosterol into 10 mL volumetric flask, use absolute ethyl alcohol dilute to scale. Fetch 1 mL above solution into 10 mL volumetric flask, use absolute ethyl alcohol to dilute to scale as the standard sample solution. Precisely move the standard sample solution 0, 1, 2, 3, 4, 5 mL into 50 mL conical flask, separately add 5, 4, 3, 2, 1, 0 mL absolute ethyl alcohol, then slowly pour the pulfate-phosphate-ferric reagent 5 mL long the cup wall into every conical flask separately, shake up, cool down in room temperature for 20 min. Then was measured the 530 nm absorbance by UV spectrometry. Each experiment was repeated 5×. Obtained absorbance vs. concentration dependency data were used to build up the standard curve. The numerical linear regression analysis was performed to obtain standard curve lineasr regression parameters. Each experiment was repeated 3×.

Determination of the sterols content procedure **(Hossain et al., 2014)**: weigh 5 samples 3 g corn silk powder, add 70% ethanol (material: liquid = 1:20), then use 200 W ultrasonic extract for 15 min, 30 min, 45 min, 60 min, 75 min for the silking, milky and mature stages. Then was used 119 W microwave extraction apparatous for 8 min, followed by addition of pulfate-phosphate-ferric reagent to process for 20 min (the same procedure as for standard curve determination), then measure the 530 nm absorbance by UV spectrometry.

Then there was used the standard curve to count the content of sterol in prepared corn silk extracts. Each experiment was repeated 5×.

Statistic analysis

Statistical analysis of the observed data was performed by application of the one way analysis of variance (ANOVA) method (Microsoft Excel, USA). This analysis allowed to detect the significance of the effect of ultrasonic-assisted extraction time and the maturity stage on extracted amounts of steroids. Five ultrasonic-assisted

extraction times and three maturity stages were considered in this study. Each experiment was replicated 5 times. Differences were considered significant at $p \leq 0.05$. Additionally, the mean values and standard errors were calculated from all measurements by application of the SigmaPlot 8.0 software (SPSS, USA). Differencies between obtained emission peaks located at 530 nm were analyzed by one way analysis of variance (ANOVA) method (Origin 8.5.0 software was used (OriginLab, USA)). Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the SEM images of the tested corn silk powders. The rectangular shape of individual particles illustrates the complex microporous structure on the intersection as a typical botanic cellulose based materials. The moisture content and thermoal analysis by TG and DTA of the samples were resulted as in Figure 2, which are the typical multistep decomposition process for all CS-S, CS-M and CS-MS samples as shown in Figure 2. The first step decomposition of CS-S was in the temperature range from 30 to 120 °C with observed weight loss 8.3% attributed to the moisture content. Total decomposition step was about 77.45% in the temperature range of 30 to 550 °C. Similarly, for the sample CS-M and CS-MS, TG data exhibited the first step decomposition of 5.9% and 10.04% in the same temperature range as CS-S followed by the total weight loss of 65.5% and 83.88% in the temperature range of 30 to 550 °C, which indicated the CS-MS contains the most thermally labile substances compared with CS-S and CS-M.

There are two endothermic peaks in Figure 2. The first one was located in the temperature of 54.3 °C for CS-S and CS-MS, 60.2 °C for CS-M attributed to the melting point of flavonoids.

The second one was observed at 397 °C (CS-MS), 415.1 °C (CS-M) and 419.7 °C (CS-S) attributed to the total thermal decomposition with the formation of a low quantity carbonaceous residues respectively. Observed exotheric process at 524 °C corresponds to the decomposition CS-MS sample.

The β-sitosterol standard curve is shown in Figure 3, the regression parameters and inset as well. The Obtained data were highly correlated as the correlation coefficient 0.999.

Figure 4 shows the effects of the Ultrasonic time and different maturity stages by the steroids concentration vs. ultrasonic time correlations. All of the CS-S, CS-M and CS-MS were a non-linear character, modeled as a third order polynomial dependencies. However, all of those three stages extraction contrations had a direct proportionality trend with the increase of the ultrasonic processing time. All of the three stages samples had an obvious increasing range of the extraction concentration from ultrasonic extraction time 15 min to 60 min. From 60 min to 75 min, three samples showed a similar steady tendency which means from 60 min to 75 min the increase of the concentration is not conspicuous anymore. Therefore, the 75 min ultrasonic extraction time can be marked as the optimum extraction time to obtain maximum extracted content for all of the three stages. Obtained maximum concentrations were as follows: CS-S 0.09 $mg.mL^{-1}$, CS-M 0.12 $mg.mL^{-1}$, CS-MS 0.37 $mg.mL^{-1}$. It is noteworthy, that the CS-MS had a much higher maximum extraction concentration as well as the increasing rate in comparison to CS-S and CS-M samples. This imply that the CS-MS has much higher content of steroids than CS-S and CS-M. Simultaneously, the ultrasonic assisted technique can be sonsidered to be much more effective to CS-MS sample extraction rather than for CS-S and CS-M. , which is in agreement with the results of UV-VIS and fluorescence excitation-emmision mapping illustrated in Figure 5, Figure 6, Figure 7 and Figure 8.

The UV-VIS as well as fluorescence spectra were measured as shown in Figure 5, Figure 6, Figure 7 and Figure 8. These were typical three major light absorption regions at 350 nm (near ultraviolet region) and visible light region of 500 nm and 650 nm. The absorption of electromagnetic radiation in the visible light region is typical for anthraquinone and phenanthrene compounds such as steroids. All of the CS-S, CS-M and CS-MS exhibited similar UV-VIS spectra.

Results of the fluorescence excitation-emmision mapping of the studied extracts are shown in Figure 8. These are characteristic similarly as the UV-VIS absorption spectra with the three distinct fluorescence enission regions at 300 nm, 430 nm and 680 nm.

There were found three distinct excitation wavelengths regions at about 275 nm, 350 nm and 380 nm. Obtained results indicate the major difference between CS-S, CS-M and CS-MS is in the fluorescence emission centered at the 300 nm and 430 nm regions. The highest intensity of the fluorescence emission at 300 nm was found for CS-MS extracted at 40 °C for 15 minutes in the ultrasonic extraction bath. Furthermore, there was found that the fluorescence emmision intensity region located at 430 nm region was of the highest intensity for CS-MS as well. Observed results were considered as statistically significant ($p \leq 0.05$). However, there was not found any major difference between fluorescence emmission intensity located at 670 nm region for all studied materials.

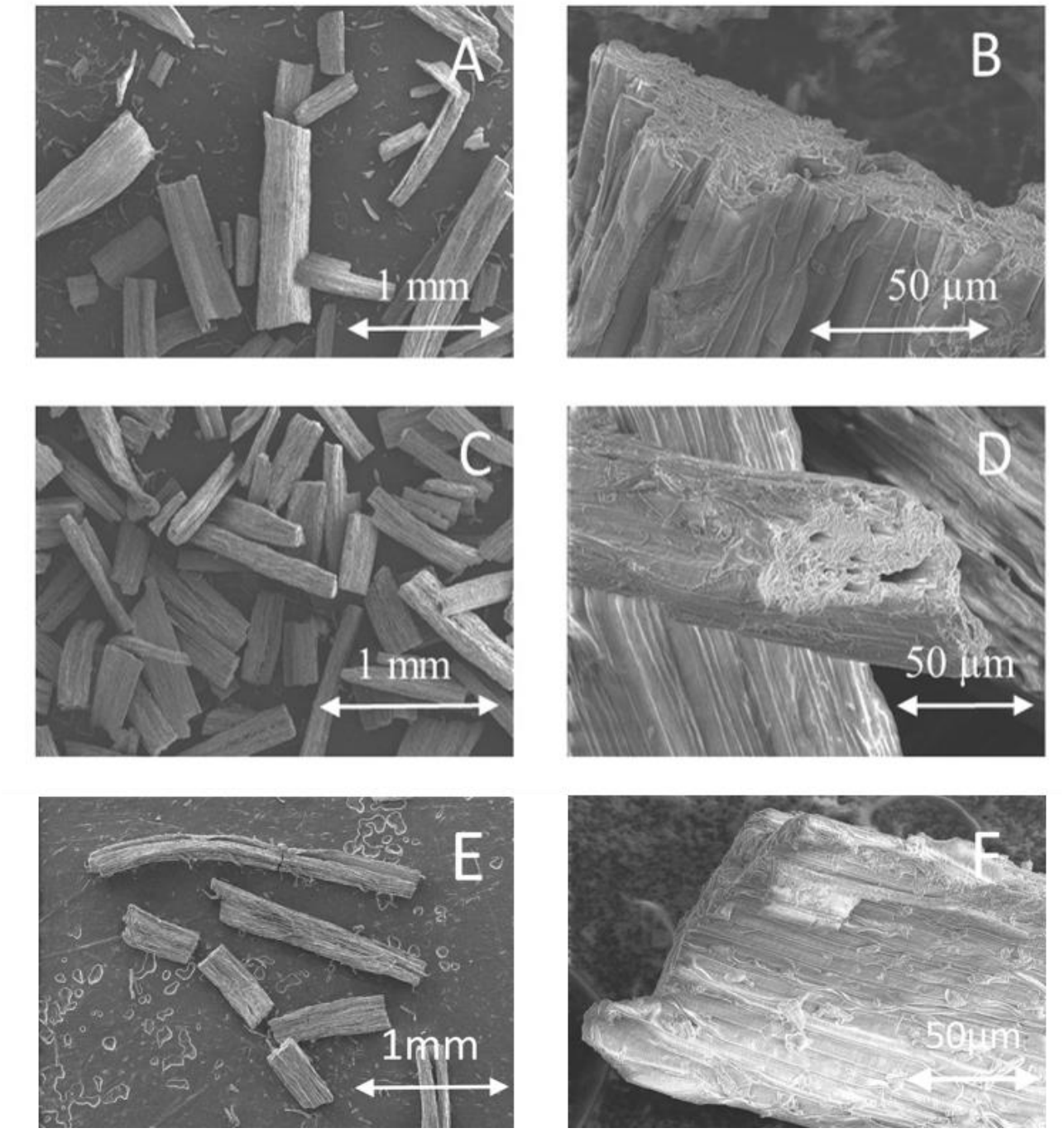

Figure 1 Studied corn silk SEM images. Note: A,B – sample CS-S, C,D – sample CS-M, E,F – sample CS-MS.

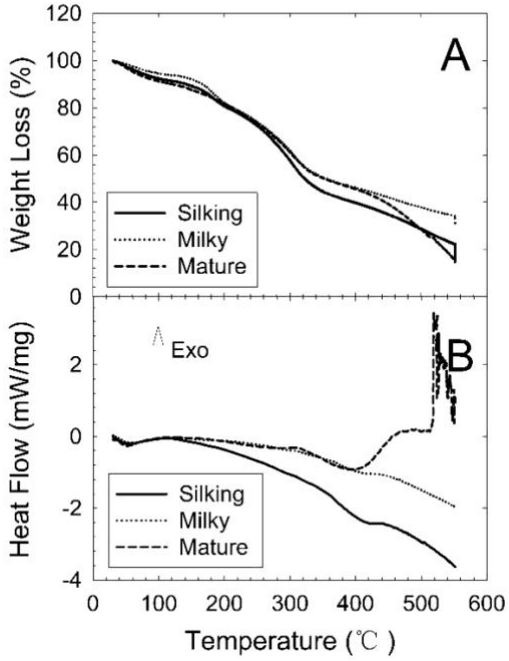

Figure 2 Thermal analysis of corn silk samples. Note: A – Thermogravimetry (TG), B – differential thermal analysis (DTA).

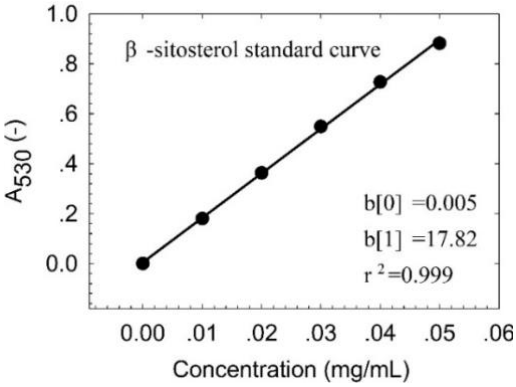

Figure 3 β-sitosterol standard curve. Note: Inset: Linear regression standard curve parameters.

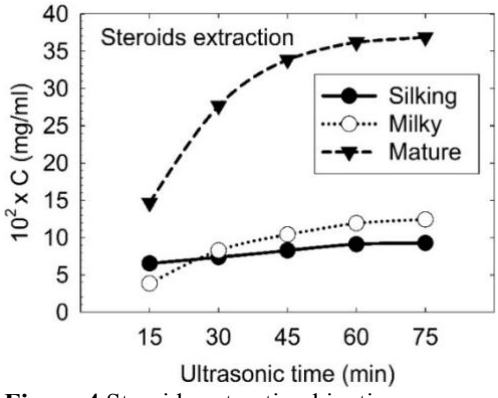

Figure 4 Steroids extraction kinetics.

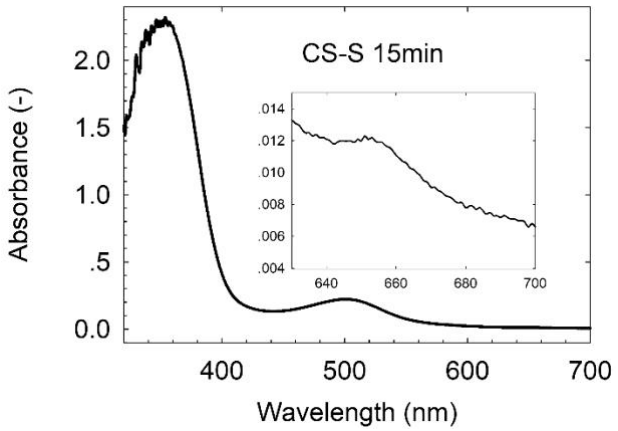

Figure 5 UV-VIS spectrum of the CS-S sample extracted at 15 min ultrasonic-assisted extraction time. Note: Inset: expanded 630 nm to 700 nm region.

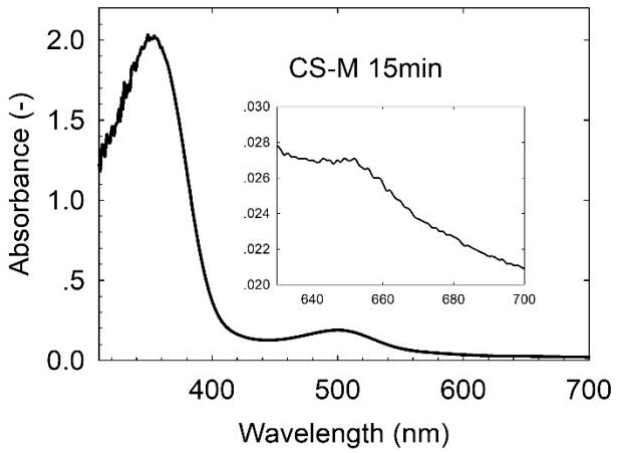

Figure 6 UV-VIS spectrum of the CS-M sample extracted at 15 min ultrasonic-assisted extraction time. Note: Inset: expanded 630 nm to 700 nm region.

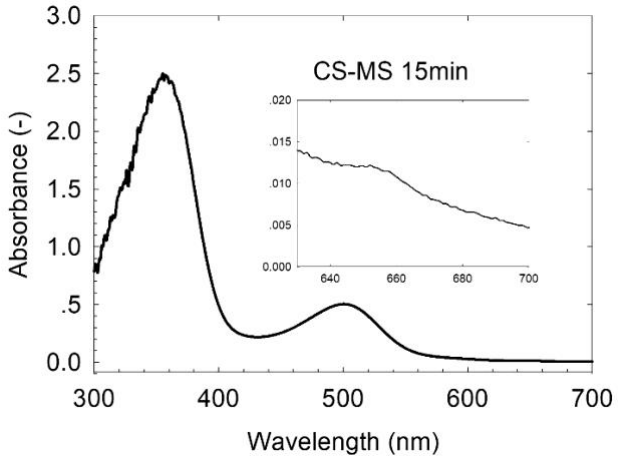

Figure 7 UV-VIS spectrum of the CS-MS sample extracted at 15 min ultrasonic-assisted extraction time. Note: Inset: expanded 630 nm to 700 nm region.

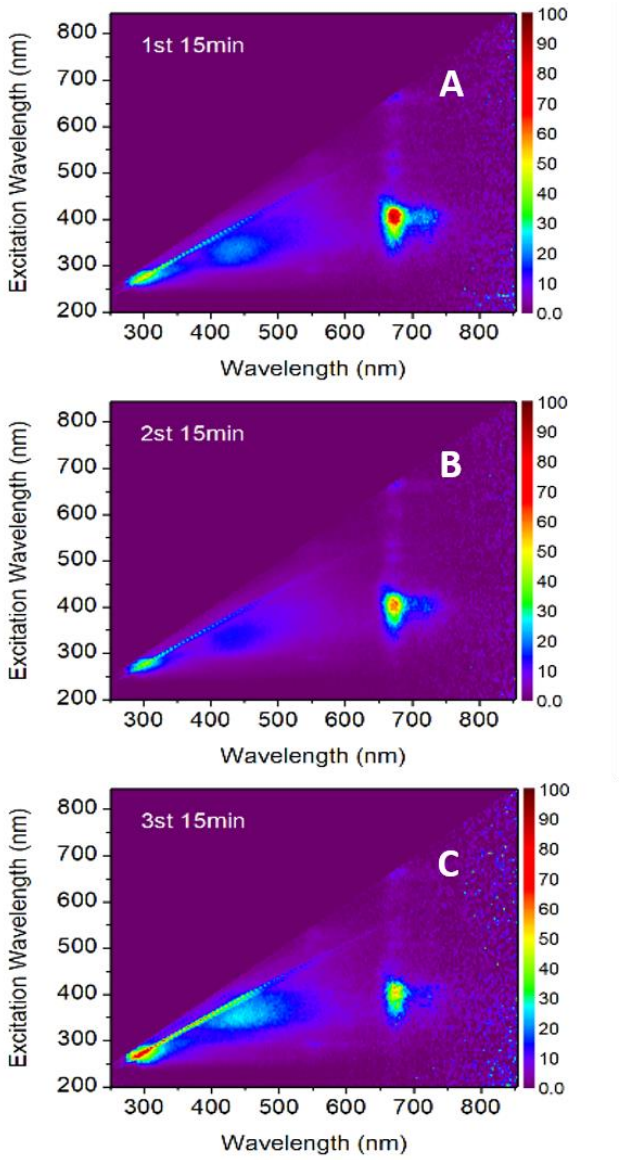

Figure 8 Results of the fluorescence excitation – emmision mapping of the studied corn silk extracts. Note: Inset legend: A – corresponds to CS-S sample, B – CS-M sample, C – CS-MS sample. Extraction temperature (40 °C or 80 °C), 15 min ultrasonic extraction time.

CONCLUSION

The ultrasonic assisted extraction procedure was ascertained to be valid for the CS steroids extraction. The total steroids contents were positively correlated with the ultrasonic assisted extraction time. The extracted steroids contents varied according to the different maturity stages of CS (silking stage, milky stage, mature stage). The β-sitosterol standardization method was applied to quantity the extracted steroids content by the 530 nm wavelength colorimetry measurements. Measured steroids concentrations range were from 38.3×10^{-3} mg.mL^{-1} to 368.9×10^{-3} mg.mL^{-1} in different extraction time and CS maturity stages. The highest concentration of steroids about 368.9×10^{-3} mg.mL^{-1} was observed for CS-MS sample after 75 min ultrasonic processing extraction time. The fluorescence mapping technique confirmed the highest extraction efficiency of steroids by ultrasonic treatment was at 40 °C from the CS-MS samples. A typical multistep thermal decomposition process was found for all of the CS-S, CS-M and CS-MS materials.

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THE IMPACT OF FREEZING METHODS ON FUNCTIONAL AND TECHNOLOGICAL PROPERTIES OF SEMI-FINISHED RABBIT MEAT PRODUCTS

Dodo Tavdidishvili, Davit Tsagareishvili, Tsira Khutsidze, Manana Pkhakadze, Lana Kvirikashvili

ABSTRACT

During storage meat and semi-finished meat products, the decisive factor is the correct implementation of freezing process, because the physical, histological, biochemical, microbiological changes that occur at this time, affect the final quality of product after defrosting procedure. Accordingly, studies of the impact of traditional and shock-freezing methods on functional and technological properties of semi-finished rabbit meat products represent scientific and practical interest. The effect of freezing temperature of natural and minced semi-finished rabbit meat products on the duration of cold treatment process has been studied, and the regression equations of their relationships have been obtained and regression curves have been constructed. It has been established that unlike traditional method, by the type of semi-finished products, the duration of cold treatment by shock-freezing method is 3 – 3.5 times shorter. The optimal freezing parameters have also been selected. It has been shown that the use of shock-freezing method contributes to the reduction of mass losses of semi-finished rabbit meat products, and by the type of semi-finished products, they are 5 – 5.3 lower as compared to traditional method of freezing. The determination of functional properties has revealed that shock-freezing allows for increasing water binding capacity-by 23.2 – 31.9%, water holding capacity-by 20 – 25% and pH by 6.7 – 10.4%. There have been studied microbiological indicators of frozen semi-finished products and their changes during the storage process. It has been established that they meet the safety and hygiene requirements for meat products. The full results obtained indicate the advantage of shock-freezing method as compared to traditional methods, as well as point to the appropriateness of its use when selecting the cold treatment modes for semi-finished rabbit meat products.

Keywords: rabbit meat; shock freezing; semi-finished products; functional properties; microbiological indicators

INTRODUCTION

The most important problem of modernity is to meet the population's needs for high quality and safe food products.

With a view to preserving the quality of foodstuffs and their safe storing for an extended period of time, of particular importance is the use of modern refrigeration technologies and the development of rational regimes cold treatment **(Bolshakov, 2003; Feiner, 2010; Hip, 2007; Erlihman, 2010; Evans, 2008; Farouk, Wieliczko and Merts, 2004)**.

During storage meat and meat products, as well as other perishable food raw materials, the decisive factor is the correct implementation of freezing process, because the physical, histological, biochemical, microbiological changes that occur at this time, affect the final quality of product after defrosting procedure **(Rogov, Zabashta and Kazyulin, 2009; Vieira et al., 2009; Gambuteanu, Borda and Alexe, 2013; Chwastowska-Siwiecka et al., 2013; Swami, Raut and Rindhe, 2015; Wang, He and Li, 2018)**.

When refrigerating meat and semi-finished meat products, the decisive factor, coupled with a temperature, is the rate of freezing. The process of crystallization of moisture existing in product, the sizes of generated ice crystals, their distribution in tissues and, consequently, maintaining the integrity of the tissue's natural structure, as well enzymatic changes in tissue and recovery of primary properties in the process of defrosting, depend on the dynamics of cold penetration into the depth of product.

Resaerch investigations of various authors have confirmed that the increase in the sizes of ice crystals in the freezing process is directly linked to disruption of a cell structure of tissue, and the most share of disruption of tissue cells takes place during freezing at slow rate. However, in some of the works, it is noted that in the superfast freezing process, there also occur substantial mechanical destructions in the tissue **(Jo et al., 2014; Onishchenko, Zheliba and Zinchenko, 2011; Leygonie, Britz and Hoffman, 2012)**.

The quality of the frozen product is achieved by the dynamics of the decrease of product temperature. In this regard, attention should be given to meat, in which 60 – 75% of the total weight is liquid. Accordingly, the freezing and defrosting processes are based entirely on the state of a liquid phase of the product.

It is known that the freezing process using traditional method takes places at temperature minus 18 °C and at an air velocity of 0.1 $m.s^{-1}$. In the case of different cooling equipment and different products, the duration of the process is 2.5 – 5 hours. At that time, due to a low rate of freezing within a temperature range from 0 to -5 °C, 70% of mosture existing in product is transferred from the liquid phase into the solid phase, the crystallization process begins, and due to slow freezing, the crystals are getting larger, which is accompanied by a substantial disruption of a cell structure of tissue.

Today, the efficient freezing and shock-freezing technologies are considered to be a promising area among the methods of cold treatment of meat products. Unlike traditional method, shock-freezing, due to high rate and low temperature, reduces 3 – 5 times the duration of freezing, prevents the increase in the sizes of crystals, considerably reduces mechanical destruction of tissue and contributes towards maintaining the structure of biological membrane. In addition, shock-freezing impedes bacteria development and activity, has a conservative impact on the natural autolytic processes of destruction of the protein structures **(Filippov, Kremenevskaya and Kutsakova, 2014; Evans, 2008; Soroko and Usenia, 2011; Qian et al., 2018)**.

Thus, the lower the meat freezing and storage temperature, the less change in the tissue structure caused by moisture redistribution and the increase in the liquid phase concentration, which is reflected in binding capacity of water and the loss of cellular fluid and nutrients dissolved in this fluid.

It should be noted that there are limited scientific data in the literature on the impact of freezing conditions on technological and functional properties on rabbit meat semi-finished products.

Based on the above, the goal of our study is to investigate the impact of different conditions of cold treatment of rabbit meat semi-finished products on the duration of freezing process and the functional-technological characteristics of semi-finished products.

Scientific hypothesis

By shortening the duration of freezing process, the shock-freezing method improves the functional-technological characteristics of product, with respect to the traditional method.

MATERIAL AND METHODOLOGY

The studies were carried out in the laboratories of the Department of Food of Akaki Tsereteli State University. Research covered semi-finished products produced from rabbit meat of Californian breed (their average age was 130 days): 1 – piece of rabbit meat – fillet; 2 – minced, made by traditional recipe; 3 – minced semi-finished product enriched with a plant-based supplement. As a plant-based supplement there was used lentil puree in the amount of 25 – 30% by mass of meat. The mass of all samples was 100 g, and the thickness was 20 mm.

The semi-finished products were frozen using the following methods: traditional freezing (freezing temperatures – T = -18 °C, air velocity – V = 0.1 $m.s^{-1}$ and relative humidity – φ = 85%), intermediate freezing (freezing temperatures – T = -30 °C, air velocity – V = 0.1 $m.s^{-1}$ and relative humidity – φ = 85%), and shock-freezing (freezing temperatures – T = -30 °C, air velocity – V = 9.4 $m.s^{-1}$ and relative humidity – φ = 87%), The freezing was carried out in a chest freezer in the wardrobe (KLIMASAN) at -18 °C, at an air velocity of 0.1 $m.s^{-1}$ and in a shock-freezing fridge (ATTILA GN 1/1 – 600x400 mm) at -35 °C, at an air velocity of 9.4 $m.s^{-1}$.

The freezing temperature in samples was determined by contact thermocouple thermodynamics (DIGITAL MULTIMETER DT9208A, the measuring range -40 °C – 1370 °C, measurement error – 1.5%). The product was considered frozen, when the temperature of product was fixed at minus 10 °C.

The mass losses during freezing and thermic treatment, we determined by the mass difference after the cold and thermic treatment of samples, water binding capacity – by press method of Grau and Hamm, water-holding capacity – by the difference between the amounts of moisture existing in semi-finished product and moisture released during thermic treatment **(Antipova, Glotova and Rogov, 2004)**, the pH medium was determined by potentiometric method.

Organoleptic indicators were determined based on a 10-point system, according to the following characteristics: appearance, color, smell, taste, consistency and succulence.

During microbiological analysis, the quantities of mesophylic aerobic and facultative anaerobic microorganisms in rabbit meat were determined according to state standard "Food products. Methods for determining the quantities of mesophylic aerobic and facultative anaerobic microorganisms" – **GOST 10444.15-94 (1994)**; number of bacteria of intestinal bacillus was determined according to State Standard "Food products. Methods for the detection and determination of the number of bacteria of the Escherichia coli group (coliform bacteria)" – **GOST 30518-97 (1997)**; Salmonella was determined according to State standard "Food products. Methods for the detection of bacteria of the genus Salmonella" – **GOST 30519-97 (1997)**.

Statistic analysis

To analyze the test parameters (mass losses, water binding and Water holding capacity) of rabbit meat, is conducted a statistical analysis of the obtained data, the reliability of the obtained data was evaluated by the mathematical statistics method T-test using the Windows IBM SPSS Statistics software program (version 20.0). To describe the ordered sample, we used statistical functions of the average arithmetic value and average standard error.

Graphical interpretation of the results was made by using Microsoft Excel. In Table 1, Table 2, Table 3, Table 4, Table 5 and Figure 1, Figure 2, Figure 3 and Figure 4, there are presented the data of typical tests, and each value is an average of at least ten determinations.

RESULTS AND DISCUSSION

In line with the target, using the different methods of freezing at the first stage of research, we determined the effect of freezing temperature of samples on the duration of their cold treatment. The results are shown in Figure 1A, Figure 1B, Figure 2A, Figure 2B, and Figure 3A, Figure 3B.

Figure 1A, Figure 1B, Figure 2A, Figure 2B and Figure 3A and Figure 3B illustrate that the process of cold treatment is significantly affected by the parameters, such as temperature and air velocity.

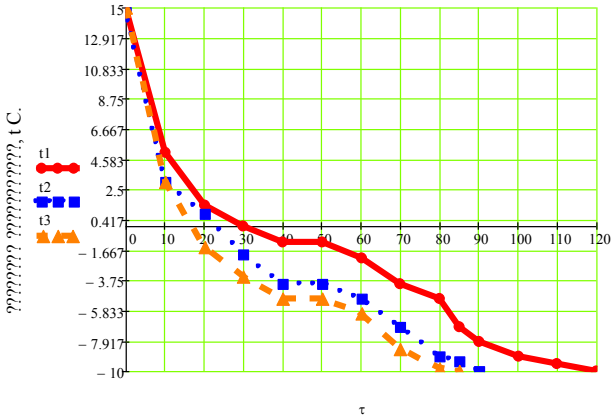

Figure 1A The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by traditional freezing method.
Note: (T = -18 ˚C, V = 0.1 m.s^{-1}, φ = 85%),
1. fillet,
2. natural minced semi-finished product,
3. minced semi-finished product enriched with a plant-based supplement.

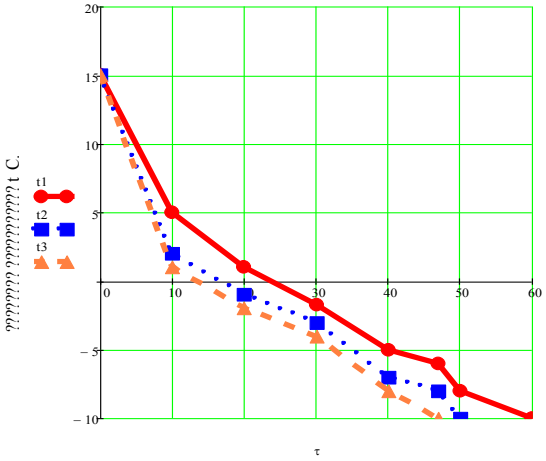

Figure 2A The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by intermediate freezing method.
Note: (T = -30 ˚C, V =0.1 m.s^{-1}, φ = 85%),
1. fillet,
2. Natural minced semi-finished product,
3. minced semi-finished product enriched with a plant-based supplement.

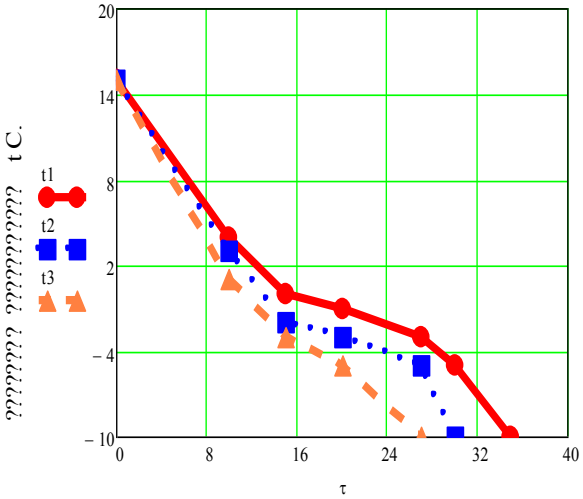

Figure 3A The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by shock-freezing method.
Note: (T = -30 ˚C, V = 9.4 m.s^{-1}, φ = 87%),
1. fillet,
2. natural minced semi-finished product,
3. minced semi-finished product enriched with a plant-based supplement.

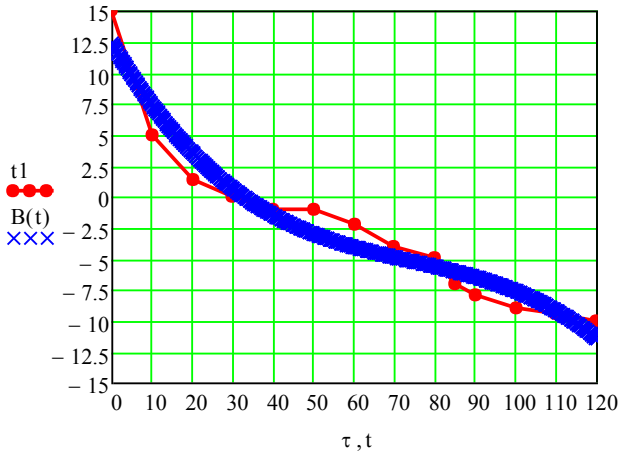

Figure 1B The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by traditional freezing method.

$b := regress(\tau, t1, 3)$

correlation coefficient

$corr(\tau, t1) = -0.961$

$b^T = (3 \;\; 3 \;\; 3 \;\; 14.619 \;\; -1.061 \;\; 0.022 \;\; -1.806 \times 10^{-4})$

Regression coefficient

$B(t) := interp(b, \tau, t1, t)$

$a := line(\tau, t1) \quad a = \begin{pmatrix} 10.857 \\ -0.379 \end{pmatrix}$

the equation of the regression line

$f(y) := a_0 + a_1 \cdot y$

Diagram of the approximation dependencies

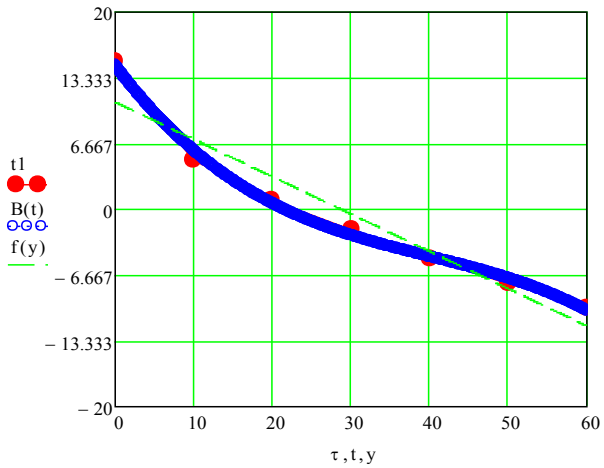

Figure 2B The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by intermediate freezing method.

$b := regress(\tau, t1, 3)$

correlation coefficient

$b^T = (3 \;\; 3 \;\; 3 \;\; 15.165 \;\; -1.631 \;\; 0.061 \;\; -9.975 \times 10^{-4})$

$$a = \begin{pmatrix} 10.857 \\ -0.379 \end{pmatrix}$$

Regression coefficient

$B(t) := interp(b, \tau, t1, t)$

$a := line(\tau, t1)$

the equation of the regression line

$f(y) := a_0 + a_1 \cdot y$

Diagram of the approximation dependencies

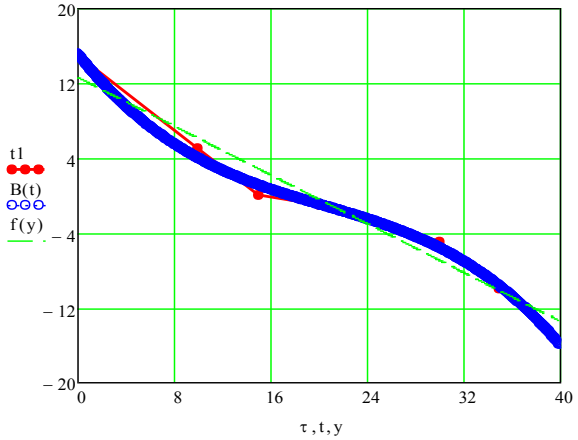

Figure 3B The effect of freezing temperature on the duration of freezing of semi-finished products during cold treatment by shock-freezing method.

We selected the value of reliability $p = 0.05$. In particular, the duration of freezing process in the case of shock-freezing is shortened relative to traditional method: 85 minutes off for rabbit meat fillet, 60 minutes off for natural minced semi-finished product, and 58 minutes off for minced semi-finished product enriched with a plant-based supplement. The difference between the freezing durations of different samples is explained by the different structures and compositions of samples.

A similar trend is reported in the data obtained by other authors **(Tsikin, 2012; Yablonenko, 2008; Ali et al., 2015; Jo et al., 2014)** in freezing similar semi-finished products produced from studied beef and pork, while in the case of rabbit meat, the obtained data are much better. This can be explained by the low content of fat in rabbit meat, the amount of which affects the duration of freezing.

Qualitative indicators of products (structure, storage stability and yield) are especially affected by the moisture content in them. Thus, at the next stage of research, we studied the functional-technological properties of samples: mass losses, water binding and water holding capacity of the study samples: mass losses, water connectivity and water retention capacity.

Table 1 and Table 2 outline the data on mass losses of the test samples under various cold treatment conditions and after thermic treatment.

The tables indicate that mass losses are as minimal in both cases of cold treatment and further thermic treatment, while using a shock-freezing method, and respectively, they are: for fillet – 0.7% and 9.5%; in the case of natural minced semi-finished products – 0.85% and 11.4%; while for minced semi-finished products enriched with a plant-based supplement – 0.75% and 10.2%.

In addition, under shock-freezing conditions, mass losses in minced rabbit meat semi-finished products are higher than in fillet, in particular, by 21.4%. In natural minced semi-finished products, while in minced semi-finished products enriched with a plant-based supplement, mass losses are 7.1% higher than in fillet, which is explained by a larger content of "free water" in fillet.

For their part, mass losses in fillet during shock-freezing are five times lower than during freezing by traditional method, while in minced semi-finished products – by 5.29 and 5.33 times, respectively. After thermic treatment of the test samples, the same data are reduced by 1.72, 1.58 and 1.69 times, respectively (Table 2).

It is well known that mass losses after thermic treatment of semi-finished products are linked to water binding capacity, while the organoleptic indicators of the finished products (succulence, consistency, appearance) depend on water holding capacity of semi-finished products.

Water binding and water holding capacities of the test samples in different freezing conditions are shown in Table 3 and Table 4.

The Table 3 and Table 4 illustrate that water binding and water holding capacities of the test samples are better in shock-freezing conditions.

The figures in tables illustrate that water binding and water holding capacities of the test samples are better in shock-freezing conditions. In comparison with traditional freezing method, water binding capacity of fillet has increased by 31.9%, while water holding capacity has increased by 25%, in natural minced semi-finished products – by 23,2% and 20%, respectively, and in minced semi-finished products enriched with a plant-based supplement – by 24,5% and 20,9%, respectively.

The better functional and technical indicators obtained by shock-freezing method show that of ice crystals formed during the fast freezing process have a minimal impact on a cell structure of rabbit meat tissue, at that time, no structural destruction of tissue and biological membranes is happening, so water crystals destroy the structure of tissues,

there is a change of hydrophilic properties of tissue and destruction of the protein-water colloidal systems, resulting in reduced water binding capacity **(Tsikin, 2012; Yablonenko, 2008)**.

In addition, as compared with other semi-finished products, higher water binding and water holding capacities of minced semi-finished products enriched with a plant-based supplement, is presumably explained by the fact that water is held due to a substantial amount of starch and fiber fetlock existing in a plant-based supplement **(Tavdidishvili et al., 2018)**.

The obtained data on water binding and water holding capacities of the test samples are in line with similar data obtained by other authors during the freezing of beef, pork and poultry meat. For example, according to **Tsikin (2012)**, the lowest water binding and water holding capacities were found in those samples, which were frozen at minus 30 °C and at an air velocity of 9.4 $m.s^{-1}$.

We have also studied the variation of pH medium of minced semi-finished rabbit meat products during different modes of freezing (Figure 4).

As shown in Figure 4 the pH medium value for both samples, in all freezing conditions is within the required limits, and besides, under shock-freezing conditions, its value is 6.7 – 10.4% higher, indicating the advantage of this method.

At the next stage of the study, we studied the organoleptic indices of frozen minced semi-finished products after their thermic treatment. The results are presented on the profilograms (Figure 5 and Figure 6).

At the same time, the organoleptic indices of minced semi-finished rabbit meat products are enriched with a plant-

The profilograms show that the organoleptic indices of both samples of minced semi-finished products are better than under shock-freezing conditions.

At the sme time, the organoleptic indices of minced semi-finished rabbit meat products are enriched with a plant-based supplement are better than the organoleptic indices of natural minced semi-finished products, particularly, their higher succulence is explained by relatively higher water holding capacity of minced semi-finished products enriched with a plant-based supplement ability.

We have determined microbiological indicators of the test samples frozen by various methods (Table 5).

Microbiological analysis was carried out on the presence of mesophylic-aerobic and facultative anaerobic microorganisms, salmonellas and bacteria of intestinal bacillus. It has been established that in test samples, the quantities of mesophylic aerobic and facultative anaerobic microorganisms varied from $3.4 \cdot 10^2$ to $2.4 \cdot 10^3$ $CFU.g^{-1}$ (colony forming $unit.g^{-1}$) that does not exceed sanitary norms and rules.

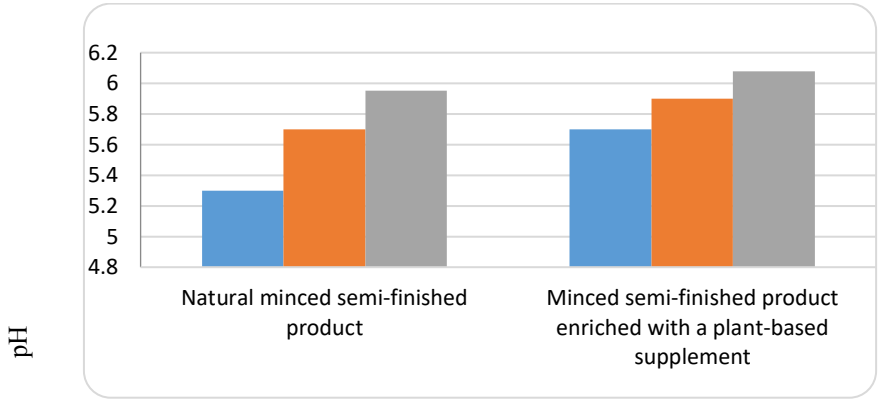

Figure 4 Change in the value of pH medium in minced semi-finished rabbit meat products in various modes of freezing.

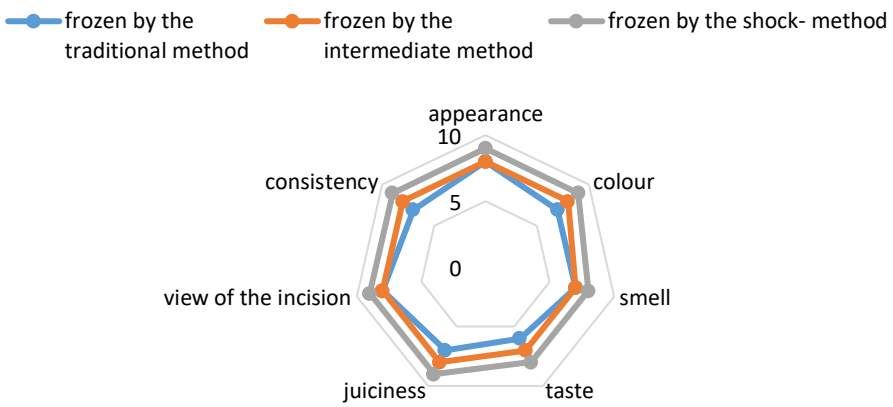

Figure 5 Profilogram of organoleptic indices of frozen natural minced semi-finished products after their thermic treatment.

Figure 6. Profilogram of organoleptic indices of frozen minced semi-finished products enriched with a plant-based supplement after their thermic treatment

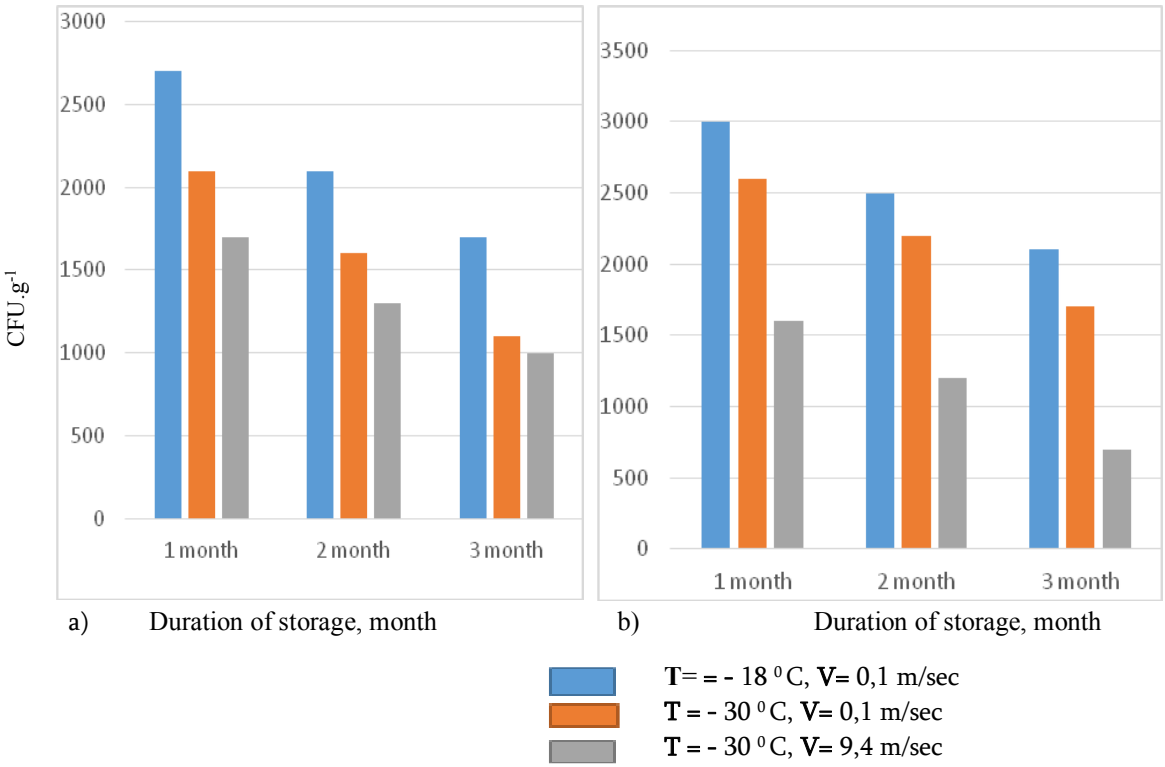

Figure 7. Changes in mezophilic-aerobic and facultative-anaerobic microorganisms (CFU.g^{-1})during the storage process: a) in natural minced semi-finished products; b) in minced semi-finished products enriched with a plant-based supplement in various modes of freezing.

Table 1 Mass losses of rabbit meat fillet and minced semi-finished products under various freezing conditions.

Semi-finished products of rabbit meat	Change of mass after freezing, %		
	T = -18 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 9.4 m.sec^{-1}
Fillet	3.5	1.6	0.7
Natural minced semi-finished product	4.5	2.15	0.85
Minced semi-finished product enriched with a plant-based supplement	4.0	1.8	0.75

Table 2 Mass losses of rabbit meat fillet and minced semi-finished products frozen under various conditions after thermic treatment.

Semi-finished products of rabbit meat	Change of mass after thermic treatment, %		
	T = -18 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 9.4 m.sec^{-1}
Fillet	16.4	13.0	9.5
Natural minced semi-finished product	18.0	14.5	11.4
Minced semi-finished product enriched with a plant-based supplement	17.2	14.0	10.2

Table 3 Water binding capacity of rabbit meat fillet and minced semi-finished products under various freezing conditions.

Semi-finished products of rabbit meat	Change of water binding capacity from the total mass, %		
	T = -18 °C, V = 0.1m.sec^{-1}	T = -30 °C, V = 0.1 m.sec^{-1}	T = -30 °C, V = 9.4 m.sec^{-1}
Fillet	47	55	62
Natural minced semi-finished product	56	60	69
Minced semi-finished product enriched with a plant-based supplement	57	63	714

Table 4 Water holding capacity of rabbit meat fillet and minced semi-finished products under various freezing conditions.

Semi-finished products of rabbit meat	Change of water holding capacity, %		
	T = -18 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 0.1 m.sec^{-1}	T = -30 ^{0}C, V = 9.4 m.sec^{-1}
Fillet	52	60	65
Natural minced semi-finished product	60	63	72
Minced semi-finished product enriched with a plant-based supplement	62	65	75

Table 5 Microbiological indicators of the test samples frozen by with different methods and their changes during the storage process.

microbiological indicators	Name of Product					
	fillet		natural minced semi-finished products		minced semi-finished products enriched with a plant-based supplement	
	before freezing	after freezing	before freezing	after freezing	before freezing	after freezing
Frozen by the traditional method (T = -18 ^{0}C, V = 0.1 m.sec^{-1})						
Number of mesophilic-aerobic and facultative-anaerobic microorganisms, cfu.g^{-1}, (colony-forming unit per gram), less than	2.4*10^3	1.7*10^3	3.6*10^3	3.2*10^3	4.0*10^3	3.4*10^3
E. coli group bacteria, in a 0.001 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified
Pathogenic microorganisms, including *Salmonella,* in a 25 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified
Frozen by the intermediate method (T = -30 ^{0}C, V = 0.1 m.sec^{-1})						
Number of mesophilic-aerobic and facultative-anaerobic microorganisms, cfu.g^{-1}, (colony-forming unit per gram), less than	2.4*10^3	1.3*10^3	3.6*10^3	2.7*10^3	4.0*10^3	3.0*10^3
E. coli group bacteria, in a 0.001 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified
Pathogenic microorganisms, including *Salmonella,* in a 25 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified
Frozen by the shock- freezing method (T = -30 ^{0}C, V = 9.4 m.sec^{-1})						
Number of mesophilic-aerobic and facultative-anaerobicmicroorganisms, cfu.g^{-1}, (colony-forming unit per gram), less than	2.4*10^3	0.7*10^3	3.6*10^3	2.1*10^3	4.0*10^3	2.0*10^3
E. coli group bacteria, in a 0.001 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified
Pathogenic microorganisms, including *Salmonella,* in a 25 g sample	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified	have not been identified

The established value of bacteria of intestinal bacillus (coliform) was not in 0.01 g sample, and met the hygienic requirements of microbiological safety, but pathogenic microorganisms including *salmonella*, have not been detected in 25 g samples, which also complied with microbiological safety norms and indicates safety of product. The data in Table 5 also confirm that the method of shock-freezing reduces the contamination of semi-finished products by mezophilic-aerobic and facultative-anaerobic microorganisms.

We have explored the changes in microbiological indicators of the test samples frozen under different conditions during the storage process, after 1, 2 and 3 months, respectively (Figure 7).

It has been established that during this period, no coliform bacteria and *salmonella* were found in the test samples, while the dynamics of changes in mezophilic-aerobic and

facultative-anaerobic microorganisms showed that, in the cases of all freezing methods, their value is reduced and is minimal when using the shock-freezing method.

Thus and so, studies have shown that method of shock-freezing allows for shortening the duration of freezing process, as compared to the traditional method, as well as for improving the functional-technological characteristics of product

CONCLUSION

With a view to preserving the quality of semi-finished rabbit meat products storing for an extended period of time, there have been studied various methods of their cold treatment. The advantage of shock-freezing method over the traditional methods has been justified and its rational parameters have been selected as follows: air temperature – not higher than -30 °C, air velocity – 9.4 m.sec^{-1} and relative humidity – 85%; the temperature in the depth of semi-finished product after freezing -10 °C.

The effect of freezing temperature on the duration of cold treatment process has been studied and the regression equations of their relationships have been obtained and regression curves have been constructed. It has been established that unlike traditional method, shock-freezing method reduces 3 – 3.5 times the duration of freezing of semi-finished rabbit meat products.

It has been shown that when using the shock-freezing method, mass losses of semi-finished rabbit meat products are minimal and are 5 – 5.3 lower as compared to traditional method of freezing.

Shock-freezing method allows for improving the functional properties of semi-finished rabbit meat products: by the type of semi-finished products, water binding capacity has increased by 23.2 – 31.9%, water holding capacity – by 20 – 25% and pH – by 6.7 – 10.4%.

There have been studied microbiological indicators of frozen semi-finished products and their changes during the storage process. It has been established that they meet the safety and hygiene requirements for meat products.

The full results obtained indicate the advantage of shock-freezing method as compared to traditional methods, as well as point to the appropriateness of its use when selecting the cold treatment modes for semi-finished rabbit meat products.

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EFFECT OF SOMATIC CELL COUNTS OCCURRED IN MILK ON QUALITY OF SLOVAK TRADITIONAL CHEESE – PARENICA

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ABSTRACT

The aim of this work was to compare somatic cell count in milk used for making steamed cheese Parenica in Slovak industrial dairies and small farm dairies and to find out whether somatic cell counts in milk affect the dry matter content of Parenica cheese. The samples of raw milk were taken from 3 industrial dairies (A, B, C) and from 3 farm dairies (E, F, G), produced traditional Slovak cheese Parenica in period from January untill December 2018. The somatic cell count in milk was determined by Fossomatic™ 5000 (*Foss*, Denmark) and dry matter of cheese by oven drying method to constant weight. There were no statistically significant differences (p >0.05) for somatic cell counts in milk processed in industrial and farm dairies. Lower somatic cell counts were determined in milk samples from industrial dairies (mean value 326.55 thousand in 1 mL) in comparison to milk samples from farm dairies (mean value 507.67 thousand in 1 mL). Statistically lower dry matter content (p <0.01) in the samples of Parenica cheese was found out in farm dairy E in comparison to other dairies. The relationship between somatic cell count in milk and dry matter in cheese was confirmed by the relatively low correlation coefficients in dairies, A = 0.22; C = 0.15 and F = -0.12 and higher correlation coefficients in dairies, B = -0.32; D = 0.45 and E = -0.48. Obtaining a more accurate effect of somatic cell count on cheese quality requires the continuation of the research on a larger number of samples and consideration of other factors.

Keywords: somatic cell count; cow milk, steamed cheese; industrial dairy; farm dairy

INTRODUCTION

To consistently manufacture high-quality dairy products, processors are demanding higher quality raw milk, which can be defined as (1) compositionally complete (e.g. protein and fat levels within norm); (2) free from off-flavors and odors; (3) free from detectable drug residues, added water, or other adulterants; (4) having low total bacteria counts; and (5) having low somatic cell counts **(Murphy et al., 2016)**.

Somatic cell count in milk is commonly used as an index of udder health in lactating dairy cattle **(Constable et al., 2016)**.

Taking cow milk as an example, most healthy cows in a dairy herd have a somatic cell count less than 5 x 10^4 cells.mL^{-1}. When somatic cell count exceed >2 x 10^5 cells.mL^{-1}, the udder is considered to be infected and mastitis is considered as subclinical **(Hachana, Znaidi and M´Hamdi, 2018)**. **Regulation (EC) No. 853/2004** of the European Parliament and of The Council of 29 April 2004 on the specific hygiene rules for food of animal origin, states that the raw cow´s milk should not contain more than 4 x 10^5 cells.mL^{-1} of somatic cells. The legal somatic cell count threshold for milk acceptance in dairy industries varies in different countries, e.g. the values for bovine milk in Germany, Canada, and the USA are 1 x 10^5 cells.mL^{-1}, 5 x 10^5 cells.mL^{-1} and 7.5 x 10^5 cells.mL^{-1}, respectively. For goat and ovine milk, the cutoff value for somatic cells is 1 x 10^6 cells.mL^{-1} in the USA but is not defined yet in the EU **(Li et al., 2014)**.

Somatic cells found in bovine milk are primarily lymphocytes, macrophages, and polymorphonuclear leucocytes, but they may also include a low percentage of epithelial cells from the gland. Somatic cells are known to be one of the major defense components of the mammary gland against diseases or intramammary infections **(Li et al., 2014; Murphy et al., 2016)**. Besides the immune defense role in the udder, somatic cells can continue their protective function in milk. For example, polymorphonuclears have bactericidal and respiratory burst activities and they can eliminate the invading bacteria by releasing reactive oxygen species and granular enzymes **(Paape et al., 2003)**. Some antibacterial proteins identified in bovine milk also arise from somatic cells such as macrophage scavenger receptor type I and II, polymorphonuclear peptidoglycan recognition protein and lymphocyte cytosolic protein I and cathelicidins. They can continue to exert their protective properties when they are in skim milk, whey, or milk fat globule membranes **(Hettinga et al., 2011)**. The role of the lysozyme, one of the somatic cell's endogenous enzymes is well known for

its ability to destroy the bacteria **(Paape et al., 2003)**. Some proteinase from polymorphonuclears, such as cathepsin G, elastase, and proteinase 3, have antimicrobial activites during phagocytosis of invading microorganisms. Catalase, an endogenous enzyme from polymorphonuclears is antioxidant enzymes in milk and is suspected of being responsible for changed redox potential of milk that limited the survival capability of microorganisms **(Hamed, El Feki and Gargouri, 2008)**.

Whether somatic cells is "fiend or a foe" in the dairy field remain a question **(Souza et al., 2012)**. Generally, somatic cells, until now, have been considered as negative **(Li et al., 2014)**. High somatic cell count is associated with an inflammatory response of the mammary gland to pathogen microorganism infection **(Bobbo et al., 2017, Potter, Arndt and Hristov, 2018)**. The negative effect of high somatic cell count includes decrease of feed efficiency, lower milk production, modification in milk composition and economic losses **(Bobbo et al., 2017; Hachana, Znaidi and M´Hamdi, 2018; Potter, Arndt and Hristov, 2018)**. Higher milk somatic cell count is associated with lower content of casein and lactose and greater pH, compared to the normal values **(Giaccone, Scatassa and Todaro, 2005; Li et al., 2014; Bobbo et al., 2017; Hachana, Znaidi and M´Hamdi, 2018)**.

Somatic cells are considered as important sources of enzymes that damage milk components and potentially result in product defects. A large range of enzymes are released into milk after lysis of somatic cells, and among them, lipases (e.g., lipoprotein lipase), oxidases (e.g., catalase and lactoperoxidase), glycosidases (e.g., lysozyme) and proteases (e.g. cathepsins, elastase, and collagenase) **(Li et al., 2014; Murphy et al., 2016)**. The role of these enzymes in dairy product quality has not been fully investigated. Elastase possibly influences coagulation properties of milk, and cathepsin B and D may play a role in cheese ripening. With increased somatic cell count is also associated increased plasmin activity. Plasmin´s role in the breakdown of caseins is significant because they are the major milk proteins that are captured in the coagulation process (e.g. cheese making). Plasmin hydrolysis of β-casein results in γ-caseins and proteose-peptones, which are lost in the whey during manufacture of cheese **(Murphy et al., 2016)**.

The negative effect of high somatic cell counts in raw milk on dairy industry include reduced shelf life of dairy products, due to undesirable sensory attributes caused mainly lipolytic and proteolytic enzymes **(Hachana, Znaidi and M´Hamdi, 2018)**. Higher levels of proteolysis have been observed in cheeses made with high somatic cell count **(Le Maréchal et al., 2011)**. Somatic cell count results in decreased cheese yield as a consequence of the low casein content and a decrease of major albumins (i.e. α-lactalbumin and β-lactoglobulin). However, increased somatic cell count induces an increase in immunoactive proteins (lactoferrin, lysozyme), as well as bovine serum albumine. Increased somatic cell count is also associated with increased rennet coagulation time, decreased of curd firmness, increased cheese moisture, decreased moisture-adjusted cheese yield or cheese yield efficiency, and reduce cheese quality **(Giaccone, Scatassa and Todaro, 2005; Litwińczuk et al., 2011; Murphy et al., 2016)**.

The aim of the work was to compare somatic cell count in milk used for making steamed cheese Parenica in Slovak industrial dairies and small farm dairies. The aim was also to verify whether somatic cell counts in milk affect the dry matter content of Parenica cheese.

Scientific hypothesis

We assume that between milk processed in industrial dairies and milk processed directly in small farm dairies will be differences in quality. We expect that milk processed in farm dairies will have a lower somatic cell count than milk in industrial dairies. We expect that higher somatic cell count in milk will affect quality of cheese and will decrease their dry matter content.

MATERIAL AND METHODOLOGY

The samples of raw milk were taken from 3 industrial dairies (A, B, C) and from 3 farm dairies (E, F, G) produced traditional Slovak cheese Parenica in period from January ´till December 2018. The samples of milk stabilized with dichroman potassium were analyzed within 24 hours of collection by Fossomatic™ 5000 (*Foss*, Denmark).

The dry matter content was determined by oven drying method (**ISO 5534:2004)** by drying to constant weight at 102 ±2 °C.

Statistic analysis

Analyses were replicated twice and they were calculated from obtained values – mean values, standard deviation, variation coefficient and correlation coefficient.

The obtained results were processed by variation-statistical method in ANOVA of Statistica CZ9.1 software (Stat Soft Ltd., Czech Republic). The differences were considered significant at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Somatic cell counts determined in raw cow's milk samples, taken from industrial dairies (A, B, C) and small farm dairies (E, F, G) during the year 2018, are in the Table 1. There were no statistically significant differences ($p > 0.05$) for somatic cell counts in milk processed in industrial and farm dairies.

Lower somatic cell counts were determined in milk samples from industrial dairies (mean value 326.55 thousand in 1 mL) in comparison to milk samples from farm dairies (mean value 507.67 thousand in 1 mL), that is in the agreement with our hypothesis. Industrial dairies buy milk from multiple vendors and it can be assumed that these dairies are interested in quality of purchased milk, while also motivating their suppliers who pay for milk on the basis, not onlyn of quantity, but also of milk quality. On the contrary, the quality of milk processed in farm dairies, which unlike to industrial dairies do not make regular milk analyzes, can vary greatly. It is in accordance with the value of variation coefficient (Table 1) and Figure 1 and Figure 2. The low values of mean somatic cell count and the variation coefficient were determined only for milk samples taken from farm dairy D. It is apparently related to the human factor (professional competence of farm staffs, interest in product quality).

Table 1 Somatic cell count x 1000 in 1 mL in milk samples from industrial and farm dairies.

mont	industrial dairies			farm dairies		
	A	B	C	D	E	F
January	268	243.5	417	140.5	24	2535
February	293.5	264.5	393	324.5	232	716.5
March	388	199.5	435	241	430	266.5
April	302	226.5	340	267	290	426
May	333.5	535.5	415	303	3288	392
June	297.5	430	443.5	587.5	712	373
September	356	134	374.5	145	243.5	277
October	270.5	244.5	399.5	320.5	189	380.5
November	264	282.5	373	476.5	260	659.5
December	280.5	261	330.5	145	227.5	357.5
mean	305.35	282.15	392.15	295.05	589.60	638.35
min	264	134	330.5	140.5	24	266.5
max	388	535.5	443.5	587.5	3288	2535
s_x	39.17	110.27	35.90	138.80	915.39	647.63
var (%)	12.83	39.08	9.16	47.04	155.26	101.45

Note: *samples were taken once per month.

Table 2 Dry matter of Parenica cheese (g.100 g^{-1}) produced in industrial and farm dairies.

month	industrial dairies			farm dairies		
	A	B	C	D	E	F
January	53.1	51.57	53.35	50.23	45.69	49.38
February	49.25	52.80	52.06	51.77	46.17	47.93
March	51.73	53.30	47.50	54.20	49.69	47.95
April	48.80	52.44	47.86	48.93	47.13	50.26
May	49.60	50.44	48.81	48.81	43.49	53.47
June	49.19	49.97	49.62	49.62	44.45	51.47
September	49.80	50.24	49.26	49.26	44.76	49.15
October	49.88	52.20	49.45	49.45	46.39	50.42
November	47.90	48.43	49.24	49.24	44.67	50.95
December	49.79	50.04	49.61	49.61	46.85	48.83
mean	49.91	51.14	49.68	49.68	45.93	49.98
min	47.90	48.43	47.50	47.50	43.49	47.93
max	53.10	53.30	53.35	53.35	49.69	53.47
s_x	1.41	1.47	1.68	1.68	1.67	1.62
var (%)	2.82	2.88	3.39	3.39	3.63	3.25

Note: *samples were taken once per month.

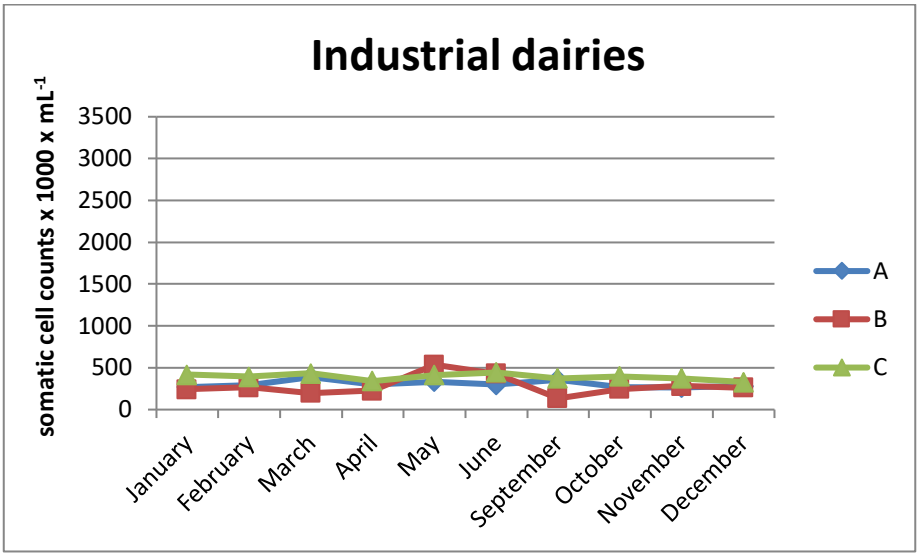

Figure 1 Somatic cell counts in samples of milk taken once per month during the year from industrial dairies (A, B, C).

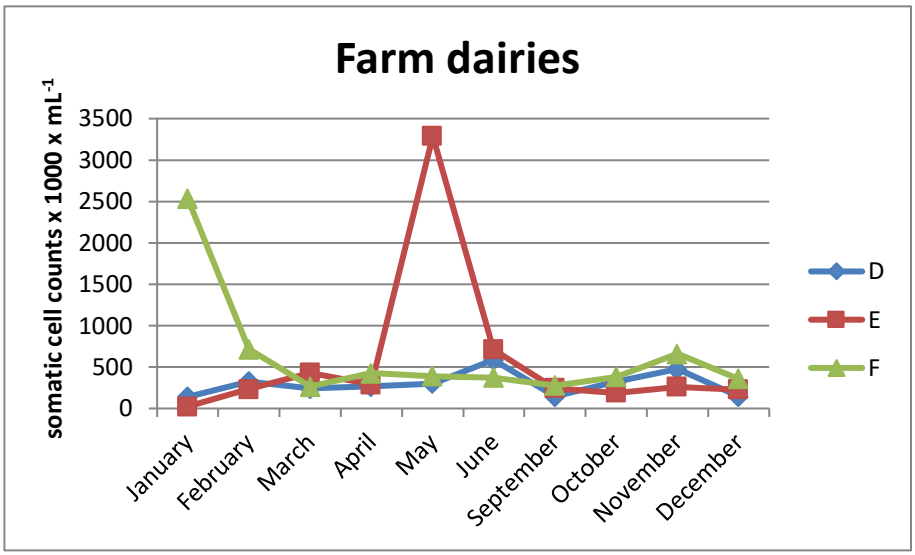

Figure 2 Somatic cell counts in samples of milk taken once per month during the year from farm dairies (D, E, F).

Kvapilík et al. (2016) determined somatic cell counts in milk from 14 experimental stables for the years 2012 – 2015 with mean value 288 thousand in 1 mL and value of variation coefficient, 3.6%. **Hristov et al. (2015)**, **Giallongo et al. (2016)** and **Giallongo et al. (2017)** reported that in cow milk samples natural logarithm of somatic cell counts x 10^3 cells in 1 mL 3.7; 4.3 and 3.2 respectively. **Litwińczuk et al. (2011)** found out that in the summer, $\log_{10}$ somatic cell count ranged from 4.68 to 6.04, whereas in the winter it ranged from 4.52 to 6.01.

Somatic cell count in milk is influenced except udder inflammation by many other factors, such as animal species, milk production level, lactation stage, individual and environmental factors, as well as management practices **(Rupp et al., 2000)**.

The somatic cell count is higher in goats and sheep milk, including milk samples from healthy udders and increases throughout the lactation period **(Šustová, Kuchtík and Kalhotka, 2016)**. **Giaccone, Scatassa and Todaro (2005)** determined in sheep's milk mean values of somatic cell count (expressed as $\log_{10}$) 6.40 and 5.56.

The dry matter content of steamed cheese Parenica from individual dairies is shown in Table 2.

Statistically lower dry matter content (p <0.01) in the samples of Parenica cheese was found in farm dairy E, in comparison to other dairies. The relationship between somatic cell count in milk and dry matter in cheese was confirmed by the relatively low correlation coefficients in dairies A = 0.22; C = 0.15 and F = -0.12, and higher correlation coefficients in dairies B = -0.32; D = 0.45 and E = -0.48.

It can be assumed that not only the somatic cell count in the processed milk, but much more factors (e.g. milk rennetability, technology of cheesemaking and others), influence the dry matter of steamed cheese.

The effect of somatic cell counts on dry matter of cheese is not unambiguous, as can be seen from the works of other authors.

For example, the authors mentioned below did not find the influence of the somatic cell count on the dry matter of the cheeses.

Cooney et al. (2000) blended milk from cows with high somatic cell count at the end of lactation with bulk tank milk with low somatic cell count. They made Swiss type

cheese with three levels of somatic cell count (113,000; 228,000 and 528,000 cells in 1 mL). They observed that there was no difference in moisture of cheese, but increased protein loss in whey.

Andreatta et al. (2007) pooled milk from cows in single herd based on somatic cell count level (<200,000 and >800,000 cells in 1 mL). They reported no difference in textural parameters and moisture of produced mozzarella cheese in relationship to somatic cell count level, but increased free fatty acids nd decreased protein during storage.

Hachana, Znaidi and M´Hamdi (2018) determined the effect of low (<115,000 cells in 1 mL), medium (422,000 cells in 1 mL) and high (>987,000 cells in 1 mL) somatic cell count on mozzarella cheese quality. Their results shown that no significant differences were observed in moisture, fat, and total protein contents among mozzarella cheese samples from milk with different somatic cell count categories. However, cheese samples produced from high somatic cell count milk had significantly higher pH (6.83), compared to samples produced with low and medium somatic cell count milk (5.58 and 5.46), respectively.

The influence of somatic cell count on sheep milk composition and cheese-making properties evaluated **Giaccone, Scatassa and Todaro (2005)**. They produced cheeses from bulk milk with somatic cell count at level 6.40 and 5.56 (expressed as $\log_{10}$). They found out that somatic cells influenced very significant (p <0.05) the lactodynamographic parameters of the milk – increase for clotting time and marked decrease of curd firmness. No statistical differences were found for Tuma and Pecorino cheeses in relationship to somatic cell count in milk.

However, there are also some works in the literature, which authors have found out that a higher number of somatic cells in milk has reduced the dry matter of the cheese produced.

Auldist et al. (1996) collected milk from herds with late lactation cows and compared cheddar cheese made from milk with 252,000 and 1,400,000 somatic cells in 1 mL. The group of cheeses produced from milk with higher somatic cell count had higher moisture (+8.1%) than cheese produced from milk with lower somatic cell count. These authors also found out that the textural defects are related to high moisture, as well as flavor defects associated with lipolysis and fat oxidation.

Vianna et al. (2008) compared quality of Prato cheese produced from milk with somatic cell count lower than 200,000 cells in 1 mL and higher than 700,000 cells in 1 mL. They found out about 3% higher value of moisture in the cheese produced from milk with higher somatic cell count, and they also reported increase of rennet coagulation time about 30%.

Klei et al. (1998) evaluated cottage cheese curd made from milk collected from the same 8 cows before and after an induced *Streptococcus agalactiae* infection with mean somatic cell count of 83,000 and 872,000 cells in 1 mL, respectively. The authors found decreased yield efficiency (4.3%), higher moisture, and increased proteolysis in cottage cheese curd made with the postinfection high somatic cell count milk.

Although from some published works seems to indicate that a particularly high number of somatic cells affects cheese production and quality, including dry matter of cheese, it is difficult to accurately determine the degree to which affect it cheeses.

CONCLUSION

The somatic cell count is currently our best industry indicator for milk quality related to udder health. From the somatic cells, which are gradually lysed, different kind of enzymes and antimicrobial agents are released. Several papers show that these compounds could negatively affect both – production yield and cheese quality.

From our results, as well as the results of several authors, the influence of the somatic cell count in milk on dry matter of cheese can not be clearly confirmed.

Obtaining a more accurate effect of somatic cell count on cheese quality requires the continuation of the research on a larger number of samples and consideration of other factors e.g. content of casein and whey protein, urea, calcium in milk, milk rennetability and other factors.

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THE CONCENTRATIONS OF PHTHALIC ACID ESTERS IN A WATER BATH AT SOUS-VIDE HEAT TREATMENT

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ABSTRACT

Esters of phthalic acid are common contaminants of the environment because of their large application in plastics. Phthalic acid esters are used as plasticizers in plastics, and they are also used in plastic intended for contact with food. In our research, we investigated the influence of heating on the migration of phthalic acid esters into the water used as a water bath. The water bath was used to heat the vacuum-wrapped meat, this heating is called the sous-vide method. The plastic thermostable bags containing phthalates were used on the meat packaging. Two esters of phthalic acid dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) have been determined. Three packaged meat samples were heated in a water bath for one hour either at 50 °C or at 60 °C. The water was analyzed always before the heating and after the heating. Average DEHP concentrations in the water dropped after heating at 50 °C in two cases and average DBP concentrations rose in one case and declined in one case. Average DBP concentrations in water declined after heating at 60 °C, while average DEHP concentrations after heating at 60 °C in water increased. The concentrations of phthalic acid esters in the water ranged from 15.2311 μg.L^{-1} to 34.5645 μg.L^{-1} for DEHP and from 0.0433 μg.L^{-1} to 2.6529 μg.L^{-1} for DBP. The heating of vacuum-packed food in plastic phthalate bags in a water bath does not pose a great risk of contamination of water with phthalic acid esters.

Keywords: water; dibutyl phthalate; di-2-ethylhexyl phthalate; plasticizer; contaminant

INTRODUCTION

Sous-vide technology is the heat treatment of vacuum-wrapped food in thermostable bags. Such a processed food has good sensory properties, juicy, fragile, tasty, and it is harmless, it kills microbes and parasites, because the temperature and the time of heating are well controlled in this technology **(Kameník, 2016)**. At the same time, studies have shown that when using the sous-vide method, food has a better nutritional quality – unsaturated fatty acids and vitamins remain in food **(Schellekens, 1996)**. The heat-treated vacuum-packed food is stored in a cold place and reheated before consumption **(Rhodehamel, 1992)**.

Conventional synthetic materials are currently synthetic polymers because of their low weight, softness and transparency, but they are difficult to biodegrade **(Siracusa et al., 2008)**. Various additives are added to the polymers to have the desired properties. However, additives have been found to migrate to water, soil, air, and food, thereby increasing unwanted exposure to humans. Additives can also be released during recycling and from products obtained by recycling plastics **(Hahladakis et al., 2018)**. Additives may be, for example, plasticizers, antioxidants, flame retardants and antistatic additives **(Brimer, 2011)**. Plasticizers provide plastic softness, brittleness, better workability and grip, for example foil. Typical plasticizers are phthalic acid esters **(Robertson, 2013)**. The legislation establishes specific migration limits for both DBP 0.3 mg.kg^{-1} food and DEHP 1.5 mg.kg^{-1} food. A specific migration limit is the amount that can be maximally released from the plastic material into the food. Material meeting specific migration limits should not pose a health hazard to the consumer **(Commission Regulation (EU) No. 10/2011)**. Migration of phthalic acid esters from materials into food accelerates the heater **(Fan et al., 2012)**. Food that was in direct and indirect contact with PVC foil and heated by microwave heating for 3 minutes increased the concentrations of both DBP and DEHP in the food. The control contained an average DBP concentration of 0.056 μg.g^{-1} and DEHP of 0.267 μg.g^{-1}, heated food without direct PVC film contact contained an average DBP concentration of 1.850 μg.g^{-1} and DEHP of 2.921 μg.g^{-1}, a heated foodstuff with a direct PVC film contact contained an average DBP concentration of 1.769 μg.g^{-1} and DEHP of 4.264 μg.g^{-1} **(Chen et al., 2008)**. The effects of temperature on phthalate migration, for example, have been investigated in the study by **Rose et al. (2012)**, where the transfer of the DEHP plasticizer from a 2 m polyvinyl chloride infusion set to a lipid-based infusion solution investigated for 6 hours at a flow rate of 12 mL.h^{-1} at 24 °C, 32 °C and 37 °C. It was found that at higher temperatures the concentration of phthalates in the

infusion solution was higher. And at 32 °C and at 37 °C may exceed the maximum recommended exposure set by EU legislation, which is 20 – 48 $\mu g.kg^{-1}$ per day, especially in infants and newborns **(Rose et al., 2012)**.

Esters of phthalic acid are produced in large quantities to produce plastics, phthalates are released from plastics and they are now ubiquitous in the environment where they are worried about their teratogenicity, hepatotoxicity and carcinogenicity **(Liang et al., 2008)**. Esters of phthalic acid also have estrogenic activity, damaging the kidneys and the liver. Acute toxicity is manifested by dizziness, nausea, cough, vision impairment, blood pressure lowering, and others. The widespread phthalate esters are DBP and DEHP. In terms of physical properties, lower phthalates are highly volatile at room temperature, while higher phthalates are non-volatile at room temperature. Esters of phthalic acid have a high boiling point **(Velíšek and Hajšlová, 2009)**. The boiling point for DBP is reported at 340 °C **(ATSDR, 2001)** and the boiling point for DEHP is 384 °C **(ATSDR, 2002)**.

The half-life of DEHP in the sediment was found out about 14 days, while the half-life in river water was found out about 10 hours for DEHP **(Yuwatini, Hata and Taguchi, 2006)**. **Shailaja et al. (2007)** found the shortest half-life of DBP when in the reactor lasted only 0.75 days; other literature reported longer periods. Degradation of DBP was investigated in the study by **Wang et al. (1997)**, when only 3% of DBP disappeared in the abiotic soil within 30 days, up to 66% of DBP concentration disappeared in soil with its own microorganisms, and up to 92% of DBP concentration was degraded in soil with own and added microorganisms. **Zhou et al. (2005)** study reported that degradation of DBP on the soil surface after 30 days was 95.7%. A high concentration above 60 $mg.L^{-1}$ of DEHP slows the degradation of both DBP and DEHP **(Gavala et al., 2003)**.

In wastewater treatment, the use of a combination of more technologies can remove a relatively high percentage of present phthalic acid esters, for example by anaerobic treatment with subsequent processing in the membrane bioreactor can remove about 95% to 97% of phthalates, while only anaerobic treatment without subsequent treatment in the membrane bioreactor can remove about 65% to 71% of phthalates from waste water **(Gao and Wen, 2016)**. Subsequent contamination with water containing phthalic esters, for example, is shown in **Tan et al. (2016)**. The study found that by irrigation of food crops with sewage, the occurrence of phthalic acid esters in crops increased. In the study investigated the effect of sewage irrigation on maize and wheat, they found higher concentrations of phthalic acid esters in wheat grains than in maize grains **(Tan et al., 2016)**.

Scientific hypothesis

The migration of phthalic acid esters from plastic bags of vacuum-packed meats into water of water bath during one-hour heating at 50 °C and 60 °C is expected.

MATERIAL AND METHODOLOGY

Vacuum packed meat, which was used for heating in a water bath, was obtained in cooperation with the University of Veterinary and Pharmaceutical Sciences Brno, with the Department of Gastronomy. Vacuum packed meat was heat-treated, so-called sous-vide technology at 50 °C or at 60 °C for 4 or 8 hours. Meat was the pork shoulder purchased in the market of Brno. The wrappings on the meat were the thermostable plastic bags Cryovac® CN300 (Sealed Air Polska Sp. z o.o., Poland).

In the study by **Jandlová, Jarošová and Kameník (2017)** was found in these plastic bags average DBP concentration of 22.47 $\mu g.g^{-1}$ plastic and average DEHP concentration of 11.76 $\mu g.g^{-1}$ plastic.

In our experiment, three pieces of vacuum-packed meat were heated at 50 °C or 60 °C per 1 hour. The used temperature corresponded to the previous temperature of the heat treatment of meat pieces. In the sous-vide technology this hourly heating represents a heating before consumption. Water samples were taken before and after heating in three replicates.

Water samples were analyzed at the Department of Food Technology at the Mendel University in Brno. The used method was by **Jarošová et al. (1999)**. Water samples were extracted 3 times with dichloromethane in a separating funnel. Subsequently, the solvent was evaporated on a rotary evaporator, the contents transferred in hexane to the vial, after drying the hexane with nitrogen, the sample in acetonitrile was determined by HPLC (mobile phase acetonitrile, UV detection at 224 nm and used column Zorbax Eclipse C8). The evaluation program was used Data Analysis (Agilent Technology).

Statistic analysis

The Microsoft Excel (Microsoft Corporation, USA) and Statistics 12 (StatSoft, USA) were used for processing and statistical analysis of data. They were used Shapiro-Wilk's test for the normality test, Grubbs' test to determine outliers and *t*-test to determine sameness of the mean values ($\alpha = 0.05$). *T*-test for dependent samples was used to compare the water sample concentrations before heat treatment with after heat treatment of the same temperature and time. *T*-test for independent samples was used to compare the water sample concentrations after heat treatment of 50 °C with after heat treatment of 60 °C, and to compare the water sample concentrations after heat treatment of the same temperature with the each other.

RESULTS AND DISCUSSION

Table 1 shows the observed concentrations of the two phthalic acid esters in water of the water bath. The concentrations of phthalic acid esters in water of water bath were for DBP from 15.2311 $\mu g.L^{-1}$ to 34.5645 $\mu g.L^{-1}$ for DEHP from 0.0433 $\mu g.L^{-1}$ to 2.6529 $\mu g.L^{-1}$. The DBP concentrations were detected higher in the water samples than the DEHP concentrations. The concentrations of DBP in water samples before heat treatment ranged from 16.0477 $\mu g.L^{-1}$ to 34.5645 $\mu g.L^{-1}$ and the concentrations of DEHP in water samples before heat treatment ranged from 0.0433 $\mu g.L^{-1}$ to 2.6529 $\mu g.L^{-1}$. While the DBP concentrations after heat treatment were in water from 15.2311 $\mu g.L^{-1}$ to 34.5645 $\mu g.L^{-1}$ and the DEHP concentrations after heat treatment was in the water from 0.0927 $\mu g.L^{-1}$ to 2.1658 $\mu g.L^{-1}$.

Figure 1 and Figure 2, show the average concentrations of DBP and DEHP in $\mu g.L^{-1}$. The average DBP

concentrations dropped in water after heat-treatment at 60 °C in both cases, whereas the average DEHP concentrations grew in water after heat-treatment at 60 °C in both cases. And the average DEHP concentrations dropped in water after heat-treatment at 50 °C in both cases, and the average DBP concentrations in water in one case grew and in one case dropped. The average concentrations of phthalic acid esters are lower before and after heat treatment than the specific migration limits set by legislation, so the amount of phthalates in water by sous-vide heat treatment should not pose a health risk **(Commission Regulation (EU) No. 10/2011)**.

The Table 2 and Table 3 show that first statistically significant difference in mean values was found in the concentrations of DBP in water before and after heat treatment at 50 °C per 1 h of meat heat treatment by 50 °C per 4 h and second statistically significant difference in mean values was found in the concentrations of DEHP in water after heat treatment at 50 °C per 1 h meat heat treatment by 50 °C per 8 h with the concentrations of DEHP after heat treatment at 60 °C per 1 h meat heat treatment by 60 °C per 8 h.

In the study by **Prokůpková et al. (2002)** in which solid phase microextraction (SPME) was used for the determination of phthalic acid esters in water and subsequently determined by GC. In water, two esters of phthalic acid esters: DBP and DEHP were found in the highest concentrations. Drinking tap water, deionized water, water in a glass bottle with a metal lid, which contains the PVC gasket (plasticized by DEHP) and water from a PET bottle were analyzed. Drinking water found DBP of 0.05 $\mu g.L^{-1}$ and DEHP of 0.66 $\mu g.L^{-1}$ in deionized water DBP of 0.15 $\mu g.L^{-1}$ and DEHP of 0.49 $\mu g.L^{-1}$ in water in a glass bottle DBP of 0.18 $\mu g.L^{-1}$ and DEHP of 9.78 $\mu g.L^{-1}$, in water in a PET bottle DBP of 0.20 $\mu g.L^{-1}$ and DEHP of 2.88 $\mu g.L^{-1}$.

Morita, Nakamura and Mimura (1974) examined the content of two phthalates dibutyl phthalate esters and di-2-ethylhexyl phthalate in Tokyo waters. They found higher concentrations of phthalic acid esters further downstream than the upper reaches of the same river. The concentrations of phthalic acid esters were detected in river water from 0.4 $\mu g.L^{-1}$ to 6.8 $\mu g.L^{-1}$, in untreated water from 1.9 $\mu g.L^{-1}$ to 8.2 $\mu g.L^{-1}$ and in tap water from 1.2 $\mu g.L^{-1}$ to 3.3 $\mu g.L^{-1}$.

Rastkari et al. (2017) examined the transfer of phthalic acid esters into acidic liquids (vinegar, lemon juice, verjuice) at different storage temperatures (4 °C, 25 °C, 50 °C). The analyses were carried out after 0, 2, 4, 6 month of storage. The concentrations of DEHP in acidic liquids stored in HDPE and PET bottles grew with higher storage temperatures, even with increasing storage times. The concentration of DBP in acidic liquids stored in HDPE and PET bottles grew with higher temperature, but only to 25 °C, and with increasing storage time but only for 2 months. After 2 months and at 50 °C the concentrations of DBP decreased.

Mousa, Basheer and Al-Arfaj (2013) measured the concentrations of phthalates in mineral bottled water exposed to direct sunlight for up to 222 hours. The concentration of DBP and DEHP rose in the water up to 36 hours of exposure to sunlight when a sample temperature of 43 °C was measured, the average concentrations of DBP in water were ranged from 14.69 $\mu g.L^{-1}$ to 22.34 $\mu g.L^{-1}$ and the average concentrations of DEHP in the water were ranged from 42.77 $\mu g.L^{-1}$ to 75.71 $\mu g.L^{-1}$.

Wang et al. (2015) investigated the concentrations of sixteen phthalic acid esters in drinking water and source water from 19 places of Zhejiang Province in China. The water samples were taken during wet and dry season. They found that phthalate concentrations were lower in the dry season than in the wet season, and lower concentrations were found in source water than in drinking water. The concentrations of DBP were ranged from 0.06 $\mu g.L^{-1}$ to 0.22 $\mu g.L^{-1}$ in source water in the wet season, and from ND to 0.08 $\mu g.L^{-1}$ in source water in dry season. The concentrations of DBP in drinking water ranged from 0.05 $\mu g.L^{-1}$ to 0.64 $\mu g.L^{-1}$ in the wet season and from 0.01 $\mu g.L^{-1}$ to 0.08 $\mu g.L^{-1}$ in the dry season. The concentrations of DEHP in source water were detected from ND to 1.68 $\mu g.L^{-1}$ in the wet season and from ND to 1.11 $\mu g.L^{-1}$ in dry season. The concentrations of DEHP in drinking water were measured from 0.12 $\mu g.L^{-1}$ to 4.58 $\mu g.L^{-1}$ in the wet season and from 0.02 $\mu g.L^{-1}$ to 1.33 $\mu g.L^{-1}$ in the dry season.

Liu, Chen and Shen (2013) examined the concentrations of phthalic acid esters in a water source in northeastern China. Most of the phthalic acid esters were detected DBP and DEHP. The concentrations of DBP were found out from
52.5 $ng.L^{-1}$ to 4 498.2 $ng.L^{-1}$ and DEHP from 128.9 $ng.L^{-1}$ to 6570.9 $ng.L^{-1}$.

In our study, we found higher DBP concentrations in water samples than DEHP, as opposed to **Prokůpková et al. (2002)**, **Mous, Basheer and Al-Arfaj (2013)**, **Wang et al. (2015)**, and **Liu, Chen and Shen (2013)**, which detected higher concentrations of DEHP than DBP. However, in our study used plastic bags also had higher DBP concentrations than DEHP **(Jandlová, Jarošová and Kameník, 2017)**.

In our study, we have not come to the conclusion that the heating cause migration of phthalic acid esters into water, since the increase of the concentrations after heating was only by DBP concentrations in one case at 50 °C and in two cases by DEHP concentrations at 60 °C. For example, **Chen et al. (2008)** reported that thermal microwave heating has resulted in the migration of phthalic acid esters into foodstuff, when the foodstuff had close or free contact with PVC film. Similarly, **Fan et al. (2012)** stated, the heating accelerated the migration of phthalates into food.

Why did not only the increase in phthalic acid ester concentrations during heating? It can be also caused by possible heat degradation, or it could occur **Velíšek and Hajšlová (2009)** vaporization to the air.

Table 1 Concentrations of phthalic acid esters, dibutyl phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP) in water samples heat-treated at 50 °C and 60 °C per 1 hour each with three samples of vacuum packed sous-vide meat.

Descriptions of water samples	DBP [μg.L^{-1}]	DEHP [μg.L^{-1}]
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	20.0267	1.3175
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	27.2050	1.5955
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	18.6800	0.5643
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	30.1417	0.3564
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	34.5645	0.9186
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h	25.5281	0.4505
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	27.3995	2.6529
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	23.5183	1.1866
before the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	25.9074	0.0433
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	26.4121	1.2342
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	20.1415	0.5508
after the heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	16.0477	0.0927
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	30.1417	0.3564
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	34.5645	0.9186
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	25.5281	0.4505
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	25.5632	2.1658
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	19.6026	0.1539
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	32.4342	0.8114
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	26.4121	1.2342
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	20.1415	0.5508
before the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	16.0477	0.0927
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	17.2808	1.7364
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	15.2311	1.9161
after the heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	16.2460	1.8160

Table 2 The results of T-test for dependent samples; phthalic acid ester concentrations in water before heat treatment are compared with concentrations in water after heat treatment.

Descriptions of campared water samples	Statistically significant difference
DBP before and after heat treatment at 50 °C per 1 h of meats treated by 50 °C per 4 h	p <0.05
DEHP before and after heat treatment at 50 °C per 1 h of meats treated by 50 °C per 4 h	p >0.05
DBP before and after heat treatment at 50 °C per 1 h of meats treated by 50 °C per 8 h	p >0.05
DEHP before and after heat treatment at 50 °C per 1 h of meats treated by 50 °C per 8 h	p >0.05
DBP before and after heat treatment at 60 °C per 1 h of meats treated by 60 °C per 4 h	p >0.05
DEHP before and after heat treatment at 60 °C per 1 h of meats treated by 60 °C per 4 h	p >0.05
DBP before and after heat treatment at 60 °C per 1 h of meats treated by 60 °C per 8 h	p >0.05
DEHP before and after heat treatment at 60 °C per 1 h of meats treated by 60 °C per 8 h	p >0.05

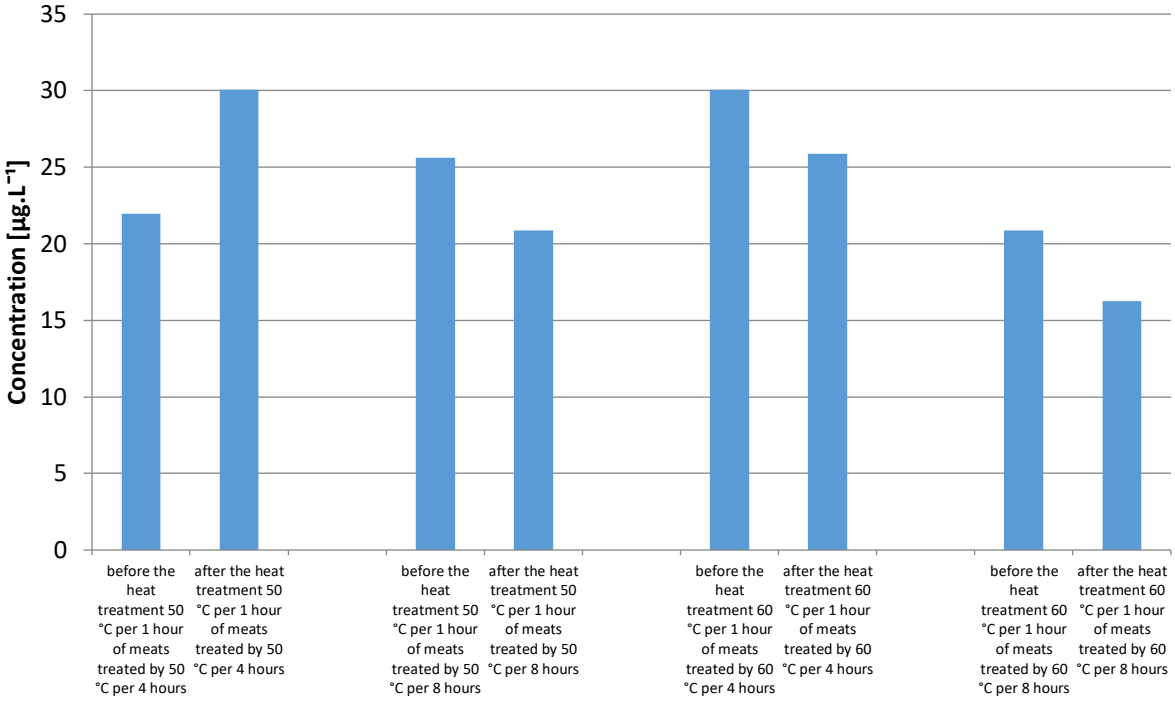

Figure 1 The average concentrations of DBP in water of water bath before and after heating.

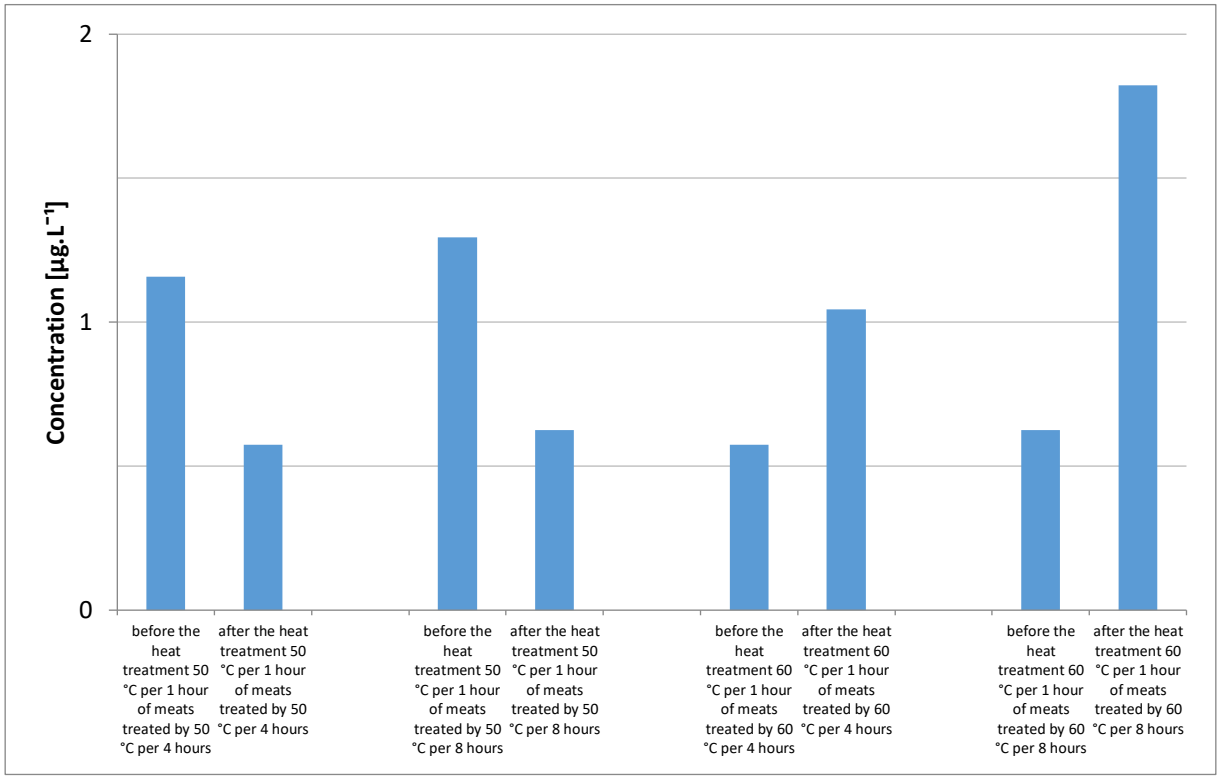

Figure 2 The average concentrations of DEHP in water of water bath before and after heating.

Table 3 The results of T-test for independent samples; comparison of phthalic acid ester concentrations in water samples after heat treatment.

Descriptions of campared water samples	Statistically significant difference
DBP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h with DBP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	$p > 0.05$
DEHP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h with DEHP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h	$p > 0.05$
DBP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h with DBP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	$p > 0.05$
DEHP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h with DEHP after heat treatment 60 °C per 1 h of meats treated by 60°C per 8 h	$p > 0.05$
DBP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h with DBP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	$p > 0.05$
DEHP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 4 h with DEHP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 4 h	$p > 0.05$
DBP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h with DBP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	$p > 0.05$
DEHP after heat treatment 50 °C per 1 h of meats treated by 50 °C per 8 h with DEHP after heat treatment 60 °C per 1 h of meats treated by 60 °C per 8 h	$p < 0.05$

CONCLUSION

In the study, we expected the migration of phthalic acid esters by heating from plastic bags to water of water bath. The increase of the average DEHP concentrations in the water of water bath was found after heat treatment at 60 °C. The increase of the average DBP concentrations was found at 50 °C, where a statistically significant difference was found. In other cases, the average concentrations of phthalic acid esters after heat treatment decreased. This can be justified by the potential volatility and degradation of phthalic acid esters during heating. The heating of vacuum-packed food in plastic phthalate bags does not pose a great risk of contamination of water with esters of phthalic acid.

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STUDYING THE PROCESSING OF FOOD DYE FROM BEET JUICE

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ABSTRACT

The manuscript describes a new method of red beet processing and the technology of manufacturing food colorant from the juice concentrate, which is natural, safe and useful analogue to existing expensive offers on the market of similar goods that have chemical origin not useful for regular consumption. Nowadays in order to give to food products a colour, close to natural coloring of fruits and vegetables, expensive synthetic dyes are used, which might have cancer-inducing effect when being accumulated by human organism. Therefore improving the technology for producing food grade dye from red beet juice is remarkably important task. Currently, there is a problem for vegetable processors – pectin substances complicate the process, like the illumination of juice and negatively affect its storage capacity. The article below reveals and substantiates the necessity of using a natural carbon-containing adsorbent shungite for the purification of beet juice from pectin substances. On the basis of the study, the authors suggest a more cost-effective way of producing a food dye from juice concentrate, which allows avoiding usage of expensive enzyme processing additives.

Keywords: food grade dye; red beet juice; natural adsorbent; shungite; studying the processing

INTRODUCTION

Red beet juice is very useful food product, because it contains significant amount of sugars, mineral substances and vitamins. It is also valuable because it's used for producing food grade dye **(Sigurdson, Tang and Giusti, 2017; Lehto et al., 2017)**.

Nowadays in order to give to food products a colour, close to natural coloring of fruits and vegetables, expensive synthetic dyes are used, which might have cancer-inducing effect when being accumulated by human organism. Therefore improving the technology for producing food grade dye from red beet juice is remarkably important task **(Scotter, 2015; Gras et al., 2017)**.

Currently for the purpose of obtaining dye from red beet juice, a part of pectin substances, which make the process of juice concentration more difficult, is removed with the help of expensive enzymatic agents **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017; Mehemdi et al., 2015)**. It is well-known that to clean juice, the following enzyme preparations are used: pectophytoside with a complex of pectolytic enzymes (pectinase) for the cleavage of pectin substances; amylorizine and glucoadamorine with an amylase advantage; protohetoidin with an enzyme complex of proteolytic (protease) and pectolytic activity for juice processing mainly for the purpose of juice illumination and removal of turbidity **(Murashev, Zhemchuzhnikova and Verzhuk, 2015; Loretz, Lopaeva and Neverova, 2016; Stich, 2016)**.

The main disadvantages of the enzymatic methods of juice cleaning are permanent repetition and long process flow **(Schweiggert, 2018; Kolyanovska et al. 2019)**.

The authors have suggested using natural carbon-bearing adsorbent shungite to purify red beet juice from pectin substances **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017; Vetrov, Akishin and Akimov, 2016)**.

Shungite is a mineral consisting of amorphous carbon and fractured graphite. Its chemical composition is not constant: shungite contains 60 – 70% of carbon and 30 – 40% of other elements.

Shungite is the only known mineral to have fullerenes (recently discovered new globular form of carbon existence). Fullerenes' structure is peculiar because carbon atoms in molecules are situated at the tops of regular pentagons and hexagons, which cover sphere's surface and present themselves as closed polygons composed of paired quantity of coordinated carbon atoms **(Rumyantsev and Sizov 2018; Kornen, 2016; Kozelová et al., 2011)**.

Fullerenes differ from particles with metallic properties due to the location of electron cloud and ability to change the form of carbon structure.

Sizing of electro-magnetic waves is determined by vibration of electrons which are divided into $\pi - \sigma$ and π – states. During adsorption on electrically neutral surface the localization of fullerenes, π – states takes place, and a particle loses its metallic properties, and because of that connected electron pair appears in the activated form. Thus mineral shows bipolar properties. Having analyzed the

features of the structure of shungite, one can be concluded that the mechanism of adsorption of pectin substances from red beet juice is explained not only by adsorption in the mineral pores, but also by ion exchange adsorption at the places of occurrence of reactively able centers of fullerenes together with the formation of hydrogen bonds with the pectin molecule. E.g. fullerene which can have properties of metal or semiconductor particles, resulting in compounds with different types of chemical bonding (exhibits bipolar properties) **(Sierra-Rosales, Toledo-Neira and Squella, 2017; Vagiri and Jensen 2017; Siva, 2007)**.

Shungites´ important characteristic is the presence of fullerene carbon nanotubes with the diameter of their cylindrical pores constituting 1-6 nanometers and the width – up to several micrometers. The cylindrical surface of tubes is formed by active carbon circles and also has empty pores.

The basis of shungite structure is a globule composed of graphitic networks, formed into packages. Each package has 6 graphitic flat networks with the quantity of carbon atoms attaining to 300 – 600 and one curved network, having 400 carbon atoms.

Colour has been measured in the food industry by subjective visual inspection, including the use of visual colour standards. Colour instruments objectively measures colour, are tools that assist the eye. With proper usage, it can provide repeatable, meaningful colour data that agree with visual measurement **(Loughrey, 2000; Nagyová, Berčík and Horská, 2014)**. The colour of any food product can be represented in terms of 'L' (lightness), 'a' ('+a', redness and '−a', greenness), and 'b' ('+b', yellowness and '−b', blueness) values or combination of these three depending upon the nature of the pigment present in the food material. It is reported that the objective measurement of colour using 'L', 'a' and 'b' system is a good indicator of the total colour change of heat treated peach puree **(Ávila and Silva, 1999)**, broccoli juice **(Weemas et al., 1999)**, mango puree, red chilli puree and paste, papaya puree and plum puree **(Ahmed, Shivhare and Kaur, 2002).**

Beet root powder or extracted pigments are used industrially for improving the red colour of tomato pastes, sauces, soups, desserts, jams, jellies, ice creams, sweets, and breakfast cereals **(Roy et al. 2004; Koul et al., 2002; Sukhenko et. al., 2017)**. The red beetroot (*Beta vulgaris* L.) is a good source of red and yellow pigments known as betalains. Betalains consist of betacyanins (red) and betaxanthins (yellow). The major betacyanin in beetroot is betanin and accounts for 75 – 95 % of the red pigment. The degradation of betanin was reported to follow first order reaction kinetics. It is reported that in the presence of excess of oxygen, betanin degradation follows pseudo first order reaction **(Attoe and Von Elbe 1982; Patkai and Barta 1996; Sukhenko et. al., 2019)**.

Betanins are reported to have some antioxidant activity and are found to be effective in inhibiting lipid peroxidation. Thus it is suggested that red beet products consumed regularly in the diet may provide protection against certain oxidative stress-related disorders in humans and also improve digestion and blood quality **(Kanner, Harel and Granit, 2001; Butera et al., 2002; Herbach, Stintzing and Carle, 2004; Azeredo, 2009)**. Therefore, the retention of colour pigments as indicated by its colour it is very important with regard to its health benefits as well as visual appeal. Knowledge of kinetic parameters of colour degradation during thermal processing is very important in optimization of the process parameter. A literature survey indicates that similar work on beet root has not been reported.

Consequently, the use of shungite makes it possible to establish binding to the pectin substances of beet juice, which will significantly improve the process of purifying the juice during the production of the dye.

Scientific hypothesis

A precedent has been published studies devoted to obtaining dye from beet juice with an adjustable amount of pectin-containing substances.

The purpose of this study was to establish the possibility of replacing expensive enzyme preparations with natural carbon-containing adsorbent shungite for cleaning beet juice.

MATERIAL AND METHODOLOGY

The method of conducting the experiment was as follows: juice was obtained from table beet and heated to a temperature of 50 – 60 °C (creating conditions as close as possible to the production), mixed with shungite powder TU U 24.6-32177584-003:2011 "Materials are shredded and ground with shungite", industrial fraction 0.001 – 0.002 m.

Shungite to be used for research was prior washed out with cold water and then termoactivated at 100 °C during 90 minutes. Cooled an adsorbent in concentrations of % mass: 2.44; 3.23; 4.76; 9.09 was put into fresh red beet juice at temperature of 20, 40, 50, 60 °C, mixed during 10, 20, 30, 60 min., filtrated. The content of pectin substances in filtrate was measured in accordance with calcium pectate method **(Sachek, Tile and Sevostyanov, 2017)** under formula:

$$ПР = \frac{(g - g_0) \cdot 100 \cdot 0.9235}{V \cdot d}$$

whereas ПР – content of pectin substances in juice, $mg.g^{-1}$;
g – weight of weighting cup with precipitate before exsiccation, g;
g_0 – weight of empty weighting cup, g;
V – juice volume, cm^3;
d – juice density, $g.s^{-3}$;
0.9235 – coefficient for conversion of calcium pectate into pectic acid.

The obtained results were compared by effect of purification (Table 1):

$$E = \frac{100 \cdot (K_1 - K_2)}{K_1}$$

where: K1 and K2 – quantity of target component in juice which was not processed by adsorbent and juice which was processed by adsorbent.

The next phase of research was determining the content of coloring substances of red beet juice, processed by shungite at temperature of 50 °C. Preparation of shungite was performed in the same way as for adsorption of pectin

substances. The content of coloring substances was determined according to standard method of Leventaal-Neubauer **(Danilova and Popov, 2004)**. Estimation of mass content of coloring substances was performed under formula:

$$X = \frac{(M_1 - M_2) \cdot K \cdot 0.004157 \cdot O_1 \cdot 100}{H \cdot O}$$

where: X – mass content of coloring substances, %;
M_1 – quantity of potassium permanganate ((chemically pure for analyzes) supplier company PrimaRia, Kiev, Ukraine) solution (0.1 mol.L^{-1}) used for the first titration, m^3;
M_2 – quantity of potassium permanganate solution (0.1 mol.L^{-1}) used for the second titration, m^3;
O_1 – volume of the primary extraction, m^3;
H – quantity of juice used for experiment, m^3;
O_2 – volume of the secondary extraction, used for titration, m^3;
0.004157 – coefficient which takes into account correlation between potassium permanganate and juice coloring substances.

Statistic analysis

The statistical evaluation of the results was carried out by standard methods using statistical software Statgraphics Centurion XVII (StatPoint,USA) – multifactor analysis of variance (MANOVA), LSD test.

RESULTS AND DISCUSSION

The obtained data shows that adsorption of pectin substances from red beet juice by shungite takes place already at temperature of 20 °C. If temperature rises the process is somewhat accelerated. The rise of temperature of processing juice over 60 °C is unreasonable because coloring components are destroyed and that causes the changes in juice colour.

Comparing the obtained results and their practical efficiency the authors determined optimal parameters, concluding that the optimal parameters for processing juice by shungite is adsorbent concentration of 4.76% mass, temperature of processing 50, 60 ° C, duration 30 min. Under such conditions 36.8 – 42.1% of pectin substances are removed.

The mechanism of adsorbing pectin substances from red beet juice by shungite is explained not only due to the fact that impurities infiltrate the mineral's pores, but also due to ion-exchange adsorption in places where reactive centers of fullerenes are formed and hydrogen bonds with pectin molecule are created.

Shungite's selectivity is explained not only because of the existence of micro-, mezzo- and macropores, but also because nanotubes participate in adsorption processes and there are pores in between them, created when packages are formed, and also because of free non-compensated charges which appear on adsorbent's surface.

Emphasizing on the receipt of natural dyes should be allocated colorant Beet Red – E162. The main colorant is betaine – an alkaloid-like compound obtained from beet juice. The molecular formula of the additive E162:C24H27N2O13 **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017)**.

The research was performed and its results are showed in Figure 1.

Analysis of data presented in Figure 1 gives grounds to state that content of coloring substances in red beet juice, after it was processed by shungite, practically does not change. This can be explained by the fact that the basis of coloring substances in red beet juice is anthocyanin **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017; Sukhenko et. al., 2020)**. By their structure they are chains of glycosides, composed of heterocyclic compounds. By their chemical nature they are surface active substances. On interphase border anthocyanin molecules are situated in such a way that hydrophilic group remains in liquid state. Hydrophobic effect takes place and thus coloring substances are not adsorbed by shungite.

The authors improved apparatuses and technological scheme for producing food grade dye from red beet juice by installing two adsorbing devices with shungite which work in regime of sorption-desorption.

Installing adsorbers will remove excess substances and do not have a temperature effect on the juice. The colour of the dye E162 may vary depending on the bright red to the blue-violet (at an elevated pH) and is destroyed by the action of a high temperature (above 65 °C). In the course of studies it was found that the optimum temperature of juice processing in the adsorber is 50 °C. So you can stabilize the colour of juice for further technological operations **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017)**.

The results of the studies allowed to develop a mathematical-statistical model of the shungite regeneration process, using second-order root-level planning.

$$y = -490.5 + 6.2t_n + 0.1\tau + 0.02\ t_n \cdot \tau - 0.02t_{n2} - 0.1\tau_2$$

where:
t_n – temperature of superheated water vapor, ºC,
t – duration of regeneration, min.

On the basis of the obtained mathematical model, optimization of the process was carried out regeneration of shungite by superheated water vapor Figure 2.

The rational parameters regeneration of shungite should be carried out with superheated water vapor with a temperature of 170 °C, pressure 0.3 MPa. Regenerated shungite is used in the technological process up to 5 times. The duration of regeneration is 15 – 20 minutes; the mass flow rate of steam is about 2.305 10^{-3} kg·s^{-1}.

As the result of regeneration, adsorption capacity of the adsorbent may recover completely or partially, depending on adsorption capacity of the desorbed components, chosen method of desorption and operating parameters of the process. In some cases it is justified to have incomplete recovery of the adsorbent activity, as this reduces operating costs. Subsequently spent shungite can be recycled by burning **(Sheiko, 2015; Sheiko, Tkachenko and Petrenko, 2017; Dhar et al., 2015)**.

Table 1 Effect of purification (E,%) from pectin substances in red beet juice by shungite at different adsorbent concentrations, temperature of mixtures, duration of interactions between adsorbent and juice, initial content of pectin substances is 1.9 mg.g-1.

Effect of purification	Adsorbent concentration in juice, % mass															
	2.44				3.23				4.76				9.09			
	Temperature, °C															
	20	40	50	60	20	40	50	60	20	40	50	60	20	40	50	60
	Duration of juice processing, 10 min															
	Content of pectin substances, $mg.g^{-1}$															
Processed juice	1.8	1.8	1.7	1.7	1.8	1.7	1.7	1.6	1.7	1.6	1.5	1.5	1.6	1.6	1.5	1.4
E, %	5.3	5.3	10.5	10.5	5.3	10.5	10.5	15.8	10.5	15.8	21.0	21.0	15.8	15.8	21.0	26.3
	Duration of juice processing, 20 min															
Processed juice	1.7	1.7	1.6	1.6	1.5	1.5	1.4	1.3	1.5	1.4	1.3	1.2	1.4	1.3	1.2	1.2
E, %	10.5	10.5	15.8	15.8	21.1	21.1	26.3	31.6	21.1	26.3	31.6	36.8	26.3	31.6	36.8	36.8
	Duration of juice processing, 30 min															
Processed juice	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.2	1.5	1.3	1.2	1.1	1.4	1.3	1.1	1.1
E, %	15.8	15.8	21.1	21.1	26.3	26.3	31.6	36.8	21.1	31.6	36.8	42.1	26.3	31.6	42.1	42.1
	Duration of juice processing, 60 min															
Processed juice	1.6	1.6	1.5	1.5	1.4	1.3	1.2	1.2	1.5	1.3	1.2	1.1	1.3	1.2	1.1	1.1
E, %	21.1	21.1	26.3	26.3	26.3	31.6	36.8	42.1	21.1	31.6	36.8	42.1	31.6	36.8	42.1	42.1

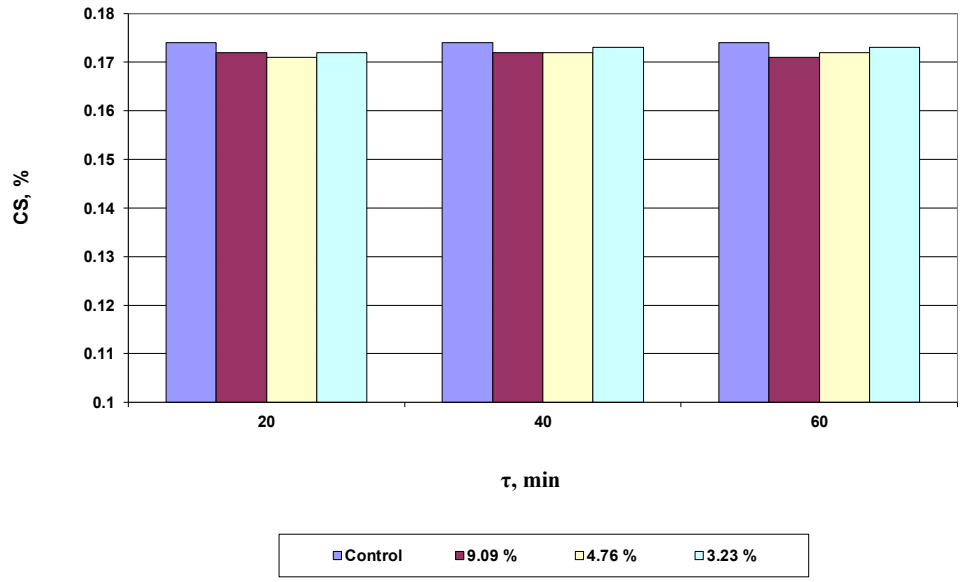

Figure 1 Quantity of coloring substances (CS) in juice processed with shungite depending on the duration of its processing at temperature of 50 °C.

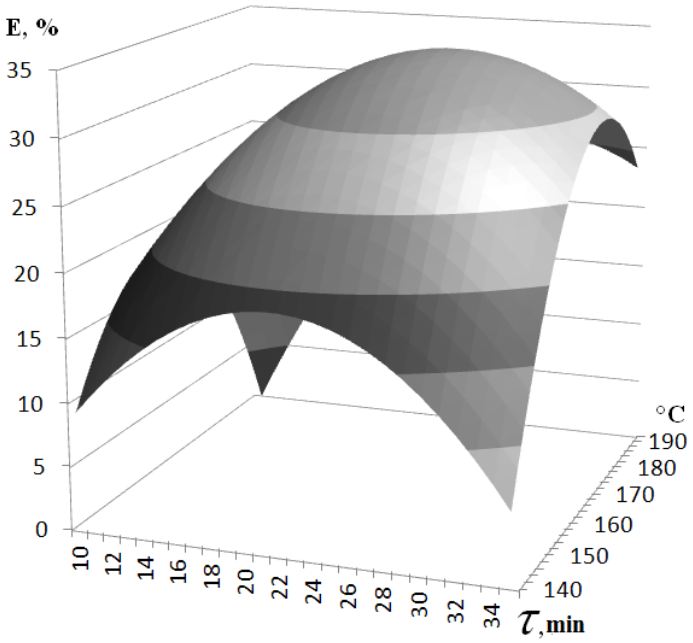

Figure 2 Optimal parameters of the shungite regeneration process.

After part of pectin substances are removed in adsorbing device, red beet juice is placed in vacuum evaporator where it is concentrated around 6 hours. Concentration of juice by its evaporation takes place with discharge of 0.055 – 0.060 MPa and temperature of 55 – 60 °C, concentrated red beet juice, already as food grade dye, is packed in the sealed container, made of dark glass. The level of pH in the obtained food grade dye does not exceed 4.5. The used adsorbent is recyclable or can be recovered.

CONCLUSION

It was established that shungite effectively adsorbs pectin substances from red beet juice and does not adsorb coloring substances. The technology of producing food grade dye was improved by additional purification of red beet juice from pectin substances by shungite. The obtained optimal technological parameters for purifying juice with shungite are as follows: adsorbent's concentration constituting 4.76% mass., temperature is 50 – 60 °C, duration of processing is 30 min. Apparatuses and technological scheme of producing food grade dye is supplemented with two adsorbing devices with shungite, which work in regime of sorption-desorption. The used adsorbent is recyclable or can be recovered.

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THE EFFECT OF REDUCTION OF NaCl CONTENT ON SELECTED PARAMETERS DURING RIPENING OF CHEESE

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ABSTRACT
The aim of this work was to observe chemical and physical changes in Dutch-type cheese during ripening depending on salt concentration. Ripening is one of the most important factors influencing the sensory quality of cheese and therefore the cheese production should be studied. Among the substances which are formed during ripening belong the biogenic amines which are produced by the decarboxylation of amino acids. These amino acids are created during proteolysis. The salt content largely affects the intensity of the ripening process, but also other cheese parameters such as dry matter content, hardness or content of biogenic amines. In the course of 3 months ripening of model cheeses with different cultures and with different salt content, the effect of the salt on pH, dry matter content, free amino acids and biogenic amines content and hardness was monitored. The concentration of NaCl affected the dry matter content and the hardness of the samples. The reduction in salt content contributes to the higher accumulation of biogenic amines during ripening.

Keywords: cheese; cheese ripening; proteolysis; biogenic amines; salt content

INTRODUCTION
Approximately one third of world milk production is used in cheese production. Cheese is a high nutritional food. In addition to other parameters, the types of cheeses also differ in salt content (NaCl) **(Farkye, 2004)**. In the case of Dutch-type cheese the salt content is between 1.5 – 3% **(Guinee, 2004)**. The salt in the cheese serves as a preservative, which contributes to the sensory properties of the cheese. Salt also serves as a source of sodium, which is important for the consumer in terms of regulation of blood pressure, water transport into and out of the cells, osmolality of tissues and cellular pulse nerve transfer **(Guinee, 2004)**. In addition to the above mentioned effects, salt content affects microbial growth, enzymatic activity or biochemical changes in cheese ripening, which affect their sensory properties **(Guinee, 2004; Shalaby, 1996)**. The current trend in food production is to find ways to reduce salt in food (reformulation) because of its high intake to the consumer and the associated health impact most often to increase blood pressure **(Guinee, 2004)**. Lowering the salt concentration, however, could affect the microenvironment, which may be more beneficial for the development of undesirable microflora. One of the undesirable manifestations of activity contaminating microflora is the production of biogenic amines. These compounds may, in case of higher intake, have an adverse effect on the health of the consumer. The most well-known effects of biogenic amines on the health of the consumer are their negative influence on the nervous and cardiovascular system **(Shalaby, 1996)**.

The aim of the work was to investigate the effect of reduction of salt content on textural properties, intensity of proteolysis and accumulation of biogenic amines in Dutch-type model cheeses during 3 months ripening.

Scientific hypothesis
Decreasing the concentration of NaCl promotes the activity of BA-producing microorganisms.

MATERIAL AND METHODOLOGY
Production of model samples
Model samples of cheese were made **(Pachlová et al., 2011)**. First, raw milk was preheated to 35 °C, followed by centrifugation of milk (Disc Bowl Centrifuge FT15, Armfield Inc., UK) and the standardization of the fat content of 2.5%. Pasteurisation with FT75 laboratory pasteuriser (Armfield Inc., UK) was performed (74 °C for 30 seconds). After pasteurisation, the milk temperature was adjusted to an inoculation temperature of 32 °C. For the production of a model batch of cheeses, 0.5 g of mesophilic culture of Flora Danica (samples marked as FD) or CHN19 (Chr. Hansen, Denmark) was added to the 20 litres of pasteurized milk. Subsequently, 10 mL of $CaCl_2$ (36% solution, Milcom a.s., Czech Republic) were applied. Culture was activated for 30 minutes. Coagulant (640 µL, Chymax M 1000, Chr. Hansen, Denmark) was used for renneting (30 minutes at 32 °C). After this process, the precipitate was carefully cut and left for 10 minutes to stand for curing. Curd was gentle stirred for

20 minutes. Before the cooking, 5 litres of whey were collected and the water at 80 °C was added to reach a temperature of 42 °C. Curd was stirred at 42 °C for 90 minutes. After this process, the curd was moved into molds where was pressed for 90 minutes. Pressed cheeses were left at 16 °C overnight. Each model batch was divided into 2 groups of cheese blocks. The first part was brined to concentration of 1.5% NaCl (samples marked as LS) and the second part of cheese blocks to 2.5% NaCl (samples marked as MS). NaCl content of brine was 20%. After brining, Delvocid (DSM, Netherlands) was applied on the surface of cheese blocks. The model cheese blocks were wrapped in a shrink foil and stored in a ripening chamber at a temperature of 12 ±1 °C. Samples of model cheeses were taken for analysis after the 14th, 28th, 56th and 84th day of ripening. Two blocks of model cheese were used for sampling from each batch. The production of cheeses with the selected culture was repeated twice.

Basic chemical analysis

The basic chemical analysis was focused on determining the dry matter content at a temperature of 102 ±2 °C (**ISO 5534:2004)**, NaCl content and value of pH according to **Flasarová et al. (2016)**. The samples were subsequently lyophilised **(Pachlová et al., 2011)** for determination of the free amino acid and biogenic amine contents.

Textural profile analysis

The texture evaluation was focused on monitoring the hardness of cheeses using TA.XT Plus (Stable Micro Systems, UK). A central cylinder of 35 mm in diameter and 20 mm in height was cut out of the cheese sample. The texture of the semi-hard cheese was evaluated by a 50 mm diameter probe by compression test. The sample was compressed by 25% of the original height at 2 $mm.s^{-1}$. The measurement was carried out at room temperature (20 ±2 °C).

Determination of free amino acids

The lyophilised samples of the cheeses were used to determine the free amino acid content. Total free amino acid content was expressed as sum of the concentrations of 30 free amino acids and their derivates (threonine, serine, asparagic acid, asparagine, glutamic acid, glutamine, proline, glycine, alanine, citrulline, valine, cysteine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, β-alanine, β -aminobutyric acid, γ-aminobutyric acid, ethanolamine, ornithine, lysine, histidine, 1-methylhistidine, 3-methyl-histidine, arginine, aminoadipic acid, α-aminobutyric acid). The extraction of the free amino acids was performed by triple extraction using Li-buffer according to **Pachlová et al. (2011)**. The resulting extract was analysed by ion-exchange liquid chromatography using Automatic Amino-Acid Analyzer AAA 400 (Ingos, Prague, Czech Republic) according to **Flasarová et al. (2016)** and **Buňková et al. (2009)**. Two blocks of cheese from each group were sampled and each extract was subjected to the chromatographic analysis twice (2 repetitions of manufacture × 2 cheese block × 2 extractions × 2 separation and determination of eluents; n = 16).

Determination of biogenic amines

The lyophilised cheese samples were used to determine the biogenic amine content (histamine, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine, spermidine and spermine). The triple extraction from the lyophilised cheeses was performed using 0.6 $mol.L^{-1}$ $HClO_4$ (Merck, New Jersey, USA) according to **Flasarová et al. (2016)** and **Dadáková, Křížek and Pelikánová (2009)**. After this process, the detection and separation of the biogenic amines was performed by means of high performance liquid chromatography (Agilent Technologies, Santa Clara, USA) using Agilent Eclipse Plus C18 RRHD column (Agilent Technologies, Santa Clara, USA) with dimensions of 3.0 × 50 mm × 1.8 μm and spectrophotometric detection at a wavelength of 254 nm and a column temperature of 30 °C **(Flasarová et al., 2016; Smělá et al., 2004)**. The extraction of biogenic amines was performed twice and each extract was subjected to chromatographic analysis twice (2 repetitions of manufacture × 2 cheese block × 2 extractions × 2 separation and determination of eluents; n = 16).

Statisic analysis

The results of the determination of free amino acid content and content of biogenic amines were statistically evaluated by means of the Kruskal-Wallis test and Wilcoxon test. Unistat® 5.5 software (Unistat, London, UK) was used for the statistical evaluation.

RESULTS AND DISCUSSION

Basic chemical analysis

After 14 days of ripening, the pH of samples inoculated with CHN19 culture was 4.91 – 5.01. Subsequently, the pH grew to 5.32 – 5.51 after 56 days of ripening. At the last sampling (84th day of ripening), a slight decrease was observed to values ranging from 5.28 – 5.38. There were no significant differences between samples with different NaCl concentration ($p \geq 0.05$). The average development of pH in FD-inoculated blocks after 14 days of ripening was 5.41. Subsequently, the pH slightly decreased to 5.35 – 5.38 (28th day of ripening). After 56 days of ripening, the pH rose to 5.46 and after 84 days of ripening reached values of 5.30 – 5.47. Differences between individual groups of model samples could arise due to the varying intensity of lactic acid production used by the culture. Non-starter lactic acid bacteria are also involved in pH change and during the ripening gradually prevail over the starter cultures and significantly contributed to the changes in matrix of ripening cheese **(Rynne et al., 2007)**.

The dry matter content slightly fluctuated during ripening. From the values found, in the case of MS cheeses (2.5% NaCl), the values on average of 2% of the dry matter content were higher than those for LS cheeses (1.5% NaCl). The dry matter content of MS samples ranged around 58.4%, while the dry matter content of LS samples was around 56.4%.

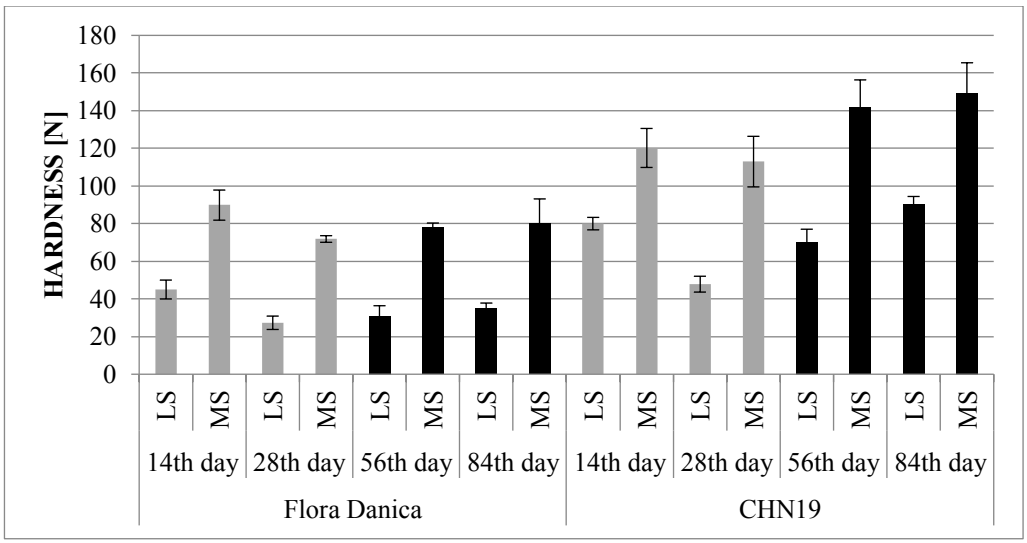

Figure 1 The development of hardness during ripening of model cheese samples: LS – model cheese with 1.5% NaCl content; MS – model cheese with 2.5% NaCl content.

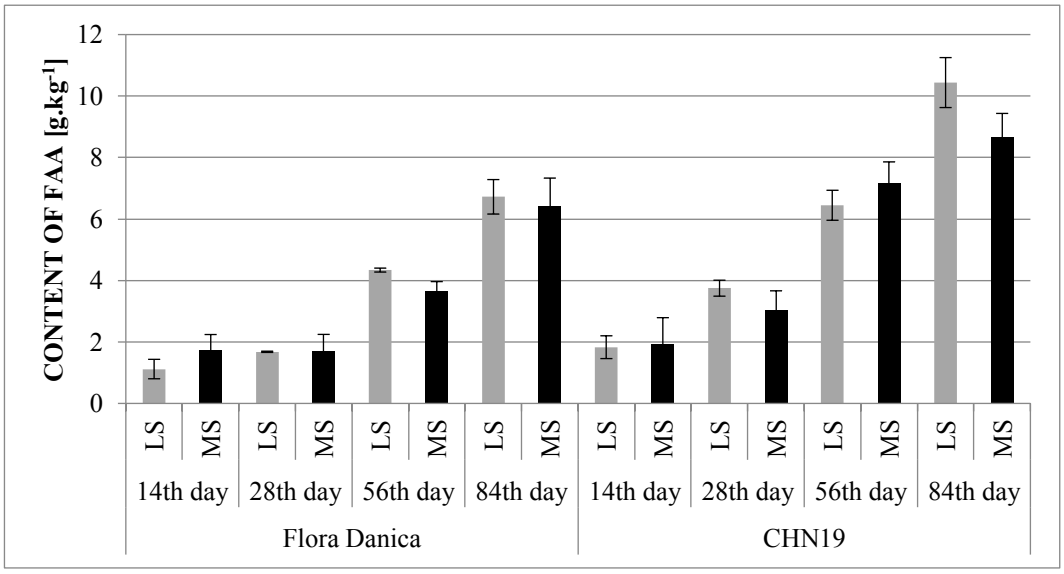

Figure 2 Free amino acid content during ripening of model cheese samples: LS – model cheese with 1.5% NaCl content; MS – model cheese with 2.5% NaCl content.

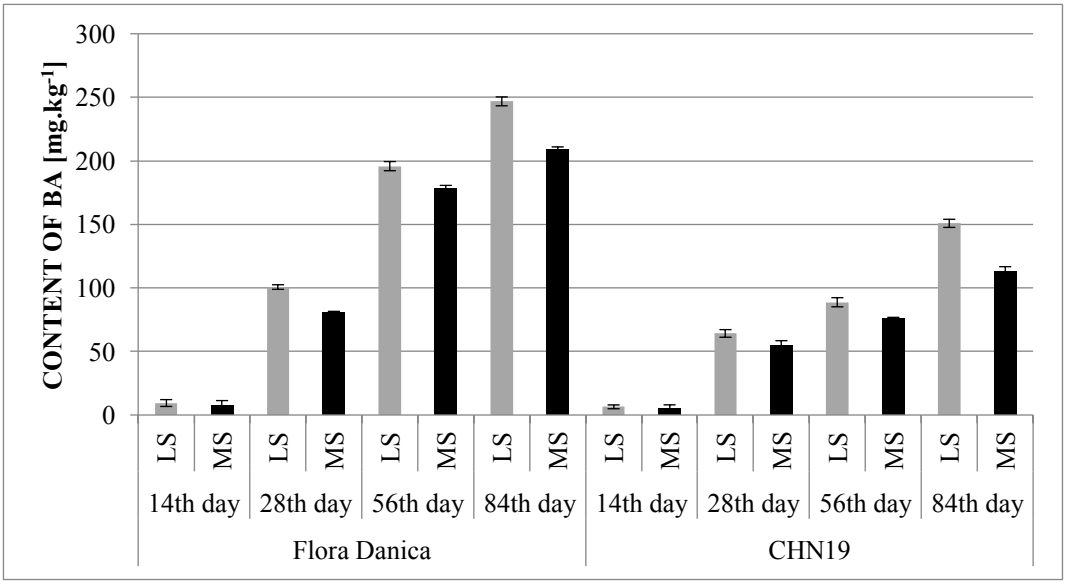

Figure 3 Biogenic amine content during ripening of model cheese samples: LS – model cheese with 1.5% NaCl content; MS – model cheese with 2.5% NaCl content.

Textural profile analysis

The effect of culture on hardness values was observed (Figure 1). In general, samples with FD culture have reached lower hardness values compared to CHN19 culture samples. After the initial decrease in hardness, a gradual increase in hardness was observed in all samples. A more pronounced trend was observed with samples of CHN19 culture. The hardness of the cheese depended mainly on the salt content and its associated dry matter content, where samples with higher NaCl concentration had a higher dry matter content and a higher hardness (Figure 1). In the case of FD-MS model cheeses, hardness was higher more than twice compared with FD-LS samples after 84 days of ripening. A similar trend was also observed for CHN19 samples. Higher hardness in the case of cheese with higher NaCl concentration was also found in Kaya's research **(Kaya, 2002)**.

Determination of free amino acids

The total content of free amino acids (FAA) in cheese blocks (Figure 2) increased during ripening as a result of proteolysis of the casein matrix **(Visser, 1993)**. In general, the free amino acid content of the MS samples was lower regardless of the culture used. This trend can be consequence of a reduction in the activity of the microflora and its proteolytic enzymes by higher salt concentration. **Murtaza et al. (2014)** also observed lower FAA contents in higher salt samples. In the case of MS cheeses, there was a limited trend in amino acid release until 28 days after production, which may be due to a slower diffusion of salt into the central parts of the blocks. Initial proteolysis is furthermore initiated by chymosin and plasmin, whose activity is not dependent on the salt content, whereas the salt content has an effect on the activity and growth of lactic acid bacteria **(Exterkate and Alting, 1995)**. In addition, different total FAA contents were determined in samples of different cultures. Higher concentrations of FAA were observed in CHN19 culture samples from 28 days of ripening. Different concentrations of FAA in model cheese with different cultures may be due to both the different proteolytic activity of the microflora present, but also by the decarboxylation of amino acids to biogenic amines **(Halász et al., 1994; Butor et al., 2017)**, as indicated by the results of the total concentration of biogenic amines (see Figure 3).

On the other hand, after 56 days of ripening the FAA content of CHN19-MS samples increased sharply. Compared with other sampling days, these FAA values are in contradiction with the trend of FAA concentrations which were determined in model cheeses with a lower salt content. The rationale can be found in extensive lysis of cells in environments with higher salt concentrations. In the case of FD-type cheeses, however, this phenomenon was not observed.

Determination of biogenic amines

The content of biogenic amines (BA) in the samples (Figure 3) increased with increasing ripening. The content of BA in the samples increases with the degree of proteolysis of the casein network, respectively by releasing their precursors – free amino acids **(Halász et al., 1994)**. The second observed trend is related to salt content in cheese blocks, where a lower total content of BA was observed in samples with higher salt concentrations. After 14 days of ripening, the content of BA in all samples was relatively low and ranged from 5.32 – 12.23 mg.kg^{-1}. After the 28th day of ripening, the BA content has increased sharply in all batches, probably as a result of the growth and activity of non-starter lactic acid bacteria **(Bover-Cid et al., 2001)**, which are referred to in many publications as the cause of the accumulation of BA during ripening of cheeses **(Shalaby, 1996; Stratton, Hutkins and Taylor, 1991; Leuschner, Kurihara and Hammes, 1999)**. For FD-MS model cheeses, the total content of BA was nearly 20% lower after 3 months ripening compared to LS samples. In the case of CHN19 cheeses, even the BA accumulation rate for MS cheeses was lower by more than 25%. This can be explained by partial inhibition of proteolysis but in particular by suppression of decarboxylase activity of bacteria due to increased NaCl content. From the established values it can be stated that the reduction in salt content may contribute to the higher accumulation of BA during ripening of the FD-MS cheese and **Gardini et al. (2001)** also measured similar results.

CONCLUSION

From the results obtained it can be stated that the salt content has a significant influence (p <0.05) on the course of biochemical processes during maturation of the cheeses. Higher concentration of salt caused the reduction of microbial activity. Subsequently lower total FAA content was determined in cheese with higher salt content. Similarly lower total content of BA was observed in samples with higher salt concentration. BA content in model cheese with 2.5% NaCl content was 20% lower after 3 months ripening copared to model cheese with 1.5% NaCl content. It has been also observed that higher concentration of NaCl increased the dry matter content, which is reflected in the higher hardness of the cheese blocks. No unambiguous trend was observed between pH values in samples with different salt contents.

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EVALUATION OF THE CONTENT OF PIGMENTS AND TOTAL SUGARS IN GROUND SWEET PEPPER

Marián Rehuš, Magdaléna Valšíková

ABSTRACT
The aim of this research was to evaluate and compare the content of pigments and total sugars in raw materials of both the domestic as well as foreign origin that are used in the production of sweet ground pepper. The tests included two samples exported from abroad, specifically from Serbia and China and the following domestic varietes of *Capsicum annum* L.: Kolora, Žitava, Dvorská (varieties that form the base of the final product called Paprika Žitava/Žitavská paprika and a mix of a number of domestic varieties called Slovenský polotovar. The tests were conducted in 2015 and 2016. The highest content of pigments in both years was contained in Dvorská and Kolora – 6.81 and 6.11 $g.kg^{-1}$. The largest amount of total sugars was recorded within Žitava in 2015 (20.5%) and in 2016 Slovenský polotovar with 24.5%. Both the exported as well as domestic products fulfilled the parameters of quality required in the production of sweet pepper.

Keywords: sweet ground pepper; total sugars; content of pigments

INTRODUCTION
Peppers, *Capsicum annum* L.var. longum, are a variety which belong to well-known commercial and industrial plants **(Lim, 2013)**. This species boasts wonderful spice qualities. It gives delicious taste, smell and colour to all different dishes. It is used both in fresh as well as dried form. There is a higher consumption of the sweet pepper, but hot pepper is also popular. Mature peppers are dried and ground until fine powder is made **(Rehuš and Valšíková, 2016)**. In Slovakia, the consumption of sweet ground pepper per capita is 100 – 200 g a year **(Valšíková, Červenka and Sudzina, 2010)**.

According to **Habán, Černá and Dančák (2001)**, the consumption of sweet pepper in Slovakia has increased from 50 to 100 g per capita a year. Usage of spices such as paprika or pepper spices are also of great economical interest **(Škrovánková et al., 2017)**. Nowadays, only around half the volume of the Slovak consumption of peppers is produced in the territory of Slovakia, the rest is transported from abroad. The production of spice pepper in the most fertile districts of Slovakia is gradually decreasing, as is the production of sweet peppers. **(Valšíková, Ryban and Srničková, 2014)**. One of the most important qualitative parameters of sweet pepper is the content of carotenoid pigments and total sugars. Pepper fruits include carotenoid pigments, mainly carotene, capsanthin, cryptoxanthin, lutein and zeaxanthin. The amount of red pigments determines the colour intensity of the final product **(Muchová et al., 2001)**. Capsorubin is the main colouring constituent. From the total amount of sugars, peppers contain 90 – 98% of dextrose, the rest is accounted for fructose and sucrose.

The final quality of the products is influenced mainly by the quality of fruits as well as by the processing technology – drying and grinding. „Paprika Žitava/Žitavská paprika“, which was entered in the register of protected designations of origin and protected geographical indications by the European Commission on the 11th of February 2014 **(Commission Regulation (EU) no. 126/2014)**, belongs to a group of products with superb quality. Paprika Žitava is sweet ground pepper made by grinding the dried spice pepper fruits harvested in the area of Podunajská nížina (Danubian Lowlands). The fruits are state-recognized varieties that are picked intact when ripe and then they undergo a special postharvest treatment.

„Paprika Žitava/Žitavská paprika“ gets its characteristic intense colour from the final stage in the grinding process on what is known as the ‘colouring stone’; as pressure is applied, the temperature rises and the oil contained in the seeds is released, which is what imparts the characteristic orange-red colour **(Council Regulation (EC) No 510/2006)**.

Scientific hypothesis
The pigments and sugars in the ground spice pepper form the taste and intense red colour. Their content depends on many cultivation and processing conditions. Therefore, we assume a significant difference between the years under review. The content of dyes and sugars is also one of the varietal properties. It is assumed that we will find differences between varieties. We expect above-average

sugar and pigments content in the final product of ground pepper called "Paprika Žitava / Źitavská paprika".

MATERIAL AND METHODOLOGY

Raw material

The Slovakian company Mäspoma, spol. s.r.o Zvolen, plant mill for processing spices – Dvory nad Žitavou provided all the following raw materials, final products and information about the production and evaluation of all samples. The company also has a laboratory, where part of all of the laboratory experiments took place. The samples were evaluated during the years 2015 and 2016.

We used strictly specified procedures of sampling, samples were taken from a number of bags. Five fractional samples of about 100 g were taken from each bag. The fractional samples were mixed, the average sample was ground in the laboratory to be consequently sieved through a 0.5 mesh size sieve. The sample, gained in the above described method was then evaluated by means of subjective as well as objective methods.

The following samples were included in the laboratory experiments:

The domestic varieties of the *Capsicum annum* L.: Žitava, Dvorská and Kolora.

The mix of a number of domestic varieties called Slovenský polotovar, which is used as a feedstock for making different spice mixtures according to the requirements of consumers.

Different transported raw materials used to make sweet pepper from Serbia and China. These two countries are the main exporters to Slovakia and their products are used in the production of spice mixtures.

The final product of ground pepper called „Paprika Žitava/Žitavská paprika“ (Figure 3 and Figure 4).

Laboratory Methods

Determination of Pigments in ground sweet pepper according to STN EN ISO 7541:1989

The spectrophotometric method was used to quantify the content of carotenoids. Sweet paprika in the amount of 0.5 g was placed into a dark reagent bottle and 50 mL of acetone was added. The sample was swirled thoroughly and left to stand for 30 minutes. 5 mL of the sample was pipetted into a 50 mL volumetric flask, which was filled up to the mark with acetone and swirled thoroughly. Absorbance was measured using a wavelength of 477 nm in a photospectrometer SPEKOL 11 (spectral colorimeter) made by Carl Zeiss Jena, Germany.

Calculation of pigments in $g.kg^{-1}$:

$$C = \frac{A \times f \times 2.5 \times 10^5}{2250 \times (100 - H) \times m}$$

where:
A – the absorbance of the test solution,
f – the correction factor,
H – the moisture content of the test sample,
m – the mass, in grams,
2250 – the absorption cofficient of capsanthin,
2.5×10^5 – a conversion factor.

Determination of Total Sugars according to Somogyi

The free hemiacetal group of sugars is characterised by its reducing properties.

The reducing sugars when heated with alkaline copper tartrate included in the Somogyi solution reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. Based on its intensity the content of reducing sugars is determined at a wavelength of 710 nm in a spectrophotometer Specord 50 Plus by Analytikjena, Germany. The distributor of this chemical is company MikroChem Trade, Pezinok, Slovakia. The acid hydrolysis then determines the amount of total sugars. The difference in both determinations gives the content of non reducing sugars **(Repčák et al., 2015)**.

Káš, Kodíček and Valentová (2006) describes the following steps of the analysis: Fill the given amounts of stock solution in the following amounts: 0; 0.2; 0.4; 0.6; 0.8; 1 mL into prepared laboratory tubes and add distilled water up to 1 mL. One mL of Somogyi solution is then added to Each laboratory tube, it is heated in a hot bath for 15 minutes. After they have cooled down, 2 mL of Nelson agent are added, swirled well and then 10 mL of distilled water is added. The intensity is then measured by the formed colour of the blue-green colour of the solution that is formed.

Statistical analysis

The results of the laboratory experiments were processed by standard statistical methods using statistical software Statgraphics Centurion XVII (StatPoint Inc, USA) – means of multiple comparison analysis testing – ANOVA test (multiple range test) and 95.0 percent LSD test .

Table 1 Average Content of Pigments and Total Sugars.

Years	Variety	Pigments in g.kg^{-1}	Total sugars in %
2015	Paprika Žitava	6.22	20.50
2015	Slovenský polotovar	5.55	20.00
2015	Chinese	6.54	18.25
2015	Serbian	5.59	17.30
2015	Dvorská	6.68	11.50
2015	Kolora	6.68	8.40
2015	Žitava	5.42	5.85
2016	Paprika Žitava	6.38	21.00
2016	Slovenský polotovar	4.93	24.50
2016	Chinese	5.29	14.50
2016	Serbian	5.01	18.00
2016	Dvorská	6.94	11.70
2016	Kolora	6.11	7.25
2016	Žitava	5.94	15.20

Table 2 Multiple Range Test for Pigments g.kg^{-1} (Method: 95.0 percent LSD).

Variety	Years	Count	LS Mean	LS Sigma	Homogeneous Group
ZI	2015	3	5.42333	0.0409768	X
SP	2015	3	5.55667	0.0409768	X
SR	2015	3	5.59333	0.0409768	X
PZ	2015	3	6.22667	0.0409768	X
CH	2015	3	6.54333	0.0409768	X
DV	2015	3	6.68	0.0409768	X
KO	2015	3	6.68333	0.0409768	X
SP	2016	3	4.93	0.0757677	X
SR	2016	3	5.01667	0.0757677	X
CH	2016	3	5.29667	0.0757677	X
ZI	2016	3	5.94667	0.0757677	X
KO	2016	3	6.11	0.0757677	X
PZ	2016	3	6.38667	0.0757677	X
DV	2016	3	6.94667	0.0757677	X
Yeas	Count	LS Mean	LS Sigma		Homogeneous Group
2015	21	5.80476	0.0767936		X
2016	21	6.10095	0.0767936		X

Notes: PZ – Paprika Žitava, SP – Slovenský polotovar, SR – serbian sample, CH – chinese sample, DV – Dvorská, KO – Kolora, ZI – Žitava.

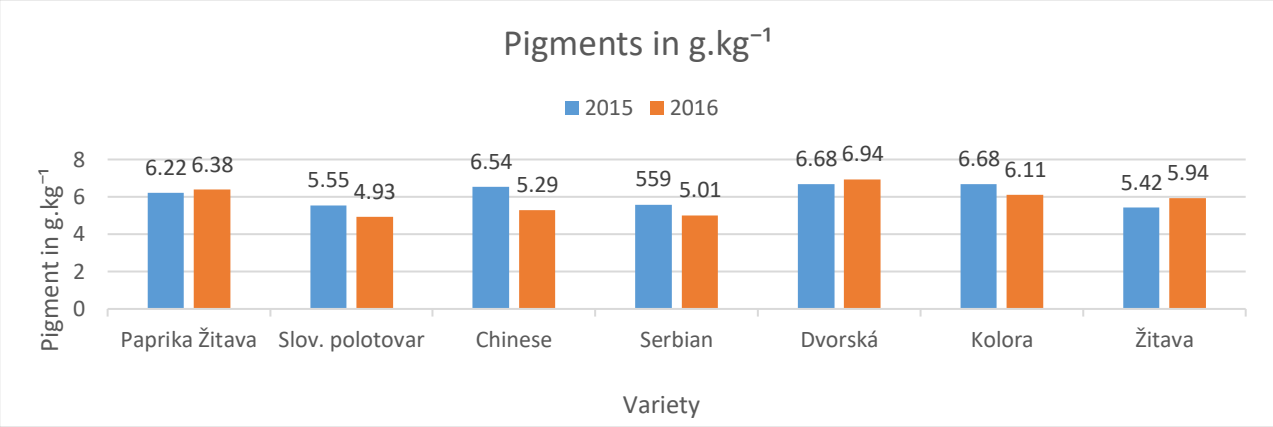

Figure 1 Contents of pigment in g.kg^{-1}.

Table 3 Multiple Range Test for Total sugars in % g.kg^{-1} (Method: 95.0 percent LSD).

Variety	Years	Count	LS Mean	LS Sigma	Homogeneous Group
SP	2015	3	5.24333	0.143668	X
SR	2015	3	5.305	0.143668	XX
ZI	2015	3	5.685	0.143668	XX
CH	2015	3	5.92	0.143668	XX
PZ	2015	3	6.30667	0.143668	XX
KO	2015	3	6.39667	0.143668	X
DV	2015	3	6.81333	0.143668	X
KO	2016	2	7.825	2.12656	X
ZI	2016	2	10.525	2.12656	XX
DV	2016	2	11.6	2.12656	XX
CH	2016	2	16.375	2.12656	XX
SR	2016	2	17.65	2.12656	XX
PZ	2016	2	20.75	2.12656	X
SP	2016	2	22.25	2.12656	X
Yeas	**Count**	**LS Mean**	**LS Sigma**		**Homogeneous Group**
2015	7	14.5429	1.13669		X
2016	7	16.0214	1.13669		X

Notes: PZ- Paprika Žitava, SP – Slovenský polotovar, SR – serbian sample, CH – chinese sample, DV – Dvorská, KO – Kolora, ZI – Žitava.

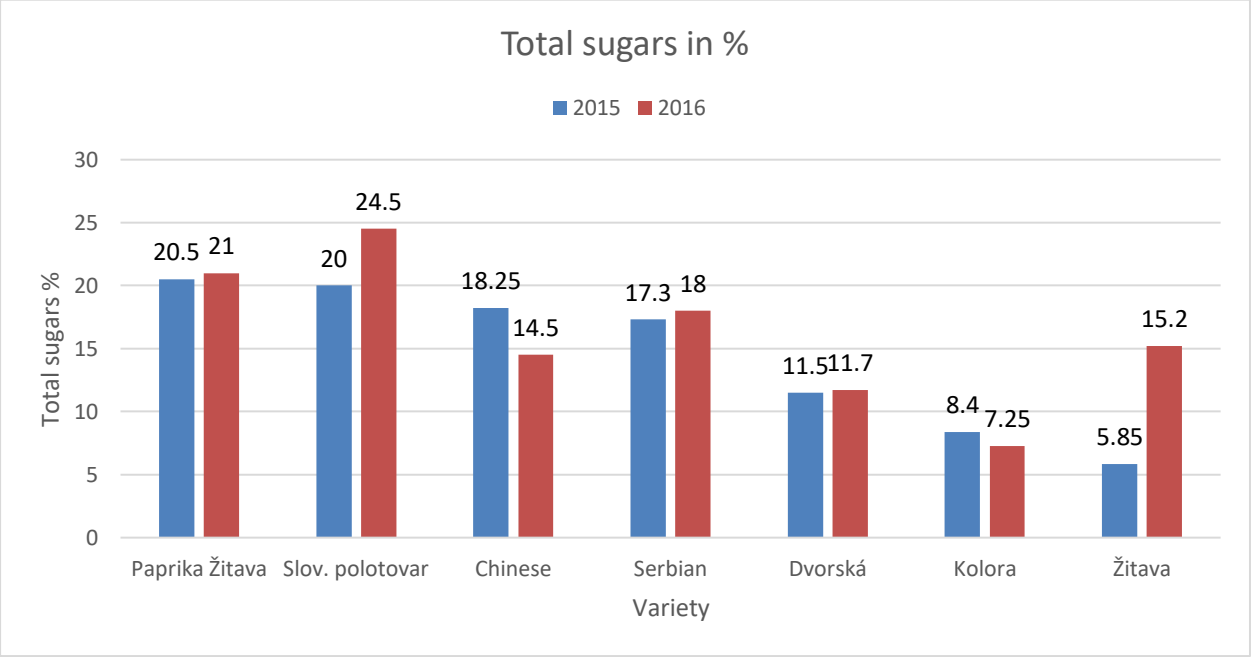

Figure 2 Contents of total sugars in %.

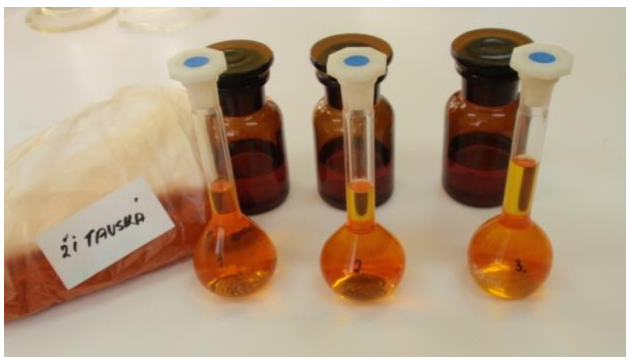

Figure 3 Varieta Žitavská – samples for testing.

Figure 4 Sample Žitavská – dry fruit, powder.

RESULTS AND DISCUSSION

The laboratory analyses determined the content of pigments and sugars in 6 samples of ground sweet pepper. In 2015 the highest content of pigments was discovered in these varieties: Dvorská and Kolora, equally 6.68 $g.kg^{-1}$ and the lowest content was determined within the variety Žitavská 5.42 $g.kg^{-1}$ (Figure 1).

In 2016 the highest content of pigments we find in the variety Dvorská 6.94 $g.kg^{-1}$ and the lowest content 4.93 $g.kg^{-1}$ in Slovenský polotovar (Figure 1).

In 2015 the highest average content of total sugars we confirmed in Paprika Žitava (20.5%) and in Slovenský polotovar (24.5%) in 2016. The lowest content of total sugars in 2015 we discovered in the variety Žitava (5.85%). In 2016 the variety Kolora showed the lowest content of total sugars (7.25%) (Figure 2). The most important requirement of ground red pepper is the pigment content. Pigment synthesis begins on the plant during growing and ripening. Later after harvest and continues throughout the drying **(Kerek et al., 2015)**.

Table 1 shows the observed average results as discovered over the duration of two years.

Evaluation of pigments content

The analysis of variance done for the samples of ground sweet pepper in 2015 showed statistically significant differences in the content of pigments (diff. = 013 – 1.26; α <0.05) in the majority of samples.

However, the samples of Dvorská – Kolora and Slovenský polotovar – Serbian did not show any statistically significant differences in the content of pigments (Table 2).

The year 2016 also proved statistically significant differences in the content of pigments of the majority of analyzed samples (diff. -1.65 – 2.02; α <0.05). The samples Kolora – Paprika Žitava and Slovenský polotovar and the Serbian sample did not show any statistically significant differences in the content of pigments (Table 2).

The multiple comparison method proved a statistically significant difference in the contents of pigments in between the years 2015 and 2016 (0.0296; α <0.05) (Table 2).

Evalution of total sugars content

The sweetness of ground paprika is a very important quality factor that affects the popularity of consumers. According to **Kyung-Hyung, Yung-A and Jae-Bok (2012)** was a total sugar content of 16.79 – 29.92%, glucose, fructose and sucrose content was 5.6 – 11.2%, 8.91 – 16.89%, and 1,78 – 2.97%. In our experiments the highest average content of total sugars was determined in 2015 within Paprika Žitava/Žitavská paprika – 20.5% and in 2016 it was the sample of Slovenský polotovar with 24.5%. The lowest content of total sugars in 2015 was recorded within the variety Žitava (5.85%) and within the variety Kolora (25%) in 2016 (Figure 2). The average results as recorded over the duration of two years are shown in the Table 1. Other authors also report the total sugar content of 20.44**% (Habán, Černá and Dančák, 2001; Bojňanská, 2004)**. **Sharma, Joshi and Kaushal (2015)** found total sugars in the dry ground pepper spice between 8.68 and 9.10%.

Comparison of the percentage content of total sugars in between 2015 and 2016 showed a statistically significant difference in the content of sugars (p = 0.019) and in the content of sugars among the following samples Chinese – Kolora, Dvorská – Paprika Žitava, Dvorská – Slovenský polotovar, Kolora – Paprika Žitava, Kolora – Serbian, Paprika Žitava – Žitavská, Slovenský polotovar – Žitavská (diff. -14.43 – 11.73; α <0.05) (Table 3).

CONCLUSION

Over the year 2015 and 2016 the laboratory tests examined the content of sweet pepper pigments as recorded from the dry matter. The average content of pigments ranged between 5.42 – 6.68 $g.kg^{-1}$.

The highest content of pigments was recorded in the following varieties Dvorská and Kolora, variete Dvorská 6.94 $g.kg^{-1}$ (2016) and 6.68 $g.kg^{-1}$ (2015), and Kolora 6.68 $g.kg^{-1}$ (2015) and 6.11 $g.kg^{-1}$ (2016). The sample Paprika Žitava also showed high contents of pigments during both years 2015 and 2016 – in 2015 it was 6.22 $g.kg^{-1}$ and in 2016 it was 6.38 $g.kg^{-1}$. The products bought abroad also showed high contents of pigments, the Chinese sample had the following average content of pigments 6.54 $g.kg^{-1}$ in 2015 and 5.29 $g.kg^{-1}$. The lowest content of pigments was recorded in the sample Slovenský polotovar (4.93 $g.kg^{-1}$) in 2016. The tests also found statistically significant differences in the average content of pigments in the majority of varieties as well as in different years.

The average content of total sugars ranged in the analysed years from 5.85% (Žitavská, 2015) up to 24.5% (Slovenský polotovar, 2016).

In 2015 it was Paprika Žitava which achieved the highest content of total sugars (20.5%) and Slovenský polotovar (24.5%) in 2016. Žitava also recorded the average content of sugars above 20% in both analyzed years and truly the sweetness is its most distinguishing feature. The majority of samples also showed statistically significant differences in the content of total sugars as well as in the analyzed years.

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TREATMENT OF POULTRY SLAUGHTERHOUSE WASTEWATER WITH COMBINED SYSTEM

Kulyash Meiramkulova, Mikhail Zhumagulov, Gulnur Saspugayeva, Zhanar Jakupova, Maral Mussimkhan

ABSTRACT
With the interest to reuse and recycle the wastewater for technological use, this project aims to test the treatment of wastewater from poultry slaughterhouse industry from three main sections of the poultry slaughtering process, defeathering, eviscerating and cooling processes. The samples for the project were obtained from Izhevskoe a Kazakhstani company. The technology used is a combination of electrocoagulation, ultrafiltration, and photochemical system and its goal is to provide treated water that can be re-utilized in the poultry industry for sterilization of technical equipment without contaminating and affecting the quality of the poultry products. The treatment of wastewater samples lasted in total for 40 min. From the results, it was found that indicators such as BOD, COD, and phosphates had removal efficiency of almost 100%, while the microbiological colonies were all eradicated from then wastewater making the treated water microbes free. Hence, proving this system to be effective for the treatment of poultry slaughterhouse wastewater and safe for technological reutilization.

Keywords: wastewater; electrocoagulation; ultrafiltration; photochemical

INTRODUCTION
Recently, with the interest in reusing and recycling the wastewater, scientists from across the globe are finding adequate and efficient treatment for poultry slaughterhouse wastewater. Electrocoagulation (EC), ultrafiltration, and photochemical treatment are only some to in the list that are possible methods that could be employed to treat the effluent not only from the poultry slaughterhouse industry but also other industries **(Meiramkulova et al., 2018c)**.

There have been several studies on the effectiveness of wastewater treatment using the EC process that proved it to be effective **(Bayramoglu et al., 2004; Kobya et al., 2003; Can et al., 2003; Lin and Chen, 1997; Meiramkulova et al., 2018a)**. Removal of organic, heavy metals, nutrients, and even pathogens from the wastewater is confirmed to effective is EC is used **(Kobya et al., 2006; Bayar et al., 2011; Meiramkulova et al., 2018d)**. In EC, coagulant ions are discharged into the solution using sacrificial electrodes and current. Reaction at the anode, cathode and consequently solution can be observed from the following reaction **(Picard et al., 2000)**:

$$\text{Anode: } M_s \rightarrow M_{aq}^{n+} + ne^-$$
$$\text{Cathode: } 2H_2O + 2e^- \rightarrow 2OH^- + H_2$$
$$\text{Solution: } M_{aq}^{n+} + nOH^- \rightarrow M(complex) \rightarrow M(OH)_{n(s)}$$

Where M is material of electrodes. Various mono monomeric species such as $M(OH)^{(n-1)}$ and also polymeric species such as $M_6(OH)_{15}^{(6n-15)}$, are formed from metal and hydroxide ions **(Sardari et al., 2018)**. Amorphous $M(OH)_{n(s)}$ in the solution as it ages which can be seen from the reaction above **(Kobya et al., 2003)**.

After that, the metal complexes $M(OH)_{n(s)}$ transforms into solid with a large surface area to trap suspended solids, organic compounds, and form flocs. Finally, the flocs will polymerize, and deposit as can be seen by the following reaction **(Rebhun and Lurie, 1993; Bayramoglu et al., 2004)**.

$$x\,M(OH)_n \rightarrow M_x(OH)_{xn}$$

Membrane technology for the treatment of slaughterhouses wastewater (SWW) is also an alternative with processes such as reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), and microfiltration. These technologies have the ability to remove colloids, particles, and macromolecules depending on the size of the pore **(Spasov and Dinkov, 2002)**. Not only that, there has been a growing use of membrane technology in the removal of microorganism, bacteria, particulates, and organic matter from the SWW. RO and NF are one of the best process that can be used to remove most of the pollutants including dissolved organics. On the other hand, the downside is their high operating cost due to large energy requirements **(Garcia et al., 2013)**. Therefore, UF is a low-pressure and cost-effective option due to its high permeate flux compared

to RO and NF. Also, UF is a mature process that has been used in the food processing industry for the past 20 years. There are numerous advantages to UF when compared to conventional treatment methods as it is low energy consuming, no added chemicals, and small footprint **(Sardari et al., 2018)**.

Similar to other membrane process, UF also suffers from membrane fouling. This causes significant decrease in efficiency of water recovery as a result of collected particles both on membrane surface and inside the pores **(Białas et al., 2014)**. Membrane cleaning and pre-treatment are some of the main strategies to minimize fouling. There have been number of studies on membrane cleaning along with sing reagent and multi-step cleaning for fouled membrane **(Mohammad et al., 2012)**. In one study it was shown that moderate flux recovery can be achieved with membrane cleaning and without using pre-treatment **(Malmali et al., 2018)**.

Several pre-treatment process prior to membrane filtration have also been considered. Some of the more popular pre-treatment methods before membrane filtration are pre-oxidation, absorption, pre-filtration, and chemical coagulation. In this study, our focus will be on the utilization of electrocoagulation (EC) as pre-treatment method before UF.

Advanced oxidation processes (AOPs) are also increasingly gaining ground as an alternative to the conventional treatment and as a complimentary treatment (pre-treatment or post-treatment), to processes such as the current biological. Advantage of such technology is that it is able to inactivate microorganism without the need of any chemicals for the treatment of slaughterhouse wastewater. This prevents the usage of method such as chlorination to disinfect the water which can possibly lead to hazardous by-product **(de Sena et al., 2009; Bustillo-Lecompte, Knight and Mehrvar**, **2015)**. Due to this AOPs is recognized as a treatment process that is showing exceptional overall results as a complimentary treatment in water reuse, pollution control, and advanced degradation. One of the most commonly used AOPs is the UV/H_2O_2 process. This method is found to be effective for the treatment of SWW. From the reaction of UV and H_2O_2, hydroxide radicals (OH) are produced which oxidizes and degrades the effluents, making this technology effective **(Bustillo-Lecompte, Knight and Mehrvar**, **2015)**. However, in this study since EC and UF are already being utilized as a treatment technology, only UV radiation will be used as a post-treatment to inactivate microorganism present is the water after the UF process.

In this study, investigation of the effectiveness of poultry slaughterhouse wastewater (PSW) treatment using a combined system of EC, UF, and UV sterilization will be analysed. This system is intended for recycling and reusing the wastewater that can ease the burden on water fresh resources and at the same time not affect the quality of the sterilization of the equipment used for the slaughtering processes. Also, since there are not many studies done on the combination of EC-UF-UV for PSW treatment, this research can provide a base for other future studies in this area.

Scientific hypothesis

The designed combined poultry slaughterhouse wastewater treatment system involving sedimentation, EC process, ultrafiltration, and UV treatment will show exceptional results as removal efficiencies for almost all indicators are within the quality standards. Also, the wastewater after treatment will be 100% free from microbe colonies. The technology is proposed for obtaining technical water from the wastewater with antioxidant properties for washing and disinfecting equipment and premises of poultry farms, not affecting the quality of poultry products (important for food safety standards **(Nagyová et al., 2019)**). The project will reduce the volume of water consumption of the poultry industry, which is one of the important indicators of "green technology".

MATERIAL AND METHODOLOGY

Water Characteristics

Table 1 below shows the characteristic of wastewater obtained from Izhevskoe a Kazakhstani company. The sample is from cooling section and was kept at 4 ℃ once delivered to the lab. To examine characteristics of the wastewater, analytical methods were used which are described in the following section. Also, before conducting the experiment, calcium chloride was added to the wastewater for the EC process.

Experimental apparatus and procedure

In this study, a combined system is used which comprises of EC process, ultrafiltration, and photochemical purification. Figure depicts the schematic of the lab combined system used. First, the mini reservoir collects the wastewater and filters large particles such as feathers or organic components, using the macro-filter added. The effluent then leaves the reservoir and enter the electrolyte cell in which the EC process occurs for about 30 min. This electrolyte cell is made from polypropylene material with the dimensions 15 x 13 x 11 cm. The electrodes plate dimension used were 10.5 x 11.5 x 0.2 cm and 10.5 x 10.5 x 0.8 cm for aluminium (anode) and graphite (cathode) electrodes respectively. These electrodes were connected to direct current supply (Xinhua electrical weld company, China) characterized by the ranges 0 – 10 A for operating current and 0 – 12 V for voltage. Usually, electrodes selected are iron and aluminium as they are cheap and readily available. However, in our previous study, we have mentioned that using graphite is a better option as it is inert and does not consumes, making it not only efficient but also cost-effective **(Meiramkulova et al., 2018d)**.

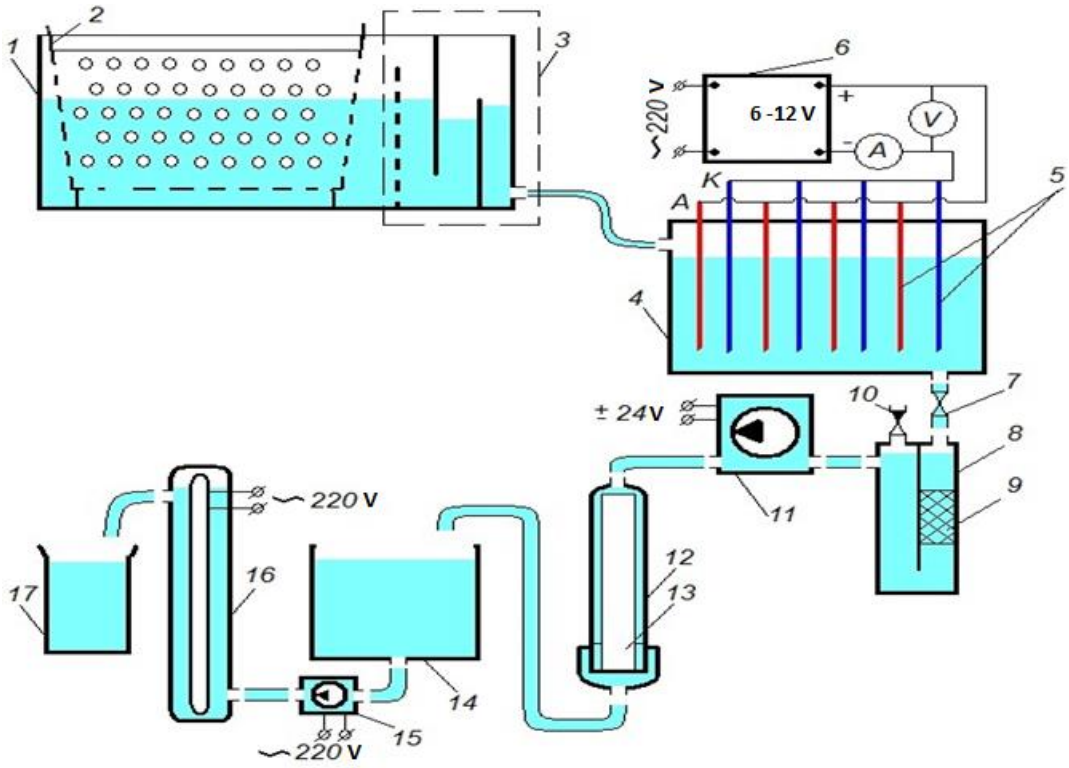

Figure 1 Combined poultry slaughterhouse wastewater treatment system.
Note: 1 – Wastewater Reservoir; 2 – Removable Macro-filter; 3 – Oil collection; 4 – Electrolyte cell; 5 – Electrodes (A – anode, K – cathode); 6 – Power supply; 7 – Valve; 8 – Sedimentary cylinder; 9 – Filter; 10 – Air vent valve; 11 – Pump (Maximum pressure of 5.5bar); 12 – Ultra-filter; 13 – Ultra-filtration membrane; 14 – Reservoir; 15 – Circulatory pump; 16 – Ultraviolet lamp; 17 – Reservoir for pure water.

Once EC is completed, processed water is then transferred to the sedimentary cylinder where any organic matter is collected. A pump with a pressure of up to 5.5 bar, allows the water to flow from the sedimentary cylinder to the ultrafilter. This ultrafilter has a pore size of 0.02 μm, sufficient to trap most sediments, bacteria, and viruses. Finally, the ultra-filtered water flows to a small reservoir where a small pump is used to recirculate the water through an UV sterilizer for 10 min to kill any remaining microorganism in the water. Overall, this process lasted for about 40 min.

Mechanism of ion transformation during EC

Calcium chloride was added as an electrolyte to all samples before electrolysis, which decreases pH level of the wastewater and prevent basic condition from occurring which can lead to formation of ammonia gas that can be harmful to the environment. Furthermore, the formation of hypochlorite ions occurs during the EC process. Then, at anode molecular chlorine consequently hydrolyses in neutral water condition, hence, reducing its concentration **(Czarnetzki and Janssen, 1992)**.

$$2Cl^- - 2e^- \rightarrow Cl_2$$
$$Cl_2 + H_2O \rightarrow HClO + H^+ + Cl^-$$

Phosphorus compounds present in the PSW are represented as polyphosphates, orthophosphates, and organic phosphorus-containing compounds. They can be present in the form of dissolved, colloidal and suspended state as phosphate ions. During EC treatment, phosphate ions released combine with a metal ion to produce metal phosphates, reducing the total phosphate presence in the treated water **(Emerson and Widmer, 1978)**.

As for nitrogen containing compounds, urea is a main by-product of the organisms. It can be found in animal-based industrial wastewater as ammonium carbonate under the impact of putrefying bacteria **(Barmaki et al., 2009)**.

$$CO(NH_2)_2 + 2H_2O \rightarrow (NH_4)_2CO_3$$

However, it degrades and results in carbon dioxide and ammonia.

$$(NH_4)_2CO_3 \rightarrow 2NH_3 + CO_2 + H_2O$$

Ammonia then transforms into nitrite and nitrate ions by consuming oxygen under the impact of nitrifying bacteria as it is shown below **(Pan et al., 2018)**.

Nitrification:

1-step $NH_4^+ + 2O_2 \rightarrow NO_2^- + H_2O + H^+$

2-step $NO_2^- + 0.5O_2 \rightarrow NO_3^- + H_2O + H^+$

Then nitrate ions combine with metal ions. Thereby, nitrates are neutralized, again reducing its concentration in the treated water.

Table 1 Poultry slaughterhouse wastewater characteristic before treatment.

Indicators	Defeathering	Evisceration	Cooling	Units
pH	7.36	7.2	6.5	-
Turbidity	49.7	98.5	167	FAU
Color	649	1371	1762	
TSS	84	180	298	mg.L-1
Chlorine free	0.14	0.03	0.80	mg.L-1
Chlorine total	0.11	0.07	6.57	mg.L-1
Nitrogen nitrites	0.109	0.06	0.122	mg.L-1
Nitrogen nitrates	18.5	8.30	24.5	mg.L-1
Phosphates	5.00	5.06	1.79	mg.L-1
Nitrogen ammonium	1.28	2.27	0	mg.L-1
COD	578	1162	1729	mg.L-1
BOD5	96.2	12.1	458.8	mg.L-1
Carbonates	0	90	0	mg.L-1
Hydrocarbonates	488	457,6	427	mg.L-1
Calcium	14	23.5	11	mg.L-1
Magnesium	2	16.5	26.5	mg.L-1

Furthermore, ammonia reacts with hypochlorite ions and produces monochloramine and dichloramine in acidic condition **(Asano et al., 2005)**. Later, mono and dichloramines results in nitrogen gas.

$$NH_3 + HClO \rightarrow NH_2Cl + H_2O$$
$$NH_2Cl + HClO \rightarrow NHCl_2 + H_2O$$
$$NH_2Cl + NHCl_2 \rightarrow N_2 + 3HCl$$

All these reactions allow the reduction of pollutants in the wastewater.

Statistic analysis

The data obtained for the pollutant level in the poultry slaughterhouse wastewater was measured locally within the university lab. The following equipment was used in the measurements of the pollutant concentration: Standard Operating Procedure for GLNPO Turbidity: Nephelometric Method and American Public Health Association 4500-Nor, were used to determine the turbidity of the samples, and total bound nitrogen (TN) and total phosphorus (TP) respectively. Spectrophotometer (Hach DR3900, HACH / LANGE, Germany), standard reagents and test kits were used for the reliability of the chemical analysis. Furthermore, lab pH-meter (Hach Co), was used to measure pH values.

In accordance with the regulatory requirements of industrial and drinking water of the Republic of Kazakhstan, sanitary and microbiological examination of water before and after treatment was done based on 3 indicators:

- Total microbial count (TBC) is an indicator for assessing total bacterial contamination.
- Total coliform bacteria (TCB) are conditionally distinguished by morphological and cultural characteristics of a group of bacteria of the *Enterobacteriaceae* family, used by sanitary microbiology as a marker of fecal contamination, belong to the group of so-called sanitary-indicative microorganisms.
- Thermotolerant coliform bacteria (TTCB) is a group of coliform organisms capable of fermenting lactose at 44 – 45 °C.

The calculation was performed through:

$$x = \frac{a * 100}{V}$$

x - number of colonies per 100 mL;
V - filtered volume of water;
a - total number of colonies counted on filters.

For the processing of the microbiological data of water before and after combined cleaning from defeathering, evisceration, cooling sections a statistical analysis Wilcoxon T-test was carried out (n = 8). This study suggested a hypothesis H_0: indicators after the experiment is less than the values of the indicators before the experiment; H_1 indicators after the treatment more than the values before the experiment.

The critical values for the Wilcoxon T-test for n = 8:
T_{cr}=1($p \leq 0.01$)
T_{cr}=5 ($p \leq 0.05$)
In this case, the empirical value of T falls into the zone of significance: $T_{emp} < T_{cr}$.
Hypothesis H_0 is accepted for all sections.

RESULTS AND DISCUSSION

The results obtained (Table 2) from the analysis proved to be impressive as the pollutant level of most indicators in the wastewater decreased by more than 90%. It is worth noting that the pH level decreased in the treated water which helped to reduce the concentration of the pollutants. The acidic condition formed from the addition of calcium chloride helped to allow the chemical reactions described above during the EC process to take place. Other studies also proved that the initial decrease in pH value during the EC process is beneficial for the pollutant removal, hence improving the overall removal efficiency.

Table 2 Poultry slaughterhouse wastewater characteristic after treatment.

Indicators	Defeathering	Evisceration	Cooling	Units
pH	6.5	6.3	5.9	-
Turbidity	-4.53	0.469	-3.80	FAU
Color	13	71.0	53	
TSS	1.0	10.0	3.0	$mg.L^{-1}$
Chlorine free	0.02	0.29	0,01	$mg.L^{-1}$
Chlorine total	0.12	0.27	0.51	$mg.L^{-1}$
Nitrogen nitrites	0.09	0.95	1.18	$mg.L^{-1}$
Nitrogen nitrates	1.74	2.42	1.25	$mg.L^{-1}$
Phosphates	11.2	51.3	20.5	$mg.L^{-1}$
Nitrogen ammonium	2.7	7.5	4.3	$mg.L^{-1}$
COD	18.5	1.1	2.8	$mg.L^{-1}$
BOD5	0.022	0.051	0.065	$mg.L^{-1}$
Carbonates	0	0	0	$mg.L^{-1}$
Hydrocarbonates	91.5	91.5	152.5	$mg.L^{-1}$
Calcium	11	14	10.5	$mg.L^{-1}$
Magnesium	9	10	14.5	$mg.L^{-1}$

Table 3 Removal efficiency from chemical pollution of treated water

Sections	Defeathering	Evisceration	Cooling	*p*-value
TSS	98.8%	94%	99.0%	≤0.05
N nitrates	90.6%	71%	94.9%	≤0.05
BOD_5	100.0%	100%	100.0%	≤0.01
COD	96.8%	100%	99.8%	≤0.05

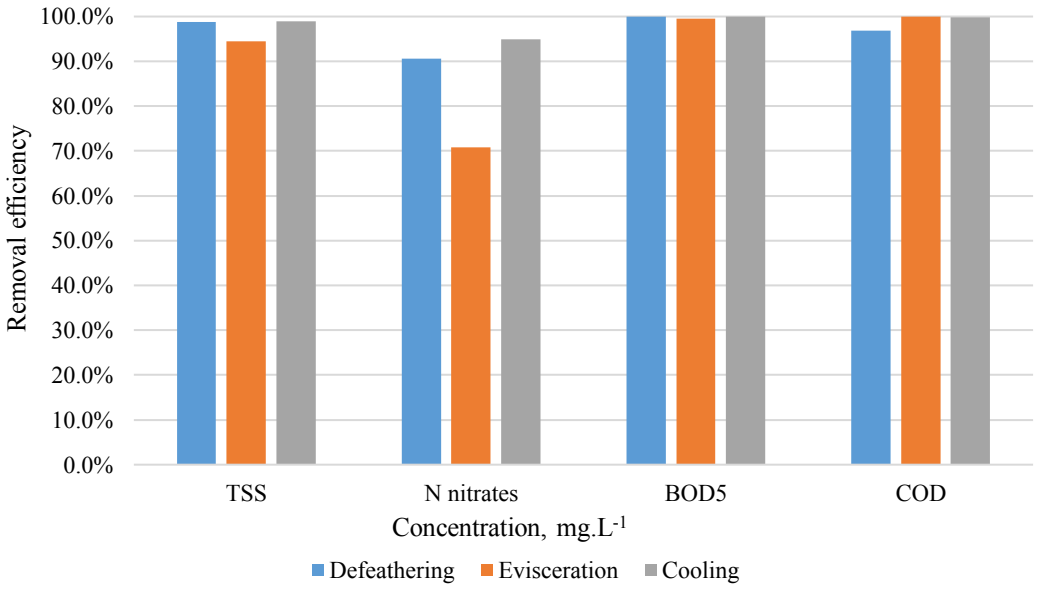

Figure 2 Chemical results for combined system.

It can be seen from the figure below that for all sections from which the wastewater was treated, the removal efficiency of COD, BOD, phosphates, TSS and nitrogen nitrates were close to 100%. Only chloride and nitrogen nitrite removal efficiency were about 80% or more. Overall, from the chemical analysis results (Table 2) it can be seen that this system is effective and can provide a technologically safe purified water. Also, from Table 3 it can be seen that the *p*-values of some indicators from the chemical pollutant analysis after treatment is ≤0.05, proving our H_0 hypothesis.

The microbiological results obtained after treatment showed that the microbiological colonies had a removal efficiency of 100%, making the water safe and hence not affecting the quality of poultry products. This again shows that the system is overall efficient **(Meiramkulova et al., 2018b)**. Once again, the H_0 hypothesis is valid as the microbes are eradicated from the treated water after treatment and the *p*-value is ≤0.01.

Observing both the chemical and microbiological results, it can be seen that this system is effective as it not only has high removal efficiency for chemical indicators, but also eradicates all microbiological colonies present in the

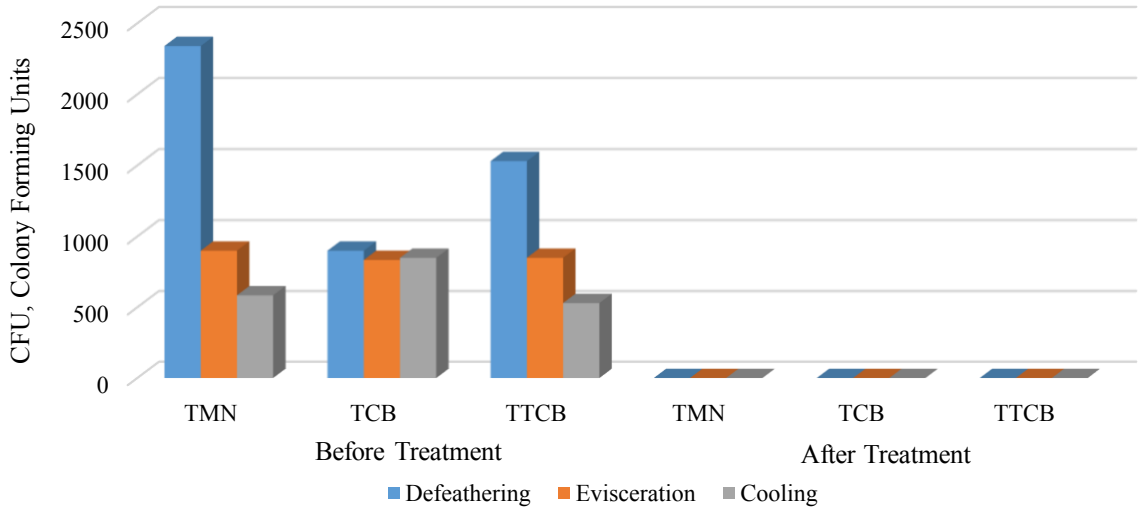

Figure 3 Microbiological results.

wastewater before treatment, making it safe to use for technological purposes within the poultry industry. Also, from the statistical analysis the indicators after the experiment do not exceed the values of the indicators before the experiment.

CONCLUSION

This project aim was to recycle poultry slaughterhouse wastewater for technological use. The results obtained for the combined system showed exceptional results. The removal rate of more than 90% of most pollutants was observed from the chemical analysis and microorganism colonies were all eliminated after treatment, the results of the microbiological analysis showed 100% efficiency of complex water purification, proving the treated water to be safe for technological use within the poultry industry. Also, the use of aluminium and graphite electrode combination proved to be effective during the EC process. For future works on this system, different electrode combination can be used to find a more effective method of treatment.

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EVALUATION OF THE ADAPTOGENIC PROPERTRIES OF THE QUARK PRODUCT ENRICHED WITH PROBIOTICS, POLYPHENOLS AND VITAMINS

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ABSTRACT

The aim of the study is to evaluate protective properties of the quark product manufactured with transglutaminase and enriched with probiotics, oligomerous proanthocyanidines and vitamins; the biological experiment on the growing laboratory Wistar stock rats has been carried out. The rats of two from three groups subjected within 21 days to the effect of low-frequency weak variable magnetic field received in semi-synthetic diet composition extra experimental and control samples of the quark product. The index of feed intake and the rats' body mass growth was registered within 32 days. At the end of the experiment blood serum biochemical index was evaluated. It was revealed that the animals consuming the experimental product substantially gained the mass before the effect (gain from the 1st up to 10th days made up 12%) as well as after effect (gain from 11th up to 32nd days – 10.3%); upon completion of the experiment the gains of these animals exceeded the gains of the rats consuming the control product by more than 28%. The experiment revealed the lipolipedemic and hypoglycemic effect of the experimental quark product that has been evidenced by the significant reduction of cholesterol (by more than 20%), glucose (up to 40%) in the rats' blood serum. On administration of the experimental dairy product in the animals' diet subjected to the impact of low-frequency weak magnetic field the effect of the broken balance recovery in antioxidant/pro-oxidant system was observed due to reduction of pro-oxidant load at the enzymatic as well as low molecular links of the antioxidant system. The identified antioxidant and adaptogenic effect of the developed dairy product promoting to reduce the intensity of free-radical oxidation at the impact of low-frequency electromagnetic field on the body make it possible recommend it in dietotherapy for correction of antioxidant/pro-oxidant status.

Keywords: curd product; transglutaminase; polyphenols; vitamins, probiotics; LF-EMF; rat

INTRODUCTION

In the modern world, a person faces a large amount of endogenic and exogenic factors negatively effecting his health and welfare. Chronic stresses, unbalanced nutrition, bad habits, radioactive, ultraviolet and electromagnetic radiation cause disbalance in the body antioxidant system that can result in chronic diseases **(Hybertson et al., 2011; Grabowska et al., 2019)**. It is known that all living organisms starting from unicellular and ending with a human are very sensitive to the impact of low-frequency electromagnetic fields (LF-EMF). Low-frequency weak intensity electromagnetic fields of natural origin present the potential threat for people health and stand in one line with such important climatic factors as temperature, pressure, etc. **(Martínez-Sámano et al., 2019; Il'chenko, 2017)**. The accumulated in the world literature data about the important role of antioxidants for prophylaxis of oxidative stress along with information about their insufficient intake in the diet show the advisability of their usage as additives enriching food products **(Tarko et al., 2015)**. Due to this fact, the scientific substantiation and development of the technologies of dairy products enriched with biologically active substances capable more or less protect the body against damaging free radicals is a very actual problem having a scientific, practical interest and social importance.

Usage of food additives complex in the technology of quark products manufacture providing the required functional-technological and prophylaxis properties including fermentative preparations, probiotic cultures, vegetable extracts, and vitamins is of great interest. **Zobkova et al. (2017)** showed that usage of the ferment preparation transglutaminase in the technology of quark production owing to protein modification promotes the increased product yield and lysine content in it.

Among the functional ingredients possessing the pronounced antioxidant properties grape stones extract has been used widely for a long time **(Tomášková et al., 2017)**.

It is generally known the significance of vitamins as antioxidants and molecules predecessor playing the important role redox reactions in cells as well as protectors

and polyphenol synergists **(Kodentsova et al., 2013; Isakov et al., 2018; Kang et al., 2019; Menshchikova et al., 2006)**.

Moreover, antioxidant enzymatic system of probiotic microorganisms enables to consider them as exogenous antioxidant protection of the human organism. The available data testify for the wide spectra of biological activity of *Lactobacillus congener*, particularly, *Lactobacillus acidophilus* which are widely used in probiotic preparations and medical food products. *L. acidophilus* immunomodulatory, antibiotical, antigenotoxical, antioxidant and many other properties have been revealed **(Hardy et al., 2013; Lin and Chang, 2000; Kim et al., 2005; Karamova and Khabibullin, 2013)**. Herewith probiotic cultures fermentation during the product manufacture is more preferably than simple addition as far as in this case besides vegetative cells their metabolites are present.

Scientific hypothesis

The quark product manufactured with transglutaminase enriched with probiotics, polyphenols, and vitamins improve the organism physiological state and increase the laboratory rats natural resistance to low-frequency electromagnetic fields impact.

MATERIAL AND METHODOLOGY

The object of the investigations were quark products – the experimental (enriched) sample prepared with transglutaminase, probiotic cultures, polyphenol-containing extract, and multivitamins premixes; the control sample prepared without the listed above functional ingredients.

The technology of the quark product manufacture

The control samples of the quark product were manufactured from skim quark and fermented product. Experimental samples were manufactured from skim quark and probiotic fermented product. The quark was produced by the acid method by skim milk fermentation with lactococcus with the following whey removal by milk protein coagulate pressing. The experimental samples of the product were prepared from the quark manufactured from skim milk with use of crosslink enzyme - the preparation of microbial tranglutaminase (BioConnecta milk, Prompostavka-M, Ltd., Russia, activity 100 $U.g^{-1}$, dosage 0.15 $g.kg^{-1}$) and probiotic fermented dairy product produced with dry extract of grape stones (Tyaga (Shanghai) Co., China, the total polyphenolics content ≥95%), multivitamin premix 730/4 containing vitamins: A, C, E, D3, B1, B2, B5, B6, B9, PP, biotin (DSM Nutritional Products Ltd., Swiss) using the *Lactobacillus acidophilus* (strain 20T supplied by the Central Laboratory of Microbiology FSANO VNIMI, Russia).

The methods of physical-chemical characteristics investigation

The following parameters were measured in the product samples: the fat – by Gerber butyrometric method (ISO 2446:2008); the total protein - by Kjeldahl method with use of KJELTEC automatic system (ISO 8968-1:2014); the carbohydrate – by IDF 028A:1974; the vitamin C – by AOAC Official method 967.21; the total polyphenolics content in terms of gallic acid – in supernatant by Folina-Ciocalteu method **(Kovarovič et al., 2017)** with use of spectrophotometer Specord M40 (Analytik Jena AG, Germany); the number of Lactobacillus – by calculation of colonies grown on agar-like selective growth media (type MRS manufactured by Pharmacotherapy Research Center, St. Petersburg, Russia) at optimal conditions (ISO 20128:2006).

The laboratory rats biological experiment

The products were studied at white male rats Wistar stock (165 ±15 g) from the Laboratory Animals Nursery "Andreevka" (Federal State Budgetary Scientific Institution "Scientific Center of Biomedical Technologies") at the base of the Experimental Clinic-laboratory of biologically active substances Gorbatov's Federal Research Center for Food Systems of Russian Academy of Sciences (Russia). After adaptation (14 days) the animals were individually marked and grouped at random: group 1 – involved the intact rats (n = 10); group 2 – control rats (n = 10); groups 3 and 4 – experimental rats (I) consuming the control quark product (n = 10) and (II), consuming the enriched quark product (n = 10). The animals of all groups during the whole experiment consumed the standard diet (310 $kcal.100g^{-1}$). The first stage of the experiment – from the first up to 10 days – was aimed at adaptation of the rats of the third and the fourth group to the introduction in the standard diet of the quark products (53 $kcal.100g^{-1}$) which were fed on the basis of 20 g per head using polysulfone vials. At the second stage starting from 11_{th} to 32_{nd} days the animals from 2, 3 and 4 groups were subjected to daily 10 minutes effect of LF-EMF with frequency 8 Hz 5 μT **(Torres-Durán et al., 2007)** at the that rats of group 3 and 4 continued to consume additionally to the diet the quark products. During the whole experiment, the rats of all groups received feed and water ad libitum. The rats were kept in polysulfone cages IV S (Tecniplast, Italy) at the temperature 22 ±3 °C, humidity – 50 – 60%, lighting – in the regime 12/12, daylight hours – from 6.00 to 18.00.

Prior to the investigation start and every fourth day the animals were weighed at the scale Ohaus (Adventurer Pro, USA). The duration of the experiment made up 32 days. At the end of the experiment the rats were put to sleep with carbon dioxide in the chamber for euthanasia (VetTech, Great Britain), sampled blood from the heart for analysis. Blood sampling for hematology research was carried out in tubes with EDTA as an anticoagulant (Aquisel, Spain). Rat plasma was obtained after centrifugation (at 2,000 g for 10 min) of EDTA tubes with blood. Rat blood serum was obtained in conformity with **Chernukha et al. (2018)**.

Hematologic, biochemical blood analysis and parameters of the animals' antioxidant system

Quantitative amount of leucocytes (LEU); limphocetes (LYM), granolucites (GRAN); mixture of monocytes, eosinophils, basophilus and immature cells (MID); erythrocytes (RBC); hemoglobin (HGB); hematocrit (HCT); mean amount of erythrocyte (MCV); mean content of hemoglobin in erythrocytes (MCH); thrombocytes (PLT); thrombocrit (PCT) in the rats' whole blood was

carried out at the automatic hematologic analyzer Abacus junior vet 2.7 (Diatron Messtechnik GmbH, Austria (using Diatron set of assay kit).

The following blood serum index was measured at the automatic analyzer BioChem FC-300 (HTI, USA) using HTI assay kit: total protein, albumin (ALB), creatinine (CREA), aspartate aminotransferase (ASF), alanine aminotransferase (ALT), glucose (GLU), total cholesterol (CHLST), trigliceride (TG).

Moreover, the following index of antioxidant organism protection was evaluated in the rats' blood serum:

- total antioxidant activity was determined by Ferric Reducing Antioxidant Power method **(Merola et al., 2009)**;
- the active products content reacting with tiobarbituric acid (TBA-RP, p.a.), by **Brazhe et al. (2014)** method;
- superoxiddismutase activity was measured (SOD) was determined by **Marklund and Marklund (1974)** method with modification of **Gatellier, Mercier and Renerre (2004)**: reaction mixture – 2850 µL 50 mM of phosphate buffer (pH 8.2), 75 µL of blood serum and 75 µL of 10 mM pirogallols (p.a.); wavelength – 340 nm;
- catalase activity (CAT) was measured by **Beers and Sizer (1952)** and **Iwase et al. (2013)** method;
- glutationperoxidase activity (GPx) was determined by **Paglia and Valentina (1967)**;
- the concentration of reduced glutathione (GSH) was determined using Ellman reagent **(Noctor et al., 2011)**.

Statistic analysis

STATISTICA 10 program was used. The results were presented as "Weighted statistical significance ±Standard deviation" (M ±*SD*). Statistical significance was calculated by one-parametric ANOVA test and Tukey criterion. 0.05 probability was chosen as a significant level.

RESULTS AND DISCUSSION

The basic parameters of the control and enriched quark product samples are specified in Table 1.

The first stage of the biological experiment didn't show any changes in the animals' clinical state in all groups.

The analysis of the consumed feed showed that from the second week in group 1, 2 and 3 the amount of the consumed calories was reduced by 5 – 7 kcal. At the second stage of the experiment the rats of group 4 relative to group 1 consumed in the average by 3 – 5% more feed and from the 2nd to 3rd week their ration caloricity made up in average 50 kcal and then to the 4th week it was reduced to 43 kcal per day. It should be noted that slight consumed calories variability was registered in rats from group 2, 3 and 4 from the second week (Figure 1, a).

Weight gain of the rats from group 2, 3 and 4 authentically didn't differ from group 1. The rats from group 1 and 4 firmly gained weight during the whole experiment. At the second stage of the experiment the weight gain of the rats from group 2 and 3 was reduced but at the 4th week, the rats weight gain was registered.

The weight of the rats from group 2 and 3 starting from the second week and up to the end of the experiment was significantly varied that is stipulated by the individual peculiarities of the organism and development of compensatory reactions in response to LF-EMF impact (Figure 1, b).

Table 1 The quark product samples parameters.

Parameter	Quark product	
	Control (*M* ±*SD*)	Experimental (*M* ±*SD*)
Fat, %	0.4 ±0.06	0.4 ±0.1
Total protein, %	10.7 ±0.3	10.8 ±0.5
Carbohydrate, %	3.3 ±0.15	3.1 ±0.15
Vitamin C, mg %	-	47 ±5
Total polyphenolics content, mg.kg^{-1}	-	246 ±14
Amount of probiotic microorganisms (*Lactobacillus acidophilus*), CFU.g^{-1}	-	8 x 10^6 ÷ 4 x 10^7

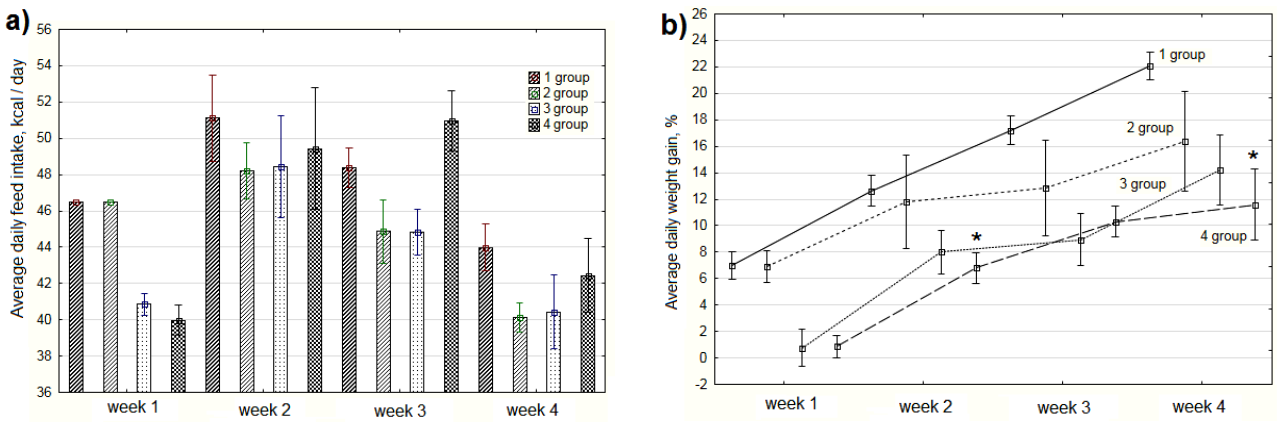

Figure 1 The dynamics of calories consumption by the rats (a) and change of the rats weight (b) during the experiment.
Note: * – significant difference by comparison between group 4 and 2.

It should be noted that at the first stage of the experiment the maximum gains were registered in rats of 1 and 4 group – 14% and 12%, in rats of 2 and 3 group – 10% and 8%.

At the second stage after LF-EMF impact the gains were: in group 1 – 9.9%, in group 2 – 4.8%, in group 3 – 5.4%, in group 4 – 10.3%. The gains at the end of the experiment relating to the initial mass of the rats made up: in group 1 – 25.9%; group 2 – 19.8%, group 3 – 14.7% and group 4 – 20.6%. Evidently, LF-EMF impact on the rats from 2nd up 4th week didn't affect significantly the rats gain from group 4 consuming the enriched product.

Statistically significant reduction of leucocytes and lymphocytes was revealed in blood serum of the rats from groups 2, 3 and 4 relatives to group 1 by 13.6% and 23.5%, 35.0% and 53.5%, 34.2%, and 45.4%, respectively.

Authentically the number of leucocytes and lymphocytes was reduced relative to group 2 in the rats of group 3 by 24.7% and 39.4% and group 4 by 23.9% and 28.6% (Table 2).

Herewith the concentration of granulocytes in the rats' blood of group 4 was reduced relative to the figures of group 1 by 41.1%, relative to the figures of group 3 by 42.7%.

There were no statistically significant changes of erythrocytes, hemoglobin content and hematocrit level in the groups. In group 2 compared to group 1, the valid reduction of MCV by 2.6% was observed. The rats from group 4 showed the valid increase MCV and MCH relative to group 1 by 2.6% and 3.1%, group 2 – by 5.3% and 4.0%, group 3 – by 3.3% and 2.7%. The reduction of thrombocytes number in the rats consuming dairy products compared to group 1 and 2 was revealed: in group 3 – by 6.8% and 8.1%; in group 4 – by 7.6% and 8.9%.

The analysis of rat blood serum biochemical index (Table 3) characterizing protein metabolism showed the lack of variations in the concentration of total protein and urea. Herewith reduction of albumin concentration in blood serum of rats group 3 and 4 was revealed compared to group 1 by 12.8% and 10.0% compared to group 2 – by 12.5% and 9.8% respectively. The rats of group 2 showed an increase of creatinine relation to group 1 by 9.5%; to group 4 – by 10.2% and to group 2 by 18.0%. AST activity in rats groups 2 - 4 was increased compared to group 1 by 22.2%, 65.8%, and 27.1%. AST activity of the rats of groups 3 compared to group 4 was increased by 20%. Herewith ALT activity was reduced in groups 2 and 3 compared to group 1 by 23.3% and 30.6%. Glucose concentration in rats blood serum of group 3 and 4 compared to group 1 by 38%, compared to group 2 up to 40% was registered. Groups 3 and 4 showed cholesterol content reduction compared to group 1 by 17.0% and 27.9%, herewith cholesterol amount in group 4 was reduced for sure compared to group 2 by 20.9%. Triglycerides content was reduced in groups 3 and 4 by 36.7% and 29.2% compared to group 1.

The analysis of antioxidant status of rats' plasma is specified in Figure 2.

It was shown that CAT activity wasn't changed in all rat groups. TBA-RP content was increased in rats of group 2 by 54.5% compared to group 1 while this index was reduced in group 3 and 4 by 31.8% and 20.4% ($p \geq 0.05$) compared to group 2. Reduction of GPx activity in rats' blood plasma of group 4 compared to group 1 by 8.9%, compared to group 2 by 11.6%, compared to group 3 by 10.8% was registered. SOD activity in the rats of group 2 was increased by 7.2% compared to group 1, in rats of group 3 and 4 it was reduced compared to group 2 by 5.2% and 7.0%. GSH concentration reduction was detected in rats blood plasma of group 2, 3 and 4 compared to group 1 by 34.4%, 33,4% and 38.4% respectively while GSH content in group 4 was also reduced compared to group 2 by 6.1% and in group 3 by 7.4%. The level of blood plasms total antioxidant activity was reduced in group 3 and 4 compared to group 1 by 35.9% and 20.2% ($p \geq 0.05$) while this index in group 3 was lower of group 2 figures by 29.4%. It was noted that the level of blood plasms

Table 2 Hematological index of the rats' blood after LF-EMF impact and feeding with fermented dairy products.

Parameter	Group			
	1	2	3	4
	(*M* ±*SD*)	(*M* ±*SD*)	(*M* ±*SD*)	(*M* ±*SD*)
LEU, 10^9/L	12.23 ±0.86	10.56 ±0.70 1	7.95 ±0.72 1,2	8.04 ±0.63 1,2
LYM, 10^9/L	10.41 ±0.61	7.96 ±0.48 1	4.82 ±0.53 1,2	5.68 ±0.24 1,2
GRAN, 10^9/L	2.41 ±0.14	1.78 ±0.15	2.48 ±0.34	1.42 ±0.11 1,3
MID, 10^9/L	0.33 ±0.10	0.54 ±0.12	0.28 ±0.05	0.40 ±0.12
RBC, 10^{12}/L	17.47 ±0.80	18.69 ±0.44	28.81 ±1.34 1, 2, 3	20.67 ±0.92
HGB, g.L^{-1}	156.9 ±1.3	155.1 ±1.9	153.6 ±1.3	156.8 ±1.3
HCT, %	43.08 ±0.42	41.64 ±0.50	41.59 ±0.37	42.68 ±0.35
MCV, mkm^3	46.15 ±0.33	44.95 ±0.38 1	45.85 ±0.27	47.35 ±0.20 1, 2, 3
MCH, pg	16.88 ±0.10	16.74 ±0.09	16.96 ±0.11	17.41 ±0.08 1, 2, 3
PLT, 10^9/L	833.3 ±12.9	844.8 ±16.0	776.3 ±13.1 1,2	769.7 ±15.0 1,2
PCT, %	0.49 ±0.01	0.50 ±0.02	0.46 ±0.01	0.47 ±0.02

Note 1: LEU – leukocytes, LYM – lymphocyte, GRAN – granulocyte, MID – other types of white blood cells not classified as lymphocytes or granulocytes; RBC – red blood cell; HGB – hemoglobin; HCT – hematocrit; MCV – mean cell volume RBC; MCH - mean cell hemoglobin; PLT – platelet (thrombocyte); PCT – plateletcrit.

Note 2: 1 – significant difference compared to group 1; 2 – significant difference compared to group 2; 3 – significant difference compared between group 3 and 4.

Table 3 The biochemical index of rats' blood serum after LF-EMF impact and the product consumption.

Parameter	Group			
	1 (*M* ±*SD*)	2 (*M* ±*SD*)	3 (*M* ±*SD*)	4 (*M* ±*SD*)
Total protein, g.L-1	76.93 ±0.95	77.12 ±1.78	72.53 ±1.76	72.60 ±1.75
ALB, g.L-1	46.70 ±1.24	46.58 ±0.76	40.73 ±0.39 1,2	42,01 ±1.12 1,2
CREA, µmol.L-1	59.50 ±1.86	65.14 ±1.49 1	59.17 ±1.85	53.43 ±1.55 1,2
Urea, mmol.L-1	8.01 ±0.30	7.94 ±0.20	8.32 ±0.31	8.05 ±0.28
AST, U.L-1	85.9 ±10.0	105.0 ±6.9 1	142.6 ±8.1 1,3	109.2 ±8.0 1,3
ALT, U.L-1	87.4 ±7.54	67.00 ±2.48 1	60.67 ±5.20 1	75.43 ±2.74
GLU, mmol.L-1	19.46 ±1.21	20.14 ±1.94	12.23 ±1.41 1,2	12.06 ±1.72 1,3
CHLST, mmol.L-1	1.47 ±0.07	1.34 ±0.04	1.22 ±0.07 1	1.06 ±0,04 1,2
TG, mmol.L-1	0.71 ±0.04	0.58 ±0.04 1	0.45 ±0.06 1	0.51 ±0.05 1

Note 1: ALB – albumin, CREA – creatinine, AST – aspartate aminotransferase, ALT – alanine aminotransferase, GLU – glucose, CHLST – total cholesterol, TG – triglyceride.

Note 2: 1 – significant difference compared to group 1; 2 – significant difference compared to group 2; 3 – significant difference compared between group 3 and 4.

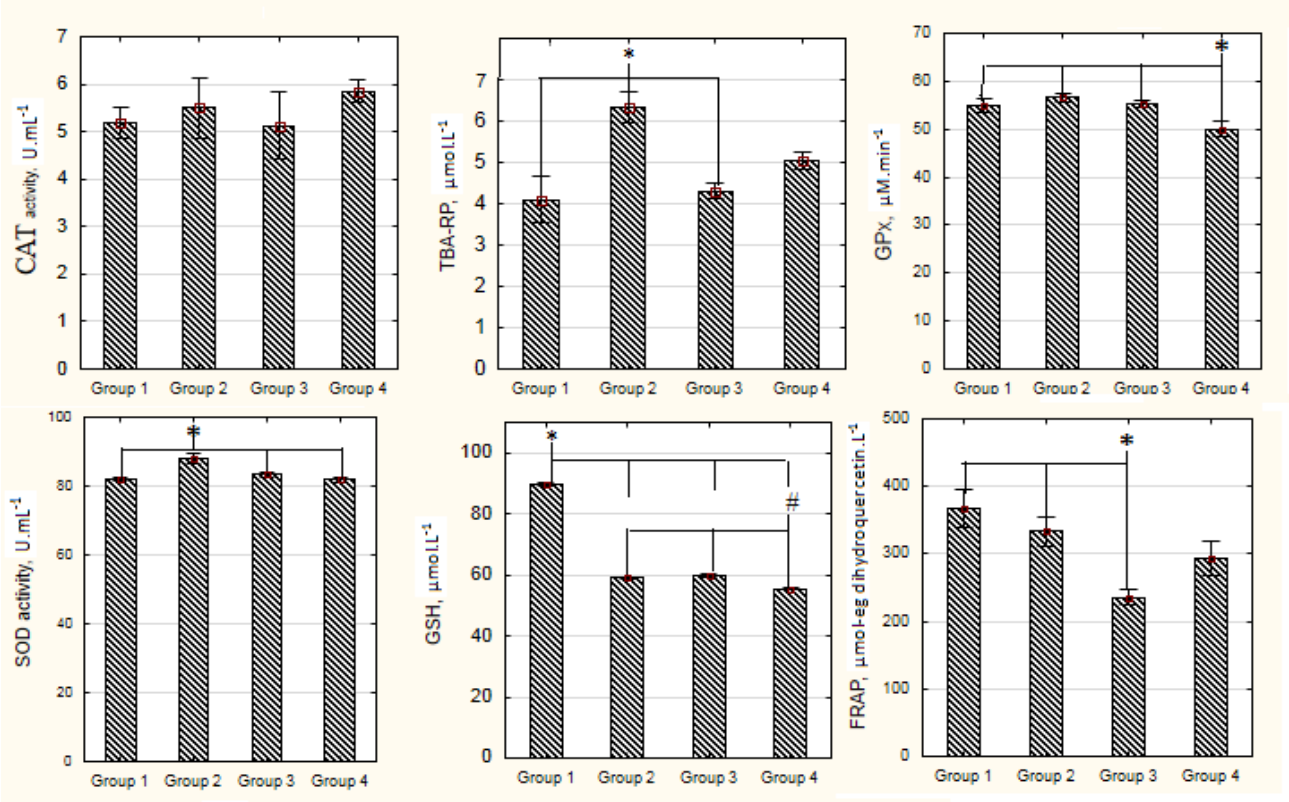

Figure 2 Antioxidant status of rats' plasma.

antioxidant activity of group 4 was increased by 24.6% ($p \geq 0.05$) relative to group 3 index.

CONCLUSION

The dairy product enriched with probiotics, polyphenols, and vitamins presented in this experiment showed the protective effect at rats' damaging impact of LF-EMF. The normalization of protein catabolism and growth rates, blood cells functional activity particularly leucocytes and erythrocytes, reduction of free-radical oxidation at electromagnetic field impact on the body were recorded. Besides, hypo-cholesterolemic (reduction of cholesterol by more than 20% relative to group 2 and 3 rats) and hypoglycemic (reduction of glucose up to 40% relative to group 1 and 2) effects were revealed.

The mentioned recovery of the broken balance in pro-oxidant-antioxidant system obviously occurs due to reduction of pro-oxidant load on enzymatic (reduction of SOD activity up to 5%, GPx by more than 10%) as well as on low-molecular chains of the antioxidant system (reduction of GSH up to 40%). These data evidence high bioavailability of self – antioxidants of milk as well as introduced additionally due to technological processes. Since any food products consumed by a human effect pro-oxidant-antioxidant state balance to a variable extent and different tendency it creates the presuppositions for correction of antioxidant potential without the usage of pharmaceutical preparations at high effectiveness due to long systematic impact of alimentary agents. Thus the enriched dairy product is of great practical interest in dieto-therapy for correction of antioxidant/pro-oxidant status.

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GENDER DIFFERENCES IN CONSUMER PREFERENCES WHEN BUYING DAIRY PRODUCTS IN SLOVAKIA AND RUSSIA

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ABSTRACT
In spite of geographical and culture differences between examined countries, there can be found similarities in consumer behavior of men and woman and also the similar tendencies on the dairy product market. In the last decade different fields of science concerns with the topic of gender differences more frequently. The article is based on a research of consumers' overall attitude to dairy products in Slovakia and Russia. The important role of gender differences underlines the outcomes of the questionnaire survey. Kruskal-Wallis test and Bonferroni correction was applied to verify the hypothesis whether there is a dependency between gender of the respondents and their attitude while choosing the dairy products in both countries. Analysis showed that in both countries women tend to buy dairy products more often than men. Also consumers prefer more domestic products, but Russian not as significantly as Slovakian. In both countries consumers consider the price of dairy products as high, but they don't outline the price as the most important factor when choosing dairy products. At the same time, both genders consider quality as the most important factor. These results indicate the existence of a niche at the Russian market, which could be used by Slovak dairy producers who can possibly penetrate Russian market. In addition, the similar marketing strategy for both, Slovak and Russian market can be used if the advertisement will be sensitively focused on the gender.

Keywords: gender difference; dairy product; preference; Slovakia; Russia

INTRODUCTION
Penetrating of foreign market is one of the main strategic decisions company's management can do. Decision making in this case is ongoing process which have to include thoroughgoing market analysis **(Crowley, Meng and Song, 2018)** and subsequently designing of marketing strategy for specific groups of products **(Yang, 2018)**. Campaign planners need to answer three questions **(Elsner, Kraft and Huchzermeier, 2004)**: "when to make an offer (timing), how often to make an offer (frequency), and whom to contact (target group). In this article we are focusing on the target group selection problem, which is widely studied under problem of direct marketing and churn management **(Zhu, Baesens and vanden Broucke, 2017)**.

The food industry has an irreplaceable status in the economy, since it is producing food for the population. Therefore, food producers are not just entrepreneurs, but they provide food security. Food security was defined by **United Nations (1975)** at World Food Summit as "availability at all times of adequate world food supplies of basic foodstuffs to sustain a steady expansion of food consumption and to offset fluctuations in production and prices" Therefore the task of food producers is not just to create profit, but provide food security and secure food too **(Golian et al., 2018)**. Their role is increasingly important within production of dairy products where every country has set the Rational Consumption Norms. Since population usually does not consume prescribed quantities **(Kubicová and Habánová, 2012; Zingone et al., 2017; Kubicová, Predanocyová and Kádeková, 2019)**, information that the consumer receives as part of the advertising campaign of companies plays increasing role in ensuring nutritionally sufficient consumption. Consumers have positive attitudes towards cause related marketing programms **(Witek, 2016)**. Nutrition educationing in this area results in an increased intake of calcium-rich foods (such as dairy products) which is important in the prevention of osteoporosis **(Melton et al., 1997)**. To popularize these products**, Kim, Reicks and Sjoberg (2003)** recommend using concepts that dairy products taste good, they can serve as beverages at breakfast or during the rest of the day, they help one to have a balanced diet, and they are foods that go well with other foods. In addition, practitioners might help older adults increase perceived control in eating dairy foods with meals by substituting milk for other beverages and enhancing cooking skills using dairy products. Nonetheless **Ajzen**

(1991) adds that, the relationship between perceived behavioral control and intention is also dependent not just on the behavior but situation as well. In addition, even in this area, we can not forget the differences between men and women. Gender differences are described from variant points of view in different fields of science, and the impact of gender on consumer behavior is increasingly being solved mainly in last decade **(Lockshin and Corsi, 2012)**. For example, research outcomes usable in marketing are that: men have higher ICT self-efficacy and hold more favorable attitudes toward technology than girls **(Cai, Fan and Du, 2017)**, women in Western societies are typically more risk averse than men in individual risk taking decisions **(Friedl, Pondorfer and Schmidt, 2019)**, or that there are differences in variations in fixation count, fixation duration, pupil diameter, and hit ratio when buying **(Qu and Guo, 2019)**. Nonetheless, not only gender should be taken into account when assessing consumer behavior, purchasing is influenced by several factors **(Kozelová et al., 2011)** among which is dominated consumer personality, income, finances and lifestyle, as well as psychological factors such as perception, motivation, learning, cognition and attitudes. But, several authors confirm specificly the role of origin **(Bryła, 2015; Kumpulainen et al., 2018a; Thøgersen, Pedersen and Aschemann-Witzel, 2019)** and gender **(Kumpulainen et al., 2018b; Broussard, 2019)** in the food marketing and consumer decisions.

Scientific hypothesis

H0: The samples come from the same population.

H1: The samples do not come from the same population

The hypothesis aply to whether there is a dependency between gender **(Mansoora, 2017; Thelwall and Stuart, 2019; Li and Zeng, 2019)** of the respondents and their attitude while choosing the brand of dairy products.

MATERIAL AND METHODOLOGY

The research was conducted from February 19, 2019 to March 20, 2019, attended by 203 respondents from Slovakia and 104 respondents from Russia. The questionary was filled by respondents of all ages, in different social situations and with different views on the issue. Questions dealt with consumers' overall attitude to dairy products.

Statistic analysis

For the collection of data, online Google form of questionnaire was used. The results of the survey were processed using XLSTAT version 2019.1 by Addinsoft.

We implied Kruskal-Wallis test **(Ruxton and Beauchamp, 2008)** and bonferroni correction on the sample of Slovak respondents and consequently on a Russian sample. A prerequisite for using this test is that all observations are independent of each other so that the variable under consideration is measured on the ordinal scale, and that all selection distribution functions are approximately the same shape. The hypothesis that all selections come from the same distribution, or from distributions with identical distribution functions were tested: H0: F1 (X) = F2 (X) = Fk (X) versus alternative hypothesis that not all distribution functions equal. The significance level α is set to 0.05, ie allowance is a 5% test error. If p-value is ≤ α, then H0 is rejected at the significance level α and we accept H1. If p-value > α, then H0 is not denied at the significance level α. Statistical hypothesis testing is based on rejecting the null hypothesis if the likelihood of the observed data under the null hypotheses is low. If multiple hypotheses are tested, the chance of a rare event increases, and therefore, the likelihood of incorrectly rejecting a null hypothesis increases **(Mittelhammer, Judge and Miller, 2000)**. The Bonferroni correction compensates for that increase by testing each individual hypothesis at a significance level of α/m, where α is the desired overall alpha level and m is the number of hypotheses **(Miller, 1970)**.

Therefore, we applied Bonferroni correction to counteract the problem of multiple comparisons between the following questions:

Question 1. How often do you buy dairy products?

Question 2. What kind of dairy origin do you prefer?

Question 3. What do you think about the prices of milk and dairy products?

Question 4. What is the most important factor for you when choosing a dairy brand?

RESULTS AND DISCUSSION

Slovakia and Russia are states with many geographic, economic (Table 1) and social differences as well. Their markets are in many ways hardly comparable. But, considering impact of Soviet block period of both countries and fact that both are “Slovanian” nations, there are many cultural similarities as well. These common signs could be fundamental for bilateral cooperation and international entrepreneurship activities between each other. Since Russian market is one of the biggest worldwide, it can be seen as a big opportunity for Slovak production sector. However, nowadays Slovak producers have to respect membership in European Union, which is visible mostly in case of quotas in primar agricultural production. These apply to selected products and in the Slovakia it is recently connected mainly with milk and situation at the market of dairy products. Slovakia with the total of 826 thousand tons produces less than 1% of the total EU milk production (Table 2). Russia, despite the total country size, in 2017 produced only more than 31 million tons of milk. While more than 154 million tons of milk was produced in the European Union. Despite this, the dairy industry is of particular importance for the economy and population of Russia. More than 21 thousand organizations and more than 1.2 million people work in the dairy industry and related industries. Milk and dairy products make up 15% of the turnover of retail chains. Strong investments from foreign enterprises, as well as government support in the form of subsidies and loans made Russia one of the world's largest producers of milk and dairy products worldwide. However, it has a fairly low share of marketable milk in total production (57%), and by the efficiency of dairy cows it loses more than twice to developed countries. But, Russian milk production is increasing constantly. Starting before the imposition of an embargo on the import of food and beverages in 2014, Russian dairy production grew on average by 4% per year. In 2017 was recorded a new high level when it exceeded

11.1 million tons of dairy products. Between 2015 and 2017, growth was especially sharp and volume of production increased by 15%. Production growth slowed to 3% in 2018 **(Rosstat, 2019)**, but the impulse is still ongoing.

Nowadays after the change in the approach of Common Agricultural Policy (CAP) milk quotas in EU gradually disappeared (after 2009) and milk production rapidly increased (in 2015) with the simultaneous decrease in price.

Milk production in Slovakia decreased by 15% from 2007 to 2017 and farmers do not cover domestic consumption of milk and dairy products. Situation is not caused just by low purchase prices for milk, but lower subsidies for Slovak farmers in comparison with farmers from other member states as well.

Consequently, Slovak dairy sector is able to produce just the 251,000 tons of drinking milk, 4,000 tons of milk powder, 9,000 tons of butter and 38,000 tons of cheese **(Eurostat, 2019)**. On the other hand, the dairy industry in Russia is capable of fully cover all major commodity groups as: 9.2 million tons of liquid milk, 935 thousand tons of cheese, 260 thousand tons of butter, 68 thousand tons of nonfat dry milk and 60 000 tons of whole milk powder. Despite the fact that the production of dairy products with high milk content increased – cheeses and cheese products (8.5%), butter (7.1%) and dry milk (28.8%), the production of milk, fermented dairy products and cottage cheese decreased (3.1%, 5.8% and 1.7%, respectively). This decrease possibly creates market niche for foreign producers and traders. Despite fact, that Slovak producers do not cover domestic consumption, penetrating of Russian market can be solid long term opportunity for them. Since Slovak market is covered by foreign producers from other EU member states with competitive advantage of higher support, market distortions could lead Slovak producers to biggest growing Eastern markets. As states **Esmerino et al. (2017)** even with their limited financial and human resources, they can focuse on new consumer markets and by using effective strategy, introduce their products with a minimal risk of failure.

This movement is also supported by the development of prices (Figure 1) which are following the same trends in Slovakia and Russia, but prices in Russia remain steadily higher ever since 2010. In Slovakia an increasing milk production, associated with the end of milk quotas, resulted in a marked decline in the milk price index. In addition, Slovaks have been for a long time Europe's weakest milk consumers.

Not only, the average Slovak drank only half of recommended 220 kilograms per year, per capita consumption here decrease from 71.5 liters in 1996 to 45.1 litres in 2016. On the contrary, in the case of cheese consumption, there was a significant increase in consumption (from 8.1 kg to 13.9 kg) and Slovaks exceed the recommended rational consumption norms (RCN) (Table 3). In Russia, devaluation risks and difficult economic situation in the country led to a decrease in the purchasing power of the population and an increase in the cost of production of dairy products in 2015 – 2016. And the consumption of dairy products in recent years is here also decreasing. In 1990 the average level of consumption of dairy products was 387 kg per person yearly, while by 2015 this number dropped to 239 kg per person in year. These values are above Slovak RCN, but Russian recommended medical norm is set on 325 kg per person per year, which creates possibility for increased consumption. In the field of dairy products, the import of dairy products and cheese annually exceeds their exports in Slovakia since 2009. While in 2016, Slovakia imported dairy products and cheese in the amount of 307 million EUR from abroad, exports amounted to 232 million EUR. However, the biggest problem for Slovakia is that only a third of the butter and cheese that can be bought in stores is made locally. The level of self-sufficiency in this area is high, but in reality foreign dairy products prevailon Slovak market. On the orher hand Slovak dairy products are exported to several countries, most of which are EU members, 25% of all exports to Hungary, another 20% to the Czech Republic, almost 18% to Germany and 15% to Italy. The remaining 35 countries account for 8% of total exports.

The introduction of the embargo contributed to a significant reduction in import volumes of dairy products into Russia. For the period from September to December 2014, the volume of imports of dairy products decreased by 27.3%, to 2.540 thousand tons. At the same time, countries that previously provided up to 38% (2013) of all imports left the Russian market. Among them for example Finland (butter and cheese), the Netherlands (cheese), Germany (cheese and cheese-like products), Lithuania (cheese), Poland (cheese), France (butter, cheese, whey), etc. The overall volume of imported goods fell from 9.4 million tons to 7 million tons per year.

The embargo also affected the export side, since export of milk and dairy products increased from 639 thousand tons in 2013 to 743 tons in 2016. The adoption of the Food Security Doctrine in Russia has also influenced the international trade of the country. Its task is to provide 90% of domestic consumption with its own products for the dairy industry. In 2013, the indicator of security of dairy industry was on the level of 76% and the indicator for commodity milk separately was lower than 66%. These results recommend hard possibility of penetration into the Russian market for foreign producers but, considering its size and possibilities it offers, this effort is highly forwarded. By 2025, the Russian dairy market is expected to reach 34.56 billion dollars. In addition, per capita income growth and increased consumption of dairy products due to health benefits are likely to contribute to the development of the market in the future. Investments into processing capacities of milk and dairy products would be effective not just in connection with the possibility of penetrating foreign (Russian) market. The volume of dairy products in the Slovak market by 2021 expects to reach 398 million kg.

The composition of respondents by gender in Slovakia shows that three quarters of respondents were women who are probably more concerned with this issue and also buy food products more often than men. Specifically, 152 women and 52 men living in Slovakia answered to the questions in the questionnaire.

In Russia, higher percentage of men responded to the questionnaire in comparison with Slovakia. Up to 38% of respondents – 40 men from Russia – participated in the survey. The remaining 62% were women. For many

questions, the Figures 2 – Figure 9 show the differences between the responses of women and men.

Figure 2 and Figure 3 shows frequency of purchases of dairy products by respondents, with possibility to see differences between genders. In Slovakia, 61% of all respondents buy dairy products several times a week, 27% once a week and 10% daily. The remaining 2% buy dairy products less frequently. However, differences can be seen between men and women. The biggest difference between answers of man and woman can be seen in case of answer "daily", when woman buy dairy products by 60% more daily than men. In Russia, almost half of respondents buy dairy products at least a few times a week. 29% of respondents buy dairy products even every day and 13% once a week. The remaining 11% of respondents buy dairy products less frequently or do not purchase them at all. All these groups are men. Thus, even in Russia, women tend to buy dairy products more often. Overall, at such a high frequency of purchases of dairy products, our respondents should have a good overview of market supply, prices and trends.

In Slovakia, the largest proportion of respondents which is 72% prefers dairy products produced in Slovakia. 27.5% of respondents do not distinguish between Slovak dairy products and those imported into the country (Figure 4). Only one respondent prefers products of foreign origin. There was a difference between preferences of men and women. While 55% of women prefer Slovak products and the rest do not make a difference between products of different origins, for men only 16% prefer Slovak products, one questioned men prefers foreign products and the rest of them don't distinguish the origin. Therefore, the preference of Slovak products could be an advantage for domestic producers.

In Russia, most respondents also prefer products made in Russia – 60% of respondents (Figure 5). The number of people preferring foreign products is higher compared to Slovakia. More than 15% of Russian customers prefer dairy products of foreign origin. Especially men are more inclined to foreign products. The remaining 25% of respondents do not distinguish where the product comes from. This creates space for importers and possible opportunity for Slovak companies to deliver their products to Russian market.

Currently, over 70% of Slovak respondents consider prices of dairy products to be high, which is 144 respondents. 26% of respondents consider prices as reasonable and only 3% as low. However, women hardly consider prices to be low. Only one woman chose this option in questionnaire. 27% of women perceive prices as reasonable and the remaining 72% think prices are high. On the other hand, 10% of men consider prices of dairy products as low, two-thirds of men as high and the remaining 23% think prices are reasonable. However, consumers' perception of prices is largely influenced by their income (Figure 6). In Russia, 70% of respondents consider the prices of dairy products to be high. The remaining 30% consider them as reasonable. However, none of the respondents perceives the prices as low (Figure 7).

For Slovak consumers, the quality of the dairy product they purchase is the most important factor. Nearly 40% of them, which is 81 interviewers, who choose this option. The second most frequently chosen factor was taste, selected by 49 respondents, which is 24%, followed by composition, which is 15%. Price was the fourth most frequently chosen factor, chosen by 14% of respondents. Only 15 respondents chose the origin of the product, which is 7%. There is a big difference between men and women in two factors: quality and taste. The most important factor for Slovak woman and man was quality followed by taste. Their preferences are different in case of next mostly preferred factor, which women consider as composition and men price. The least answered factor for chosen dairy products for women was origin and composition for men. According to the results of the survey, the majority of Slovak respondents appreciate if the product is of high quality, it is tasty, has the appropriate composition and origin of production (Figure 8).

Also in Russia, the most important factor was the quality of the product, which was selected by 40% of respondents. This was followed by the taste and composition of the product chosen by 20 respondents, which is 19.23%. Only six respondents have chosen the origin of the product. It is also possible to see compliance between Russian women and men. While 30% of men chose the product's quality as the most important factor, also 43.75% of women chose the same factor. On the contrary, 21.88% of women chose the price of the product as the most important factor, but only 5% of men. The composition of the product was also more important for men, while the overall quality of the product for women. From the founded similarities between Slovak and Russian respondents we can recommend for Slovak exporters to Russian market to copy the gender approach to Slovak market at Russian market as well (Figure 9).

For the statistical evaluation firstly Kruskal-Wallis test on the sample of Slovak respondents and consequently also on Russian sample was used (Table 4). The results of the analysis of the 203 samples for Slovakia already described above were proved by calculated means and the significance of the variables were verified by Kruskal-Wallis test. From its *p*-value (Table 5) we can see this as highly significant. Therefore, we have accepted alternative hypothesis, and thus there is a dependency between the most important variables for choosing a brand of dairy products and gender of the respondents.

From the multiple comparison of selected variables using Bonferroni correction, we can see the significance between gender and every included variable. According to this we can conclude, that gender has significant impact on the answers concerned with the preferences of buying dairy products in Slovakia. Also there are significant differences between the selected questions and surprisingly we can see connection of origin and all the other variables.

Table 1 Economic performance of Russia and Slovakia (as a part of European Union) in 2017.

	Russia	Slovakia	EU
GDP per capita/USD	11 441	19 897	36 593
Area/km2	17 125 200	49 036	4 475 757
Population/millions	144.5	5.4	513
Average income per capita/EUR	685	1096	1.520

Note: Source: Own processing based on the **World Bank (2019)**.

Table 2 Selected indicators of milk production in Russia and Slovakia (as a part of European Union), 2007 – 2017.

RUSSIA.											
	2007	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**
Milk production/thousand tons	31998	32363	32570	31847	31646	31756	30529	30791	30781	30759	31184
Dairy cows/thousand heads	9320	9127	9026	8844	8976	8859	8661	8531	8379	8250	8200
Milk yield per cow per kg	3433	3546	3608	3601	3526	3585	3525	3609	3674	3728	3803
SLOVAKIA											
Milk production/thousand tons	964	946	852	800	812	851	827	844	865	823	826
Dairy cows/thousand heads	180	174	163	159	154	150	145	143	139	133	130
Milk yield per cow/kg	5351	5439	5245	5023	5266	5665	5706	5897	6210	6204	6360
EU											
Milk production/thousand tons	133812	135281	133700	135528	138859	139951	141247	147847	151632	153275	154792
Dairy cows/thousand heads	24287	24406	23871	23314	23053	23193	23468	23559	23594	23525	23311
Milk yield per cow/kg	5510	5543	5601	5813	6024	6034	6019	6276	6427	6515	6640

Note: Source: own processing based on data of **SÚSR (2019)**, **Eurostat (2019)** and **Rosstat (2019)**.

Table 3 Per capita consumption of milk and dairy products in kg.year-1, 2007 – 2016.

	RCN	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Slovakia	220	153	153	154	163	159	159	158	167	169	177
Russia	325	242	242	244	247	246	249	248	244	239	239

Note: Source: Own processing based on data of **SÚSR (2019)** and **Rosstat (2019)**. RCN – Rational consumption norms.

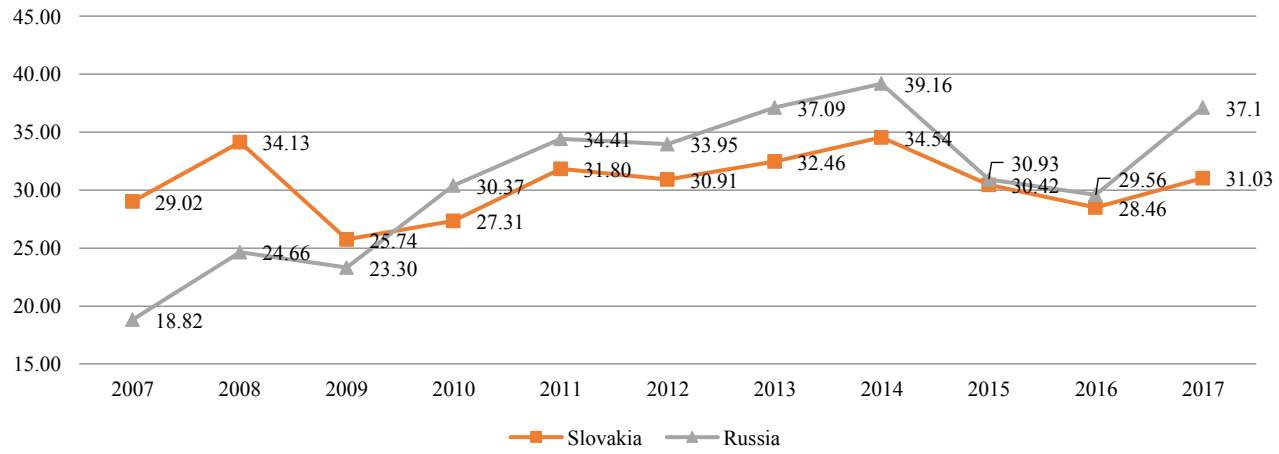

Figure 1 Development of milk prices in EUR.100kg-1, 2007 – 2017. Note: Source: Own processing based on data of **SÚSR (2019)** and **Rosstat (2019)**.

Table 4 Summary statistics for Slovakian respondents and Kruskal-Wallis test.

Variable	Observations	Minimum	Maximum	Mean	Std. deviation	Kruskal-Wallis test:	
Gender	203	0.000	1.000	0.251	0.435	K (Observed value)	512.284
1. Frequency	203	1.000	5.000	2.236	0.713	K (Critical value)	9.488
2. Origin	203	1.000	3.000	1.557	0.896	DF	4
3. Price	203	1.000	3.000	2.232	0.488	*p*-value (Two-tailed)	**<0.0001**
4. Factor	203	1.000	5.000	2.443	1.407	alpha	0.05

Note: Source: Own processing.

Table 5 *P*-values: Bonferroni corrected significance level: 0.005, Slovakia.

	Gender	1. Frequency	2. Origin	3. Price	4. Factor
Gender	1	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
1. Frequency	**<0.0001**	1	**<0.0001**	0.725	0.622
2. Origin	**<0.0001**	**<0.0001**	1	**<0.0001**	**<0.0001**
3. Price	**<0.0001**	0.725	**<0.0001**	1	0.399
4. Factor	**<0.0001**	0.622	**<0.0001**	0.399	1

Note: Source: Own processing.

Table 6 Summary statistics for Rusian respondents and Kruskal-Wallis test.

Variable	Observations	Minimum	Maximum	Mean	Std. deviation	Kruskal-Wallis test:	
Gender	104	0.000	1.000	0.385	0.489	K (Observed value)	234.058
1. Frequency	104	1.000	5.000	2.115	1.036	K (Critical value)	9.488
2. Origin	104	1.000	3.000	1.654	0.856	DF	4
3. Price	104	2.000	3.000	2.308	0.464	*p*-value (Two-tailed)	**<0.0001**
4. Factor	104	1.000	5.000	2.308	1.330	alpha	0.05

Note: Source: Own processing.

Table 7 *P*-values: Bonferroni corrected significance level: 0.005, Russia.

	Gender	1. Frequency	2. Origin	3. Price	4. Factor
Gender	1	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
1. Frequency	**<0.0001**	1	**0.004**	0.019	0.694
2. Origin	**<0.0001**	**0.004**	1	**<0.0001**	**0.001**
3. Price	**<0.0001**	0.019	**<0.0001**	1	0.050
4. Factor	**<0.0001**	0.694	**0.001**	0.050	1

Note: Source: Own processing.

Figure 2 Answers to the Question 1: How often do you buy dairy products (SK answers). Note: Source:Own processing.

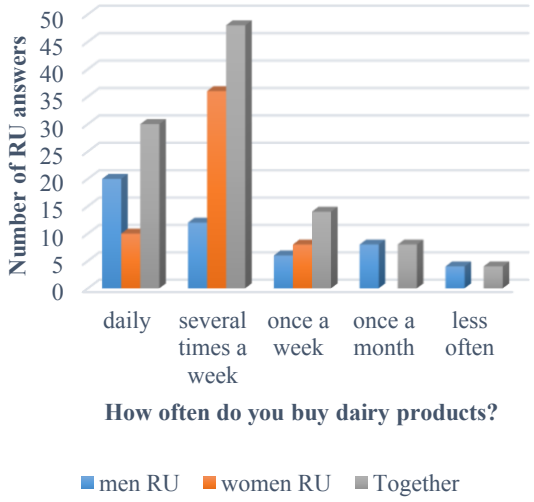

Figure 3 Answers to the Question 1: How often do you buy dairy products (RU answers). Note: Source:Own Processing.

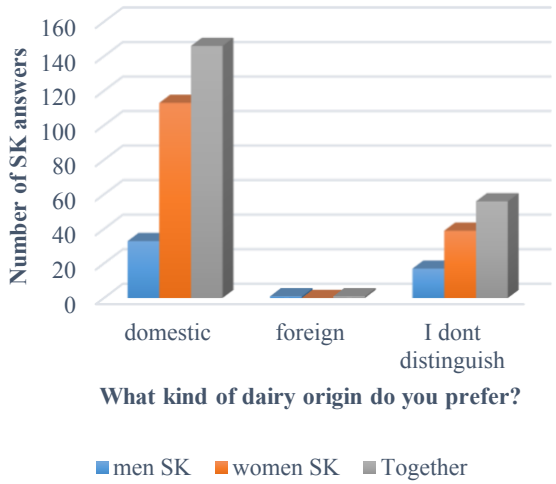

Figure 4 Answers to the Question 2**:** What kind of dairy origin do you prefer (SK answers)? Note: Source: Own processing

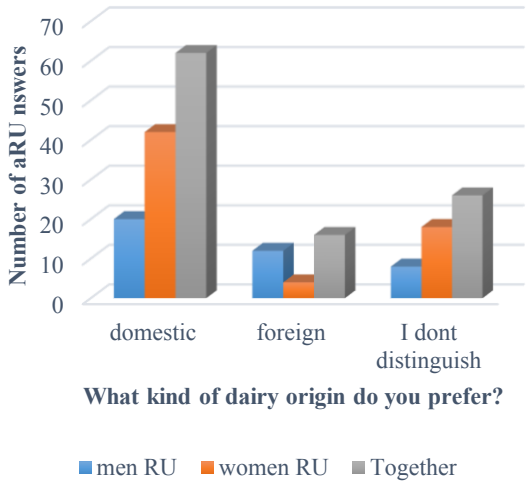

Figure 5 Answers to the Question 2**:** What kind of dairy origin do you prefer (RU answers)? Note: Source: Own processing.

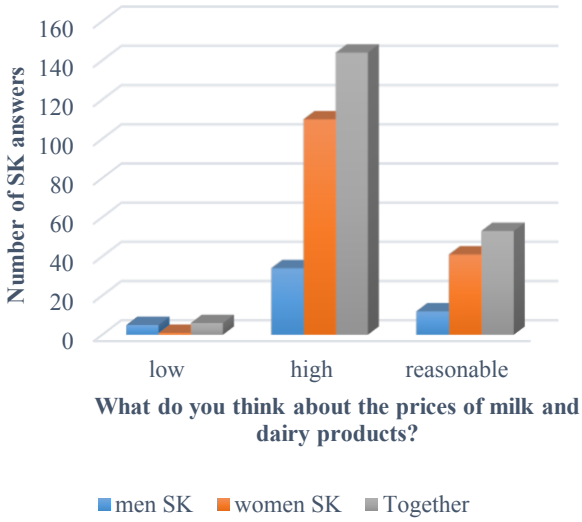

Figure 6 Answers to the Question 3**:** What do you think about the prices of milk and dairy products (SK answers)? answers)? Note: Source: Own processing.

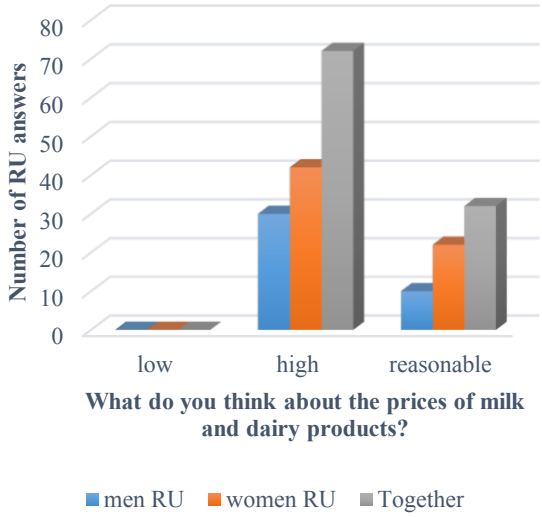

Figure 7 Answers to the Question 3**:** What do you think about the prices of milk and dairy products (RU answers)? Note: Source: Own processing.

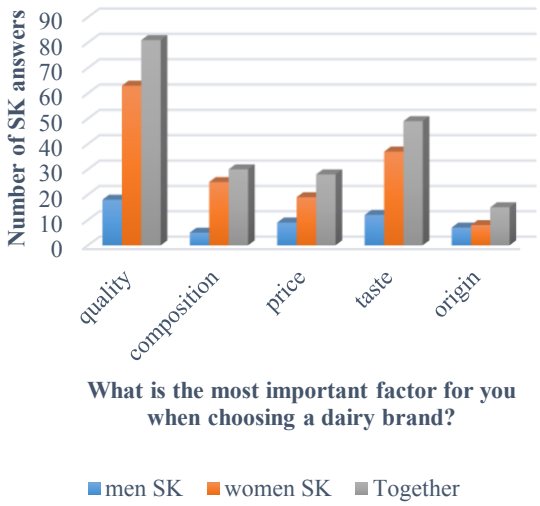

Figure 8 Answers to the Question 4: What is the most important factor for you when choosing a dairy brand (SK answers)? Note: Source: Own processing.

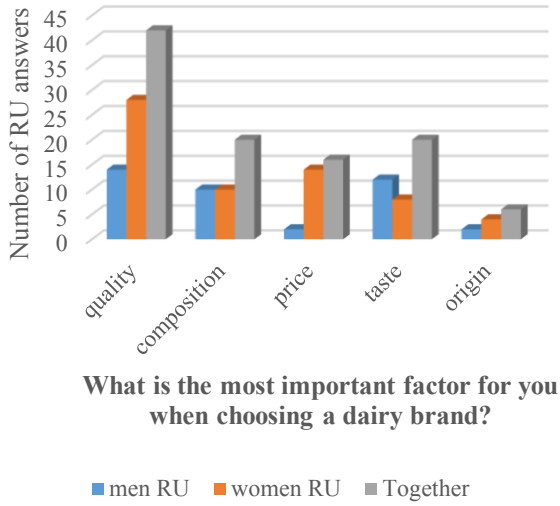

Figure 9 Answers to the Question 4: What is the most important factor for you when choosing a dairy brand? (RU answers)? Note: Source: Own processing.

From the statistics for Russian respondents the significance according to Kruskal-Wallis *p*-value was also proved and we accept H1, which shows a dependence of chosen variables to gender (Table 6). Significant importance is seen from the *p*-values (Table 7) of Bonferroni correction in the question of origin. This can indicate that the Russian market can be more open to the foreign producers of dairy products. Outcomes mean, that for both Slovak and Russian market the strategy should be aimed regarding to gender and thus, the similar competitive marketing strategy **(Valdani and Arbore, 2015)** can be used on both markets.

CONCLUSION

Analysis of the survey on 104 Russian and 203 Slovak respondents showed that the sample of respondents was diverse enough to show their overview of market supply, prices and trends. In both countries women tend to buy dairy products more often than men. Slovak consumers prefer more domestic products and this trend is also noticeable in Russia but not as significantly as in Slovakia. For 70% of consumers in both countries the price of dairy products was high and almost the same percentage of Slovak and Russian respondents consider them as reasonable. Despite the fact that majority of respondents consider price as high, the price is not the most important factor when choosing dairy products. In Slovakia for both genders the price is even on a fourth position behind quality as first, followed by taste and composition. In Russia the same order of preferred factors can be seen, but considering gender separately, Russian women don't copy the overall order, and after quality the price was second most frequent answer.

The fact that most Slovak consumers prefer domestic dairy products, results in support of domestic producers. Despite the same preferences in Russia, but slightly less significant, in both countries the origin was chosen as the last option. This can be used for benefit of Slovak producers who can export their dairy products to Russian market.

Based on this outcomes, the hypothesis that gender has significant impact on the answers concerned with the preferences of buying dairy products was set. The Kruskal-Wallis test proved the dependence of chosen variables by gender for both countries. When comparing the differences between selected questions we can see the connection of origin with all the other variables. Our results indicate the same importance of origin and gender as a key factors for respondents to buy dairy products. The implementation of this fact to the marketing strategy would mean that the advertising shouldn't be aimed just on the gender, but the domestic origin should be highlighted too. According this, it can be recommended, that the same marketing strategy of producers used in Slovakia can be applied without major changes also on the Russian market. Last but not least, it is very important to take gender into consideration and form this universal marketing strategy with the focus on man and woman separately.

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RHEOLOGICAL PROPERTIES OF TOMATO KETCHUP

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ABSTRACT
The objective of this paper was to determine the rheological properties especially shear stress and apparent viscosity vs. shear strain rate, and density of commercially available but also homemade tomato ketchup. The effect of tomato content of density and apparent viscosity of tomato ketchup was also described. Shear stress and apparent viscosity were observed in shear strain rates range from 0.1 s^{-1} up to 68 s^{-1}. All measurements were carried out at a constant temperature of 22 °C. Experimental results were modeled using a power-law (also known as Ostwald-de Waele) model (R^2 ranged from 0.9508 up to 0.9991). The flow behaviour of all measured tomato ketchup samples exhibited non-Newtonian pseudoplastic (shear thinning) behavior where the flow index (n) showed values between 0 and 1. Flow index (n) and consistency coefficient (K) can be used especially in numerical simulation of the flow behaviour of pseudoplastic (shear thinning) liquids.

Keywords: tomato; ketchup; rheological properties; viscosity; flow curve

INTRODUCTION
Production of tomato (*Lycopersicon esculentum*) is worldwide the second largest among the vegetable crop. Tomatoes may be consumed raw, but due to its perishable nature are processed on tomato juice, puree or paste **(Ray et al., 2016)**. A large part of the world tomato crop is processed into tomato paste, which is subsequently used as an ingredient in many food products, mainly soups, sauces, and ketchup. The main source for producing ketchup is tomato paste described as a dispersion of solid particles (pulp) in an aqueous media (serum) resulting from the concentration of tomato pulp, after the removal of skin and seeds containing 24% or more of natural soluble solids **(Gould, 2013)**. Tomatoes have also been recognized as a source of carotenoids (lycopene), a very important class of bioactive compounds especially known for their anti-inflammatory properties and supporting prostate health. The quality of the processed tomato product is dependent upon processing conditions. It is important for tomato processors to know how to obtain high viscosity products to prevent loss of flavor and nutritional quality by preventing loss and increase the bioavailability of lycopene and appropriate evaluation of the tomato products **(Xu, Adyatni and Reuhs, 2018)**. Tomato ketchup is a product made from strained tomatoes with spices, salt, sugar and vinegar with or without starch, onions and garlic and contains not less than 12% of tomato solids. It is the most important product of tomato and is consumed extensively **(El-Desouky, 2016)**.

There are many kinds of ketchup in the market, such as baby ketchup, fine, sharp, ketchups with various types of flavors, etc. These ketchup differ mainly in the content of the basic ingredient, ie tomatoes and the spices used, as well as stabilizers (modified starch, pectin) which are often used extensively **(Koocheki et al., 2009)**.

Rheology is the study of the deformation and flow of matter. Rheological properties are based on flow and deformation responses of foods when subjected to stress. Technology processes and simultaneously the texture of the final product significantly affect the flow properties of food products **(Fischer and Windhab, 2011)**. Viscosity is one of the main quality aspects that should be considered to determine the comprehensive quality and consumer acceptability of many tomato products. Consistency is related to non-Newtonian or semi-solid fluids (sauces, purees, and pastes) with suspended particles and long-chain soluble molecules, and is measured practically by distribution or flow of the product **(Dak, Verma and Jaaffrey, 2008)**. The consistency of the product also depends on the bio-availability of various compounds, such as hydro-colloids (pectin, hemicelluloses). If the initial product is rich in these hydro-colloids, and subsequently evaporated to produce tomato paste, it results in a high consistency tomato paste **(Xu, Adyatni and Reuhs, 2018)**.

Several parameters contribute to the flow behavior of tomato ketchup, including the quality of the raw material (i.e. tomato paste) and the processing conditions. Different agronomical and processing conditions cause that there are difficulties in quality control arise from great variation in flow behavior in commercial tomato paste **(Sobowale et al., 2012)**. Degree of maturity, the temperature of processing, the content of solids, particle size and particle interactions number play a role in determining the

viscosity of tomato products **(Xu, Adyatni and Reuhs, 2018)**.

This work is aimed at determining of rheological properties of tomato ketchup, such as viscosity and flow curves for six different tomato ketchup.

Scientific hypothesis

The main hypothesis of this work is confirmation of assumption non-Newtonian behavior of tomato ketchup. Rheological behaviour will be modeled using mathematical model. With these mathematical model and their coefficients is possible to predicts flow behaviour of tomamto ketchup.

MATERIAL AND METHODOLOGY

The research was focused on evaluating rheological properties of tomato ketchup. Five tomato ketchup were taken from commercial distribution and one was homemade ketchup (Table 1). Homemade ketchup contains tomato-apple-onion (in ratio 6:4:1), sugar, salt, vinegar, and herbs. Denstity of tomato ketchup was carried out using digital densitometer Densito 30 PX (Mettler Toledo, USA). Rheological measurements were carried out using the DV-3P rotary viscometer (Anton Paar, Austria) equipped with a coaxial cylinder sensor system with precision small samples adapter and standard spindle TR9 according to Anton Paar (number 27 according to Brookfield). In the first step viscosity and flow curves (shear strain rate versus viscosity/shear stress) of tomato ketchup were measured in shear strain rate between 0.1 s^{-1} and 68 s^{-1} in the standard room temperature 22 °C.

The value of viscosity was measured ten times and resulting in an average dynamic viscosity value. Power-law model also known as Ostwald-de Waele model was used. This widely used equation takes the form $\boldsymbol{\tau = K \cdot \dot{\gamma}^n}$, where τ is a shear stress, K is a consistency coefficient, $\dot{\gamma}$ is a shear strain rate, and n is a flow index that indicates the type of liquid **(Burg et al., 2018; Meher, Keshav and Mazumdar, 2019)**. For a Newtonian liquid $n = 1$; for a dilatant fluid $n > 1$, and for pseudoplastic fluid $0 < n < 1$. The most non-Newtonian foods are pseudoplastics shear thinning liquid ($0 < n < 1$) **(Bourne, 2002)**.

Statistic analysis

Statistical analysis of differences was based on Statistica12 (TIBCO, CA, USA), namely single-factor ANOVA - Duncan's test. Software MATLAB® R2018b with toolboxes (MathWorks, USA) was used to modelling of the experimental results. The satistically inconclusive difference was considered to be a result whose probability value reached $p > 0.05$.

RESULTS AND DISCUSSION

The viscosity of tomato ketchups was determined relative to four different shear rates. The first important physico-mechanical properties of tomato ketchup, which were measured, are the apparent viscosity at 20 s^{-1}, results see Table 2. The highest density, as well as the apparent viscosity, was shown by the sample marked K4, ie by the gentle curl of Otma Gurmán, which is attributed to its highest proportion of tomato share, which significantly influenced these parameters.

For modeling dependencies density and apparent viscosity of tomato ketchup on the tomato content it was used linear models: $\boldsymbol{\rho = 0.6112 \cdot tc + 1031.7}$ [kg.m^{-3}] (R_2 = 0.86) and $\boldsymbol{\eta_{app} = 14.544 \cdot tc + 632.11}$ [mPa.s] (R_2 = 0.77), where ρ is a density, tc is a tomato concent, and η_{app} is an apparent viscosity, see Figure 1.

Koocheki et al. (2009) reports the rheological behavior of tomato ketchups as non-Newtonian in all measured samples, using the power-law model and Herschel-Buckley. In our Ostwald-de Waele model, all ketchup samples also showed non-Newtonian, pseudoplastic behaviour, see Figure 2 and Figure 3.

Mirzaei et al. (2018) reports the highest sample viscosity with the addition of natural thickeners in the combination of glucomannan and xanthan (1:3), but the results did not show a significant statistical difference from the sample viscosity with the addition of a thickener without combination. These differences may be due to the synergistic interaction between these thickeners.

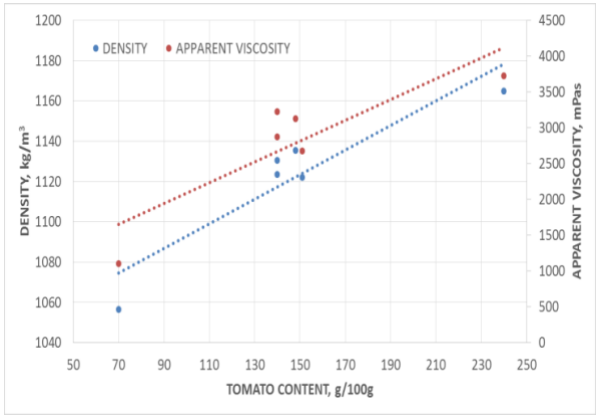

Figure 1 Influence of tomato content on density and apparent viscosity.

Table 1 Overview of meaured ketchups.

Sign	Manufacturer	Tomato g.100g^{-1}	Energy kJ.100g^{-1}	Fats g.100g^{-1}	Carbohydrates g.100g^{-1}	Proteins g.100g^{-1}	Salt g.100g^{-1}
K1	Hellmann's	151	440	0.1	25.0	1.0	1.8
K2	Heinz	148	435	0.1	23.2	1.2	1.8
K3	Otma	140	508	0.4	28.1	0.9	1.2
K4	Otma - Gurman	240	637	0.7	34.4	1.6	1.7
K5	COOP Klasik	140	535	0.4	29.9	0.8	2.1
K6	Homemade	70.4					

Table 2 Physico-mechanical properties of tomato ketchup at shear strain rate 20 s^{-1}.

	K1	K2	K3	K4	K5	K6
Density, [kg.m^{-3}]	1122.1 ±3.25	1135.4 ±2.80	1123.6 ±2.96	1165.0 ±3.11	1130.6 ±1.78	1056.6 ±2.04
Apparent viscosity, [mPa.s]	2675 ±8.6	3130 ±7.4	3222 ±9.0	3726 ±6.8	2870 ±9.7	1099 ±6.6

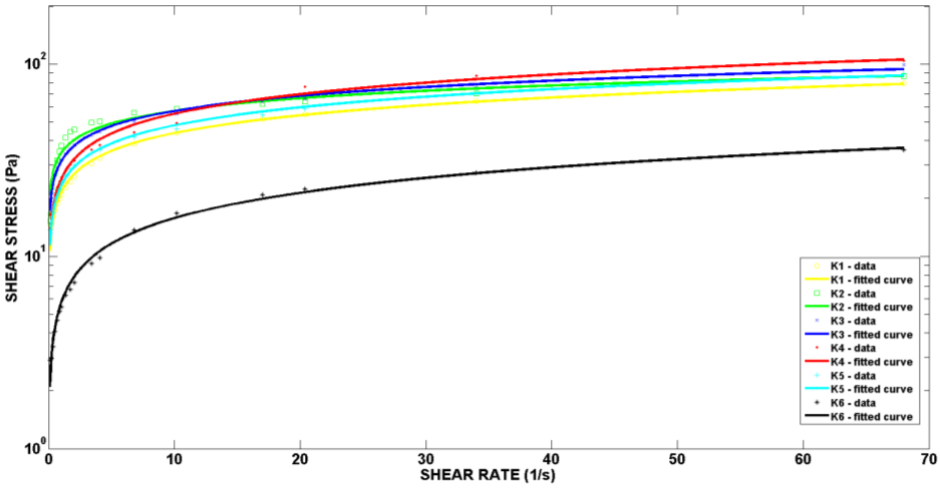

Figure 2 Experimental records of the flow curves (shear stress vs. shear rate) – Ostwald-de Waele model.

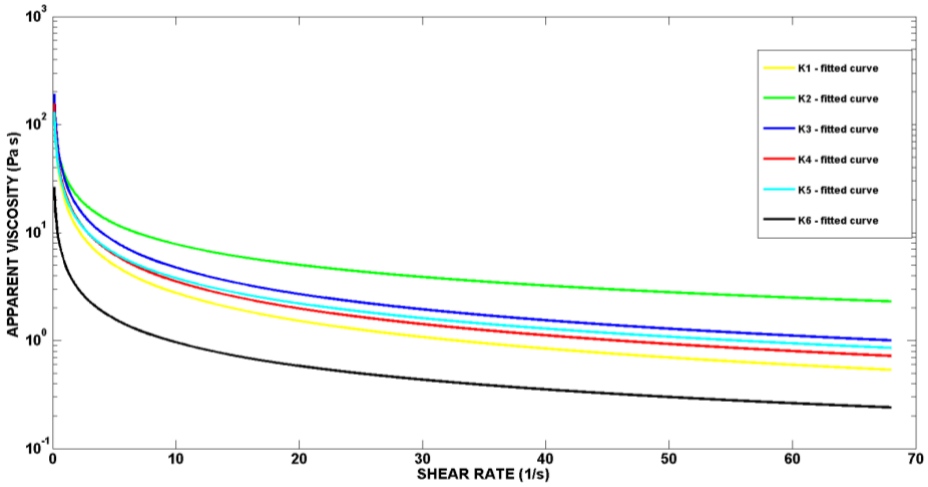

Figure 3 Experimental records of the flow curves (apparent viscosity vs. shear rate) – Ostwald-de Waele model.

Table 3 Coefficients of the Ostwald-de Waele rheological model.

Sign	FLOW CURVES			VISCOSITY CURVES		
	***K*, Pa.s^n**	***n*, –**	**R_2**	***K*, Pa.s^n**	***n*, –**	***R_2***
K1	21.55	0.3070	0.9956	19.63	0.1463	0.9969
K2	34.46	0.2187	0.9508	33.58	0.3648	0.9913
K3	31.19	0.2615	0.9933	30.35	0.1918	0.9991
K4	25.21	0.3392	0.9868	23.61	0.1728	0.9959
K5	23.36	0.3123	0.9965	22.37	0.2261	0.9976
K6	5.74	0.4392	0.9962	5.08	0.2768	0.9871

The consistency and thus its coefficient K can be influenced by the addition of hydrocolloids, their type, and also by the temperature **(Bozikova et al., 2018)**. The consistency coefficient values measured for flow curves ranged from 5.74 Pa.s$_n$ (for K$_6$) up to 34.46 Pa.s$_n$ (K$_2$), and for viscosity curves from 5.08 Pa.s$_n$ (K$_6$) up to 33.58 Pa·s$_n$ (K$_2$), see Table 3. For **Koocheki et al. (2009)** results, the consistency coefficient ranged from 6.56 Pa.s$_n$ up to 27.22 Pa.s$_n$. The flow behavior index n ranged from 0.2187 up to 0.4392, respectively from 0.1463 to 0.3648 for viscosity curves. **Koocheki et al. (2009)** lists values between 0.189 and 0.288. However, since all flow behavior index values are in interval between 0 and 1, which indicates a pseudoplasticy (shear thinning) of tomato ketchup samples. These results are consistent with **Gujral, Sharma and Singh (2002)** and **Bayod, Willers and Tornberg (2008)**. The coefficient determination R_2 values ranged from 0.9508 (K$_2$) up to 0.9968 (K$_4$) (see Table 3), and almost identical results were obtained when compared to the **Koocheki et al. (2009)** study where the determination coefficient values were between 0.990 to 0.999 using the Herschel-Bulkley model. This implies that the closer the value of R_2 is to 1, it gives us proof of the suitability of using the model in the determination.

CONCLUSION

The practical importance of knowledge of rheological parameters was outlined. Experimental data were successfully fitted with the Ostwald-de Waele model. We can conclude that the content of tomato in ketchup significant affect (p <0.05) the density and apparent viscosity of ketchup. With increasing tomato content is density and apparent viscosity of ketchup increases. These increases can be described using linear model. Obtained results also demonstrated non-Newtonian (pseudoplastics) behaviour of tomato ketchup - all of coefficients n (flow behaviour index) are less than 1. The pseudoplastic behaviour of tomato ketchup was successfully modeled using power-law (also known as Ostwald-de Waele) model (R_2 ranged from 0.9508 up to 0.9991). The coefficients of power-law model can be used for used in various software application dealing with a numerical simulation of flow parameters as calculate volume flow, friction factor, mean and maximal flow velicity, Reynolds number, 2D and 3D velocity profiles, and other flow properties of tomato ketchup.

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THE COMPARATIVE STUDY OF MEDICINAL PLANTS UTILIZATION AS HERBAL ANTIBIOTICS BY COLLEGE STUDENTS

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ABSTRACT

The medicinal plant utilization has become more and more popular and increasing number of consumers prefer alternative medicine to synthetic antibiotic. Research dealing with evaluation of medicinal plant usage as herbal antibiotics including the sample of 584 quizzed college students aged 19 – 25 years (337 women, 217 men) originated from Slovak Republic (n = 338), Czech Republic (n = 112) and Hungary (n = 134). According to university and the study programme the following groups were evaluated: Constantine the Philosopher University **CPU** (**PEES** – Pre-school and elementary education in Slovak language, **PEEH** – Pre-school and elementary education in Hungarian language, **BI** – Biology, **RT** – Regional Tourism), Mendel University in Brno **MU** (**H** – Horticulture), Slovak University of Agriculture **SUA** (**H** – Horticulture), University of Pécs **UP** (**PE** – Physical education), Comenius University **CU** (**PE** – Physical education). The study was aimed at the evaluation of the significance of the country and the study programme for the use of the most commonly used herbs: plantain, elderberry, stinging nettle, ginger and coneflower (*Echinacea*). Our results showed that the choice of preferred medicinal plants as herbal antibiotics during illness had not been clearly influenced by country or field of study programme. Plantain was the most frequently used herb by students of UP/PE (51.5%), CPU/PEES and CPU/PEEH (47.9%; 41.1%). Elderberry was the most popular herb among the students CPU/BI (52.9%), CPU/RT and SUA/H (37.8%). Stinging nettle was preferred as the most popular herb in groups of CPU/RT (46%). The significantly lower consumption of *Echinacea* was noticed in MU/H 4.5% in comparison with groups, CU/PE 26.4% (p <0.05), CPU/PEEH 27.4% (p <0.01), UP/PE 17.2% (p <0.05) and CPU/RT 28% (p <0.05). Regularly, all the year round the highest utilization of *Echinacea* was evident in CPU/BI 30.0%. The highest percentage formed respondent's utilized *Echinacea* only during illness. Otherwise, the differences between the frequencies of *Echinacea* usage cannot be considered as statistically significant. Generally, a significantly higher level of ginger usage was assayed within groups SUA/H 80.0% (p <0.001), CPU/PEEH 66.3% (p <0.001), UP/PE 36.6% (p <0.001), CPU/BI 58.8% (p <0.001), CPU/RT 56.0% (p <0.001), MU/H 78.6% (p <0.001) and CPU/PEES 77.1% (p <0.001) in comparison with the rest of the groups. Daily the respondents from CU/PE 20.8% consumed ginger significantly more often than students belonging to CPU/BI 0.0% (p <0.05) and MU/H 0.0% (p <0.05). Respondents from CPU/PEEH consumed statistically significantly more ginger once a week in comparison with students belonged to MU/H 0.9% (p <0.05). To sum up the research results, we can claim that state or study programme had no clear statistically significant evidence on the regular consumption of medicinal plants as herbal antibiotics.

Keywords: herbal antibiotic; medicinal plant; college student; frequency of usage

INTRODUCTION

An increased number of multidrug resistance of pathogens forces us to look for natural sources as alternative therapy for treatment of infectious diseases. Due to the high effectiveness of essential oils, terpenoids, polyphenols and other biologically active substances isolated from medicinal plants against microbes the researches on antimicrobial activity of plants have been more and more actual **(Mbosso et al., 2010; Jakubcova et al., 2014; Juríková et al., 2016a; Juríková et al., 2017)**. Phytotherapy has a rich tradition in Slovakia including widely grown or cultivated herbs. Nowadays between 150 and 200 medicinal plants are actively used as a part of therapy **(Salamon, 1995; Salamon, 2014)**. The leading position among medicinal plants in Slovakia with antimicrobial effect has traditionally used herbs **(Candan et al., 2003; Modarresi-Chahardehi et al., 2012; Juríková et al., 2016b; Juríková et al., 2016c)**. For example, extract of broadleaf plantain (*Plantago major*) display high antibacterial properties against *Staphylococcus aureus* and *Bacillus cereus*, moderate against *Pseudomonas aeroginosa* and *Acinetobacter bowi*, narrow leaf plantain (*P. lanceolata*) teas demonstrate good antimicrobial activity *in vitro*, *in vivo* test showed

a significant decrease in growth of *Streptococcus* strains **(Betoni et al., 2006; Ferrazzano et al., 2015)**. Extract isolated from flowers of elderberry (*Sambucus nigra*) exhibited strong antimicrobial effects on various nosocomial pathogens notably upon methicillin-resistant *Staphylococcus aureus* MRSA **(Hearst et al., 2010)**. Stinging nettle (*Urtica dioica*) displayed the highest inhibition against some pathogenic bacteria such as *Bacillus cereus*, MRSA and *Vibrio parahaemolyticus* **(Saffidine, Sahli and Zerroug, 2015)**. The microbial spectrum of the *Echinacea* extracts was broad, with activity against all microbial type **(Juríková et al., 2016b; Juríková et al., 2016c)** especially against *Clostridium difficile*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Legionella pneumophila* and *Propionibacterium acnes* **(Sharma et al., 2008)**. Extract isolated from coneflower (*Echinacea purpurea*) showed also a significant growth inhibition of *Candida albicans* **(Stanisavljevič et al., 2009)**. Except for traditionally used medicine in Slovakia it has become more and more popular to consume of ginger **(Juríková et al., 2016c)**. It is common for ginger (*Zingiber officinale*) to be used in the treatment of flu and colds. Moreover, the plant is known for soothing and antibacterial properties too **(Hara et al., 1998; Park, Bae and Lee, 2008; Costa et al., 2009)** especially against *S. aureus*, *S. pyogenes* and *P. aeruginosa* and also *Helicobacter pylori*. This strong antibacterial activity authors explain by the content of resins and volatile oils such as borneol, camphene, citral, eucalyptol, linalool, phellandrene, zingiberine and zingiberol phenols **(Ahmad et al., 2008; Rosato et al., 2007)**. Ginger (*Zingiber officinale*) possess effective anti-bacterial activity against multi-drug resistant clinical pathogens causing nosocomial infection **(Ponmurugan and Shyamkumar, 2012)**, especially against drug resistant *Escherichia coli* **(Rahman et al., 2011; Ushimaru et al., 2012)**, *Bacillus subtilis* **(Alzoreky and Nakahara 2003)**, *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Shigella sonnei, Staphylococcus epidermidis* and *Salmonella typhi* **(Betoni et al., 2006; Gull et al., 2012)**.

Due to the fact that during the last two decades there has been evident return towards the alternative sources of medicine, our research has been dealing with the mapping of traditionally used (elderberry, stinging nettle) and historically important (ginger, *Echinacea*) medicinal plants displayed antibacterial effect **(Sikkema, de Bont and Poolman, 1994)**. Furthermore, it has been statistically evaluated that the differences between three countries and research focused on the evaluation of study programme the importance in relation to usage of medicinal plants displayed antibacterial effect. The results represented in the second part of research and continue the evaluation of herbal antibiotic – vegetable among college students.

Scientific hypothesis

H1

We suppose that there have been statistically significant differences in consumption of herbs as natural antibiotics among assayed countries.

H2

We suppose that there have been differences in consumption of evaluated herbs as natural antibiotics among college students with different field of study.

MATERIAL AND METHODOLOGY

The data were obtained by questionnaire method. Cross-sectional study was conducted among 584 college students aged 19 – 25 years (337 women, 217 men) from 3 countries: Slovak Republic, Czech Republic and Hungary. The overview of evaluated universities, field of studies and group number, number of students together with abbrevations is given in Table 1. College students were asked for consumption of medicinal plants generally – plantain (*Plantago*) sp., elderberry (*Sambucus nigra*), stinging nettle (*Urtica dioica*), ginger (*Zingiber officinale*) and coneflower (*Echinaceae sp.*) with mapping the frequency of usage in case of *Echinacea* and ginger.

Statistic analysis

The statistical evaluation was provided on programme STATISTICA 6.0 by method of ANOVA and post-hoc tests Tamhane and Dunett T 3 on the level of probability 99 and 95%.

RESULTS AND DISCUSSION

In the first part of study we focused our attention on mapping of usage of traditionally utilized herbs for the treatment of common respiratory diseases.

Comparison of the utilization of traditionally used herbs

The traditional used herbs involved are represented by elderberry, stinging nettle, plantain (narrowleaf and broadleaf) and coneflower – *Echinacea*.

The college students generally preferred to use plantain (34.7%) followed by elderberry (26.7%) and stinging nettle (25.3%) (Figure 1). Our results are similar to study of **Juríková et al. (2015)** mapping the consumption of the same medicinal plant sources with antimicrobial effect among the college students of Constantine the Philosopher University in Nitra. On contrary, the usage of coneflower (*Echinacea*) was the lowest (19.7%). It can be caused by the controversial studies pointed on the health benefits against microorganism only in prevention **(Juríková et al., 2016c)**.

The second part of research focused attention on mapping the usage of the most popular concrete herbs displaying antimicrobial activity in relation to country and study programme of students. The results of statistical evaluation showed that plantain was in the lowest amount utilized by study group CPU/BI 5.9% and CU/PE 13.2% (Figure 2). On the contrary, the highest one was noticed in respondents belonged to UP/PE 51.5% and CPU/PEES 47.9%. In comparison with the CPU/BI and CU/PE the statistically higher consumption was evident in group CPU/PEEH 41.1% (p <0.001), UP/PE 51.5% (p <0.001) and CPU/PEES 47.9% (p <0.001). There were proved statistically significant differences between UP/PE, CPU/RT 18% (p <0.001) and MU/H 28.6% (p <0.001). The hypotheses 1 and 2 have been proved.

Elderberry was used in significantly lower amount among quizzed college students of MU/H 2.7% than in groups of students belonging to the study programme SUA/H 38% (p <0.001), CPU/PEEH 27.4% (p <0.001), UP/PE 35.8% (p <0.001), CPU/BI 52.9% (p <0.001), CPU/RT 40% (p <0.001) and CPU/PEES 33.3% (p <0.001). It means that statistical hypotheses have been confirmed partially. In accordance with our results **Juríková et al. (2015)** found out the highest percentage of college students from Constantine Philosopher University (42.33%) preferred elderberry for treatment of respiratory illnesses. **Balla et al. (2013)** studied the utilization of the most frequent medicinal plants among 550 pupils from the Nitra region. They found out that the stinging nettle was the second most frequently used herb in case of respiratory diseases otherwise the **elderberry** belonged to non-popular medicinal plant.

It is interesting that no usage of stinging nettle was noticed in group of respondents belonged to MU/H. Statistically significantly higher amount of this herb was evident in groups CPU/PEEH 23.2% (p <0.001), CPU/PEES 39.6% (p <0.001) and UP/PE 40.3% (p <0,001). Statistically lower amount of stinging nettle was used within CU/PE 15.1% in comparison with college students belonged to UP/PE 40.3% (p <0.001) and CPU/RT 46% (p <0.05). It means that statistically significant differences have been confirmed among different field of studies and **countries** as well.

Coneflower (*Echinacea*) (Figure 5) was consumed in significantly lower amount within MU/H than in groups SUA/H 33.3% (p <0.001), CPU/RT 28.0% (p <0.05), CPU/PEEH 27.4% (p <0.001), CU/PE 26.4% (p <0.05) and UP/PE 17.2% (p <0.05). So hypotheses 1 and 2 were confirmed partially. On contrary, in previous study of **Juríková et al. (2015)** mapping the consumption of herbal antibiotics among college students in Nitra pointed to the highest percentage of respondents with non utilisation of the mentioned herb.

As we can see in Figure 6, only in winter time was *Echinacea* used in the highest amount within SUA/H (17.8%), in the lowest in CPU/PEES (2.1%) and MU/H (2.7%). Regularly, all the year round, the highest utilisation of echinacea was evident only in group CPU/BI 11.8% (Figure 7). On the contrary, non consumption all year was noticed in MU/H. The differences among evaluated groups can not be considered as statistically significant ($p \geq 0.05$). So hypotheses 1 and 2 have not been confirmed. Students of CPU/BI, CPU/PEEH consumed higher amount of *Echinacea* all year in comparison with CU/PE, UP/PE, CPU/RT, MU/H and finally CPU/PEES. So the differencies between selected groups have not represented the clear evidence the influence of field of study of students and **countries** as well.

The highest percentage of respondents claimed that they utilized *Echinacea* only during illness, except for respondents belonged to group UP/PE 17.2% (Figure 8) with no significant differences among groups and **countries**. Similarly, **Juríková et al. (2015)** mapping the frequency of *Echinacea* utilization among college students from Nitra (CPU and SUA) found out the highest percentage of college students utilized this herb only during illness. On the contrary, the majority of students at secondary schools stated that they did not like this herb and have never utilized it in common life (boys 56.06%; girls 65.71%) **(Juríková et al., 2016c)**. The decreasing popularity and tendency of using is given by controversial studies. They pointed to the fact that *Echinacea* could not prevent illness or reduce the length of symptoms **(Keith et al., 2003)**.

Significantly lower usage of ginger was noticed in UP/PE 36.6% in comparison with groups SUA/H 80.0% (p <0.001), MU/H 78.6% (p <0.001), CPU/PEES 77.1% (p <0.001) and CPU/PEEH 66.3% (p <0.001) (Figure 9).

In the same way the consumption of ginger twice a week can be evaluated as very low (Figure 12). Significantly higher level of consumption was registrated in group MU/H 14.3% in comparison with groups CU/PE 1.9% (p <0.05).

Daily the respondents from CU/PE 20.8% consumed ginger significantly more often than students belonging to CPU/BI and MU/H 0.0% (p <0.05). The differences between the rest of the groups cannot be considered as statistically significant ($p \geq 0.05$) (Figure 10). The hypotheses 1 and 2 have been confirmed only partially.

Respondents from CPU/PEEH consumed statistically significantly more ginger once a week in comparison with students of MU/H 0.9% (p <0.05). The highest usage with frequency once a week was evident in SUA/H 17.8% (Figure 11).

According to Figure 13 the highest percentage of college students preferred the monthly usage of ginger among another forms of usage. Our results are corresponded with the results of research study **Juríková et al. (2015)** in which college students from Nitra preferred to use ginger monthly (38% of quizzed students). It is evident that statistically a significantly lower consumption was noticed in groups UP/PE 27.6%, than in group MU/H 50% (p <0.001). Our results of research are more positive in comparison with **Juríková et al. (2016c)** evaluated the utilization of herbal antibiotics among students of secondary school. They found out that they used ginger only during the illness (16.66% – girls) or rarely (39.39% – boys). Our results are in conflict with another research study of **Sloand and Vessey (2001)**. According to study the majority of the adolescents (89%) have access to the medicine in their households, and most popular and frequent was ginger usage.

Once a year used the ginger 35.7% quizzed from groups MU/H that can be considered as significantly higher amount in comparison with respondents of CU/PE 5.7% (p <0.001), CPU/PEEH 9.5% (p <0.001) and UP/PE 21.6% (p <0.05) (Figure 14).

Explanation of groups for Figures 2 – 14: Group **1** – Slovak University of Agriculture **SUA** (**H** – Horticulture), **2** – Comenius University **CU** (**PE** – Physical education), **3** – Constantine the Philosopher University – **CPU** (**PEEH** – Pre-school and elementary education in Hungarian language, **BI** – Biology, **RT** – Regional Tourism), **4** – University of Pécs **UP** (**PE** – Physical education), **5** and **6** – Constantine the Philosopher University – **CPU** (**PEEH** – Pre-school and elementary education in Hungarian language) **BI** – Biology and **RT** – Regional Tourism, **7** – Mendel University in Brno **MU** (**H** – Horticulture), **8** – Constantine the Philosopher University – CPU (PEES – Pre-school and elementary education in Slovak language. Mean values (+/- 95% CI).

Table 1 Overview of college students according to university, field of study and number of students (with group designation and number in figures).

University/Abbreviation	Field of study with group designations	Number of students	Group number in figures
Constantine the Philosopher University **CPU**	**CPU/PEES** – Pre-school and elementary education in Slovak language	48	8
	CPU/PEEH – Pre-school and elementary education in Hungarian language	95	3
	CPU/BI – Biology	47	5
	CPU/RT – Regional Tourism	50	6
Mendel University in Brno **MU**	**MU/H** – Horticulture	112	7
Slovak University of Agriculture **SUA**	**SUA/H** – Horticulture	45	1
University of Pécs **UP**	**UP/PE** – Physical education	134	4
Comenius University **CU**	**CU/PE** – Physical education	53	2

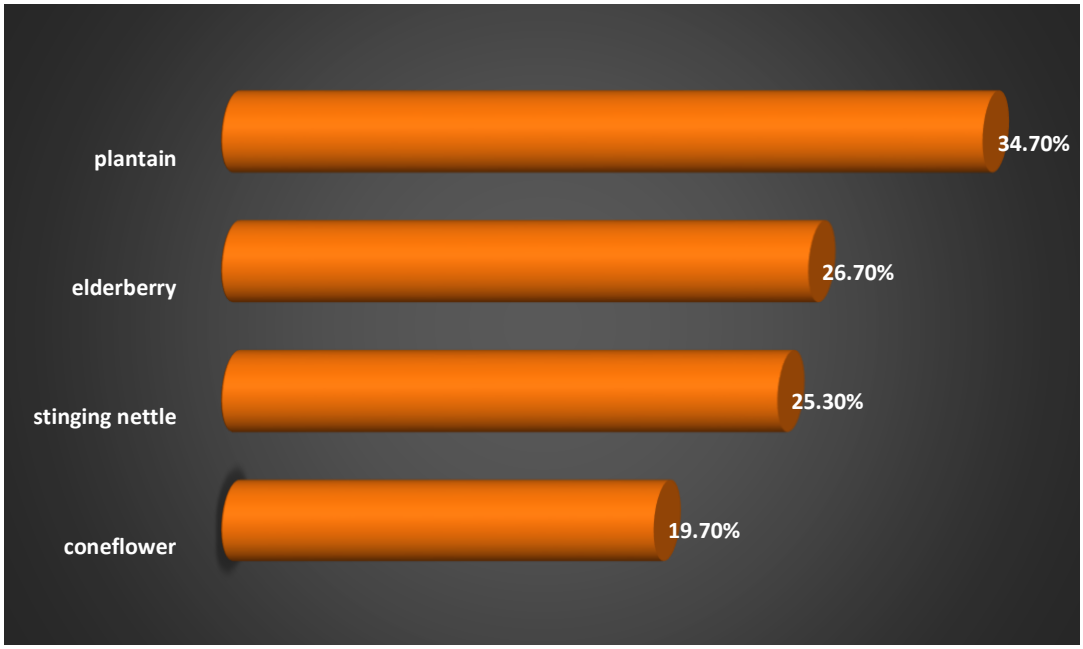

Figure 1 The comparison of traditionally utilized medicine plants with antibiotic effect.

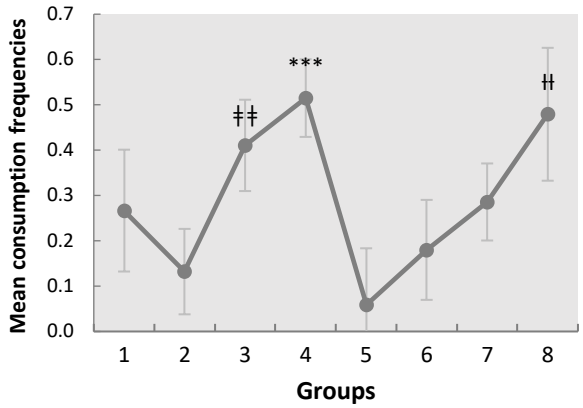

Figure 2 Consumption of plantain.
Note: *** p <0.001 group 4 vs. groups 5,6,7; ‡‡ p <0.01 group 3 vs. groups 2, 5; ‡‡ p <0.01 group 8 vs. groups 2, 5.

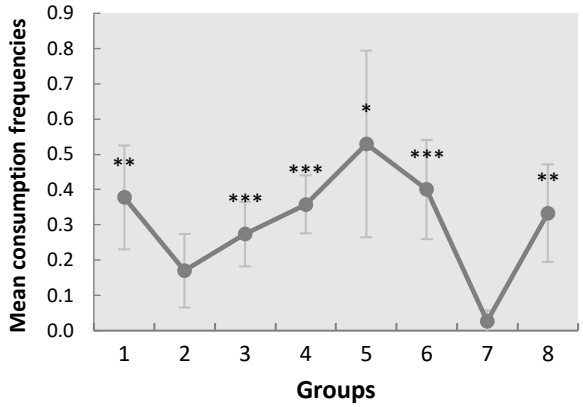

Figure 3 Consumption of elderberry.
Note: *** p <0.001 group 7 vs. groups 3,4,6; ** p <0.01 group 7 vs. groups 1, 8; * p <0.05 group 7 vs. group 5.

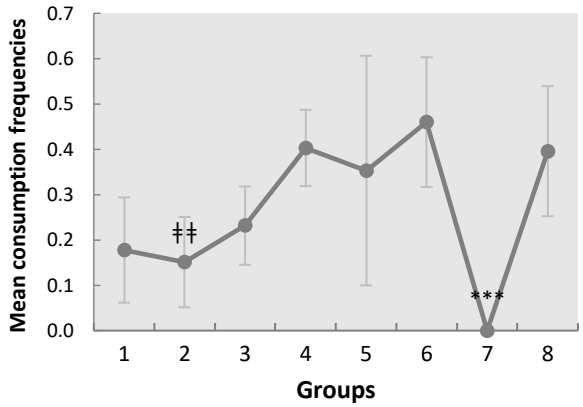

Figure 4 Consumption of stinging nettle.
Note: *** p <0.001 group 7 vs. groups 3, 4, 6, 8; ‡‡ p < 0.01 group 2 vs. group 4; ‡ p <0.05 group 2 vs. group 6.

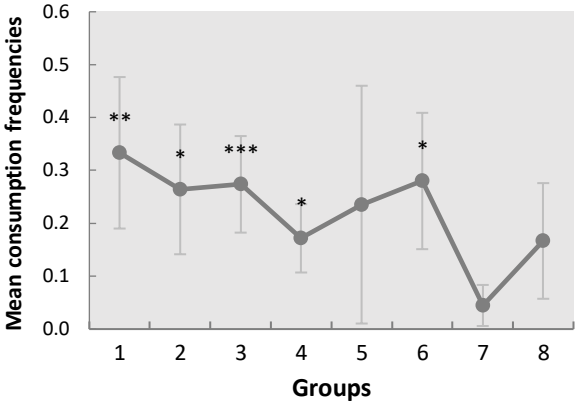

Figure 5 Consumption of coneflower (*Echinacea*).
Note: *** p <0.001 group 7 vs. groups 3; ** p <0.01 group 7 vs. group 1; * p <0.05 group 7 vs. groups 2, 4, 6.

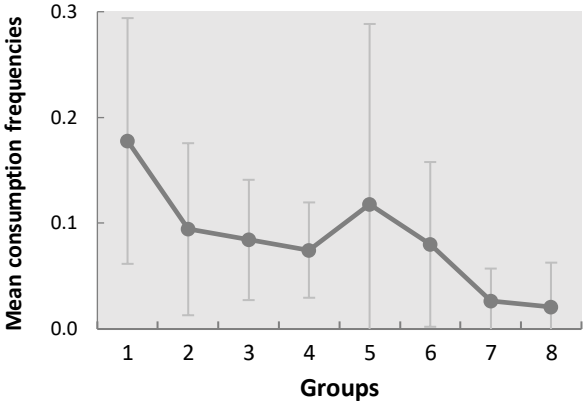

Figure 6 Consumption of *Echinacea* during winter time.
Note: $p \geq 0.05$.

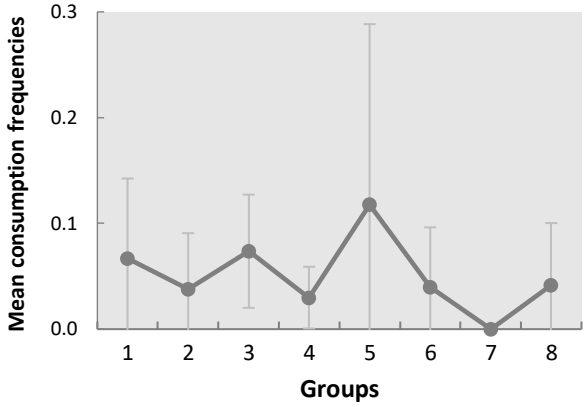

Figure 7 Consumption of *Echinacea* all year.
Note: $p \geq 0.05$.

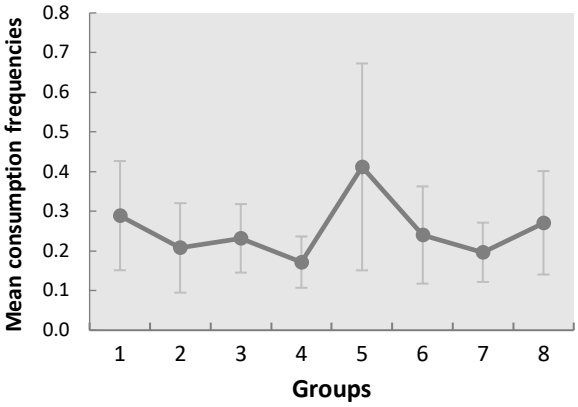

Figure 8 Consumption of *Echinacea* during illness.
Note: $p \geq 0.05$.

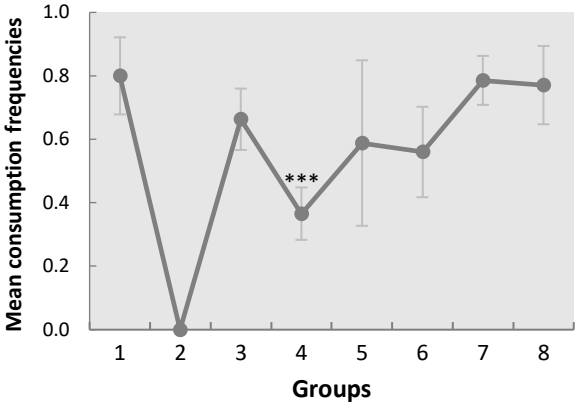

Figure 9 Consumption of ginger.
Note: *** $p < 0.001$ group 4 vs. groups 1, 3, 7, 8.

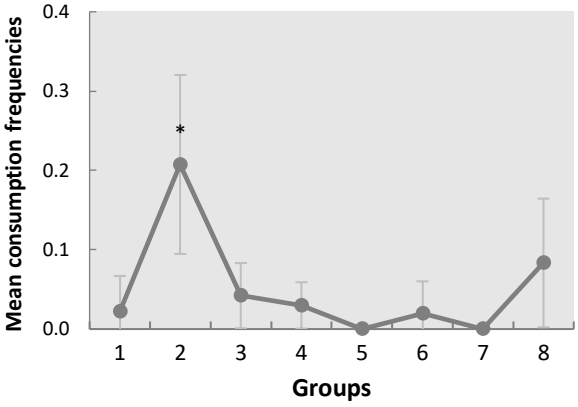

Figure 10 Daily consumption of ginger.
Note: * $p < 0.05$ group 2 vs. groups 5, 7.

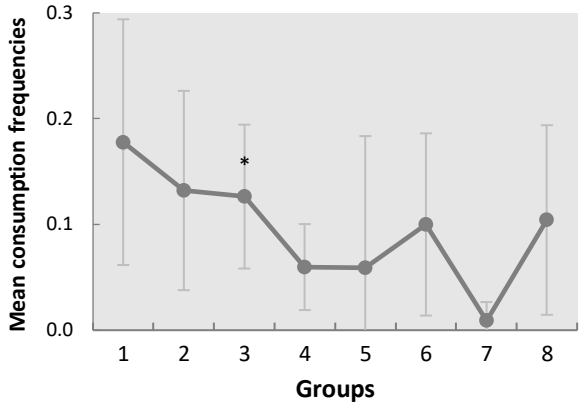

Figure 11 Consumption of ginger once a week.
Note: * $p < 0.05$ group 3 vs. group 7.

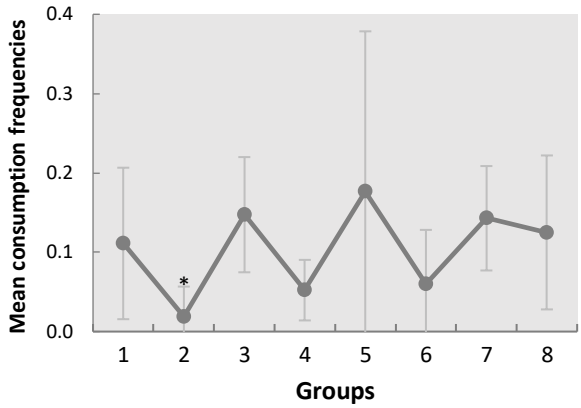

Figure 12 Consumption of ginger twice a week.
Note: * $p < 0.05$ group 2 vs. group 7.

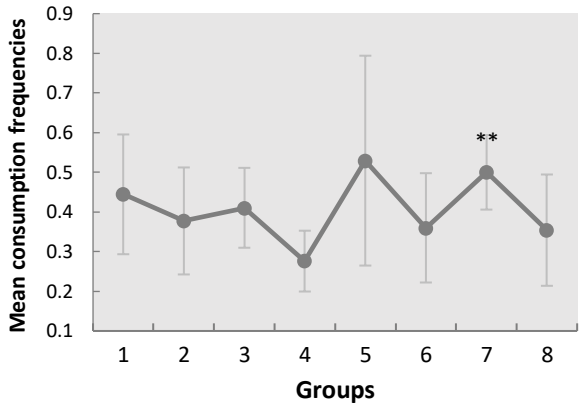

Figure 13 Consumption of ginger monthly.
Note: ** $p < 0.01$ group 7 vs. group 4.

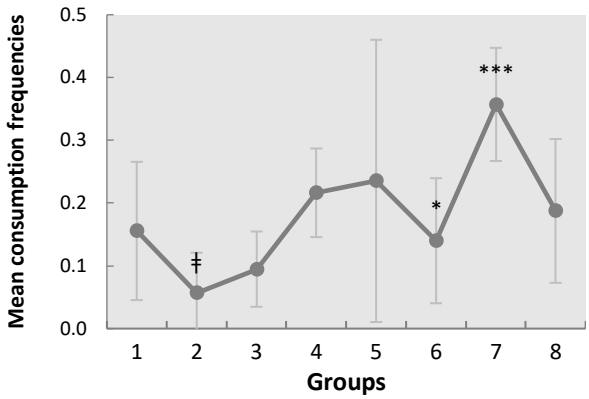

Figure 14 Consumption of ginger once a year.
Note: *** p <0.001 group 7 vs. groups 2, 3; * p <0.05 group 7 vs. group 6; ǂ p <0.05 group 2 vs. group 4.

CONCLUSION

To sum up the research results the assayed species of medicinal plants had different frequency of usage among college students in relation to countries and field of study. Plantain was preferred by students UP/PE (51.5%), CPU/PEES and CPU/PPEH (47.9% and 41.1%), elderberry by quizzed of CPU/BI (52.9%), CPU/RT (40%) and SUA/H (37.8%). Stinging nettle was popular among students of CPU/RT (46%) and as well as UP/PE (40.3%). *Echinacea* was used in preference of SUA/H (33.3%) and CPU/RT (28%). Despite the differences in usage of *Echinacea* they have not be statistically significant differences between assayed groups of students. On contrary, within the group UP/PE we noticed 80.6% usage of ginger that can be considered as significantly lower amount in comparison with the rest of the evaluated groups. The highest frequency of daily ginger utilization was noticed in group of students CU/PE, non usage was registered in groups MU/H a CPU/BI. The mentioned groups of students significantly differed from the rest of the evaluated groups. To sum up all assayed species, we can claim that country or study programme had no clear statistically significant evidence on the regular consumption of medicinal plants as herbal antibiotics. According to our opinion, the public popularity, preference in household, in the family have the greatest influence on usage biologically active substances isolated from medicinal plants.

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THE EFFECT OF FATTY ACID PROFILE ON THE STABILITY OF NON-TRADITIONAL AND TRADITIONAL PLANT OILS

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ABSTRACT

The effect of fatty acid composition on the autoxidation of selected plant oils (rapeseed (canola) oil, corn oil, frying oil, grapeseed oil, pomace olive oil, rice bran oil, sunflower oil and high oleic sunflower oil) during their storage was studied. Oils were purchased in retail food stores. Oxidative stability of plant oils was monitored during the storage under the Schaal test conditions at 60 °C in 100 mL beakers and the dark for 40 days. The weight changes, the peroxide and acid values were analysed during the storage. Changes in the composition of fatty acids were analyzed by the gas chromatography-mass spectrometry. The results obtained by monitoring the weight changes of oils correlated with their peroxide values. The induction period in case of grapeseed and sunflower oils was 27 and 28 days respectively. The induction period for frying and rapeseed oils were around 35 days. The remaining four oils had induction periods over 40 days. The acid values at the end of experiment correspond to both the relative weight gain and the the peroxide values. The stability of oils depended mainly on the degree of fatty acids unsaturation. A strong negative correlation between oleic acid content and oil stability expressed as the peroxide value was found. The significant positive correlation was found in case of linoleic acid. The relative content of polyunsaturated fatty acids decreased during the storage while the content of saturated and monounsaturated fatty acids increased. The highest relative increase in oleic acid was found at the least stable oils, grapeseed and sunflower oils, by 37.5% and 25.3% respectively. The initial content of free fatty acids monitored by the acid value did not affect the oxidation rate. With consideration to all monitored parameters the grapeseed and the sunflower oils were the least stable. The most stable ones were olive pomace and high oleic sunflower oils.

Keywords: plant oil; autooxidation; oxidative stability; fatty acid; Schaal test

INTRODUCTION

Vegetable oils differ in both their origin and composition. They are made from seeds, grains, sprouts, nuts, etc. They consist mainly of triacylglycerols (97%) which serve as a solvent for other lipophilic substances present in oils (sterols, fat-soluble vitamins – mainly tocopherols and tocotrienols, pigments including chlorophylls and carotenoids, phenolic compounds, phospholipids, free fatty acids and mono- and diacylglycerols). Oils differ in the degree of unsaturation, the type of triacylglycerol-forming fatty acids as well as the amount and type of unsaponifiable substances. These differences in composition are responsible for differences in oxidative stability of oils and their sensory and technological properties **(Kamal-Eldin, 2006)**.

Oil stability is a resistance to oxidation during the processing and storage **(Guillen and Cabo, 2002)**. It can be expressed as the time required to reach the critical point of oxidation, either in terms of sensory properties or the sudden acceleration of the oxidation process. Oxidation stability is an important indicator to determine the oil quality and its shelf life as the oxidation produces low-molecular compounds that affect sensory quality. These compounds cause oil to become less acceptable or totally unacceptable both for consumers and for industrial use. During the oxidation, essential fatty acids are degraded and toxicologically undesirable compounds and oxidized polymers could be formed. Oxidation has a great effect on the taste, nutritional quality and safety of oils **(Choe and Min, 2006; Matthäus, 2010; Angelovič et al., 2015)**.

Oil oxidation is influenced by many factors including fatty acid composition, processing, energy of heat or light, the presence, concentration and type of oxygen (triplet – $^3O_2$ and singlet – $^1O_2$), free fatty acids, mono- and diacylglycerols, transition metals, peroxides, thermally oxidized compounds, pigments, and antioxidants. These factors interactively affect the oxidation of oil and it is hard to differentiate the individual effect of each of them. Edible oil is oxidized during autoxidation (triplet oxygen – $^3O_2$ reacts with the oil) and photosensitized oxidation (singlet oxygen – $^1O_2$ reacts with the oil). Autoxidation of oils requires radical forms of acylglycerols, whereas photosensitized oxidation does not require lipid radicals – $^1O_2$ reacts directly with double bonds **(Choe and Min, 2006; Matthäus, 2010; Žabčíková and Červenka, 2015)**.

In order to minimize the oxidation of edible oil during the processing and storage, it is advisable to lower the temperature, exclude light and oxygen, remove metals and oxidized compounds, and use appropriate concentrations of antioxidants such as tocopherols and phenolic compounds **(Choe and Min, 2006)**.

Scientific hypothesis

The stability of plant oils during the storage under increased temperature is affected by the composition of fatty acids.

MATERIAL AND METHODOLOGY

Assessment of oil stability using the Schaal test

The oils were stored in open 100mL beakers at 60 °C and the weight changes indicating the amount of oxygen absorbed in the oil were recorded. The increase in mass reflects the degree of oxidation of the monitored oil.

Materials and analytical equipment

Oils were purchased in retail food stores. They were in one-liter clear plastic or colored glass bottles. All samples were odourless without any rancidity off-flavour and their expiry date was more than 10 months.

Corn oil: Made by Olitalia, via Meucci 22/A, Forli, Italy. The declared composition (per 100 mL of product) – 91 g of total fat, 13 g of saturated fatty acids (SFA), 23 g of monounsaturated (MUFA) and 55 g of polyunsaturated fatty acids (PUFA).

Grape seed oil: Made by Olitalia, via Meucci 22/A, Forli, Italy. The declared composition (per 100 mL of product) – 91 g of total fat total, 10 g of SFA, 19 g of MUFA and 62 g of PUFA fatty acids.

Frying oil: Made by Palma Group, a.s., Račianska 76, Bratislava, Slovak Republic. Mixture of low-erucic rapeseed oil (canola oil) and high oleic sunflower oil. The declared composition (per 100 mL of product) – 91.3 g of total fat, 7.2 g of SFA, 75.8 g of MUFA and 8.3 g of PUFA.

Olive-pomace oil: Made by Ondoliva, Urzante S.L., Ciudad, Agroalimentaria, Tudela (Navarra), Spain. The declared composition (per 100 mL of product) – 100 g of fat total, 13 g of SFA, 79 g of MUFA and 8 g of PUFA.

Rice oil: Made by Olitalia, via Meucci 22/A, Forli, Italy. The declared composition (per 100 mL of product) – 91 g of total fat, 22 g of SFA, 38 g of MUFA and 31 g of PUFA, 200 mg of γ-oryzanol and 16 mg of vitamin.

Rapeseed (canola) oil: Made by COP, Prins Albertlaan 12, Izegem, Belgium. The declared composition (per 100 mL of product) – 100 g of total fat, 8 g of SFA, 62 g of MUFA and 30 g of PUFA.

Sunflower oil: Made by Fabio Produkt spol. s.r.o., Holin 92, Jičín, Czech Republic. The declared composition (per 100 mL of product) – 92 g of total fat, 10 g of SFA.

Sunflower oil with increased oleic acid content (organic farming product): Made by Rapunzel Naturkost, Legau, Germany. The declared composition (per 100 mL of product) – 100 g of total fat, 6 g of SFA, 79 g of MUFA (of which 79 g is oleic acid) and 15 g of PUFA.

Analytical equipment

Analytical balances AND, FR 200 MK II; capacity 210 g, resolution d = 0.1 mg; thermostats Memmert 54853 and POL-EKO ST.

Procedure

Approximately 25 g of oil was weighed (with the accuracy to four decimal places) into the 100 mL beakers (two parallel samples from each oil). The beakers were placed in the thermostat set at 60 °C for 40 days. One series of samples was reguralry weighed and another series of the same samples was reguralry sampled for the peroxide and acid values determinations. The relative weight gain (Δ m) was calculated according to the formula:

$$\Delta\, m = \frac{m_x - m_p}{m_x}$$

where m_x is the weight of the oil at the day of weighing and m_p is the weight of the oil at the beginning of the storage (day = 0).

Peroxide value determination

Peroxide value was used to monitor the amount of primary oxidation products produced during the Shaal tests. The determination is based on the reaction of the sample with potassium iodide solution in acetic acid and chloroform solution according to the **ISO 3960:2017**. The released iodine is then titrated with standard sodium thiosulphate. Determination is based on the reactions:

$$ROOH + 2I^- + 2H^+ \rightarrow I_2 + ROH + H_2O$$

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}$$

The results were expressed in millimoles of active oxygen per kilogram of oil. The peroxide number was measured at 0; 5; 10; 20; 30 and 40 days of storage.

Chemicals

Glacial acetic acid p.a. 99.8% (PENTA), chloroform p.a. (Lachner), potassium iodide p.a. (PENTA), sodium thiosulphate pentahydrate p.a. (Lachner), potassium dichromate, hydrochloric acid 35% (Lachin), starch soluble p.a. (Lachema), distilled water.

Acid value determination

The acid value determination was used to monitor the changes in the amount of free fatty acids during the Shaal test. The method is based on the neutralization of free fatty acids by ethanolic potassium hydroxide solution according to the **ISO 660:2009**. The acid value was measured at days 0; 20 and 40.

Chemicals

Diethylether p.a. (PENTA), ethanol 99.8%, potassium hydroxide p.a. (Lachema), phenolftalein (Lachin), oxalic acid dihydrate p.a. (Lachema).

Determination of fatty acids profile by gas chromatography

Gas chromatography was used to determine the fatty acid representation in fresh oils and oils stored for 40 days at 60 °C and to determine the changes of the fatty acid saturation during the oils rancidification. The base esterification method using 0.5M methanolic potassium hydroxide (KOH) was used for fatty acid derivatisation. Methyl esters of the fatty acids were then analysed using gas chromatography-mass spectrometry Agilent 7890A GC coupled to Agilent 5975C single-quadrupole mass detector equipped with an Rt-2560 column (100 m × 0.25 mm ID, 0.25 μm film, Restek Corporation, Bellefonte, USA). Hexane was used as the solvent and 1 μL of the sample was injected in the split mode (ratio 50:1) into the injector, which was heated to 225 °C. Starting at 70 °C for 2 min, the oven temperature was increased at a rate of 5 °C.min^{-1} to 225 °C where it was kept constant for 9 min, and then subsequently increased at a rate of 5 °C.min^{-1} to a maximum of 240 °C where it was maintained for 25 min. Helium was used as the carrier gas at a flow rate of 1.2 mL.min^{-1}. The MS analysis was carried out in full scan mode with a mass range of 40 – 400 m.z^{-1}, and the electron ionization energy was set at 70 eV. The methylated fatty acids were identified using a Restek Food Industry FAME mix (cat. No. 35077) and by comparing their mass spectra with those reported in the National Institute of Standards and Technology Library (NIST, USA). The proportions of the fatty acids were calculated using the area normalisation method and expressed as relative percentage of all fatty acids.

Chemicals and equipment

Methanol (Scharlau), potassium hydroxide p.a. (Lachema), n-heptane (MERCK), sodium chloride p.a. (PENTA), sodium sulphate anhydrous pure (Lachema).

Statistical analysis

The data were processed using Microsoft Excel 2007 (Microsoft Corporation, Seoul, Korea) and statistically evaluated using the Statistica 13.2 software (StatSoft, Inc., Tulsa, OK, USA) using a correlation matrix with a significance level of $\alpha = 0.05$ and a tree-clustering analysis.

RESULTS AND DISCUSSION

The difference in weight increase of individual oils during the storage could be seen in Figure 1, the peroxide and acid values changes are in Figure 2 and Figure 3. Representation of SFA, MUFA and PUFA in oils at the beginning and at the end of the storage are in Figures 4 and Figure 5. Relative contents of the main fatty acids at the beginning (day 0) and at the end of experiment (day 40) are given in Table 1. The results of tree-clustering analysis are seen in Figure 6.

Figure 1 shows that according to the relative weight increase the tested oils could be devided into three groups: very oxilable unstable oils – grapeseed and sunflower oils, medium stable oils – frying, rapeseed, corn and rice oils, and very stable oils – olive pomace and sunflower-HO oils. Peroxide number values (Figure 2) again clearly separated very stable oils from the others. Figure 3 shows that though rice and olive oils had higher acid value before the storage test these oils were not subject to such great hydrolytic changes as sunflower and grapseed oils.

The results of the Schaal tests showed the lowest increase in weight gain in olive oil and sunflower oil with increased oleic acid content. On the contrary, the highest increase was in traditional sunflower oil and grape seed oil. **Foster, Williamson and Lunn (2009)** explained the low oxidative stability of sunflower oil by high levels of linoleic acid and low γ-tocopherol content. Low γ-tocopherol content may also contribute to the low stability of grape seed oil as well as its high linoleic acid representation. The increased stability of sunflower-HO oil is due to its high content of oleic acid and the low content of polyunsaturated fatty acids **(Smith, King and Min, 2007)**. The high oleic acid content supported by the presence of a number of non-saponificable components is responsible for the high stability of olive oil **(Gunstone, 2005; Firestone, 2005)**. Olive oil, compared to conventional sunflower oil, also shows good stability under oxidation at 180 °C for 60 minutes, which **Silva et al. (2010)** attributed to the high content of phenolic compounds.

The results obtained by monitoring the weight changes of oils correlated with their peroxide values ($R_2 = 0.83$). It is seen from Figure 2 that sunflower and grape seed oils showed a peroxide value decrease after reaching its maximum at about 30 days, i.e., hydroperoxides (primary oxidation products) have already begun to convert to the secondary oxidation products. Corn and frying oils seemd to reach their peroxide value maxima while the rapeseed and rice oils still exhibited the increase in the peroxide number at the day 40. On the other hand, the increase in the peroxide value of olive oil and high oleic sunflower oil was very small and at the end of the measurement it was far from the propagation stage of the radical chain autooxidation reaction.

It was possible to determine the induction period (IP) in case of grapeseed and sunflower oils, which was 27 (grapeseed oil) and 28 (sunflower oil) days. The IP for frying and rapeseed oils could be estimated around 35 days. The remaining four oils did not show the clear beginning of fast radical chain reaction and had IP over 40 days.

The acid values at the end of experiment correspond to both the relative weight gain ($R_2 = 0.97$) and the the peroxide values ($R_2 = 0.90$). Oils exhibited faster oxidative decomposition have achieved significantly higher acid value (indicating the hydrolytic changes extension) than stable oils at the end of the measurement. Sunflower-HO and olive oils did not show a significant increase in the acid value. On the other hand, the increase between the 20th and the 40th day was significant for other oils. Although the low content of free fatty acids is one of the factors influencing the stability of oils, it is clear from the high initial acidity value of olive oil that this is not the only one determining factor.

Regarding to fatty acid composition (Table 1), the largest decrease during the storage was in PUFA. On the contrary, oleic and palmitic acids relative contents increased. For olive oil, which showed high stability in all previous tests, the change in composition was minimal compared to other oils. It has been confirmed that oils containing less than 10% of PUFA are significantly more stable than those containing more double bonds **(Velíšek, 2014)**.

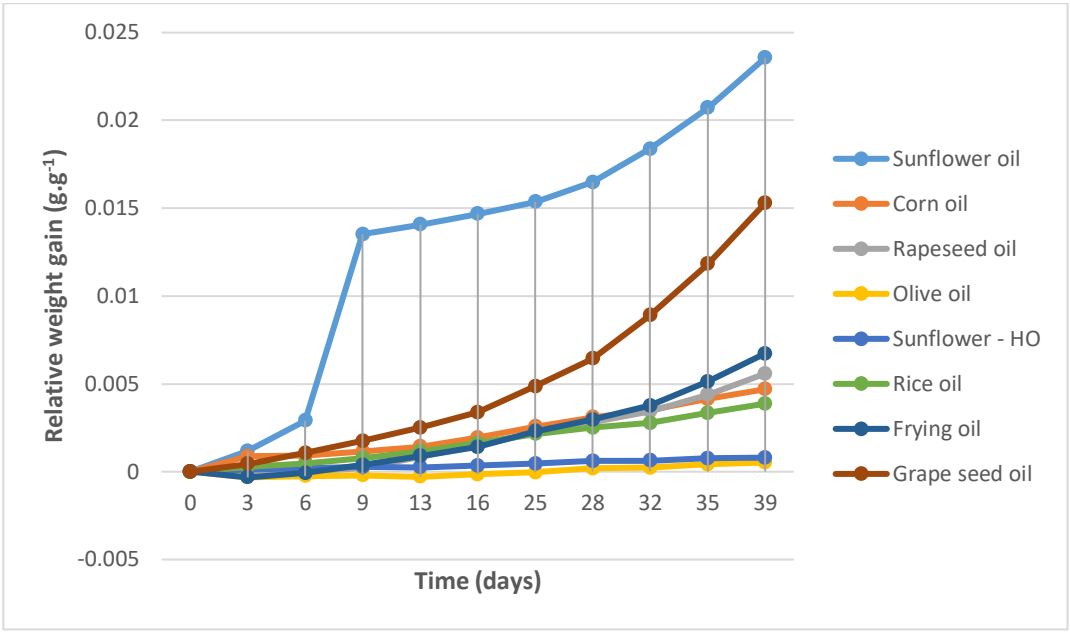

Figure 1 The relative weight changes of individual oils during the Schaal test.

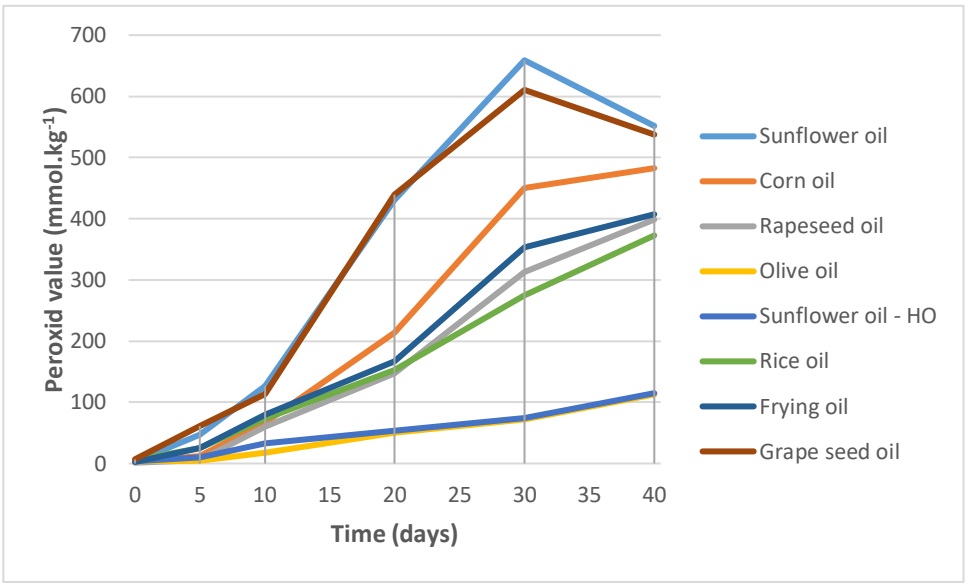

Figure 2 The peroxide value changes of individual oils during the Schaal test.

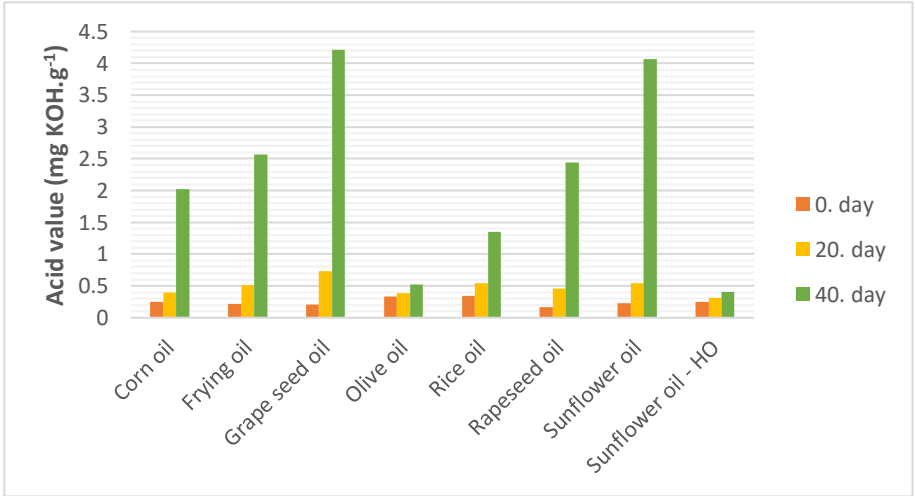

Figure 3 The acid value changes of individual oils during the Schaal test.

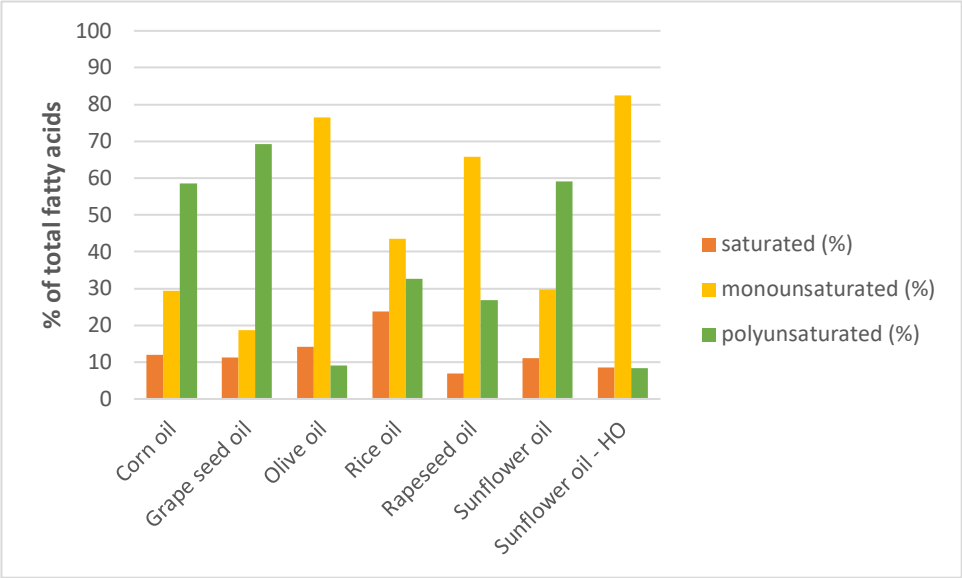

Figure 4 Representation of fatty acids – day 0.

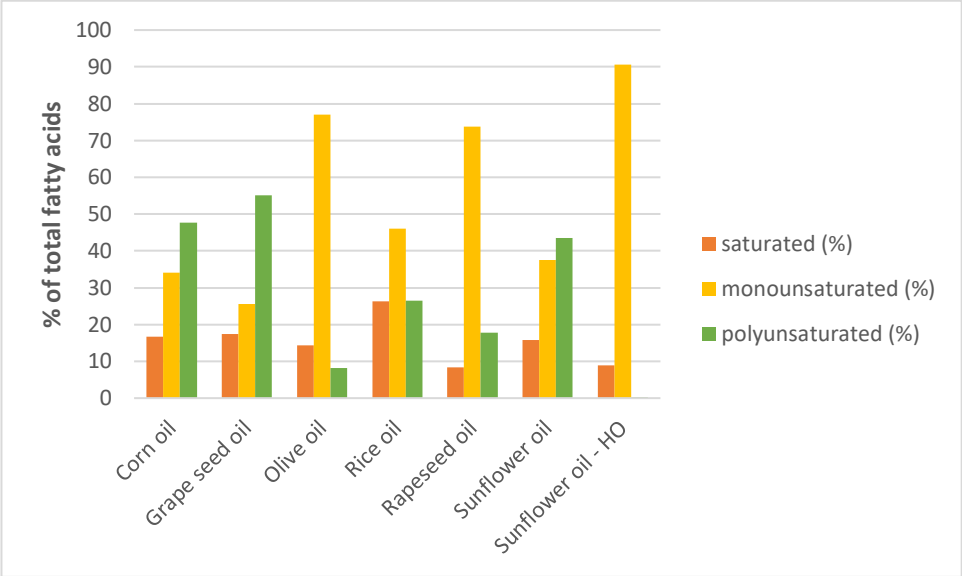

Figure 5 Representation of fatty acids – day 40.

Table 1 Changes of the main fatty acids in oils during the Schaal test.

Oil	Fatty acid content (% of all FA)				
	C16:0 day 0/day 40	C18:0 day 0/day 40	C18:1 cis-9 day 0/day 40	C18:2 cis 9.12 day 0/day 40	C18:3 cis-9.12.15 day 0/day 40
Corn	9.3/13.7	1.8/2.2	22.9/33.6	57.2/47.2	0.7/0.4
Grapeseed	6.8/10.3	4.3/6.4	18.4/25.3	69.1/54.8	0.1/<0.1
Olive	10.1/10.3	3.1/3.1	75.5/76.0	8.4/7.6	0.6/0.5
Rice	19.3/21.9	2.2/2.3	42.6/45.2	31.5/25.8	1.1/0.7
Rapeseed	4.3/5.3	1.6/1.9	63.4/71.5	18.6/13.8	7.8/3.8
Sunflower	5.7/8.8	3.8/5.0	29.5/37.2	58.4/42.9	0.6/0.3
Sunflower-HO	3.8/4.3	2.8/2.9	82.6/90.6	8.1/0.3	0.3/<0.1

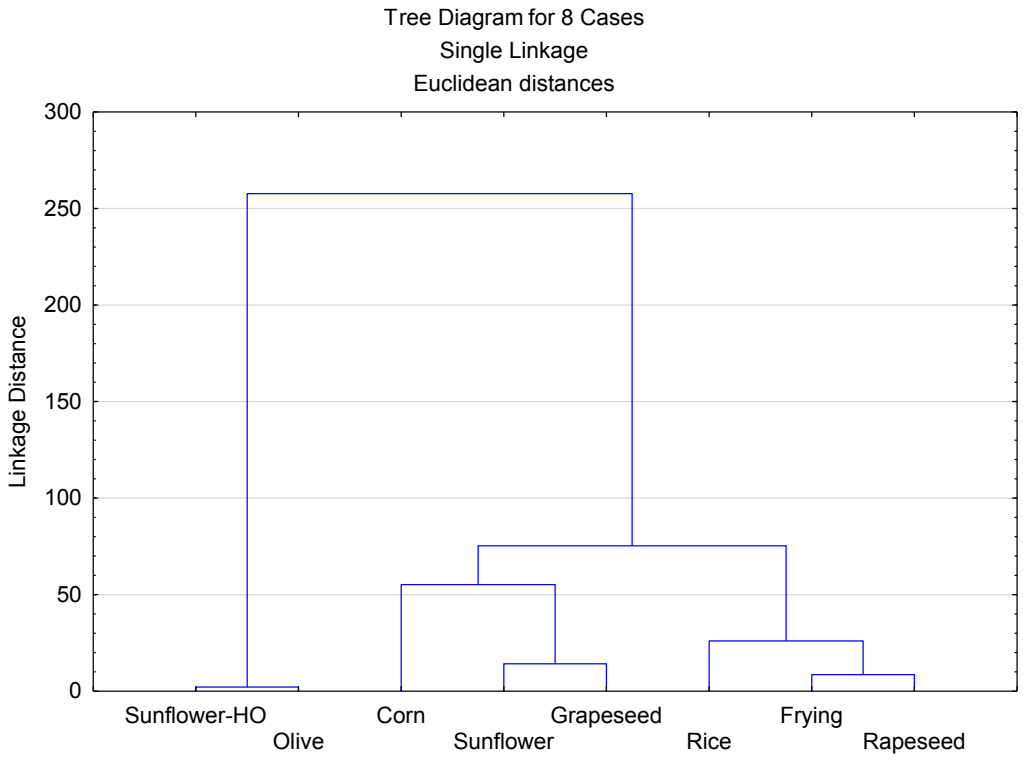

Figure 6 Cluster analysis results of oils - day 40.

A strong negative correlation (R_2 = -0.93) between oleic acid content and oil stability expressed as the peroxide value was found. The significant positive (R_2 = 0.93) correlation was found in case of linoleic acid. This confirms the assumption that the oil unsaturation decreases its stability.

Considering the weight gain, peroxide and acid values at the end of experiment alltogether in cluster analysis (Figure 6) it can be clearly seen that oils with dominating oleic acid (olive and sunflover-HO) and low degree of polyunsaturation are very distant from all other samples.

CONCLUSION

The decrease in polyunsaturated acids and the increase in saturated and monounsaturated acids were observed in different kind of oils during their storage at 60 °C. There was a strong negative correlation between oleic acid content and stability expressed by the peroxide value. As well as a strong positive correlation between linoleic acid content. The stability of vegetable oils therefore decreases with increasing amounts of polyunsaturated fatty acids. The initial free fatty acid content does not significantly affect the stability of the oil. In case of the least stable oils (sunflower oil and grapeseed oil), the hydroperoxides began to change to some secondary oxidation products during the monitoring period.

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SUBSTANTIATION OF REGIME PARAMETERS OF VIBRATING CONVEYOR INFRARED DRYERS

Igor Palamarchuk, Mikhailo Mushtruk, Volodymyr Vasyliv, Marija Zheplinska

ABSTRACT

The analysis of constructive-technological schemes of vibrating conveyor machines for heat-exchange processing of bulk technological masses in the current mode is carried out, which allowed to substantiate the effectiveness of using a new modification of infrared dryers of vibration-wave execution with a flexible transporting body. For this scheme, a vibrating system is developed which mathematical modelling allowed to determine and substantiate the main parameters of the operating mode of the drive mechanism of the projected device. The experiments, using the developed research model, confirm and refine the results of theoretical analysis, energy efficiency and comparatively low metal consumption of the design structure. The difficulty of working with such a large number of factors led to the application of the second similarity theorem and the introduction of a mathematical model of the criteria of Stanton, Froude, Burdo, whose magnitudes are reflected through the main factors of influence and were found experimentally. After using the "dimension theory" and graph-analytic analysis of power functions, a criterial equation of the investigated process was obtained. This allows recommending the regime parameters and the design series of projected thermo-radiation dryers with vibration-wave transport of products when varying the main factors of influence.

Keywords: vibration; wave conveyor; loose products; system unbalance; infrared drying

INTRODUCTION

The infrared irradiation method is one of the most promising physical methods for processing food products. By using advantages over traditional methods of heat treatment, it is increasingly being used in various branches of the food industry and restaurant industry. Infrared radiation treatment is used for blanching, roasting and drying of fruit and vegetable raw materials, pasteurization of milk, juices, wines and beer, heat treatment of meat products, preparation of grilled products from various types of food raw materials **(Krasnikov et al., 1985; Kolyanovska et al., 2019)**. At the same time, this research raises the task of ensuring uniformity of layer-by-layer processing of bulk products in conditions of such intensive thermal action as infrared, as well as the organization of the current form of agricultural raw materials processing. In order to solve the set problems, an original design solution for the application of a wave conveyor is proposed, and the parameters of the non-vibrating nature of the vibrating system designed to provide the necessary trajectory of the flow of bulk materials with the required speed **(Bubelová et al., 2017; Zverev and Sesikashvili, 2018)**.

Energy is used 2 – 3 times in heat-exchange processes of processing and food technologies of agro-industrial production more than it is physically necessary for a process that determines the energy intensity of production and the quality of products. The drying processes of agricultural products are among the most energy intensive. When using these processes, the share of energy in the cost of production is up to 30%. Therefore, there is a problem of fundamentally new approaches in processing and food technologies, the creation of new energy-saving and intensive technologies, the development of principles and equipment that realize the advantages of combined and targeted supply of infrared and vibro-mechanical energy, pulse and mechanical processing methods **(Krasnikov et al., 1985; Kolyanovska et al., 2019; Czako et al., 2018)**.

In the article it is proposed to realize the process of sufficiently intensive infrared drying in the conditions of the vibrated layer of loose products for continuous processing regimes. The process of transportation of raw materials in the working area is planned to be performed by a vibration-wave method by changing the non-vibrant nature of the vibrating system without the use of additional mechanical devices. At the same time, the use of a deformable transporting body with a vibrowave action will provide significantly less energy consumption than the most common infrared vibrating convection dryers, including Russian ones such as (SWIC), which currently dominate the market of infrared dryers for processing loose agricultural raw materials and are marked by a rather massive and correspondingly metal-intensive vibrational

surface **(Palamarchuk et al., 2015; Sukhenko et al., 2017)**, requires considerable energy consumption for its reduction to motion. In the projected drier, vibration to the mass of production is indirectly transmitted through the deformable surface of a special belt, which serves as a wave conveyor in the drying device. Such approach, together with a significant intensification of the process, with a lower energy consumption (7 – 8 times less as in the SWIC device) and material sorption (the metal consumption is less by 9 – 10 times as in the SWIC devices) will significantly improve the quality indices of products, in particular uniformity of the layered processing and maximum preservation of raw materials' initial qualities. The relevance and uniqueness of such results for agro-industrial production lies in the effective maintenance of the level of consumer value of products in the stages of storage and primary processing of raw materials **(Krasnikov et al., 1985; Kolyanovska et al., 2019; Sukhenko et al., 2020)**.

Scientific hypothesis

Research results have been published on the creation and justification of the operating modes of the newest energy-efficient and material-intensive drying equipment. The aim of this scientific work is creation and justification of the operating modes of the newest energy-efficient and material-intensive drying equipment that realize the advantages of combined and targeted energy supply on the basis of a combination of vibro-mechanical and infrared technological action and the current vibrowave organization of transportation of raw materials in the work area.

MATERIAL AND METHODOLOGY

Theoretical research uses modern concepts of physical and mathematical modelling of granular media, the theory of similarity, the theory of vibrational processing of freely granulated masses, the theory of vibrational and wave transport of loose masses, the methods of Lagrange and Cauchy in the mathematical analysis of the equations of motion of the machine's executive organs. MATHCAD and STATGRAPHICS CENTURION XVII software is used to process the data.

During the implementation of the experimental studies, the German Robotron equipment, modern complexes for estimating the kinematic, power and energy characteristics of the vibrowave transport of the rapeseed and soybean masses are used.

Statistic analysis

The statistical evaluation of the results was carried out by standard methods using statistical software Statgraphics Centurion XVII (StatPoint, USA) – multifactor analysis of variance (MANOVA), LSD test.

RESULTS AND DISCUSSION

At the present stage of development of drying equipment and technologies, vibrational and electrophysical methods of processing raw materials are widely introduced, among which the heat treatment with infrared heating is important **(Krasnikov et al., 1985; Kolyanovska et al., 2019; Sukhenko et al., 2020)**. Its use makes it possible to significantly intensify the drying process, reduce the specific energy consumption and processing time, provide rational temperature regimes, improve product quality by reducing mass losses and the rate of undesirable physical and chemical changes, and yields significant economic benefits. In addition, the use of vibration in the process of drying by infrared radiation provides an intensive mixing of the raw material particles among themselves, a constant renewal of the product layer that is in the range of the infrared emitter.

Considering the large number of conducted studies of the application of infrared radiation in various technological processes, let's concentrate on analysing the equipment of combined processes, which include infrared heating and the vibrational effect on the product.

According to the complex of structural and technological classification characteristics in infrared dryers, the following main groups can be noted: chamber, conveyor, vibrating and combined. In modern processing agricultural production, it is the latter that are becoming increasingly popular due to their high productivity and technological flexibility. Depending on the features of the transporting body, conveyor vibratory dryers contain deformable or non-deformable surfaces. The use of conveyor dryers with deformable working surfaces can significantly reduce the energy consumption and metal capacity of the structure that predetermines the choice of such scheme for further research **(Rachmat et al., 2010)**.

When developing the design scheme of the dryer with the given characteristics, vibrating conveyor units are first designed, the characteristic features of which are a perforated conveyor belt covering the system of support rollers (Figure 1 – 3); centrally built-in mechanical vibration exciter; a system of elastic elements that provide the necessary restoring action in vibration, in particular the elements 5 (Figure 2), 13 (Figure 3) or reduce dynamic loads on the supporting rollers (elements 17 in Figures 2 and 12 in Figure 3) **(Krasnikov et al., 1985; Palamarchuk et al., 2013)**. These vibrating conveyor machines have the following features: the installation shown in Figure 1 allows us to divide the working zone into two streams; the water remover in Figure 2 contains a blade mechanism for intensifying the processing process; the unit diagram in Figure 3 allows multi-stage processing of products. However, these mechanical solutions unite mechanical complexity for the realization of the assigned technological tasks, in particular for the fouling of loose agricultural raw materials. Installation of a mechanical vibration exciter in the support rollers of the vibrating conveyor system allowed to level the shortcomings and open a promising direction for the design of vibration-wave dryers that fit organically into the schemes of infrared drying of bulk products in the current processing mode.

Figure 4 shows the developed unit that combines these positive features and contains a flexible load-carrying body on which a traveling or standing wave is created when a mechanical exciter is operating 2, 3 **(Nowak and Lewicki, 2004; Palamarchuk, Turcan and Palamarchuk, 2015; Palamarchuk Bandura and Palamarchuk, 2013)**.

Such wave promotes both the transportation of products coming from the feeder 7 and its intensive mixing. This

reduces the thermal intensity to the surface layer while maintaining a sufficiently high flow velocity. In this case, it is sufficient to ensure that only rollers 5, 6 vibrate to maintain the high kinetics of the investigated process, which significantly reduces energy consumption per drive compared to vibrating conveyor systems.

In order to select effective operating conditions, an analysis of the dynamics of the vibration excitation power circuit of the investigated drier is made. At the same time, the following tasks are solved: compilation of the design scheme of the machine with a mechanical unbalance vibration exciter; compilation of equations of motion of mobile links of vibration drives with approximate and numerical methods of analysis; investigation of amplitude-frequency characteristics; determination of drive power **(Palamarchuk Bandura and Palamarchuk, 2013)**.

The following mass distribution takes place for the investigated vibration drier (Figure 5).

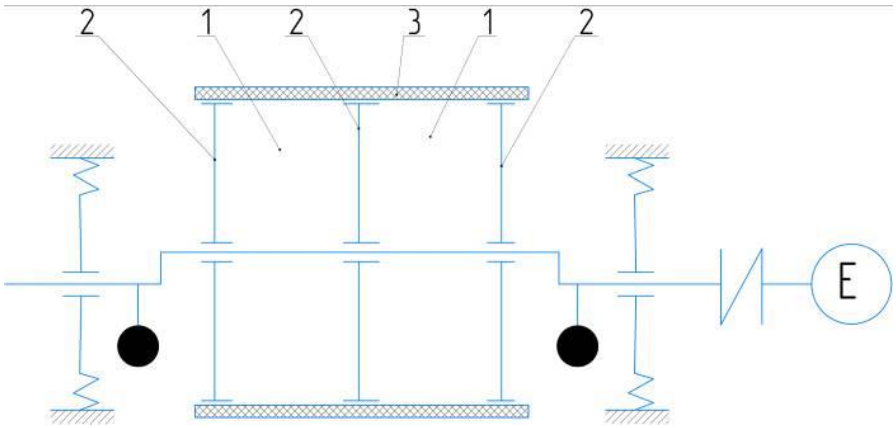

Figure 1 Multi-chamber vibrating conveyor machine with mechanical combined vibration excitation. Note: 1 – working chambers; 2 – side discs; 3 – flexible belt.

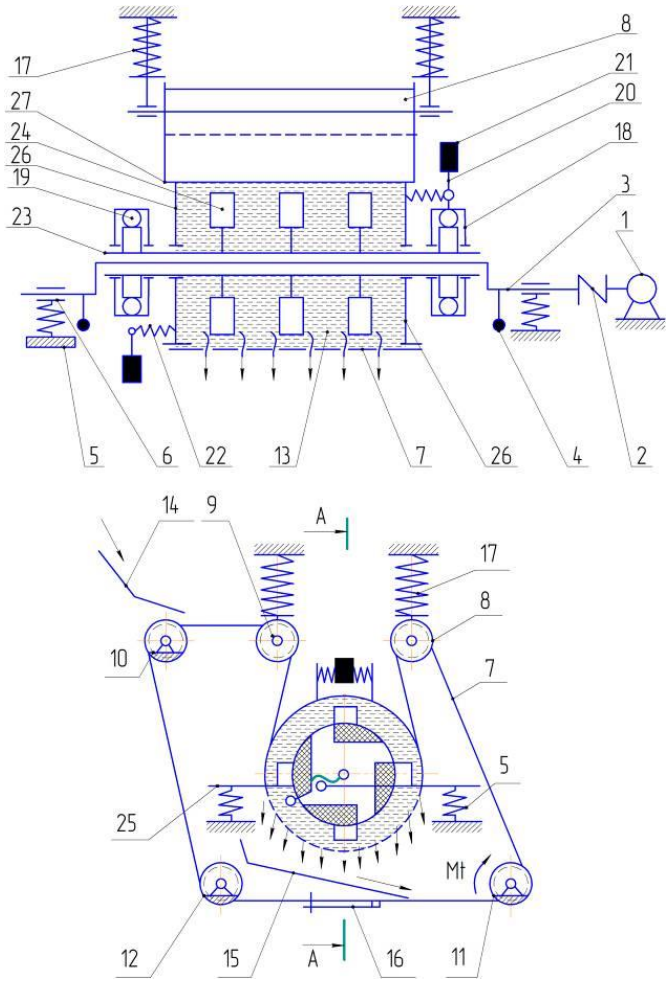

Figure 2 Conveyor vibrating vane machine. Note: 1 – engine, 2 – elastic coupling, 3 – drive shaft of vibration exciter, 4 – counterweight, 5 – elastic elements of vibration exciter; 6, 19 – support nodes, 7 – belt; 8, 9, 10, 11, 12 – support rollers, 13 – loose raw materials; 14, 15 – trays, 16 – door for additional charging, 17 – elastic suspension elements, 18 – freewheel mechanism body, 20 – rod, 21 – pendulum, 22 – elastic elements, 24 – blade, 26 – side disk, 27 – flange.

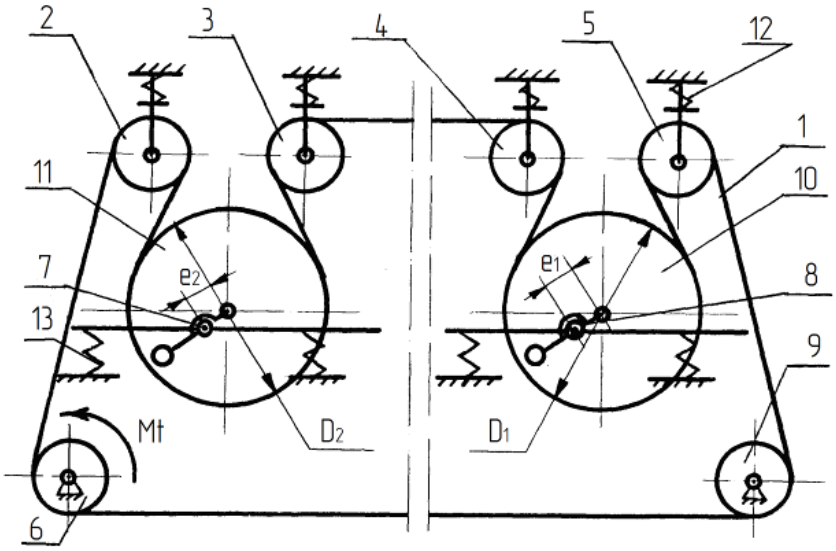

Figure 3 Vibrating conveyor machine for multi-stage processing of products with mechanical combined vibration excitation. Note: 1 – flexible belt; 2, 3, 4, 5, 9 – supporting rollers; 6 – drive roller; 7, 8 – drive shafts; 10, 11 – side discs of the working container; 12 – elastic suspension; 13 – elastic elements of a power shaft.

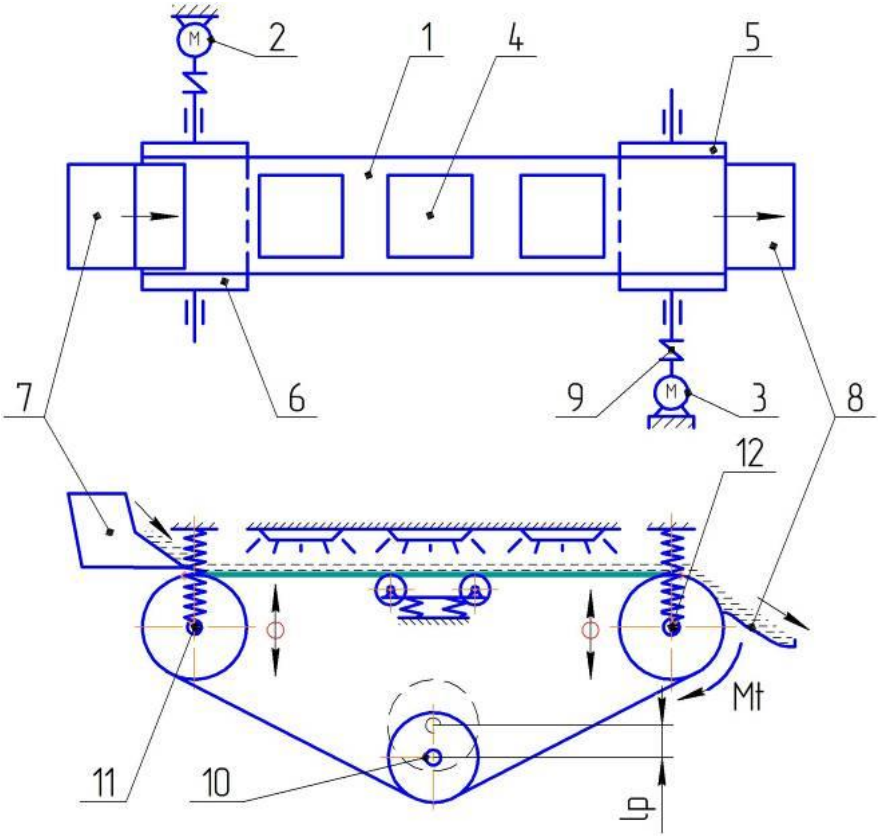

Figure 4 Scheme of the conveyor vibrowave infrared dryer: 1 – belt; 2, 3 – exciter engines; 4 – infrared emitters; 5, 6 – rollers; 7 – feeder; 8 – receiving hopper; 9 – flexible coupling; 10 – tensioning roller; 11, 12 – unbalance vibration exciter.

In order to select effective operating conditions, an analysis of the dynamics of the vibration excitation power circuit of the investigated drier is made. At the same time, the following tasks are solved: compilation of the design scheme of the machine with a mechanical unbalance vibration exciter; compilation of equations of motion of mobile links of vibration drives with approximate and numerical methods of analysis; investigation of amplitude-frequency characteristics; determination of drive power **(Palamarchuk Bandura and Palamarchuk, 2013; Krasnikov et al., 1985; Kolyanovska et al., 2019)**.

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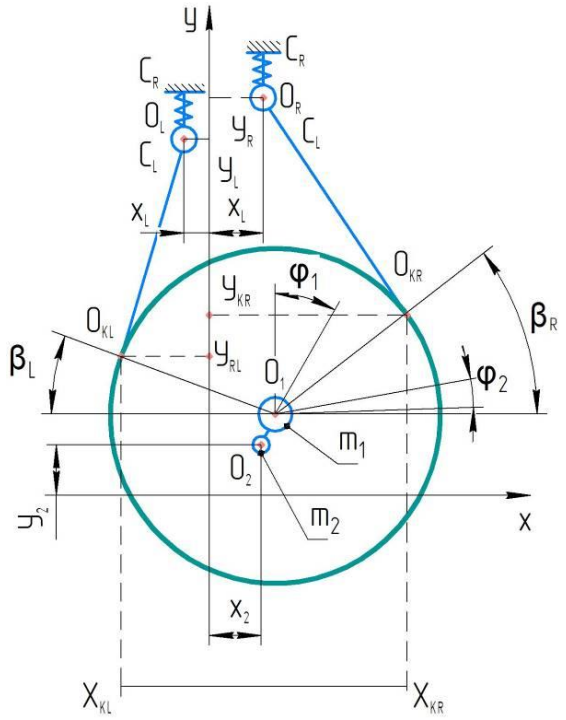

Figure 5 Calculation scheme for vibrating conveyor drier drive with a deformable transporting body.

$$m_1 = m_{unb};\ m_2 = m_{d.b} + m_s + m_{d.s} + m_{t.c};\ m_0 = m_1 + m_2 . \quad (1)$$

where, m_s – masses of the supporting units of the drive shaft; $m_{d.b}$ – mass of side discs and bushings; $m_{d.s}$ – mass of the drive shaft; m_{unb}- mass of unbalances; $m_{t.c}$- mass of technological charging (filler).

The mass distribution in the system is:

$$m = m_1 + m_2;\ m_1 = m_{unb};\ m_2 = m_{d.s} + m_s. \quad (2)$$

The degrees of freedom of the system include: x_1 - horizontal displacement of the drive shaft; y_1 – vertical movement of a drive shaft; ϕ_1 – angular displacement of the drive shaft; ϕ_2 – angular displacement of the side disk of the support roller.

Analysing the geometry of the system, let's found dependencies for the forces of the belt tension.

$$T_L = C_0\sqrt{(X_L - X_2)^2 + (Y_L - Y_2)^2 - R^2} - \sqrt{X_L^2 + Y_L^2 - R^2} + R\varphi_2 \quad (3)$$

$$T_R = C_0\sqrt{(X_L - l_{12}\sin\varphi_1)^2 + (Y_L - l_{12}\sin\varphi_1)^2 - R^2} - \\ - \sqrt{X_L^2 + Y_L^2 - R^2} + R\varphi_2 \quad (4)$$

Using Lagrange's method, let's compose the equations of motion of the working bodies of vibration drives.

$$\ddot{X}_2 + \frac{c_X}{m_0}X_2 = \frac{m_1 l_{12}}{m_0}\left[\ddot{\varphi}_1\cos\varphi_1 - \dot{\varphi}_1^2\sin\varphi_1\right] + \\ + \frac{1}{m_0}\left[T_L\sin B_L - T_R\cos B_R\right] \quad (5)$$

$$\ddot{Y}_2 + \frac{c_Y}{m_0}Y_2 = \frac{m_1 l_{12}}{m_0}\left[\ddot{\varphi}_1\sin\varphi_1 - \dot{\varphi}_1^2\cos\varphi_1\right] + \\ + \frac{1}{m_0}\left[m_0 q - T_L\sin B_L - T_R\cos B_R\right] \quad (6)$$

$$\ddot{\varphi}_2 - \left[T_R - T_L\right]R/J_2 \quad (7)$$

$$\ddot{\varphi}_1 + \frac{\ddot{X}_2}{l_{12}}\cos\varphi_1 + \frac{\ddot{Y}_2 + m_1 q}{l_{12}}\sin\varphi_1 + \frac{M_t}{m_1 l_{12}} \quad (8)$$

When solving these equations, let's use the following assumptions:

$$\beta_L = \beta_R = \text{const};\quad Y_L = Y_R;\quad |Y_L| = |Y_R|;\quad \dot{\varphi}_1 = \text{const}.$$

Using the assumptions and the Cauchy method for solving linear inhomogeneous differential equations with constant coefficients, the equation of the trajectory of the working bodies of the vibration drives and their graphical dependences is obtained (Figure 6).

$$X_2 = \frac{A_1[\omega_1^2 - k_X^2]}{[\omega_1^2 - k_X^2] + \varphi_X^2\omega_1^2}\sin\omega_1 t + \frac{A_1\varphi_X^2\omega_1^2}{[\omega_1^2 - k_X^2] + \varphi_X^2\omega_1^2}\cos\omega_1 t \quad (9)$$

$$Y_2 = -\frac{A_1[\omega_1^2 - k_Y^2]}{[\omega_1^2 - k_Y^2] + \varphi_Y^2\omega_1^2}\cos\omega_1 t + \frac{A_1\varphi_Y^2\omega_1^2}{[\omega_1^2 - k_Y^2] + \varphi_Y^2\omega_1^2}\sin\omega_1 t \quad (10)$$

Using the obtained equations, the dependencies for the main parameters of the investigated unbalance vibration exciter are formulated.

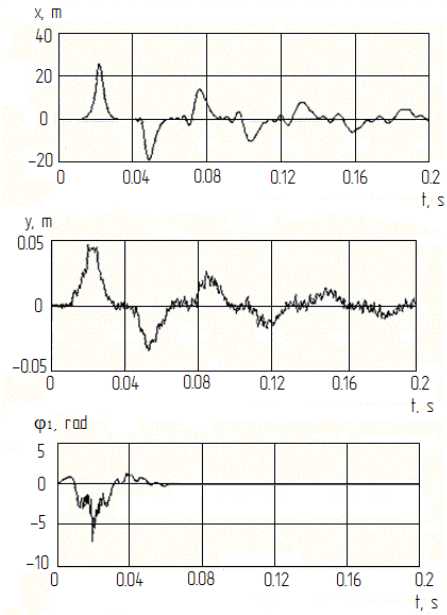

Figure 6 Motion trajectories of the working bodies of dynamic imbalance vibration drive of the flat vibrations of a machine with flexible guide: X, Y – linear movements of the working container; φ – angular displacement of the drive shaft; ω – angular displacement of the platform.

Amplitude-frequency and power characteristics of the machine with a flexible container and dynamic drive are presented as follows.

Absolute vibration amplitude of vibration exciter:

$$A = m_0^{-1} m_1 l_{12} \omega_1^2 \sqrt{\left[\left(\omega_1^2 - k_X^2\right)^2 + \alpha_X^2 \omega_1^2\right]^{-1} + \left[\left(\omega_1^2 - k_Y^2\right)^2 + \alpha_Y^2 \omega_1^2\right]^{-1}} \quad (11)$$

Machine drive power:

$$N_{dr} = m_1 l_{12} \omega_1^2 \eta_{np}^{-1} \left(0{,}5 d_u \mu + m_1 l_{12} \omega_1^3\right) \cdot \cdot m_0^{-1} \sqrt{\left[\frac{\left(\omega_1^2 - k_X^2\right)\cos\omega_1 t - \alpha_X \omega_1 \sin\omega_1 t}{\left[\left(\omega_1^2 - k_X^2\right)^2 + \alpha_X^2 \omega_1^2\right]}\right]^2 + \left[\frac{\left(\omega_1^2 - k_Y^2\right)\omega_1 \sin\omega_1 t - \alpha_Y \omega_1 \cos\omega_1 t}{\left[\left(\omega_1^2 - k_Y^2\right)^2 + \alpha_Y^2 \omega_1^2\right]}\right]^2} \quad (12)$$

Load on the support units of the drive shaft:

$$F_{unb} = m_1 l_{12} \omega_1^4 \sqrt{\left[\frac{\left(\omega_1^2 - k_X^2\right)\cos\omega_1 t - \alpha_X \omega_1 \sin\omega_1 t}{\left[\left(\omega_1^2 - k_X^2\right)^2 + \alpha_X^2 \omega_1^2\right]}\right]^2 + \left[\frac{\left(\omega_1^2 - k_Y^2\right)\omega_1 \sin\omega_1 t - \alpha_Y \omega_1 \cos\omega_1 t}{\left[\left(\omega_1^2 - k_Y^2\right)^2 + \alpha_Y^2 \omega_1^2\right]}\right]^2} \quad (13)$$

Energy of motion of vibrating masses of vibration drive:

$$E_{vib} = 0{,}5 m_0^{-1} m_1 l_{12} \omega_1^6 \left(\left[\frac{\left(\omega_1^2 - k_X^2\right)\cos\omega_1 t - \alpha_X \omega_1 \sin\omega_1 t}{\left[\left(\omega_1^2 - k_X^2\right)^2 + \alpha_X^2 \omega_1^2\right]}\right]^2 + \left[\frac{\left(\omega_1^2 - k_Y^2\right)\sin\omega_1 t - \alpha_Y \omega_1 \cos\omega_1 t}{\left[\left(\omega_1^2 - k_Y^2\right)^2 + \alpha_Y^2 \omega_1^2\right]}\right]^2\right) \quad (14)$$

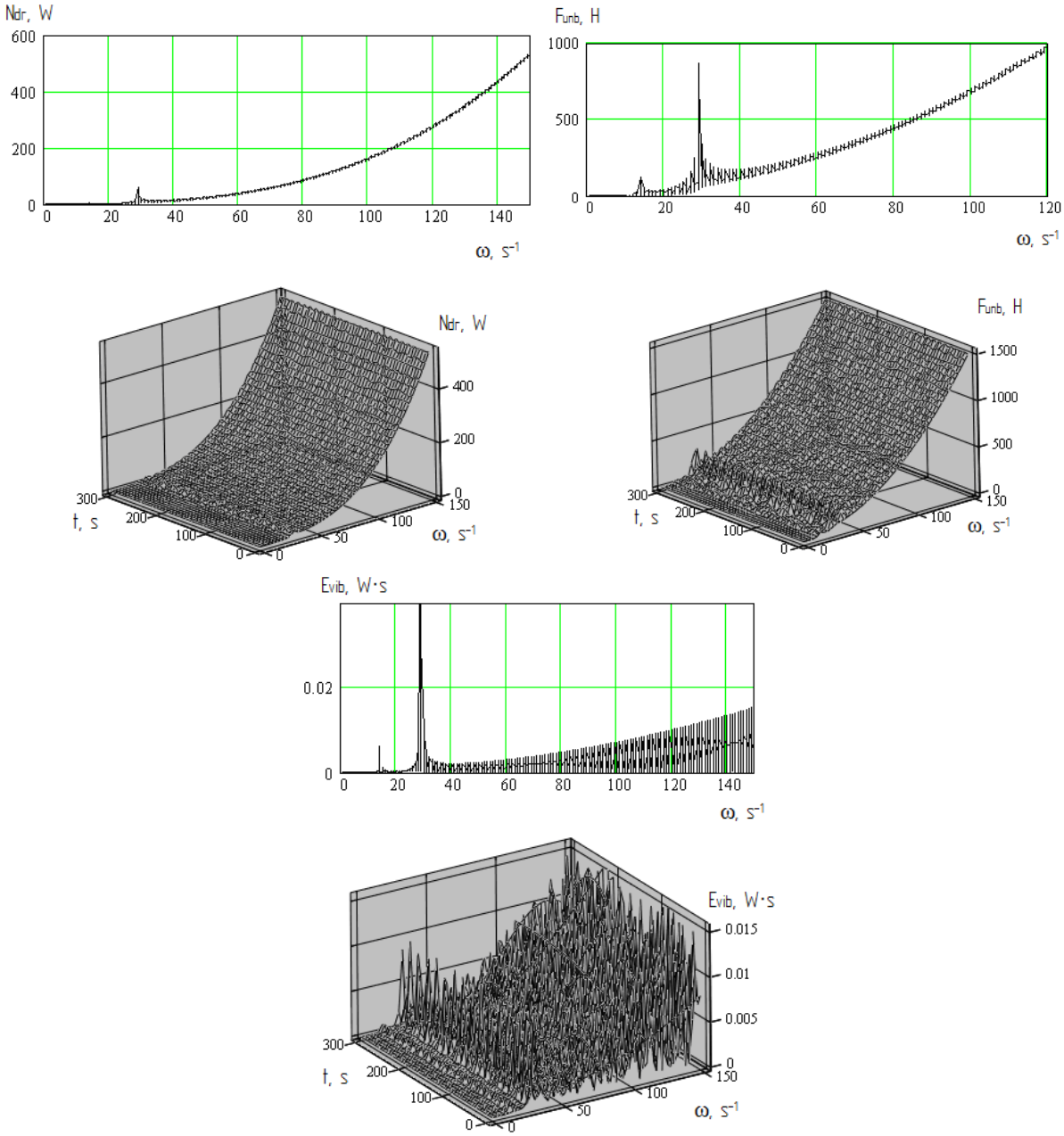

Figure 7 Selection of operating modes of vibration drive operation: A – amplitude of vibrations of the working container; N_{dr} – drive power; F_{unb} – unbalanced forces, loading the support nodes; E_{vib} – the energy of vibrational masses.

Thus, during the study of the dynamics of the conveyor vibration machine, kinematic, power and energy characteristics are obtained: the dynamic unbalanced vibration drive (Figure 7), which allows rate of the effective modes of operation of the vibration unit, in order to ensure the most intensive mode of transmission of the inertial pulse at moderate energy consumption.

The resulting inertial mode should provide the desired wave motion to the belt and is determined by the amplitude-frequency and power characteristics of both vibration exciters that are aggregated in the support rollers of the conveyor vibration machine. The modes are: 2 – 2.5 mm of the vibration amplitude, the angular velocity of the drive shaft of the vibration exciter within 60 – 90 rad/s **(Zavialov et al., 2015; Yanovich et al., 2015)**.

To confirm and refine the theoretical data, experimental studies are carried out using the developed research model of the infrared vibrowave dryer (Figure 8).

Figure 8 The developed design of a vibrowave conveyor infrared dryer. Note: 1 – belt; 2, 3 – exciter engines; 4 – infrared emitters; 5,6 - rollers; 7 – feeder; 8 - receiving hopper; 9 – flexible coupling; 10 – tensioning roller.

During the analysis of the main kinematic and energy characteristics of the vibrating system, an estimation of these parameters is performed at different angles of the location of the unbalances relative to each one. This allows changing a given angle from 0 to π, vary the value of the inertia force of the unbalance elements from the maximum value to zero. The change in such positions of the unbalances makes it possible to obtain the graphical dependences of the basic vibration parameters shown in Figure 9 – 10. The change in the positions of the unbalance elements relative to the vertical axis of the unit makes it possible to obtain variants of the power, moment and combined unbalance of the investigated vibration system.

The developed vibrating conveyor dryer with infrared irradiation of products (Figure 8) is a combination of a belt conveyor and a vibrating transport-technological machine with a dynamic vibration generation method, creating conditions for continuous processing of products, ensuring its suspended state, reducing energy consumption and the metal content of the structure **(Bandura et al., 2015; Palamarchuk et al., 2016)**.

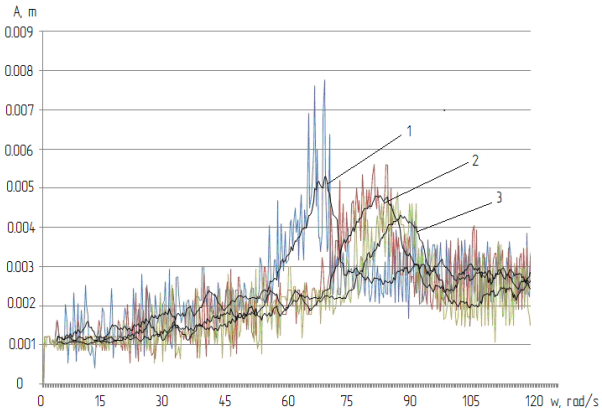

Figure 9 Amplitude-frequency characteristic of the investigated unit, depending on the angular speed of rotation of the drive shaft and the angle of dilution of the unbalances. Note: 1 – at 0 degrees; 2 – at 45 degrees; 3 – at 90 degrees.

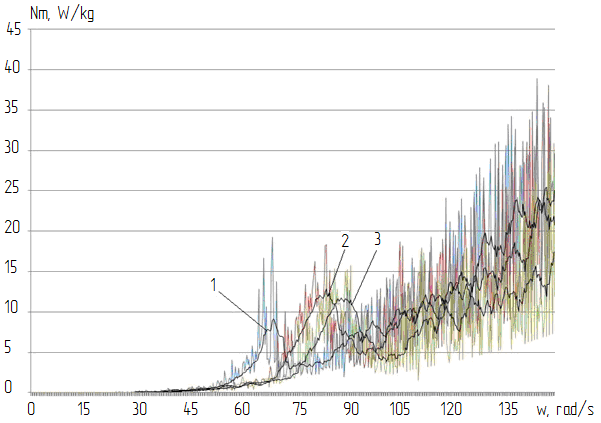

Figure 10 The intensity of the vibrations of the investigated unit as a function of the angular velocity of rotation of the drive shaft and the angle of dilution of the unbalances. Note: 1 – at 0 degrees; 2 – at 45 degrees; 3 – at 90 degrees.

CONCLUSION

1. Based on the analysis, the main trends in the development of conveyor technology machines are formed, the study of the structural and technological schemes of which allows justifying the wave conveyor machine for drying loose agricultural products. Such design makes it possible to significantly increase the surface heat and mass transfer and create conditions for reducing energy consumption during the processing.

2. Graphical representation of the amplitude-strength and energy dependencies allows substantiating the theoretical range of operating modes of the investigated vibration transport-technological machine: 2 – 2.5 mm of the vibration amplitude, angular velocity of the drive shaft of the vibration exciter within 60 – 90 rad/s.

3. The design of the vibrowave infrared drier is developed in the presented scheme. The previous tests confirm the results of the above studies and found the prospects for significant energy savings in this modification of the machine compared to conventional vibrating conveying drying units having a non-deforming transporting body.

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THE INFLUENCE OF COOKING ON THE ANTIOXIDANT PROPERTIES AND POLYPHENOL CONTENT IN BUCKWHEAT, BARLEY AND MILLET GROATS AND THE TRANSFER OF THE COMPOUNDS TO THE WATER

Barbara Krochmal-Marczak, Barbara Sawicka

ABSTRACT

The research subject was the influence of the cooking process on the polyphenol content and antioxidant properties of groats (buckwheat groats, barley groats, millet groats) and on the colour parameters of the products. After groats cooking, also the water was tested for the polyphenol content and antioxidant properties that permeated into the decoction of the cooked raw material. The evaluation of the antioxidant properties of groats was conducted with the DPPH radical assay, the polyphenol content was determined with the Folin-Ciocalteu reagent, and the colour was determined with the trichromatic colorimetry method using the Konica Minolta CM-5 colorimeter (Osaka, Japan). The cooking process significantly lowered the content of polyphenolic compounds and antioxidant properties of the ready products. The best antioxidant properties after cooking were found in buckwheat groats and the weakest in millet groats. The polyphenol content in cooked products depended on the type of groats. Cooking significantly decreased the polyphenol content, but only in buckwheat and barley groats, as well as causing a change of groats colour as compared to uncooked samples. The most antioxidant properties were found in the water from cooking barley groats, and the least – from cooking millet groats. The most polyphenols permeated into the water from cooking buckwheat groats, and the least from cooking millet groats. All groats, except millet groats, darkened after cooking. In all types of groats, the correlation coefficients between colour parameter and general polyphenol content and the ability to scavenge DPPH radicals showed very strong negative dependences. The conducted research can help in designing the technological process of cooking buckwheat, barley and millet groats, and a way of using the water from cooked groats for consumption.

Keywords: buckwheat groats; barley groats; millet groats; antioxidant propertie; cooking; polyphenol

INTRODUCTION

Due to their health properties, barley, buckwheat and millet groats are a vital element of human diet **(Subramanian and Viswanathan, 2007; Sedej et al., 2010; Paszkiewicz et al., 2012; Liu et al., 2012; Danihelová and Šturdík, 2013; Hęś et al., 2014; Wronkowska, Honke and Piskula, 2015; Mateo-Gallego et al., 2017; Kerienė et al., 2015; Hung, 2016; Skotnicka Ocieczek and Małgorzewicz, 2018; Kuznetsova et al., 2018)**. They contain a number of valuable nutrients, such as: high-value protein, fats, mineral salts, and vitamins **(Ragaee, Abdel-Aal and Noaman, 2006; Singh, Mishra and Mishra, 2012; Aoe et al., 2017; Mateo-Gallego et al., 2017)**. Groats have a variety of applications in human nutrition due to the availability of different preparation techniques. They can be consumed with meats, fish, vegetables, as well as sweet desserts, warm or cold. They are recommended in human nutrition, especially to persons suffering from acute gastroenteritis, convalescents with strict diet plans, and children **(Liu et al., 2012; Subramanian and Viswanathan, 2007; Ragaee, Abdel-Aal and Noaman, 2006; Kalinova and Moudry, 2006; Skotnicka Ocieczek and Małgorzewicz, 2018)**. According to **Górecka et al. (2009)** and **Huth et al. (2000)**, the fibre contained in these products is capable of binding water and bile acids, absorbing metals, encourages bowel movement, lowers glucose and cholesterol levels in blood, increases fecal weight. Groats are also the source of bioactive ingredients, including polyphenols. Such compounds include: polyphenols (phenolic acids and a large group of flavonoids with anthocyans), vitamins A and C, tocopherols, carotenoids, as well as organic acids, macro- and micro-elements, including selenium, chlorophyllins, glutathione, indoles, phytates and thiocyanates. Antioxidant properties are also found in the products of transformations in technological processes **(Singh, Mishra and Mishra, 2012; Skotnicka Ocieczek**

and Małgorzewicz, 2018). According to **Hollman, Geelen and Kromhout (2010); Karamać (2010)** such compounds can improve health, facilitating mainly the treatment and prevention of cardiovascular diseases. According to **Marchand (2002); Mateo-Gallego et al. (2017)** and **Skotnicka Ocieczek and Małgorzewicz, (2018)** polyphenols also play an important role in preventing cancer and neurodegenerative diseases, and the content of those compounds in food improves the antioxidant potential of food, which is an additional protection of the organism against the activity of an excess of free radicals accumulated due to oxidative stress. However, when prepared for consumption, groats vary significantly in terms of the content of these ingredients. One of the ways of preparing groats for consumption is cooking. It is generally believed that cooking can have a positive effect on the polyphenol content and antioxidant activity, but some authors have a different opinion and argue that during the cooking process, some of the ingredients transfer to the stock, decreasing the nutritional value of groats, whereas the water from cooked groats is usually disposed of into the drain **(Ismail, Marjan and Foong, 2004; Jeong et al., 2004; Jiratanan and Liu, 2004; Puupponnen-Pimia et al., 2003; Sharma, Gujral and Singh, 2012; Hęś et al., 2014; Kerienė et al., 2015)**. Cooking also causes a change of colour, which is often a quality that determines consumer desirability of the product. Considering the aforementioned factors affecting the quality of cooked groats, research was conducted in order to determine the influence of the cooking process on the polyphenol content and antioxidant properties of groats, as well as their colour parameters. Moreover, the polyphenol content and antioxidant properties were tested in the water from cooked groats.

Scientific hypothesis

The aim of this study was to ascertain that the cooking process reduces the polyphenol content and antioxidant properties in buckwheat, barley and millet groats, and that it results in a colour change of cooked groats. Furthermore, the aim of the study was to determine the amount of polyphenols and antioxidant properties that transfer to the water when cooking buckwheat, barley and millet groats, and whether there is any correlation between the colour of the groats and the general polyphenol content and antioxidant properties.

MATERIAL AND METHODOLOGY

Materials

The research material included three types of groats: barley, buckwheat and millet purchased in 2017 at one of the supermarket chains in Krosno, Poland (49°49′ N, 21°46′ E).

Preparation of sample extracts

Groats samples were ground in a grinder, then 1 g of each sample was extracted in 10 mL of 70% ethanol for 15 minutes on the Model Sonic 6 (Polsonic, Poland) ultrasonic bath. Following the extraction, the samples were filtrated, and the obtained filtrate was used to determine the polyphenol content and antioxidant properties of groats **(Sánchez-Rangel et al., 2013; Brand-Williams, Cuvelier and Berset, 1995)**. The water from cooking was tested for antioxidant properties and polyphenol content **(Kerienė et al., 2015; Hęś et al., 2014)**.

DPPH radical scavenging assay

The antioxidant activity of the extracts was determined with the DPPH radical assay **(AOAC International, 2009)**, and the results were expressed in the percentage of DPPH radical scavenging. For this purpose, the extracts were diluted to 1:1, and then, a DPPH ethanolic solution was prepared with a concentration of 0.05 $mg.mL^{-1}$, whose absorbance prior to analysis was 1.5. Then, 500 µL of the diluted sample was combined with 500 µL of the DPPH in three replicates. The samples were vortexed and left in a dark place for 30 minutes at room temperature. The control sample contained 500 µL of 70% ethanol and 500 µL of the DPPH solution. After that time, the absorbance of the samples was measured in relation to ethanol at a wave length of 515 nm. The results were expressed as the percentage of antioxidant activity.

$$\%AA = ((Ak - Ap)/Ak))*100\%$$

Where: Ak – absorbance of the control sample, Ap – absorbance of the tested samples.

The antioxidant activity of the tested groats was determined with the use of the Jenway 6850 UV/VIS spectrophotometer (Jenway, England).

Determination of total polyphenol content

The total polyphenol content in the groats extracts was determined with the use of the Folin–Ciocalteu reagent **(Aanchez-Rangel et al., 2013)**. For this purpose, calibration solution of gallic acid in 70% ethanol was prepared with specific concentrations (0.45 – 0.01 $mg.mL^{-1}$) in order to determine the calibration curve. Then, 20 µL of each solution of the standard of gallic acid and the tested extract were sampled in three replicates, 1.58 mL of distilled water and then 100 µL of the Folin-Denis reagent were added to each of them. The samples were mixed and after 1 minute, 300 µL of a 20% sodium carbonate solution was added to each; the samples were vortexed and placed in a thermostat set at 40 °C for 30 minutes. Afterwards, the absorbance of the extracts and standards was measured at a wave length of 765 nm against the reference sample (instead of 20 µL of the extract/standard, 20 µL of 70% ethanol was added). The polyphenol content in the extracts was determined on the basis of the calibration curve for gallic acid [mg $GAE.mL^{-1}$]: $y = 1.0425x + 0.1277$, $R^2 = 0.888$. Analyses were carried out in three replicates.

Polyphenol determination in a water sample after cooking

Groats were cooked according to the manufacturer's instructions on the packagings. The polyphenol content in the water from the cooked groats (buckwheat, barley, millet) was determined with the spectrophotometric method with the use of the Folin–Ciocalteu reagent, by adding to 100 µL of the water extract from cooked groats: 7.9 mL of distilled water, 0.5 mL of the Folin–Ciocalteu reagent, and 1.5 mL of 20% sodium carbonate. The samples were thoroughly mixed and incubated at room

Table 1 Polyphenol content in different types of groats (mg.100g^{-1} DM of groats).

Groat type	Type of groat treatment		Mean
	Raw	Cooked	
Buckwheat	127.04	59.58	93.31
Millet	0.37	0.01	0.19
Barley	109.80	26.98	68.39
Mean	79.07	28.86	53.96
$HSD_{0.05}$	Kind of groats (R) – 3.29; type of groats processing (F) – 2.19; interaction R x F – 6.58		

Table 2 Polyphenol content in the water after cooking different groat types (mg·100g^{-1} DM of groats).

Groat type	Type of groat treatment		Mean
	Raw	Water	
Buckwheat	127.04 ±15.28	28.93 ±0.42	77.99
Millet	0.37 ±0.56	1.75 ±0.64	1.06
Barley	109.80 ±7.04	4.49 ±0.26	57.15
Mean	79.07	11.73	45.40
$HSD_{0.05}$	Kind of groats (K) – 2.50; groats form (F) – 1.66; interaction K x F – 4.98		

temperature for 1 hour, then their absorbance was measured with the use of the UV/VIS spectrophotometer at a wave length of 760 nm. The total polyphenol content in the tested samples was calculated as mg of the equivalent of gallic acid in 100 mL of water from cooked groats based on the calibration curve for gallic acid, with the following formula: y = 1.1799x + 0.0847, where R^2 = 0.9996. The concentration range of gallic acid for the purpose of drawing the calibration curve was from 0.001 to 0.5 mg.mL^{-1} **(Kerienė et al., 2015)**.

Determination of antioxidant properties in a water sample after cooking

The ability of water extracts from cooked groats to scavenge free radicals was determined with the spectrophotometric method using a stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). For this purpose, the tested extracts were diluted as follows: 0.1 of the water extracts from cooked groats and 2 mL of ethanol, then 0.25 mL of DPPH ethanolic solution with a concentration of 1 mM was added to them, and they were incubated in darkness at room temperature for 30 minutes. After that time, the absorbance of the samples was measured for ethanol at a wave length of 517 nm and % AA was calculated in relation to the absorbance of the control sample, which contained ethanol instead of water extract **(Hęś et al., 2014)**. The antioxidant activity of the tested groats was determined with the use of the Jenway 6850 UV/VIS spectrophotometer.

%AA = ((A0 – Ap)/ A0)*100%
A0 – absorbance of the control sample
Ap – absorbance of the tested samples

Colour determination

The colour of the samples of raw and cooked groats was determined with the trichromatic colorimetry method using the Konica Minolta CM-5 colorimeter (Osaka, Japan). The colour was expressed in the CIE L*a*b* system. Positive a* parameter stands for the amount of red, and negative for green. Positive b* parameter stands for the amount of yellow, and negative for blue. Parameter L* has a value from 0 (black) to 100 (white) **(CIE, 2007)**.

Statistic analysis

All analyses were carried out in three replicates and the results were subject to the analysis of variance (ANOVA) using the Statistica package, ver. 10. The significance of differences between the averages was calculated in Tukey's test at a level of significance p <0.05. Further analysis involved Pearson linear correlation coefficient and multiple regression (StatSoft, USA).

RESULTS AND DISCUSSION

The commodity-science-based evaluation of groats did not show any deviations from the relevant norms. All groats had a characteristic odour and a characteristic colour: brown in the case of roasted buckwheat groats, light grey with a greenish shade – of barley groats, and green-and-yellow – of millet groats. Moisture content in groats was in line with the norm and within a range from 8.9% (barley groats) to 10.40% (millet groats) **(PN–EN ISO 712:2012)**.

The polyphenol content depended on the type of groats and varied from 0.19 to 93.31 mg·100g^{-1} DM of groats (Table 2). Thermal processing (cooking) significantly lowered the polyphenol content, but only in buckwheat and barley groats (Table 1).

Groats demonstrated various antioxidant activity measured by the ABTS•+ free radical scavenging assay, ranging from 9.11% to 82.00% for raw groats, and from 5.22% to 67.55% for cooked groats. The type of groats significantly influenced the antioxidant properties of the product. The best antioxidant properties were found in buckwheat groats, and the lowest in millet groats. Barley groats had better antioxidant properties than millet groats, but significantly worse than buckwheat groats (Table 2).

The polyphenol content in the water from cooking varied from 1.75 to 28.93 mg.100g^{-1}.

Table 3 Antioxidant properties of groats, DPPH, expressed as (% unreduced radical).

Groat type	Type of groat treatment		Mean
	Raw	Cooked	
Buckwheat	82.00	67.55	74.78
Millet	9.11	5.22	7.17
Barley	48.84	13.63	31.24
Mean	46.65	28.80	37.73
$HSD_{0.05}$	Kind of groats (K) — 1.91; type of groats processing — 1.27, interaction (R x F) — 3.81		

Table 4 Antioxidant properties of groats, DPPH, expressed as (% unreduced radical).

Groat type	Type of groats treatment		Mean
	Raw	Water	
Buckwheat	82.00 ±0.79	55.99 ±2.00	68.99
Millet	9.11 ±2.24	17.09 ±0.26	13.10
Barley	48.84 ±1.64	57.21 ±1.67	53.02
Mean	46.65	43.43	45.04
$HSD_{0.05}$	Kind of groats (K) – 2.30; groats form (F) –1.53; interaction K x F – 4.46		

Note: Content of antioxidants in the water after cooking different forms of groat, DPPH, expressed as (% unreduced radical).

Table 5 Colour parameters of raw and cooked groats in the CIELab system.

Specification		L*	a*	b*
Groat type	Buckwheat	41.9	9.0	15.9
	Millet	71.6	2.9	26.9
	Barley	65.9	4.0	17.9
	$HSD_{0.05}$	3.9	0.3	1.3
Type of groats treatment	Raw	61.2	6.0	21.6
	Cooked	58.4	4.6	18.8
	$HSD_{0.05}$	2.6	0.2	0.9
	Mean	59.8	5.3	20.2

Note: L * – brightness (luminance), a * – green to red colour, b * – blue to yellow colour.

The most polyphenols permeated into the water from cooked buckwheat groats, and the least from cooked millet groats. The water from cooked barley groats had a higher polyphenol content than from cooked millet groats (Table 2).

Thermal processing resulted in decreased antioxidant activity of groats ready for consumption as compared to raw groats. The greatest decrease of antioxidant capability – of 3.5 times – in comparison to the raw product was observed in barley groats. Cooked millet groats showed a decrease in the antioxidant value as compared to the raw product. Cooked buckwheat groats demonstrated the least change in antioxidant properties – 1.2 times smaller than the value obtained before cooking (Table 3).

The type of groats significantly influenced the antioxidant properties of the water from cooking. The most antioxidant properties were found in the water from cooking barley groats, and the least from cooking millet groats (Table 4).

Cooking had a significant influence on the change of selected colour parameters of groats. The highest L* value, i.e. the lightest colour among raw groats was observed in millet groats, and the lowest in buckwheat groats. Barley groats were lighter than buckwheat groats, but significantly darker than millet groats. After cooking, the value of this parameter decreased significantly (Table 5).

In each case, cooking resulted in a change of the a* colour parameter towards green, as compared to raw groats samples. The highest a* value was found in buckwheat groats, and the lowest (from blue to yellow) in millet groats (Table 5).

After cooking, the L* parameter was slightly higher in millet groats, i.e. they went brighter, and in the case of buckwheat and barley groats, the value decreased by 1.9% and 14% respectively. A significant change, however, was only observed in barley groats as compared to the colour determined prior to cooking (Figure 1).

After cooking, in all of the examined groats, the a* parameter was lower as compared to raw groats. The significantly lowest value of his parameter was observed in millet groats (Figure 2).

The highest value of the b* parameter, i.e. the share of the red colour after cooking, was demonstrated by buckwheat groats. In the other groats, the b* parameter decreased towards blue (Figure 3).

In all types of groats, the Pearson's correlation coefficients between colour parameter and general polyphenol content and the ability to scavenge DPPH radicals showed very strong negative dependences. Only the a* parameter value was found to be significantly positively correlated with groats colour (Table 5).

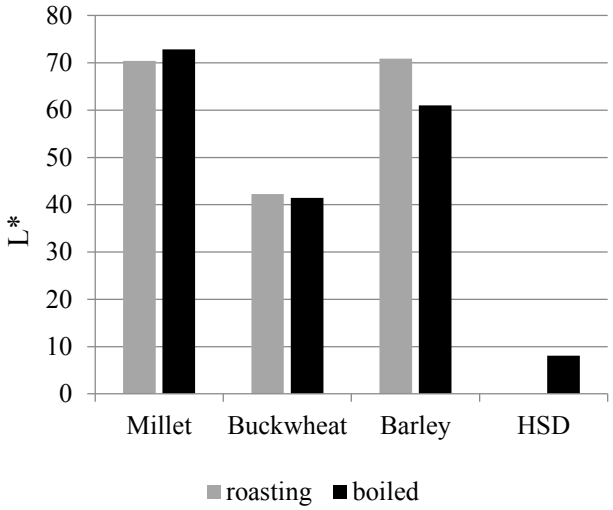


Figure 1 Impact of grout processing on buckwheat brightness (L*).

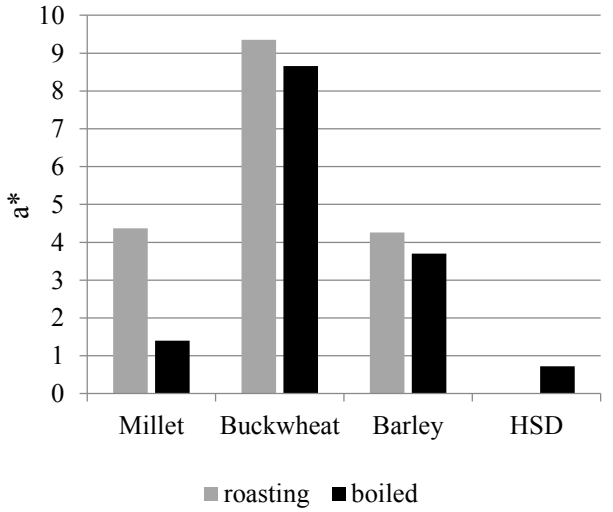
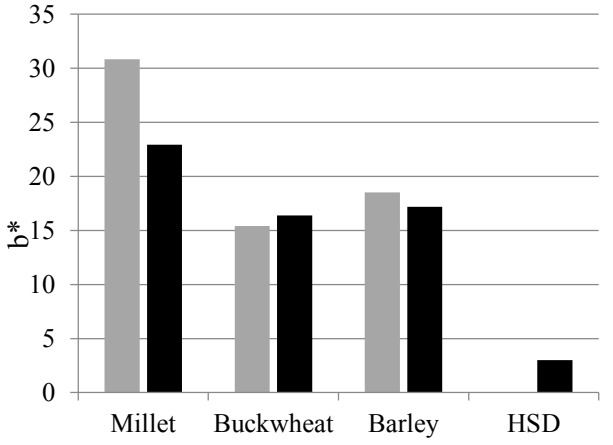


Figure 2 Impact of a* value – green to red colour .



The groats colour showed the strongest negative correlation with antioxidant properties, expressed as the capability to neutralise the free radical $ABTS^+$ (r = -0.79), and with the b* colour parameter (r = -0.78) (colour change in the range from blue to yellow) (Table 6).

The polyphenol content turned out to be positively, highly significantly correlated with the L* parameter value (r = 0.95), and the b* parameter value (r = 0.90), and with the antioxidant properties of groats (r = 0.88), which means that an increase in the polyphenol content coincides with an increase in the antioxidant properties and in the shade of the examined products (Table 6).

Polyphenols are compounds of plant origin belonging to the group of basic antioxidants. Along with other antioxidants (e.g. phytates, microelements and vit. E), they are the so-called natural non-nutritive substances, and their function is to scavenge free radicals initiating the oxidation process and the chelation of metal ions catalysing oxidation, reducing the activity of oxidative enzymes and antioxidant enzymes, and acting as reducing substances and compounds binding singlet oxygen **(Filipiak-Florkiewicz and Florkiewicz, 2016)**. The cooking process in the conducted research significantly lowered the content of polyphenolic compounds in grouts. The polyphenol content in cooked buckwheat groats decreased 2.1 times, and in the case of barley groats – over 4 times as compared to the raw product. Raw millet groats showed a minimum content of phenolic compounds, whereas after cooking, there were practically no such compounds at all. This might have resulted from the permeation of water-soluble polyphenols into the solution during cooking. The results of the research are concurrent with the research by **Majkowska, Klepacka and Rafałowski (2015)**, who believes that the content of polyphenolic compounds is the characteristic differentiating types of groats.

The groats cooking process had an influence on a significant decrease in the polyphenol content as compared to raw groats. According to **Gumula, Korus and Achremowicz (2005**), most compounds belonging to antioxidants show high lability and small resistance to environmental factors, that is why processing results in significant losses of natural antioxidants.

In the in-house research, the polyphenol content in cooked buckwheat groats decreased by approximately 53%, and in the case of barley groats – by 75% as compared to the raw product. In cooked millet groats, practically no polyphenolic compounds were detected. Similar results were obtained in the research by **Worobiej and Koleński, (2013)**, where the cooking process of buckwheat groats caused a decrease in the determined content of polyphenolic compounds by 53% and 27% respectively as compared to raw groats. According to **Worobiej et al. (2017)**, this may be a result of the permeation of water-soluble polyphenols into the solution during cooking, when the product has contact with water at a high temperature, which was confirmed in the in-house research. **Dietrych-Szóstak and Oleszek, (2001**) argue that as temperature and hydrothermal processing time increase, the content of bioactive compounds decreases, whereas **Zieliński et al. (2006**) showed that depending on the processing temperature , their content may change to a varying degree. The authors claim that the changes depend on the form of polyphenols in food products and the consequential thermal stability. In the in-house research, the greatest antioxidant capability of raw groats was found in buckwheat groats, and the smallest in millet groats. In the case of all groats types, the cooking process decreased the antioxidant activity of the product. The best

Table 6 Correlation coefficients between colours in the CIELab system, and the total polyphenol content and antioxidant properties of particular types of groats**.**

Variables	*Y*	x_1	x_2	x_3	x_4	x_5
Y	1.00					
x_1	-0.49**	1.00				
x_2	-0.79**	0.88**	1.00			
x_3	-0.36*	0.95**	0.84**	1.00		
x_4	0.38*	-0.94**	-0.85**	-1.00**	1.00	
x_5	-0.78*	0.90**	0.99**	0.86**	-0.87**	1.00

Note: ** significant at $p_{0.01}$, * significant at $p_{0.05}$. y – groats colour, x_1 – polyphenol content; x_2 – antioxidant properties; x_3 – L* parameter value; x_4 – a* parameter value; x_5 – b* parameter value.

antioxidant properties were found in buckwheat groats, both roasted and cooked. Similar results for buckwheat groats were obtained by **Górecka et al. (2009)**, where the ethanolic extract made up 80.8%, with a high capability of scavenging free radicals $DPPH^{+\bullet}$.

Thermal processing resulted in a decreased antioxidant activity of groats ready for consumption as compared to the raw products. The greatest decrease of antioxidant capability – by 72% – in comparison to the raw product was observed in barley groats. Buckwheat groats demonstrated the smallest change in antioxidant properties after cooking – a decrease of 19% as compared to the value obtained before cooking. According to **Şensoy et al. (2006)**, most compounds belonging to antioxidants show high lability and small resistance to environmental factors, that is why processing results in their significant losses. According to **Gumula, Korus and Achremowicz (2005)**, heating of plant products in water results in a relatively quick heat transfer into the tissues, which in turn causes a longer exposure to that factor of the entire volume of the heated product and high antioxidant losses. Other processing types which slightly increase or do not affect the antioxidant activity include: blanching, freezing or short thermal processing. **Stempińska et al. (2007)** conducted research using the cationic radical ABTS+ and found out that after thermal processing, buckwheat seeds had significantly lower antioxidant capability than unprocessed seeds. The research results show that cooking causes a decrease in the antioxidant capability of groats extracts obtained in 70% ethanol. **Şensoy et al. (2006)** concluded that the roasting of food products at 200 °C for 10 minutes decreases the antioxidant activity of buckwheat seeds. **Zieliński et al. (2006)** examined the antioxidant capability of buckwheat seeds prior to and after hydrothermal processing. They applied two methods of testing the capability of buffer and methanolic (80%) extracts to neutralise superoxide anion radicals, and both experiments showed a decreased antioxidant capability of the products. According to **Filipiak-Florkiewicz and Florkiewicz (2016)**, during heating in the air, the internal temperature of the product is lower than the surface temperature, which results in lower antioxidant losses, so it is recommended to steam plant products, which involves better heat transfer and shorter thermal processing time. In the in-house research, the greatest antioxidant capability of raw groats was fund in buckwheat groats, and the smallest in millet groats. In the case of all types of groats, the cooking process decreased their antioxidant activity. This was confirmed by **Filipiak-Florkiewicz and Florkiewicz (2016)**.

CIELab is presently the most popular way of describing colours and is the foundation of modern colour management systems. The difference between two colours in the CIELab colour space is a simple Euclidean distance between two points in a three-dimensional space. The conducted research showed that the brightest colour was characteristic for millet groats, and the darkest for buckwheat groats. Cooking of groats significantly differentiated their colours. The analysis of relationships between two determined features pointed to statistically significant correlations between groats colour and the total polyphenol content and the ability to scavenge radicals $DPPH^{+}$. However, in order to determine the exact influence of cooking on the polyphenol content, antioxidant properties and colour of selected types of groats, further research is required, considering various time combinations of thermal processing.

CONCLUSION

The cooking process significantly lowered the content of polyphenolic compounds and antioxidant properties of groats. The best antioxidant properties after cooking were found in buckwheat groats – 67.55 $mg.100g^{-1}$, and the weakest in millet groats – 5.22 $mg.100g^{-1}$. The most antioxidant properties were found in the water from cooking barley groats, and the least – from cooking millet groats. The most polyphenols permeated into the water from cooking buckwheat groats, and the least from cooking millet groats. The cooking process resulted in a colour change of groats as compared to raw products. After cooking, all groats, except millet groats, had a darker colour. It was shown that there are strong negative relations between the colour parameter and the total polyphenol content and the ability to scavenge radicals DPPH. Only the a* parameter value turned out to be significantly positively correlated with groats colour. Moreover, the polyphenol content was highly positively correlated with the value of the L* parameter and the b* parameter, as well as the antioxidant properties of groats, which proves that the antioxidant properties increase as the polyphenol content increases. The conducted research can help in designing the technological process of cooking buckwheat, barley and millet groats, and a way of using the water from cooked groats for consumption.

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IDENTIFICATION OF LARD ON GRILLED BEEF SAUSAGE PRODUCT AND STEAMED BEEF SAUSAGE PRODUCT USING FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY WITH CHEMOMETRIC COMBINATION

Any Guntarti, Mustofa Ahda, Neng Sunengsih

ABSTRACT
Many issues are spreading about the use of lard in food, one of it is sausage. Sausage is one of the processed foods which are prone of containing pork. In Indonesia, grilled and steamed sausages are popular for children and adults. One of the method which is developed to analyze fat in grilled and steamed sausage products was FTIR spectrophotometry combined with chemometrics. This research aims to develop an analysis method using FTIR spectrophotometry combined with chemometrics to analyze lard content in grilled and steamed beef sausage. Reference sausage made from a mixture of pork and beef. This research was designed by making each 7 concentrations variants of pork and steamed sausage samples (100%, 75%, 65%, 50%, 35%, 25% and 0%). Five samples from market were taken from various beef sausage traders. The fat extraction used n-hexane solvent at the temperature of 70 °C for 5 hours. The extracted fat was analyzed by FTIR spectrophotometry combined with chemometrics. The results of spectrum were analyzed using Horizon MB^{TM} to obtain optimum wave number of steamed sausages in the range of 1000 – 791 cm^{-1} and grilled sausages in the range of 1070 – 796 cm^{-1}. The analysis of steamed sausage with Partial Least Square (PLS) is obtained the equation y = 0.9977x + 0.1166; and the value of R2 0.9977; RMSEC 1.22%; RMSEP 0.22%; and RMSECV 1.26%. The PLS analysis of grilled sausage is obtained the equation y = 0.9972x + 0.1379; and the value of R2 0.9972; RMSEC 1.27%; RMSEP 0.42%; and RMSECV 0.18%. The conclusion of this research is that the FTIR method combined with chemometrics is the proper method for analyzing fat in sausages. The analysis result using *Principle Component Analysis* (PCA) was obtained from 3 of 5 samples in the market which had the similar physicochemical properties of lard sausage.

Keywords: Beef sausage; FTIR; PCA; PLS; pork sausage

INTRODUCTION
Consuming halal food both physically and spiritually is an obligation for every Muslim. Halal and thayyib food are rights for every Muslim consumer **(Regenstein, Chaudry and Regenstein, 2006)**. Nowadays, there are many issues spread about the use of lard in food, one of it is sausage **(Garcia, 2012)**.

The term sausage comes from the Latin word "salsus", which means salt, so sausages can be interpreted as ground meat which is preserved with salt. The grilled sausage is, first, steamed sausage which is, then, grilled and given spices to add its flavor. Generally, the food processing by heating causes oxidation reactions and will cause damaged fat that is contained in these foods. In the process of oxidation, most of the unsaturated fat acids will be damaged and the results of the damage can mostly be evaporated **(Che Man, Syahariza and Rohman, 2010)**. The fat in these food products can be damaged due to the process of steaming (dehydration) and burning (oxidation) **(El-Gindy, Emara and Mostafa, 2006)**. The pork, which the price is relatively cheaper than beef, is often used by the producers for substituting the beef ingredients in producing beef sausages because of its low price **(Guntarti et al., 2015)**.

According to the research result which is conducted by **Jaswir et al. (2003)**, it states that the FTIR method (fourier transform infrared) is potential to be used as a lard detector that is quick and has a consistent result. The FTIR method can give the analysis result of lard acids which mix with the other animal fats consistently, even the very low content **(Jaswir et al., 2003; Rohman and Che Man, 2012)**. In addition, in its development, the FTIR spectroscopic method has succeeded in analysing the difference in profiles and characteristics of animal fats (chicken, beef, and pork) **(Marikkar et al., 2005; Rohman and Che Man, 2010)**. Beside, the FTIR method is also combined with chemometrics for analysing the rat meat (*Rattus diardi*) in beef meatballs **(Guntarti and Prativi, 2017)**. Based on the description above, this research aims to develop the method of analysis using FTIR spectrophotometry which is combined with

chemometrics to analyze lard content in grilled and steamed beef sausage products **(Schieber, 2008)**.

Scientific hypothesis

FTIR Spectrophotometry method can be used to analyze pork in sausage quantitatively as well as for the classification of beef and lard in sausages.

MATERIAL AND METHODOLOGY

Ingredients

Beef, pork and spices which was obtained from Kranggan Market, Yogyakarta. The production sample was some brands of beef sausages that had been circulating in the market, both traditional and modern markets. N-hexane solvent (technical) (Merck), and Na2SO4 anhydrous.

Tools

Tools for sausage production, Soxhlet, ABB Analytical brand of FTIR spectrophotometer: MB3000 (Canada) with DTGS detector (deuterated triglicine sulfate).

The Research Progress

Making reference sausages: Sausages were made by mixing 90% of the ingredients in the blended meat (can be pork, beef or a mixture of pork and beef) with 10% seasoning in sausages production (Table 1).

Fat extraction of the reference sausages and market sausages: some grams of sample (±120 g) sausages in various concentrations (steamed and grilled sausages) and also sausages from the market were mashed using mortars, then in the Soxhlet with n-hexane solvent at the temperature of 70 °C for 5 hours **(Rahmania, Sudjadi and Rohman, 2015)**. The extraction result was added the Na_2SO_4 anhydrous. The fat obtained was weighed and calculated.

Statistic analysis

Then, the fat was analysed by FTIR spectroscopy with the ATR crystals at controlled temperature (20 °C). All spectra were recorded from 4000 – 400 cm^{-1}, it was recorded in the form of absorbance. The data from FTIR analysis were processed using PLS and PCA chemometrics programs with ABB Inc. Horizon MB™ QA software. Microsoft Excel 2007 was used for validation and calibration. The root mean square error of cross validation (RMSECV), root mean square error of prediction (RMSEP) and determination coefficient (R2) value were used as criteria for the calibration and validation model **(Zhao et al., 2014)**.

RESULTS AND DISCUSSION

The fat extraction is done by Soxhlet, n-hexane solvent for 5 hours in the maintained temperature (70 °C). The fat extract which obtained is, then, added with anhydrous Na_2SO_4 sufficiently to attract some water which may still be contained in the n-hexane **(Rowe, Sheskey and Quinn, 2009)**. Then fat extracts are analyzed with FTIR to identification of fungtional groups and analysing of fingerprint field that differentiate between steamed sausage and grilled sausage sample. The reading of IR spectra of extracted fat from steamed sausage and grilled sausage samples is done in the middle wave number area between 4000 – 650 cm^{-1}. The selection of the middle wave region is due to FTIR spectroscopy which will provide information about the types of functional groups in detail which is contained in pork derivatives **(Rohman and Che Man, 2010)**, and fingerprints in the wave numbers of 1500 – 600 cm^{-1} **(Stuart, 2004)**.

The spectra (Figure 1a) are spectra between steamed pork reference sausages of 100% and steamed beef reference sausages of 100%, and the spectra between grilled pork reference sausages of 100% and grilled beef reference sausages of 100% (Figure 1b) which are read by the Horizon MBTM application. In Figure 1 (a) and (b), it shows that between the fat spectra of steamed pork and beef sausages, also pork and beef sausages both have almost the same spectrum pattern due to the main components of the two fats' types are the same, which are triglycerides and both are animal fat **(Rohman and Che Man, 2011)**. The shift of the functional group spectrum of steamed and grilled sausages is presented in Table 2.

In Figure 2, Spectrum A, B, and C are fingerprint areas which become the difference between steamed sausages and grilled sausages. Spectrum A shows a spectral change in the extracted fat that occurs between steamed sausage and grilled sausage 1234 – 1233 cm^{-1} that the C-O Group in the ester has a stretch vibration. Spectrum B was the isolated trans-olefins bending vibration of -CH functional group which gives the m peak in the wave number 964 – 963 cm^{-1}. Spectrum C in the wave number 721 – 719 cm^{-1} is an overlapping vibrations of methylene shake (-CH2) and vibrations out of field by the cis- is substituted. The quantitative analysis in this research was done by Horizon MB™ chemometric application with 2 methods, which are Partial Least Square (PLS) and Principle Component Analysis (PCA). The optimal wave number for steamed sausage is 1000 – 791 cm^{-1} and the grilled sausage is 1076 – 796 cm^{-1}. In the research which was conducted by **Sari and Guntarti (2018)**, the wild boar fat wave number optimization of steamed sausages at 1250 – 900 cm^{-1}. There is a shift in the wave number because of the different types of animal fat (Figure 3).

Based on Figure 3, the R2 value is close to 1. Beside the R2, PLS modelling can use the RMSEC calibration (root mean square of error calibration), RMSEP (root mean square error of prediction), and RMSECV (root mean square error of cross validation), which is a precision parameter that is used to evaluate errors in the calibration model. Table 3 presents the PLS calibration and validation parameters.

When the R2 value is close to 1 the more linear the relation between the actual value (x axis) and the predicted value (y axis). The low RMSEC, RMSEP, and RMSECV values indicate the errors that occur in the analysis are lower or smaller, so the method is more valid **(Zhao et al., 2014)**. Steamed and grilled sausages are also given the same treatment and the same modeling that is done as the original sausage, because the modeling which is done on the original sausages is successfully separated lard and cow fat in the different quadrants. The number of samples that is analyzed consists of 5 products. Next, it is done the analysis by the PCA chemometrics method for grouping for the aim of finding out the existence of pork in steamed

and roasted sausage samples. The sample grouping is done at the wave number area of 1000 – 791 cm^{-1} (Figure 4). The PCA analysis of steamed production sausage samples and grilled production sausages shows the separation or grouping of lard and beef fat in the two different quadrants, which are quadrant A and quadrant B (Figure 5).

In Figure 4, the pork and beef quadrants is far apart, it means the grouping lard and beef fat in steamed sausages (a) and grilled sausages (b) works well. Then, the products on the market (A, B, C, D and E) are taken. In Figure 5, the products on the market C, D, E both on the steamed and grilled sausages are located in the quadrant of lard sausage. The products on the market are steamed and grilled sausages of A, B located in the quadrant of beef fat. It means that the products of steamed and grilled sausage C, D, E have the same physicochemical properties as pork sausage, while the products of steamed and grilled sausage of A, B have the same physicochemical properties as beef sausage. Steamed sausage which will be grilled, based on PCA chemometrics grouping, gives the same results. To ensure the content of pork, it is needed a more sensitive and specific method.

Table 1 The Formula of reference grilled and steamed beef sausages in various concentration.

Concentration (%)	Pork (g)	Beef (g)	Spices (g)
Beef 100	-	225.00	25.00
Pork 100	225.00	-	25.00
(B-S) 25	56.25	168.75	25.00
(B-S) 35	78.75	146.25	25.00
(B-S)50	112.50	112.50	25.00
(B-S) 65	146.25	78.75	25.00
(B-S) 75	168.75	56.25	25.00

Table 2 The Shift of the Functional Group and Vibration Model of Lard and Beef Fat in the Steamed and Grilled Sausage.

Wave Number of Steamed Sausage (cm^{-1})	Wave Number of Grilled Sausage (cm^{-1})	Functional Group Vibration	Intensity	Desc
3003	3010	Stretched *Cis* C=CH	Weak	Shift to right
2951	2956	Methylene group of asymmetrical stretching vibration (-CH3)	Average	Shift to right
2920 and 2850	2918 and 2852	Vibration Asymmetrical and symmetrical stretch of methylene group (-CH2)	Strong	Shift to left, Shift to right
1744	1745	The carbonyl (C = O) function group of triacylglycerol ester bonds	Strong	Shift to right
1655	1653	*Cis* C=C	Weak	Shift to left
1465	1463	Bending vibration of the aliphatic CH2 group	Strong	Shift to left
1374	1377	Symmetrical bend vibration of the CH3 group	Average	Shift to right
1234 and 1156	1233 and 1156	Vibration stretch of the C-O group in ester	Strong	Shift to left, No shifting
1113 and 1096	1112 and 1095	Bended -CH vibration and change -CH form of fat acid	Weak	Shift to left
964	963	The isolated *trans*-olefins bending vibration of –CH functional group	Weak	Shift to left
719	721	Overlapping vibrations of methylene shaking (-CH2) and vibrations out of the cis- dis-substituted field	Strong	Shift to right

able 3 The PLS calibration result on steamed and grilled sausage.

Parameters Value	Steamed Sausage	Grilled Sausage
Linear Similarity	y = 0.9977x + 0.1166	y = 0.9972x + 0.1379
R^2	0.99776	0.99772
RMSEC	0.12	1.27
RMSEP	0.22	0.42
RMSECV	1.26	0.18

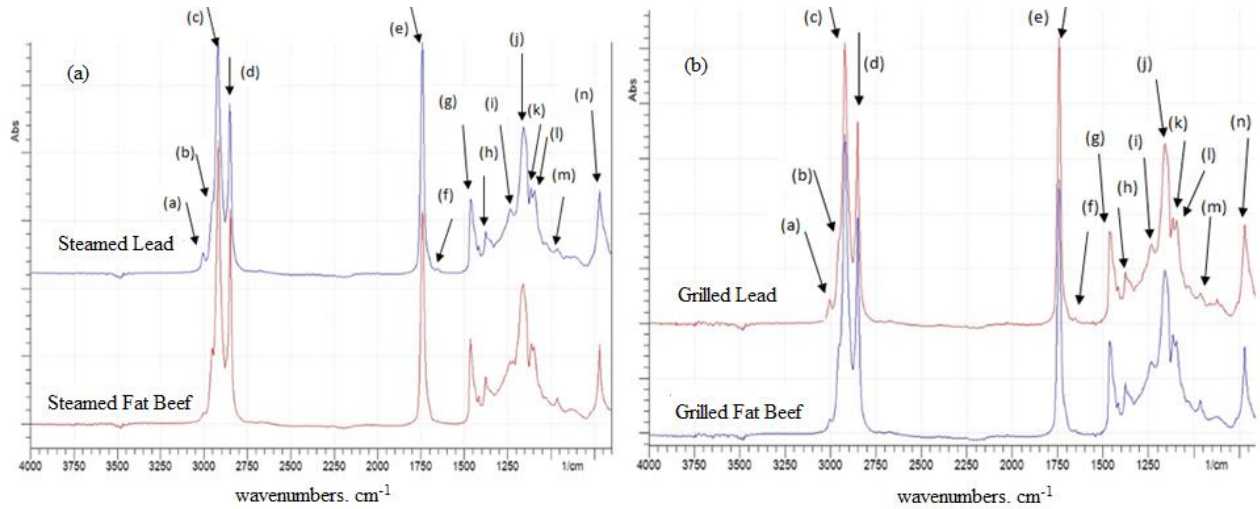

Figure 1 (a) Spectra of Steamed Pork Sausage Fat of 100% and Steamed Beef Sausage of 100 %. (b) Spectra of Grilled Beef Sausage Fat of 100% and Grilled Pork Sausage Fat of 100%

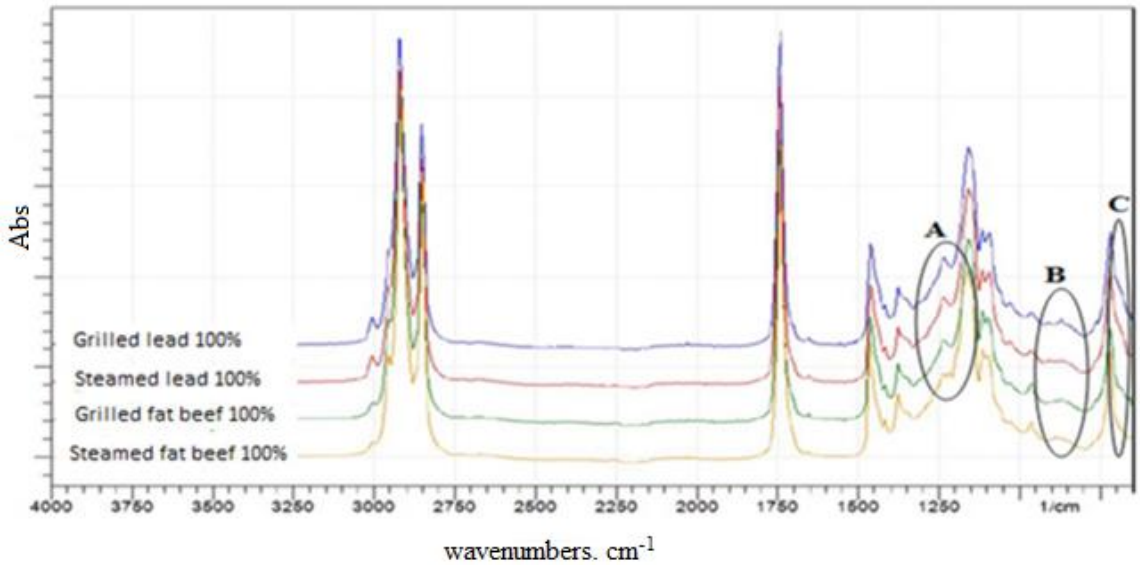

Figure 2 The infrared spectrum of Steamed and Grilled Pork Sausage.

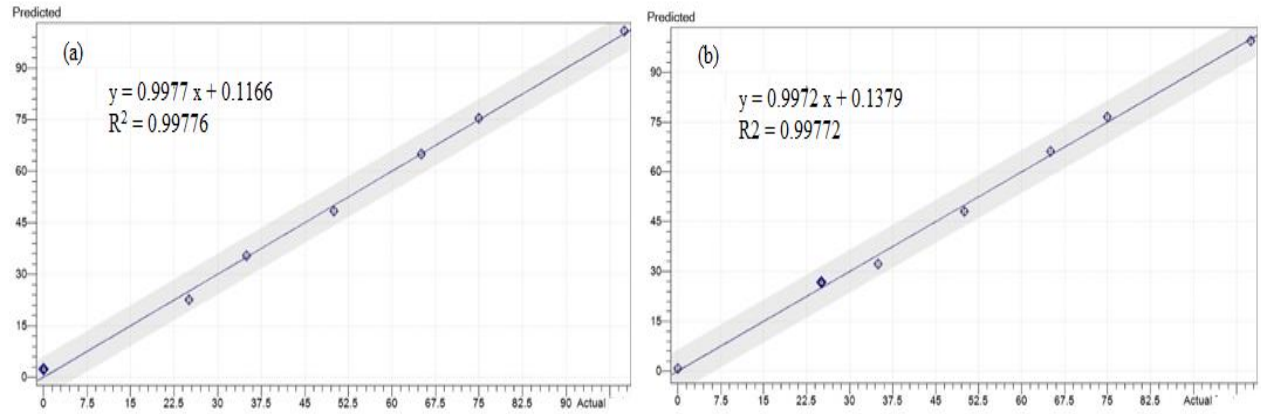

Figure 3 The Result of PLS Curve Relation between The Actual Value (x Axis) and Prediction Value (y Axis) on the Steamed Sausage Reference (a) and Grilled Sausage Reference (b) with Levelled Concentration.

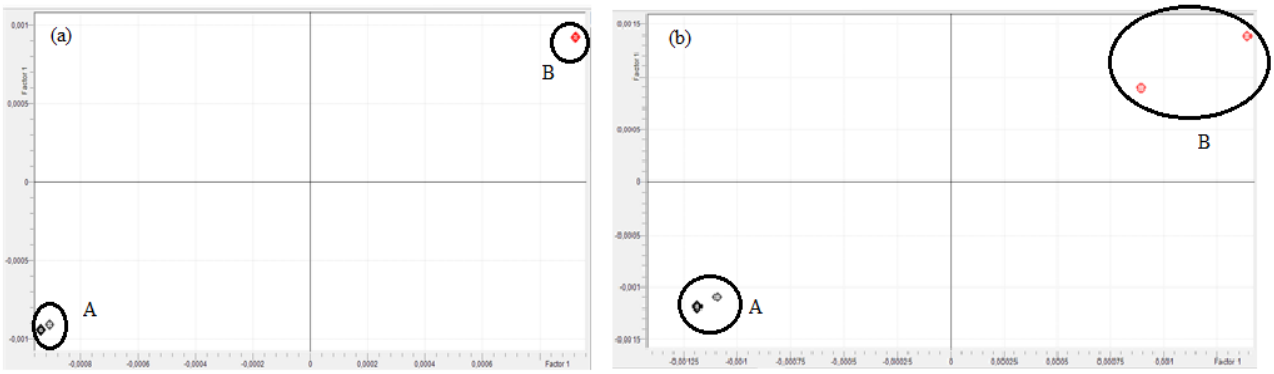

Figure 4 Plot score PCA of Beef Fat (A) and Lard (B) from steamed sausage (a), and grilled sausage (b).

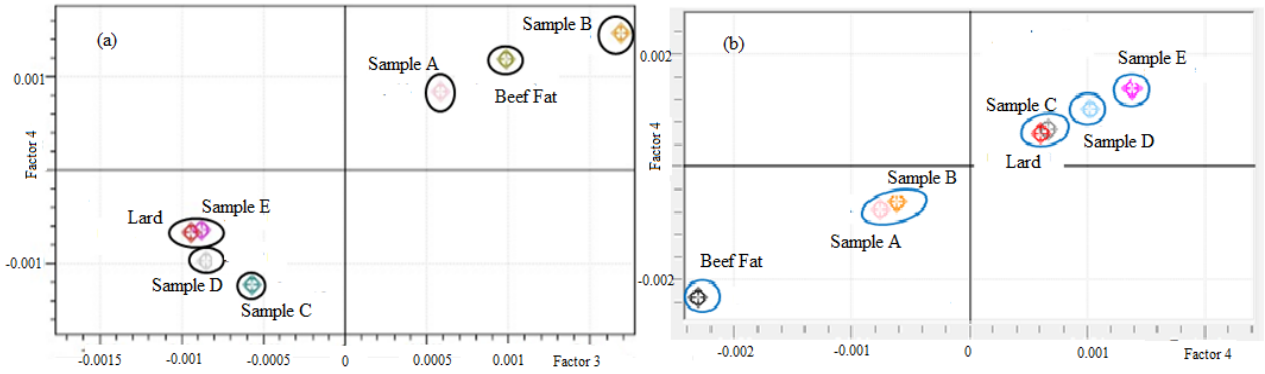

Figure 5 The PCA Result of Products in the market of steamed sausage (a), and Product on the market of grilled sausage (b).

CONCLUSION

Partial Least Square (PLS) chemometrics can be used for quantitative analysis of lard in steamed beef sausages and grilled sausages products. The combination with PCA chemometrics can grouped the lards in the steamed beef sausages and grilled beef sausages products in the market.

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THE ISOLATION AND CHARACTERIZATION OF LIPASE FROM *CARICA PAPAYA* LATEX USING ZWITTERION SODIUM LAUROYL SARCOSINATE AS AGENT

Dang Minh Nhat, Phan Thi Viet Ha

ABSTRACT

Most of industrial lipases are derived from microbial sources, following by a wide variety of plants. Among plant lipases, lipase from *Carica papaya* latex has been the focus of intense and growing research due to low cost, easy acceptance by consumers and its unique characteristics. This enzyme has been successfully applied for lipid modification and synthesis of some organic compounds. However, research for its molecular structure has been limited due to the difficulty to isolate the enzyme from the latex matrix. In this study, we suggested a modified approach using sodium lauroyl sarcosinate to solubilize the latex, then the protein was precipitated by ammonium sulphate. We also carried out the characterization of the lipase obtained from *Carica papaya* latex. The results showed that freeze-drying the fresh latex could improve significantly lipase activity of latex powder in comparison with sun-drying or oven-drying. The zwitterion sodium lauroyl sarcosinate could solubilize nearly 50% of the latex and the achieved supernatant exhibited great lipase activity. There was no need to use an organic solvent to delipidate the latex prior to solubilization with sodium lauroyl sarcosinate due to possible denaturation of enzymes. The proteins which were fractionally precipitated with 50 – 60%, 60 – 70% and 70 – 80% ammonium sulphate saturation showed lipolytic activity. The fraction from 50 – 60% saturation with the greatest mass was subjected to ion exchange chromatography, SDS electrophoresis and kinetic parameter determination. The results showed the presence of two proteins with molecular mass ranging from 35 kDa to 55 kDa and both presented lipase activity. The K_m and V_{max} of the lipase fraction from 50 – 60% saturation was 1.12 mM and 1.2 x 10^{-6} mM.min^{-1}.mL^{-1} respectively. So, the freeze-drying of papaya latex could help to preserve its lipase activity and the usage of sodium lauroyl sarcosinate could improve the isolation of the lipase from the papaya latex and pave the way for research on the molecular structure of *Carica papaya* latex lipases.

Keywords: lipase; papaya; enzyme isolation; enzyme purification

INTRODUCTION

Lipases are the most applied enzyme in organic synthesis due to their broad substrate acceptance and availability. They are serine hydrolase defined as triacylglycerol acyl hydrolase (E.C.3.1.1.3) and should be distinguished from esterase by the nature of their substrates. While lipases possess the ability to hydrolyse long-chain acylglycerols (>10 carbon atoms), esterases are capable of hydrolysing short-chain ones (<10 carbon atoms) **(de María et al., 2006; Casas et al. 2012).** Lipases in the industry are mainly isolated from microorganisms and partly from plants. Plant lipases with the advantages of low cost, easier acceptance in food and medicine products by consumers and unique characteristics have attracted more and more attentions in research, especially lipases from *Carica papaya* latex (CPL) **(Campillo and Tovar, 2013)**. The *Carica papaya* latex was well known to contain various proteases and chitinases long time ago **(Azarkan et al., 2003)**, however the lipolytic activity of *Carica papaya* was discovered by Frey-Wyssling until 1935. The more information about this hydrolase emerged in the early 1990s mostly on their characterization and utilization **(de María et al., 2006)**. It has been reported that the papaya latex contains several hydrolases which could hydrolyse triacylglycerol of both short- and long-chain fatty acids with preference to the short-chain ones. Despite of the great success in the discovery of possible utilization of CPL lipase in industry, research in the molecular structure of CPL lipase has been limited because of the "naturally immobilized nature" of the enzyme, i.e. the enzyme is contained in a complex matrix of the papaya latex, which is difficult to dissolve. There were some unsuccessful researches to solubilize the enzymatic activity of this latex fraction **(Abdelkafi et al., 2011)**. In 2009, **Abdelkafi et al. (2009)** used zwitterion CHAPS and sonication to solubilize the latex and then separated protein fractions with lipolytic activity. The obtained enzymes were determined to be esterase rather than lipase and therefore latex lipase could lose its activity during separation**.**

In this study, we applied the freeze-drying to the latex as soon as possible to limit solidification of papaya fresh laticifer. The modified separation procedure from **Abdelkafi et al. (2009)** were used to isolate lipase from CPL latex and to characterize the obtained lipase.

Scientific hypothesis

The hypothesis was that drying method would affect the solidification of latex laticifer and the entrapment of lipase. Using freeze-drying to obtain papaya latex powder could improve the lipase isolation. The zwitterion sodium lauroyl sarcosinate was expected as an effective agent in latex solubility to produce protein fractions with high lipase activity.

MATERIAL AND METHODOLOGY

Chemicals

Sodium lauroyl sarcosinate (purity 97%), *p*-nitrophenyl palmitate (purity 98%), *p*-nitrophenol (purity 99%), NaCl (purity 99.5%), *n*-hexane, *2*-propanol (purity 99.8%) and ammonium sulfate (purity 99%) were purchased from Sigma–Aldrich, Singapore; Tris base (purity 99.9%) and Triton X-100 were provided by Bio Basic, Canada. All other chemicals used were of analytical grade.

Preparation of CPL powder

Papaya latex was collected between 6 am and 8 am from unripe fruits grown in Quang Nam province, Vietnam. The 4 – 6 incisions were made along with the fruits by stainless steel knife and the latex was let drain into plastic bottles and stored in cold box with ice and frozen within 4 hours for freeze-drying. The CPL powder was obtained after grinding of dried matter. The latex was also sun dried and dried in an oven at 40 °C until constant weight and then ground into CPL powders. These powders were compared for their specific lipase activity and the most active powder was used for other experiments.

Preparation of crude CPL lipase

The 3 grams of CPL powder were weighed into 100 mL of distilled water and mixed for 3 minutes. The solution was centrifuged at 6000 rpm at 4 °C in 20 minutes using Hettich universal 320R centrifuge (Germany). The supernatant was disposed, and the precipitate was collected. These steps were repeated for 3 times and the final precipitate was freeze-dried in Alpha 1-2 LDplus freeze dryer (Germany) at -43 °C, 24 h to obtain crude CPL lipase.

Solubilisation of crude CPL lipase

One gram of crude CPL lipase was suspended in the 50 mL of the mixture of n-hexane and 2-propanol (1:1 v/v) and shaken in 30 minutes at 4 °C to extract lipid from the latex. It was then centrifuged at 7500 rpm in 20 minutes. The solvent residue was eliminated from the precipitate in vacuum rotary evaporator to obtain the lipid-free CPL lipase. This residue was then suspended into 50 mL solution of Tris–HCl 0.1M pH 8, prior mixed with the 0.5% (w/v) sodium lauroyl sarcosinate. The suspension was shaken for 30 minutes and then centrifuged at 7500 rpm in 20 minutes at 4 °C to obtain both supernatant and precipitate. These 2 fractions were used in other experiments.

Separation of lipolytic fractions from partially purified CPL lipase

Protein in supernatant collected after solubilizing crude CPL lipase in SLS was fractionally precipitated by using ammonium sulfate crystal. The precipitation was separated by ammonium sulphate saturation in the range of 0 – 90% (v/v). Precipitation was allowed to occur at 4 °C for 1 h and followed by centrifugation at 7500 rpm for 20 mins to obtain protein precipitate. All of the precipitate samples were dissolved in the solution of Tris HCl 0.1M, pH 8 and lipase activity was measured **(Burgess, 2009)**. The fraction with the highest activity was subjected to dialysis using cellophane to eliminate salts at 4 °C for 48h. The obtained solution was used for chromatographic and electrophoretic analysis.

Chromatographic separation of lipase

Samples after dialysis were loaded on the HiTrap Q Sepharose Fast flow column (5.0 mL, 1.6 cm × 2.5 cm) of the flash protein liquid chromatography system (GE Akta Purifier 100, Sweden). The column was firstly equilibrated with starting buffer of Tris-HCl pH 9. The sample was then eluted with an increase of NaCl concentration from 0 to 1 M at rate 0.1 M NaCl.min^{-1}. The flow rate was set at 1 mL.min^{-1}. The eluent was measured for absorbance at 280 nm and collected in fraction tubes of 1 mL volume.

SDS polyacrylamide electrophoresis

The molecular mass of the purified lipase was determined by SDS-PAGE as described by **Minaev and Makhova (2019)** using 15% acrylamide gel, prestained dual colour protein molecular weight marker (10 – 170 kDa).

Determination of K_m and V_{max} of purified CPL lipase

The Lineweaver–Burk plots were used to determine the Michaelis–Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) of lipase for *p*-nitrophenyl palmitate at pH 8.0, using a spectrophotometric method. Briefly, assays with lipases were performed in 0.1M Tris–HCl buffer, pH 8.0 at 40 °C with increasing concentrations of *p*-nitrophenyl palmitate from 0.397 mM to 3.973 mM, to calculate K_m and V_{max} **(Holme and Peck, 1998)**.

Lipase activity measurements

Lipase activity was measured spectrophotometrically using an assay based on the enzymatic hydrolysis of *p*-nitrophenyl palmitate (pNPP) to form *p*-nitrophenol (pNP) whose absorbance was measured at 410 nm (Jenway 6305, UK) **(Palacios et al., 2014)**. One activity unit (U) of lipase was defined as 1 µmol *p*-nitrophenol produced per minute under the assay condition.

Briefly, solution A containing 30 mg pNPP in 10 mL propan-2-ol and solution B containing 180 mL Tris-HCl buffer (0.1 M; pH 8.0), 720 µL Triton X-100 and 180 mg gum Arabic were prepared. The mixture of 4860 µL of solution B and 540 µL of solution A were incubated at 40 °C, then added with 0.0015 g enzyme for the reaction to take place. The absorbance was measured spectrophotometrically at 410 nm after 10 minutes and the

amount of hydrolysed *p*-nitrophenol was determined from the standard curve prior built with pNP standard.

Proximate analysis of papaya latex

Proximate composition of papaya latex was analysed using appropriate methods: AOAC 927.05 for moisture, AOAC 425.06 for ash, AOAC 2011.04 for protein and AOAC 948.15. for lipid.

Statistical analysis

Experiments were done in triplicates and the results were expressed as mean values. ANOVA and the Duncan Test was applied to assess significant difference at a significant level of α 0.05 using software Minitab 16.0.

RESULTS AND DISCUSSION

The impact of drying method on lipase activity of latex powder

Papaya latex collected from the fruit was analysed for its proximate composition. The results were shown in Table 1.

Table 1: Proximate analysis of papaya latex.

Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
77.53	2.07	14.6	1.86

Table 2: Effect of drying method on lipase activity of papaya latex powder.

Drying method	Sun-drying	Oven-drying	Freeze-drying
Moisture (%)	11.8	11.3	9.13
Specific activity ($mU.g^{-1}$)	27.68	18.22	120.35

Table 3 Lipolytic activity of fractions from crude CPL lipase after delipidation and centrifuging.

Samples	Crude lipase	Precipitate	Supernatant
Mass/ Volume	1.0120 (g)	0.5632 (g)	45 (mL)
Specific activity	108.55 ($mU.g^{-1}$)	17.30 ($mU.g^{-1}$)	0.19 ($mU.mL^{-1}$)
Total activity	109.85 (mU)	9.74 (mU)	8.55 (mU)

Table 4 Lipase activity of fractions from crude CPL lipase after centrifuging without delipidation.

Samples	Crude lipase	Precipitate	Supernatant
Mass	1.016 (g)	0.498 (g)	97 (mL)
Specific activity	115.07 ($mU.g^{-1}$)	20.83 ($mU.g^{-1}$)	50.16 ($mU.mL^{-1}$)
Total activity	116.91 (mU)	10.37 (mU)	4865.52 (mU)

The fresh latex contained a high amount of water, protein and low lipid. High protein content could be linked to the presence of various enzymes and the high water content may explain for its flowability. This result was in accordance with **Macalood et al. (2013)**.

However, the papaya latex tends to solidify quickly in the air, and this may entrap lipase and affect the lipolytic activity of the latex. In this study, the papaya latex was dried in 3 different methods until its constant mass as described in 2.2.1. The dried latex was ground to powder and measured for their lipolytic activity. The results were presented in Table 2.

The results showed that long time of air exposure at high temperature had a significant negative impact on the lipase activity (p <0.001). The freeze-drying gave the highest quality powder, which achieved four-time higher of specific activity than those from sun-drying or Oven-drying. Freeze-drying was also confirmed to be a good method for preserving activity of lipase from *Yarrowia lipotica* **(Darvishi et al., 2012)**.

Partial purification of crude CPL lipase

Partial purification of crude CPL lipase including delipidation

The crude CPL lipase was partially purified according to method described by **Abdelkafi et al. (2009**) using hexane:propanol (1:1) to delipidate. After centrifuging, 45.0 mL of supernatant and 0.5632 g of the precipitate was measured for lipolytic activity, which is illustrated in Table 3.

The data showed that sodium lauroyl sarcosinate could solubilize about 44.3% of the crude lipase mass. Both supernatant and precipitate possessed lipolytic activity, but their specific activities were apparently reduced by approximately 80.6% in comparisons with the origin of crude lipase. The reason for this loss could be the denaturation of lipases in precipitate or in supernatant caused by solvent during the lipid extraction step.

This result suggested that the zwitterionic SLS could solubilize nearly half crude CPL lipase and extraction lipid step might be ineffective and unnecessary.

Partial purification of crude CPL lipase without delipidation

The previous procedure was repeated with the modification of omitting lipid extraction step. The results are presented in Table 4. The data show that precipitate and supernatant contained lipolytic activity. The activity remained in precipitate was just a small portion of the origin activity in crude lipase. However, the total lipolytic activity in the supernatant was 40-fold increased, compared to the activity of crude lipase. This sharp increasing suggests that a significant amount of lipase in the latex was isolated from the matrix by zwitterionic SLS to dissolve part of the latex matrix. The enzyme was more active in free form than in the immobilized form in the matrix and might not be negatively affected by the zwitterionic nature of SLS at used concentration.

Effect of sodium lauroyl sarcosinate concentration on the extraction yield of lipolytic activity from crude CPL lipase

Crude CPL lipase was suspended in zwitterionic SLS in 30 minutes at different concentrations. Figure 1 presents the

effect of SLS concentration on supernatant lipolytic activity.

It could be confirmed that SLS concentration affected significantly on the lipolytic activity of the obtained supernatant (p <0.005). The higher specific lipolytic activity was achieved with the increasing SLS concentration from 0.25% to 0.5% but then decreased with concentration ranging from 0.5% to 1%. SLS is a surfactant with ionic nature.

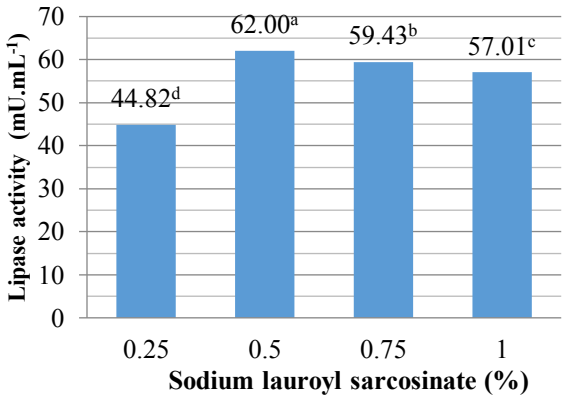

Figure 1 Effect of SLS concentration (%) on lipolytic enzyme extraction.

Table 5 Lipolytic activity of fractions from crude CPL.

Saturation (%)	Precipitate (g)	Lipase activity
10 – 40	0.000	-
40 – 50	0.050	-
50 – 60	0.300	+
60 – 70	0.102	+
70 – 80	0.031	+
80 – 90	0.000	-

"+": present; "- ": absent

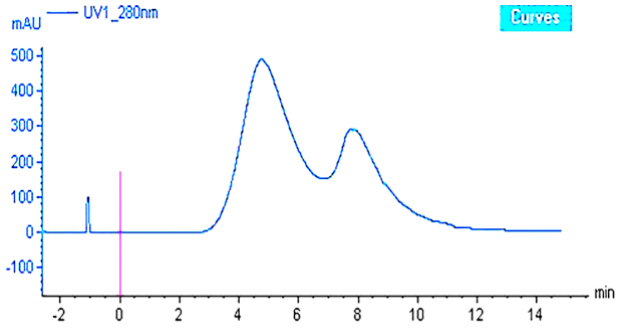

Figure 2 Chromatograph of 50 – 60% fraction precipitate.

The increasing concentration until 0.5% could solubilize the latex matrix to liberate lipase to increase the activity of the supernatant. However, the higher concentration than 0.5% of this zwitterion might cause denaturation of lipase, leading to the decrease of lipolytic activity of the supernatant. The highest lipolytic activity of 62.00 mU.mL-1 in the supernatant was achieved with 0.5% SLS solution.

Isolation of lipase from crude CPL lipase

Fractional precipitation of CPL lipase with ammonium sulphate

The ammonium sulphate was used at different saturation levels to precipitate fractionally the CPL lipase from the supernatant. Table 5 presents the weights and lipolytic activity of different precipitates.

No precipitate was visible after adding $(NH_4)_2SO_4$ to 40% of saturation. All proteins were sedimented in ammonium sulphate solution with saturation ranging from 50% to 90%. Only the fractions by 50 – 60%, 60 – 70% and 70 – 80% saturation showed lipolytic activity.

This result complied rather well with the work of **Paques et al. (2008)**, regarding to saturation level of ammonium sulphate used for precipitation of lipase from papaya seed and skins. Lipases were also found in fraction of 40-80% saturation.

As the first attempt in this study, the fraction achieved by 50 – 60% saturation presented good lipolytic activity and the highest weight was examined for further purity by ion exchange chromatography, electrophoresis and kinetic determination.

Chromatographic analysis of isolated lipase fraction 50-60%

The Figure 2 illustrates the chromatograph of lipase fraction 50 – 60%. It showed the presence of at least 2 proteins in the sample.

The first peak was eluted from 2.58 min to 6.87 min, following by the second peak appeared from 6.87 min to 11.29 min. Accordingly, the first and the second peak fractions had the lipase specific activity of 0.316 mU.mL-1 and 0.338 mU.mL-1 respectively.

Electrophoretic analysis of isolated lipase fraction 50-60% from $(NH_4)_2SO_4$ precipitation

The proteins from fraction 50 – 60% were also subjected to SDS electrophoresis. The result in Figure 3 showed the presence of 2 bands from all 3 triplicate samples of dialyzed 50 – 60% fraction, suggesting there were 2 proteins in every sample. One protein had a molecular weight in the range of 35 – 40 kDa, while this value in the second protein was 40 – 55 kDa. This result complied very well with the previous result of chromatographic analysis. It can be concluded that there were 2 lipases with a molecular weight ranging from 35 – 55 kDa in the fraction 50 – 60%.

The molecular weights of these papaya lipases are similar to lipases of some microbial and plant lipases, such as 40 kDa of purified lipase from *Microbacterium* sp. **(Tripathi et al., 2014)** or 40 kDa of lipase from Tunisian *Euphorbia peplus* latex **(Lazreg et al., 2014)**. Meanwhile the native molecular weight of lipase from *Raphia* mesocarp was 35 kDa **(Okunwaye et al., 2015)**.

Determination of K_m and V_{max} of lipase fraction 50 – 60%

To study the enzyme–substrate affinity, the kinetic parameters of the lipases to *p*-nitrophenyl palmitate were determined. The K_m and V_{max} values for lipase activity with *p*-nitrophenyl palmitate were calculated from Lineweaver–Burk plots constructed by using activity values depending on substrate concentrations. The Lineweaver–Burk plots

were linear and indicated that hydrolysis of *p*-nitrophenyl palmitate by the tested lipase followed Michaelis–Menten kinetics (Figure 4).

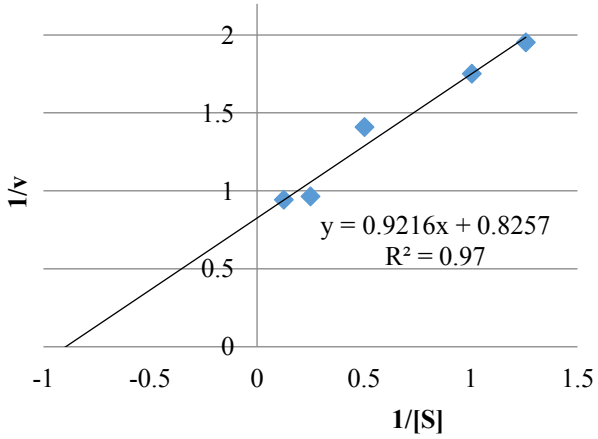

Figure 4 Lineweaver-Burk plot for determination of K_m (mM) and V_{max} (mM.min^{-1}.mL^{-1}) of papaya lipase.

The result of K_m and V_{max} calculation for purified lipase from papaya latex from Lineweaver-Burk plot were 1.12 mM and 1.2 x 10^{-6} mM.min^{-1}.mL^{-1}, respectively. The value of K_m depends on the type of substrate and on environmental conditions such as pH, temperature, ionic strength and polarity. K_m is also a measure of the strength of the ES complex or the affinity of the enzyme for substrate. The smaller the K_m, the bigger the enzyme-substrate affinity, the faster the reaction rate. The K_m of the purified papaya lipase showed its higher affinity to pNPP than lipase from *Microbacterium* sp. which had K_m of 3.2 mM as determined in the work of **Tripathi et al. (2014)**. However, its ability to form the complex with pNPP was not as good as that from the lipase of the thermophilic *Bacillus stearothermophilus* MC 7, whose K_m was only 0.33 mM **(Kambourova et al., 2003)** or from *Acinetobacter sp.* AU07 whose lipase K_m was determined to be 0.51 mM **(Gururaj et al., 2016)**. In the other work, the K_m and V_{max} of lipase from raphia palm fruit mesocarp were calculated as 0.01 mM and 20.5 µmol.min-1.mL^{-1} respectively **(Okunwaye et al., 2015)**.

CONCLUSION

Papaya latex lipase is so far the only plant lipase used commercially in many applications. However, the molecular characteristics of this lipase are less known due to its immobilized nature in the papaya latex. This study has shown the possibility to liberate substantially lipolytic activity from the latex. It is suggested to freeze-dry the latex as soon as possible to preserve lipase activity and then use sodium lauroyl sarcosinate to dissolve the latex without delipidation step to avoid possible denaturation of lipase. The purified enzyme could be obtained using traditional fractional precipitation of protein with ammonium sulphate. In this study, the concentration of sodium lauroyl sarcosinate determined of 0.5% (w/v) gave the highest lipolytic activity. Two lipases were found in the papaya latex with molecular mass ranging from 35 – 55 kDa.

The K_m and V_{max} of lipase fraction obtained from precipitation using 50 – 60% saturation of ammonium sulphate was 1.12 mM and 1.2 x 10^{-6} mM.min^{-1}.mL^{-1} with *p*-nitrophenyl palmitate as substrate.

Further study should be carried out to isolate lipases from other precipitate fractions with ammonium sulphate and characterize isolated lipase.

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MICROBIOLOGICAL COMPARISON OF VISIBLY DIRTY AND VISIBLY CLEAN MATURE GREEN TOMATOES BEFORE AND AFTER TREATMENTS WITH DEIONIZED WATER OR CHLORINE IN MODEL OVERHEAD SPRAY BRUSH ROLLER SYSTEM

Oleksandr Tokarskyy, Mykhaylo Korda

ABSTRACT
The purpose of the current study was to compare natural microflora counts of mature green tomatoes as influenced by visual cleanness, and investigate ability of chlorine sanitizer to reduce different groups of natural microflora on the surface of tomatoes using overhead spray brush roller system. We hypothesized that natural microflora might not be equally affected, with vegetative Gram negative bacteria being more sensitive and soil-related Gram positive sporoforming bacilli and molds more resistant. Microflora from untreated visibly clean and visibly dirty tomatoes, as well as from visibly clean tomatoes after 30 seconds deionized water or 100 ppm chlorine treatments, was recovered and spread plated on Tryptic Soy agar, MacConkey agar, and acidified Potato Dextrose agar. Microflora from untreated and chlorine-treated tomatoes was non-specifically enriched and plated on agar with chlorine paper disc diffusion assay applied to check for inhibition zone differences. Interestingly, there was no significant difference in plate counts between visibly clean and dirty tomatoes (p >0.05). Chlorine was more effective than water alone to reduce microbial counts on tomatoes for all microbiological media tested. Based on similar relative reductions of microorganisms in each group, it was concluded that chlorine may have no preferential kill for investigated groups of microorganisms. High counts remaining after treatment with chlorine solution suggested possibility of resistant microbial biofilm formation on the surface of tomatoes.

Keywords: tomatoes; cleanness; natural microflora; overhead spray brush roller system

INTRODUCTION
Tomatoes are an important agricultural fruit, placing nine per production volume among most popular agricultural produce in the Ukraine. Referencing **FAOSTAT (2017)**, top ten tomato growing countries were China, India, Turkey, USA, Egypt, Iran, Italy, Spain, Mexico, and Brazil, with Ukraine present in the top twenty and producing as much as 2,267,460 tonnes in 2017 alone. The visual appearance of the tomato surface as "clean" may give a false feeling of safety of its consumption. However, two large groups of microorganisms, which are of concern on fresh produce, are spoilage and pathogenic, which may cause either spoilage or foodborne illnesses, are invisible to human eye **(Jay, 1998)**. Enteric Gram-negative pathogens, including *Salmonella* and *Escherichia coli* O157:H7, may be present on fresh tomatoes as contamination from environment and may persist on the surfaces **(Tokarskyy et al., 2018; Tokarskyy and Schneider, 2019)**. Common tomato spoilage microorganisms include Gram-negative rods (*Erwinia carotowora)*, Gram-positive sporeformers (*Bacillus* spp.), yeasts and molds **(Shi et al., 2009; Jay, 1998)**. Although novel methods to decontaminate surface of edible foodstuff are available **(Tokarskyy and Marshall, 2010)**, they remain expensive comparing to the use of low-cost alternatives, such as chlorine sanitizers **(Dychdala, 2001; Tokarskyy et al., 2015)**. One of the approaches to reduce microbial load and prevent cross-contamination on tomatoes before retail sale is through their washing with low concentration chlorine sanitizer **(Chang and Schneider, 2012; Gereffi, Sreedharan and Schneider, 2015)**. For example, one of the most common tomato processing system in the United States is a flume tank with 150 ppm free chlorine (pH 6.5 to 7.5) and a maximum of a 2 minute treatment **(Gereffi, Sreedharan and Schneider, 2015)**. **Gereffi, Sreedharan and Schneider (2015)** have shown that even 25 ppm of chlorine may be adequate to prevent cross-contamination of tomatoes with *Salmonella* if the concentration is properly maintained, chemical oxygen demand does not exceed 500 ppm, and tomatoes are treated for at least 120 seconds in a flume tank. Such tank may be terminally equipped with an additional overhead spray and brush roller system, where increased physical removal of bacteria with brushes in conjunction with antimicrobial efficacy of sanitizers may greatly improve decontamination step **(Chang and**

Schneider, 2012). The primary purpose of chlorine sanitizer is to prevent cross-contamination and bacterial build-up **(Gil et al., 2009)**. Though chlorine is believed to have non-specific mode of action, bacterial spores have innate resistance at concentrations used in food industry **(Davidson and Harrison, 2002)**. Some of the naturally occurring bacteria found on tomatoes do include *Bacillus* spp., in addition to *Cyanobacterium* spp., *Erwinia* spp., *Enterobacter* spp., *Pantoea* spp., *Pseudomonas putida,* among others **(Shi et al., 2009)**. Chlorine is capable to reduce natural microbial contamination level on produce, but never eliminate it completely **(Allende et al., 2009; Rahman, Ding and Oh, 2010)**. **Chang (2011)** found that with initial population of natural microflora on tomato surfaces of 5.31 log units, 100 ppm chlorine significantly reduced more natural microflora than water with a 1.41 log units reduction after 30 seconds treatment (p <0.05), but never below detection limit. Increasing treatment time to 60 seconds did not significantly affect efficacy.

The purpose of the current study was to compare microbial loads of "visibly clean" and "visibly dirty" tomatoes, to evaluate influence of 100 ppm chlorine wash on different groups of natural microflora on tomato surfaces using overhead spray-brush roller system, as well as to evaluate resistance to chlorine of residual microflora in order to better understand surviving natural microorganisms after treatment.

Scientific hypothesis

We hypothesize that visibly dirty tomatoes will have significantly higher microbial counts on all microbiological media tested, comparing to visibly clean tomatoes. We hypothesize that 100 ppm chlorine treated tomatoes will have significantly lower microbial counts comparing to water treated and untreated tomatoes. We hypothesize that residual microflora, regrown from 100 ppm chlorine treated tomatoes, will be more resistant to chlorine in paper disc diffusion antimicrobial assay comparing to untreated tomatoes, with smaller inhibition zone diameter.

MATERIAL AND METHODOLOGY

Brush roller machine and chlorine preparation

Two rotating (180 rpm) nylon rollers (46 cm long and 12 cm diameter) sat alongside in a 46 cm by 34 cm box (Figure 1). Five tomatoes at a time were placed between two brush rollers and revolved in directions depending on their size and shape while being brushed by rollers. Simultaneously, three spray nozzles released a cone shaped spray (16 psi pressure) with a flow rate of ca. 21 mL.second^{-1} on the surface of rotating tomatoes. Treatment solution was fed to the nozzles using 20 L bucket, piping, and centrifugal pump.

Chlorine sanitizer was prepared by mixing 22 mL of 5.65 to 6.00% sodium hypochlorite (Thermo Fisher Scientific, Waltham, MA, USA) with ca. 10 L of deionized water. The sanitizer pH was adjusted to 6.50 ±0.05 with 1N HCl (Thermo Fisher Scientific, Waltham, MA, USA). Free chlorine concentration was measured using Hach DR/890 colorimeter, method 8091 (Hach Co., Loveland, CO, USA) by diluting treatment solution 1:100 in chlorine-free DI water to get to the required range of 0.98 to 1.02 ppm, corresponding to 100 ±2 ppm chlorine of undiluted solution.

The brush roller machine rotating brushes and pump were switched on and the system was flushed/rinsed with deionized water for 3 minutes. Following initial flushing, the cleanness of each brush was evaluated by swabbing it four times from one end to another with sterile cotton-tipped applicator (Thermo Fisher Scientific, Waltham, MA, USA) and swabbing the Tryptic Soy agar plate followed by confirmation of absence of microbial growth (32 °C, 48 hours). The first tomato treatment was deionized water (30 seconds), followed by chlorine treatment (30 seconds).

Tomatoes preparation and treatment

Green mature unwashed round tomatoes (*Lycopersicum esculentum*) variety 602 were acquired from a single local packinghouse on three different days late May-early June in Florida, USA. Five tomatoes were selected as "visibly dirty" ("D") based on their appearance and presence of adhered soil, leaves, and dirt. Fifteen tomatoes were classified as "visibly clean" ("C") based on their appearance, for each round of experiments. These fifteen "C" tomatoes were rubbed each in three rounds with sterile nitrile gloves to "normalize" microbial flora among them.

Five of each "D" and "C" tomatoes were analyzed immediately untreated, while second set of five "C" tomatoes was treated with deionized water and third set – with 100 ppm chlorine wash.

Deionized water treatment was applied to five visibly clean tomatoes ("C-W") for 30 seconds by placing them simultaneously on the rollers, and pH of the liquid and absence of chlorine was verified using sample solution from nozzles as described previously. This set of clean and water-treated tomatoes was removed for microbiological analysis. Following deionized water treatment, the system was flushed for 1 min with prepared 100 ppm chlorine sanitizer (pH 6.5) and concentration of the chlorine and pH were verified using sample solution from the nozzles. The third set of five visibly clean tomatoes ("C-CHL") was placed on the rollers and treated with chlorine sanitizer for 30 seconds before microbiological analysis.

Microbiological analysis of tomatoes

Each tomato was transferred to 50 mL Bacto™ Tryptic Soy Broth (TSB, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in a stomacher bag and was vigorously shaken for 20 seconds, rubbed for 20 seconds, and shaken again for 20 seconds. The rinsate was serially diluted in buffered peptone water and 0.1 mL aliquots were immediately spread plated on Tryptic Soy agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for Total Plate Count (TPC, 32 °C, 48 hours), MacConkey agar (MCA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for Gram-negative bacterial counts (GNC, 37 °C, 48 hours), and acidified Potato Dextrose agar (aPDA, pH 3.5, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for Yeast and Mold Count (YM, 25 °C, 5 days). The countable agar plates contained preferred 25 to 250 CFU per plate range and conversion from CFU.mL^{-1} rinsate to CFU.tomato^{-1}

was done by multiplication factor 50. Therefore, the detection limit was 2.7 $\log_{10}$ CFU.tomato^{-1} (est.).

The TSB rinsates with tomatoes ("C" and "C-CHL") were further incubated for 10 hours at 32 °C to non-specifically enrich natural and residual microflora after tomato treatments for chlorine selective bactericidal activity evaluation.

To prepare paper discs soaked in chlorine, 6 mL of sodium hypochlorite (5.65 – 6%) was mixed with 41 mL of autoclaved deionized water and 3 mL 1N HCl, resulting in plates were incubated for 48 hours at 32 °C before inhibition zones were measured and pictures of the plates were taken **(Duran and Marshall, 2005)**.

Statistic analysis

The experiment was repeated three times and counts were analyzed for each microbiological medium (TSA, MCA, aPDA) using one-way ANOVA with a single factor of treatment ("D", "C", "C-W", "C-CHL"). Means were separated using Fisher LSD method if influence of the factor was significant (p <0.05). Chlorine inhibition zones for enriched microflora from "C" and "C-CHL" treated tomatoes around chlorine-soaked paper discs were measured with the ruler and data were analyzed using one-way ANOVA. Mean values of the inhibition zones were separated using Fisher LCD method. Statistical analysis of the obtained data was performed using commercial software Statistica ver. 10.0 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

There was a significant influence of analyzed factor with variations as "C", "D", "C-W", and "C-CHL" on microbial counts of all three microbiological media plated (p <0.05).

Surprisingly, there was no significant difference in total plate count (6.01 ±0.50 vs. 6.33 ±0.52 $\log_{10}$ CFU.tomato^{-1}), Gram-negative counts (5.71 ±0.65 vs. 5.80 ±0.60 $\log_{10}$ CFU.tomato^{-1}), and yeast and mold counts (4.42 ±0.54 vs. 4.56 ±0.41 $\log_{10}$ CFU.tomato^{-1}) between clean and dirty tomatoes, respectively (p > 0.05, Figure 2). This can be attributed to smaller sample sizes, comparing to other studies **(Schneider et al., 2017; De et al., 2018)**. Water alone decreased TPC by 0.40 $\log_{10}$ CFU.tomato^{-1}, GNC by 0.48 $\log_{10}$ CFU.tomato^{-1}, and YM by 1.24 $\log_{10}$ CFU.tomato^{-1}on water-treated tomatoes (Figure 2).

Chlorine wash was more effective with corresponding average reductions of 1.12, 1.19, and 1.66 $\log_{10}$ CFU.tomato^{-1} for TPC, GNC, and YM, respectively (Figure 2, Table 1). Interestingly, the highest reduction was observed in YM counts, while **Dychdala (2001)** noted that higher chlorine, 135 to 500 ppm, is required to inactivate molds. Based on water alone data, it can be concluded that yeasts and molds might have been simply washed off tomatoes without kill step. Similarly, **Schneider et al. (2017)**, while analyzing pre- and post-processed tomatoes from Florida, New Jersey and Maryland packinghouses in spring, have found that average microbial TPC per untreated tomato was 6.25 $\log_{10}$ CFU.tomato^{-1} and 5.31 $\log_{10}$ CFU.tomato^{-1} for chlorine water flume tank treated tomatoes, corresponding to 0.94 $\log_{10}$ CFU.tomato^{-1} reduction. Considering large number of analyzed samples, overall range for TPC for tomatoes collected year-round was 2.3 to 12.1 $\log_{10}$ CFU.tomato^{-1} with median of 6.9 $\log_{10}$ CFU.tomato^{-1} **(Schneider et al., 2017)**.

MacConkey agar is selective and differential medium for bacteria, formulated to selectively isolate Gram-negative and enteric bacilli. Therefore, it may be argued that GNC is a subset of TPC, and though similar relative log reductions of microorganisms in each group of these two groups were found, absolute reductions in counts as CFU.tomato^{-1} suggest that chlorine may have had an "all kill" approach, reducing not only Gram-negative bacteria counts, but also Gram positive (Table 1). Similarly, **Schneider et al. (2017)**, while analyzing larger sets of pre- and post-processed tomatoes, have found total coliforms counts on CHROMagar™-ECC to be 5.13 $\log_{10}$ CFU.tomato^{-1} and 4.70 $\log_{10}$ CFU.tomato^{-1} for untreated and chlorine flume tank treated tomatoes, respectively.

This observation, together with no significant difference between inhibition zones by chlorine for untreated and chlorine-treated tomato residual microflora (p >0.05), 18.4 ±1.7 and 19.9 ±2.3 mm, respectively, suggested that chlorine may have had no preferential kill, but rather a shotgun approach (Figure 3).

However, concentrated circle patterns were observed on disc diffusion plates, suggesting that certain microorganisms on the tomato surface might be indeed more sensitive to chlorine (Figure 4).

High counts remaining after treatment with chlorine suggested resistant biofilm formation on the surface of tomatoes. Another suggested explanation by **Fatica and Schneider (2009)** is that natural microflora is hiding in crevices and pockets of the hydrophobic, waxy cuticles of the produce, where aqueous chlorine sanitizer cannot enter.

Table 1 Average values of $\log_{10}$ and absolute reductions in microbial population counts on TSA, MCA, and aPDA of water-treated (C-W) and chlorine-treated (C-CHL) tomatoes comparing to visually clean tomatoes (C) used for overhead spray brush roller experiments.

Microbial population	Ave log reduction, $\log_{10}$ CFU.tomato^{-1}		Ave absolute reductions, CFU.tomato^{-1}	
	C-W	C-CHL	C-W	C-CHL
TPC/TSA	0.40	1.12	608,095	939,583
GNC/MCA	0.48	1.19	342,430	480,962
YM/aPDA	1.24	1.66	24,815	25,773

Figure 1 Overhead spray brush roller system used in the experiments, manufactured by Agri Machinery Inc. (Orlando, Fla., USA).

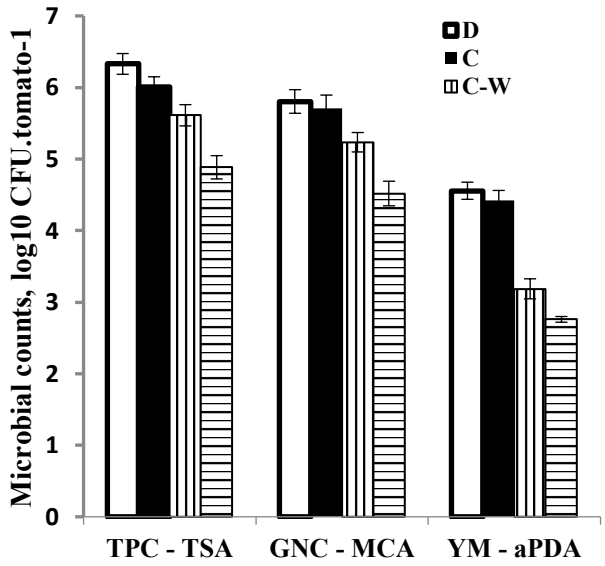

Figure 2 Microbial counts of visibly dirty (D), visibly clean (C), visibly clean treated with water (C-W), and visibly clean treated with chlorine (C-CHL) tomatoes on Tryptic Soy agar (TPC-TSA), MacConkey agar (GNC-MCA), and acidified Potato Dextrose agar (YM-aPDA). Counts are expressed as $\log_{10}$ CFU.tomato^{-1}. Note: Error bars reflect standard errors of mean. Means within the same microbiological medium with the same letters are not significantly different (p >0.05).

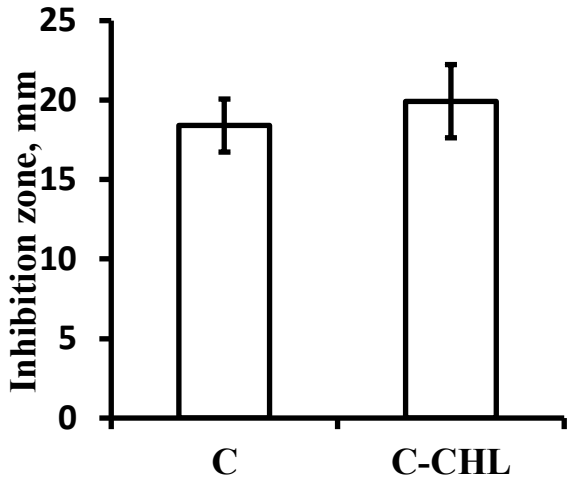

Figure 3 Inhibition zones of 7,200 ppm soaked paper discs on non-selective enrichments of natural microflora and residual microflora of untreated and chlorine-treated tomatoes. Note: Error bars reflect standard deviation. Same letters mean non-significant difference (p >0.05).

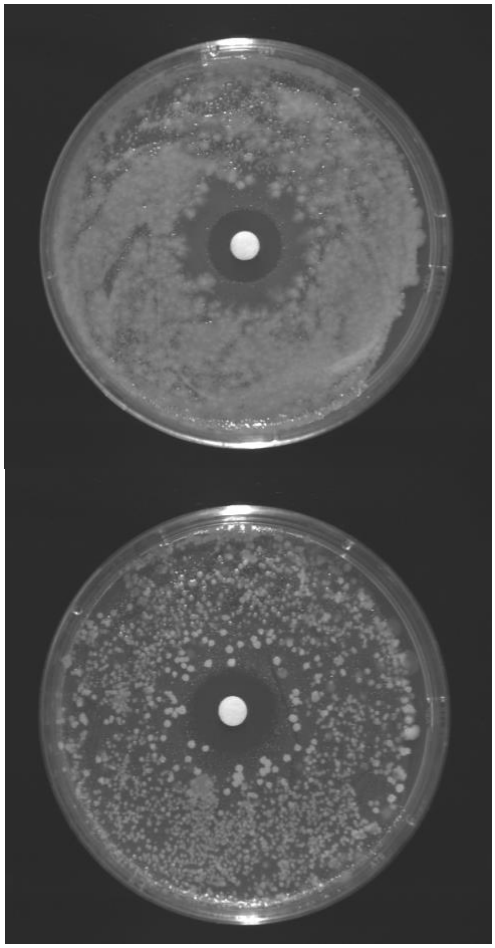

Figure 4 Examples of paper disc diffusion assays (7,200 ppm chlorine) on non-selective residual microflora enrichments of chlorine treated tomatoes. Note: Circles of bacterial populations with different sensitivities are shown with arrows.

CONCLUSION

To summarize, cleanness may attribute to lower counts on the surface of tomatoes, irrespective of microbial group analyzed, though microbial counts were not significantly different. Larger sets of tomatoes are needed to fortify this statement. Although 100 ppm chlorine treatment reduced all microbial counts significantly better than water alone, it failed to bring them below detection levels, suggesting strong interaction such as biofilm formation, between natural microflora and tomatoes. Comparing reductions of microorganisms in each group, it was concluded that chlorine may have no preferential kill but rather a shotgun approach.

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HOUSEHOLD FOOD WASTE BEHAVIOUR: SUBJECTIVE AND OBJECTIVE EVIDENCE

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ABSTRACT
This paper is concerned with the issue of quantifying food waste as a basic assumption for an effective measure to achieve the lowering of its volume. From literary sources one can see great differences in the amounts recorded, caused, among other reasons, by the unclear methods of monitoring and the unclear terms used for description of the term food waste. From questionnaire research carried out on the opinions and causes of waste among 1582 respondents it was found that it is regarded as a significant problem by society but the everyday behaviour of the individual does not correspond to this. Changes in the behaviour of the individual occur during their realisation of this waste issue, for instance by means of objective research into wasted food (through the weighing of the individual types of food thrown out). The average value of wasted food reached approximately a quarter of the amount given for EU and corresponds to the amount reported in Finland.

Keywords: Food waste; consumer behaviour; wasting of food; waste motivations; causes of waste

INTRODUCTION
Food is an inseparable and daily part of people's lives. The issue of food waste is a problem for the whole society, which is addressed in the entire food chain – from the level of primary manufacture to consumption of food by households. This issue has varying forms in various parts of the world. In one part of the world there are almost 800 million people suffering for undernourishment, in another part of the world, in economically developed countries, wasting of food has a large environmental and economic impacts and also introduces ethical aspects **(FAO, IFAD and WFP, 2015)**. **Nunley (2013)** adds that among other things, the inhabitants of cities in developed countries are today de facto separated from the production of food and waste infrastructure and that many people consume food without any knowledge and awareness of their role in that system. The impacts of food waste however, are often worse than the general consumer imagines. The results are not just large economic losses, but also the wasting of natural resources vital for people's existence or damaging the environment as a result of the raising of emissions of carbon gases and the consumption of water.

Society is realising this fact more and more, and thus currently lowering food waste is one of the key points of sustainable global development. Food wastage can be regarded as an ecological, economic and moral problem. In 2015, one of the aims for sustainable development (SDG 12.3) which was adopted at the UN summit, on the basis of which it is necessary to lower the amount of food losses and waste in the entire food chain by half by 2030 **(European Parliament, 2017)**. In light of this fact, the issue of the production of food waste and searching for possibilities to limit it has been given great attention in many differing countries. Compulsory reporting on the food waste generated by households should be introduced from 2020 at state level in the individual member states of the EU, in later years the other links in the food chain should also join in compulsory reporting of data on food waste **(DG SANTE, 2018)**. On the basis of the data from 2012, a voluntary pilot project was carried out at Eurostat level, the so called "food waste plug in" within the framework of which member states could provide Eurostat with data to estimate the amount of food waste **(Schrör, 2015)**. Using this data (it relates to data divided according to individual categories of waste according to the Waste Catalogue under which food wastage can occur and in accordance with CZ-NACE) Eurostat then processes the data and creates estimates from it. The purpose of the project is to evaluate whether the existing data can be used to express food waste. However, the carrying out of this project immediately drew attention to a number of inadequacies – a unified system for collecting data is missing at the level of individual states (every state can choose from a number of prescribed methods and on that basis then provide data to Eurostat). Another limitation is

the fact that the selected categories of waste do not include only food waste, but also other organic waste components, which should not be treated as food waste. Any comparison of data across varying states is thus problematic **(Stenmarck et al., 2016; Hanssen et al., 2013)**. Currently the issuance of a common method for measuring food waste is awaited which should result from a discussion by experts involved in a working group dedicated to losses of food and food waste. The form of the method should be published during 2019 **(Directive (EU) 2018/851)**.

This decision, even though well meant, and definitely needed, will meet one big problem, which is the lack of unification of methods and in particular the ambiguous interpretation of terms in the individual countries.

The terms connected with the issue of food waste and biological waste, which are dealt with by European law in **Directive (EC) no. 98/2008** concerning waste, article 3:

‘’waste’’ is any substance or item which its holder gets rid of, or intends getting rid of, or of which they are required to dispose of’’.

‘‘Biological waste’’ is biologically degradable waste from gardens or parks, food or kitchen waste from households, restaurants, catering and retail outlets and comparable waste from food industry facilities’’. These terms however are inadequate to define the issue of food waste.

From the point of view of the term food waste generally a valid definition does not exist. It is questionable whether it is possible to find a unified definition for wasting food over the whole extent of the food chain, that is from the level of farmers to the level of households **(European Union Committee, 2014)**. The UN’s specialized agency, the Food and Agriculture Organisation (FAO) has been trying to contribute to creating a common definition, in particular due to global harmonization of the issue, improvement in the collection of data, the comparison of data and at the same time the creation of regulation measures to reduce waste. Currently the FAO defines food wastage as lowering the mass of edible food originally intended for human consumption. Wasting of food includes losses arising during manufacture, harvesting and the phase of processing of food and waste from food, which occurs at the point where retailers are involved and during consumption’ **(FAO, 2014)**.

In EU countries, as for the Czech Republic, the term food waste is not legally fixed and defined. The fixing of the term in the EU should not be undervalued, as it has an effect on the creation of the politics and quantification across all sectors of the food chain **(Östergren et al., 2014)**.

In the opinion of the European economic and social committee related to the limitation food losses and food waste it is stated that, *“food losses and food wastage can be defined as “any food originally intended for human consumption (with the exception of products which do not serve nutritional purposes), which are thrown out or destroyed at all levels of the food chain from agricultural companies to the consumer.”*

In the studies and publications of certain authors we can find and alternative terms for food losses and food waste. **Parfitt, Barthel and Macnaughton (2010)** mention that the wasting of food after harvest is usually identified as food losses. These according to him are related to a lowering of quantity or quality of food which makes them unsuitable for human consumption. In the later phases of the food chain it is more common to use the term food waste, which according to him is more related to the behaviour of consumers. We will not find terms related to food waste in either European or Czech legislation. The differentiation between food losses and waste is however also mentioned in the FAO's document Toolkit: Reducing the Food Wastage Footprint **(FAO, 2013)** and **Priefer, Jörissen, Bräutigam (2013)**:

Food loss means the depletion of the amount of food intended for human consumption, which is lost from the supply chain for differing reasons. They are usually related to the production phase, harvesting and the post harvesting manufacturing processes. Food losses are related to and caused by ineffective food chains – Infrastructure and logistics, technologies, inadequate skills, knowledge, management capacity in the subject in the food change and inadequate access to markets. In the case of food losses natural disasters also play a role.

Food waste is a subset of food losses. It concerns food which was intended for human consumption, but was thrown out because they had exceeded their expiration date or they were thrown out due to human action or inactivity. The reasons can be an excessive offering from the market or the individual buying and consumption behaviour of the inhabitant.

The scientific community differentiates between the terms avoidable, unavoidable and potentially avoidable food waste as defined by WRAP **(Quested and Johnson, 2009)**. Avoidable food waste is food and drink, which at the point at which it was thrown away was still suitable for human consumption, or would be still edible, if it was consumed in time (for instance a slice of bread, meat, apple). Potentially avoidable waste is food and drink which some consumers consume and others don’t because of preferences (for instance the crust of bread, apple peel). Unavoidable waste is related to products and raw materials which are already not suitable for consumption. These include the inedible parts of food such as banana skins, bones or egg shells, but also products which are so damaged either due to bad weather, illnesses or pests, that they cannot be consumed.

Gillick and Quested (2018) then tend to use the definition of food waste not on the basis of avoidability in three categories (as is described above), but rather on the basis of the edibility of part of the food waste which is edible and non-edible. Both parts thus form food waste. Another change in the altered definition of food waste is the fact that food, which were fed to animals (but sold for human consumption) are not considered food waste according to this altered definition any more.

The ambiguity of terms around the issue of food waste over the entire extent of the food chain from production to the final consumer leads to very different values of food losses and food waste from that the data about the economic costs connected to the disposal of waste and protection of the environment. Due to the fact that food losses are related to many technologies used during the production of food, the level of losses can be better quantified than in the case of food waste. Food waste has one basic difference – the amount of food is dependent on the behaviour of the consumers, from the behaviour of

every individual. Even though the wasting of food is a global problem, every individual is able to influence their own consumption of food and the amount of waste produced.

Quested and Johnson (2009) sum up three of the most often used methods of determining food waste in households. This is the waste composition analysis in which a community waste analysis is carried out. Then there is keeping diary recording the type of food and drink waste, their amount and reason why they become waste. The third is the subtraction method which compares the difference in the amount of sold and consumed food and drink, while the difference in their values is considered waste.

In general it can be said that the most significant reasons for limiting food waste are personal benefits because reasons of a societal and ecological character are overshadowed in daily human behaviour. The personal level of wastage comes to the fore in daily behaviour (time savings, money) and is very different based on the identifiable features of the consumer (amount of income, place of residence, age, economic activity and so on). In everyday life the Czech public realises the effects of waste primarily through statements but the result of the complex effects of throwing out food in the areas of the environment, waste disposal, social health and the ethical and economic sides are issues which do not affect them. They are more likely to be sceptical as to whether their behaviour can change the position of society as a whole.

The aim of the paper is to discover the amount of wasted food in households. To compare information on the amount of food waste from various data sources with estimates of the wasted amounts in kg per person per year on the basis of questionnaire surveys and with the results of objective checks carried out through weighing and recorded daily for a period of one month by actual households in the Czech Republic. The authors of the paper, on the basis of the information obtained during their study of the issue of food waste, see the main issue as not only in the quantification of food waste but also in recognising the behaviour of each individual, their standpoint and opinion on the issue of waste. Thus they regard it as beneficial to widen the aims of the paper by the investigation of subjective opinions of individuals to the issue of food waste and on the basis of this knowledge seek out incentives for changes in behaviour in the sense of lowering the amount of wasted food.

Scientific hypothesis

There were formulated few hypotheses which were tested with appropriate statistical methods. Hypothesis was formulated as follows:

There is no relationship between the amount of food waste and qualitative variables such as specific shopping habits or attitudes to the problem of food waste (Table 4 with results of hypotheses testing).

MATERIAL AND METHODOLOGY

Secondary data concerning the production of food waste are taken from the sources of a number of companies and organisations active in the world, European and national level. The majority of these companies use data from Eurostat and the FAO as a basic source of data. These data will be used to compare the following results concerning the wasting of food in households.

The primary data concerning consumer behaviour, their opinions and standpoints in relation to the issue of food waste was obtained in a questionnaire survey carried out in 2017 among 1582 respondents in the Czech Republic. The chosen set was a representative set of the Czech Republic from the point of view of sex and age. The questionnaire contained 21 questions relevant to the issue of food waste and 9 identification questions. A scale with 10 point steps was used for expressing opinions and standpoints on the issue of food waste. These data are regarded as subjective, as they were obtained on the basis of the subjective estimates of the respondents themselves.

Other primary data come from a diary survey that is from an objective determination of food waste in households. This investigation was carried out in 99 Czech households, for a period of one month in the period of September to October 2018. The task of the respondents was daily weighing of the amount of waste food while also including a listing of the composition of the waste food and the method of its further processing (thrown into communal waste, feeding animals, compost).

Descriptive statistical tools were used to process the primary data. After carrying out the individual analyses the results are compared and commented in light of the stated aim of the paper.

Statistic analysis

χ^2 test (1) was used to check the relationship of the amount of food waste and chosen qualitative variables. The strength of the potential dependence was expressed by the Pearson coefficient (2). The relations used are shown below:

$$\chi^2_{\alpha,(k-1)\cdot(m-1)} = \sum\sum \frac{(n_{ij} - o_{ij})^2}{o_{ij}} \quad (1)$$

$$P = \sqrt{\frac{\chi^2}{\chi^2+n}} \quad (2)$$

The SPSS Statistics and Statistica programs by Statsoft were used for work with the data. The *p*-value used to test the null hypothesis in order to quantify the statistical significance is provided in Table 4.

RESULTS AND DISCUSSION

Lowering the amount of food waste is one of the main aims of sustainable development. Determining the amount of food wasted in households and achieving a reduction of that amount, is related not only to the lack of a method of how to monitor wastage, but also the ambiguity of terms which the issue of food wastage uses. After carrying out the comparison (Table 1) of the listed results for food waste from various sources and their differences an argument merged that, the problem of food waste is not addressed just by the objectification of the quantification of waste, but it is also necessary to consider the behaviour of the individual.

Table 1 Existing data sources concerning the amount of food waste.

Countries, groups of countries	Year measured	Source	Amount of food waste	Definition	Method
Europe	2007	**FAO (2011)**	95 – 115 kg/person/year (data summarised for Europe and North America)	Food waste is only the edible part, not animal food or its parts, which are not edible, it also includes food originally intended for consumption but redirected to non-food use (animal feed, bioenergy and similar).	The estimates used data from the FAO food balance, also supplemented by literary research and estimates from the SIK institute according to the similarity of regions, the steps in the food chain and commodity categories.
EU-28	2012	**Stenmarc et al. (2016)**	92 ±9 kg/pers*	Food waste also includes the non-edible part of food (FUSIONS definition) - the edible part is estimated at 60% on average..	Data from the waste statistics of selected member states (11 states provided data), the values were later recalculated for the entire EU-28**.
EU-27	2006	**Monier et al. (2012)**, BIO Intelligence service, study for the EC	76 kg/person	Taken from catalogue waste numbers (EWC codes).	Altered EUROSTAT data and national sources.
Czech Republic	2006	**Monier et al. (2012)**, BIO Intelligence service, study for the EC	25 kg/pers/year	Taken from catalogue waste numbers (EWC codes).	Altered EUROSTAT data and national sources.
UK	2015	**Gillick and Quested (2018)**	108 kg/person (32 kg inedible part and 77 kg edible part)	The division of food waste into a inedible part (does not include wrappings) and an edible part (does not include food fed to animals, a difference compared to FUSIONS definition).	Analysis of the composition of waste in combination with food diaries.
Finland	2010	**Katajajuuri et al. (2014)**	23 kg/pers/year	Includes only avoidable waste (not unavoidable like skins, shells and similar), it includes milk among fluids .	Diaries (380 households selected from an online panel).
Denmark	2012	**Halloran et al. (2014)**	76 kg/person (42 kg edible part and 34 kg inedible part)	Only hard waste is studied, divided into its edible and inedible parts.	Analysis of the composition of waste in households.

Note: *95% reliability interval.
**the data were provided in particular by states with a higher GDP than the EU-28 average. If the amount of food waste is related to the level of GDP, then the data given are overvalued.

Table 2 The amount of food wasted weekly in households (% of respondents).

The amount of food thrown out (grams)	Questionnaire survey	Diary survey
less than 50	19.53	0.00
51 – 500	48.76	48.48
501 – 1,000	19.91	23.23
1,001 – 1,500	8.69	12.12
1,501 – 2,000	2.60	6.06
2001 and more	0.00	10.10

Table 3 Place of disposal of food waste.

Calculated values from the diary survey	The amount of food thrown out (diary survey)	Which were placed in: waste/bins	animals	compost heaps
Average (grams per household per month)	3892.1	2183.1	901.2	566.4
Total for all respondents (grams per month for all households)	385317.0	216126.3	89214.0	56074.7
Percentage*	100%	56%	23%	15%

Note: *Some of the respondent do not fill the place, where the food is frown, therefore the sum of all variants is not 100%.

Table 4 The dependence of the amount of food waste on selected variables.

Variable	*p*-value	Dependence	Pearson's chi-squared test
I buy less often and in larger amounts.	0.42675	NO	
I go food shopping regularly.	0.31627	NO	
Before buying food I check the fridge, cupboard etc., so that I can find out my needs.	0.00010	YES	39.0866
I buy in accordance with a list prepared in advance.	0.02688	YES	23.10111
Price is the most important factor for me when buying food.	0.55191	NO	
I give preference to buying large packages of food because they are cheaper per unit.	0.02589	YES	23.22268
I use discounts and I often buy foodstuffs which are discounted	0.06336	NO	
Planning purchases and the preparation of food so that nothing gets thrown out	0.00000	YES	54.83071
I consume all food bought.	0.00000	YES	132.3506
Food wastage is a current issue.	0.00000	YES	59.44576
Food waste represents a great threat to us in the future.	0.00000	YES	56.85539

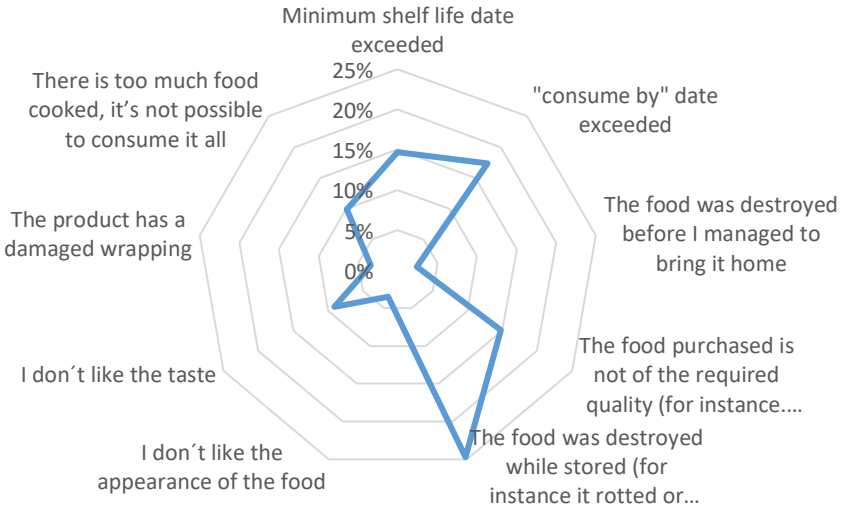

Figure 1 Causes of waste.

Data on the amount of food waste and the differences arising from these data concerning waste produced by households raises doubts about their descriptive capabilities and these inaccuracies are transferred to the estimates of the environmental and economic effects of food wastage. Even this fact does not contribute to enlightenment concerning waste and the need to lower food waste and also raises doubts about meeting the government resolutions of individual countries about the need to lower the amount of food waste by half by 2030 and so on. Data from various sources concerning the amount of food waste at household level are given in Table 1 below.

These primarily concern data which are for Europe as a whole, or for groupings in the European union and from chosen countries. Apart from the Czech Republic, there are also values shown for states such as Denmark, Finland and the UK, where the issue of food waste is given great attention. In Denmark for instance, Selina Juul, the great proponent of the fight against food waste is active. She spread the understanding about the problem of food waste and founded the Stop Wasting Food movement, a movement which is also supported by the Ministry of Environment and Food of Denmark and also The Danish Environmental Agency. Within the framework of the UK a group experts is active as part of the WRAP program, which is working on the issue of food waste as part of the framework of various sectors of the entire food chain just the same as Finland where the issue of food waste is given great attention (for instance the Wastestimator project).

The estimates by respondents about the amount of food waste produced by them and objectively determined (by weighing) amount of waste and recorded each day in household diaries are given in Table 2. The daily records of food wastage carried out over a period of a month in 99 households contained identification data (related to households), data on the amount of food wasted and data on the food composition (Table 2).

Interesting findings arise from the Table 2. When required to estimate the amount of wasted food, consumers give very underestimated amounts. Consumers have no understanding of the weight of the individual types of food wasted, most (approximately 70% of the answers) give values of 500 grams, 20% give up to 1000 grams of food thrown out per week in the questionnaire. The average amount of food waste according to the subjective estimates of respondents after recalculation corresponds to 10.5 kg per household per year. It is a value which does not even approach the levels given in literary sources.

From the survey into the amount of food waste, carried out by weighing the individual types of food wasted and recorded in the diary, it was found that the average value of the amount of waste comes out at around a value of 46.7 kg per household per year. This value differs considerably from the estimates given for the EU from literary sources (approximately 90 kg per person per year), but it corresponds to the amount of food waste reported in Finland.

The reasons for the differences of food waste amounts reported could be various definition of food waste and methods for its measurement as well as the fact that the individual who has been asked to collect data has their wastage influenced. It gets into their consciousness and they behave differently than if they had not been informed about the issue of waste and had not realised the complexity and effort which went into their production. This fact strengthens the opinion on the process and methods of influencing individuals but also society as a whole. Society must create an understanding of the issue of food waste and everything related to it.

But the behaviour of the individual when trying to lower food wastage and the costs related to their disposal is a deciding element of the protection of life on the planet.

The diaries also allowed the discovery of how the wasted food was handled. Another use was found for almost 40% of it (feeding animals and composting). Almost 60% of the total food waste ends up in mixed communal waste, which represents not only financial costs for its disposal, but this waste is also regarded as a significant source of climatic change, as the manufacture of an excessive amount of food and its potential disposal is accompanied by the production of a large amount of CO_2. The place of disposal of food waste, determined from diary records is in Table 3.

From the questionnaire survey, which focussed on the behaviour of individuals in relation to the buying, consumption and wasting of food, carried out with 1582 respondents in the Czech Republic in 2017 it was clearly found that the approach to the issue of wastage of food is shaped over the entire life of an individual with a significant influence from family and upbringing on the individual.

Is was further found from the research that more than 90% of respondents regard waste as a great threat to society, particularly due to the threat to human life on Earth and the high costs of disposal of waste. The everyday behaviour of individuals however does not correspond to these views with the exception of the segment of consumers which is formed from people aged 65 years and over, people living in the countryside, equipped with knowledge about the demands of work related to the production of agricultural products, aware of the process of their production and the necessity to connect it to nature. This segment has a positive relationship to nature and their daily behaviour is based on respect for nature. They do not consider the issue of waste; their own nature is to not waste food.

The opinions and positions on the issue of food waste taken from the answers to the questions asked which indicate daily behaviour, allowed the grouping of consumers into three segments with similar approaches to the issue of wastage. Almost all respondents from all three segments believe finding a solution to solving this significant problem is important. The segment with the greatest numbers is the one, where respondents showed an interest in the issue, for whom the problem is important, who want to learn more about the issue, speak more about waste and use the media, but have not however yet changed their daily behaviour.

These stances towards waste however do not appear in the daily behaviour towards the amount of food wasted, they continue to waste. Despite this it is a group which has a great potential to change their behaviour. They are primarily young people who have a certain understanding of life on Earth. It is necessary that an upbringing which promotes nature and life on Earth begins at the earliest possible age, both through the education system as well as

at the same time examples within the family. At school it is necessary to receive information about products which are used to feed people and within the family learn habits in relation to the approach to food. The second segment which was mentioned before, has lesser numbers and is formed of people with a relationship to nature, mostly living in the countryside for whom it is normal not to waste food. Attention must be given to the third segment which is formed from individuals of a productive age, financially secure but who don´t consider the issue of wastage and are not even interested and where the economic means of these families allow them to live in a food excess. The re-education of these individuals is very difficult, it is almost unrealistic, because in their thoughts, economic thoughts dominate and they are only willing to consider a change of behaviour in relation to a threat to their health and the quality of life on Earth.

The dependence of the amount of wasted food and the consumer behaviour of individuals in the market for food is shown in the Table 4 above.

From Table 4 it is found that the lowering of food waste is positively affected by, preparations for the purchase of food (current state of supplies, necessity, amount purchased), the approach to food as a raw material and making an effort related to its production and processing. The amount of food waste is not affected by the frequency or size of the purchases or the current price of food.

The authors **Giordano et al. (2019)** draw attention in their research on a sample of 385 households in Italy to the fact that the frequency of purchase is one of the variables that affects the amount of food waste. According to the conclusions of their study, households have more food waste that buy less often in comparison with households that buy more often. In this the authors do not agree with our results. **Giordano et al. (2018)** also concluded that the positive or negative effect of shopping for cheap food on food waste cannot be confirmed, in this respect they agree with our results since our survey shows that the purchase of food at lower prices has no effect on the amount of food waste.

However the price of food is often reffered to a significant factor affecting consumer decision process when buying food – as was also confirmed by research conducted in Slovakia where more than 80% of respondents perceived the price as the most important factor in their purchasing decision-making **(Golian et al., 2018)**.

Koivupuro et al. (2012) have found that the amount of food waste is affected not only by selected household socio-demographic indicators (such as the type and size of household), but for example also by the purchase of cheap food, their results showed that people who buy discounted food or take up special food offers produce less food waste (this explains their tendency to be more economical and save money, appreciate the value of food and throw less of it away). On the other hand, these authors did not manage to show the clear effect of shopping habits, such as shopping frequency and handling food, on the amount of generated food waste.

However it must be stressed that our outputs were created on the basis of testing hypotheses on data obtained from questionnaire research on the sample of 1582 respondents in the Czech Republic, while the conclusions of the study of **Giordano et al. (2019)** are based on the diary research. The study carried out by the team of authors **Koivupuro et al. (2012)** is based on data collection in the form of questionnaire research and the addition of outputs from the diary research. **Giordano et al. (2018)** discuss the appropriateness and reliability of the outputs of the questionnaire research used to quantify the amount of food waste in one of their older studies.

The conclusions of this older study by **Giordano et al. (2018)** show in a sample of 30 Italian households that the amount of food waste differs according to their findings – specifically speaking they compared the amount of food waste obtained from respondents from the questionnaire research, from the diary research and from waste sorting. To obtain more reliable results they are more inclined towards the method of gaining food waste data from the diary research, particularly in case that waste sorting can be used which provides objective and credible results. **Richter and Bokelmann (2017)** talk of the appropriateness of the diary research method as they carried out diary research on a sample of 25 households in Germany. Their research showed that the storage of food, shopping and waste is correlated and supplemented, that when determining a campaign focusing on creating awareness, findings about individual behaviour concerning food handling and food waste are also required.

Respondents expressed the most frequent causes of food waste with the aid of a 10 point scale. The intensity of the individual causes is shown in Figure 1 below. The most frequent reason of food waste mentioned was that the food was spoiled during storage or that the food sell by date or shelf life had expired.

Authors **Richter and Bokelmann (2017)** or **Silvennoinen et al. (2014)** came to the same conclusions stating that their studies showed that spoiled food and the sell by date or shelf life had expired as the most common reasons for the creation of food waste.

CONCLUSION

For the Czech Republic there is a favourable finding that the amount of waste food determined based on the diaries maintained on the amount and type of food wasted (46.7 kg per household per year) while not approaching the information on the amount of wasted food in EU countries (92 ±9 per person and year) but is close to the values achieved in countries who are concentrating on this issue and are successfully reducing the amount of food wasted. It is a country which pays systematic attention to the environment, landscape protection and this approach to nature is the practice of each individual. It is precisely the lower values of food waste found which the authors attribute to the fact that at the point where the respondents were requested to make daily records of food thrown out that they started to think about the issue and began to change their behaviour. Despite the importance of recognising the behaviour of individuals on the market for food, their decisive role during their daily behaviour (regardless of their opinion and stance on waste) it is not possible to rely on estimated quantification of food waste. As the comparison of the results of the estimated amount and the objectively determined amount in households showed, these data can be proven to differ significantly.

However the questionnaire survey confirmed that for most Czech households food waste is a significant issue which society should take an interest in. In the everyday behaviour of the individual however this opinion does not appear, based on the individuals behaviour it is found that "it is an overall issue" and the behaviour of the individual does not influence it or they do not realise it. From the results of the research it is clear how important the daily behaviour of the individual is and not only his opinion. The achievement of changes in behaviour requires the systematic re-education of society as a whole, beginning not only with the actions of educational institutions but also examples within the family and most importantly a change in the relationship of society to the products of nature and life on Earth.

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MULTIPLE LINEAR REGRESSION MODEL OF GOLDEN APPLE'S FAILURE CHARACTERISTICS UNDER REPEATED COMPRESSIVE LOAD

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ABSTRACT

In this paper, the multiple linear regression model of mechanical properties related to the failure mechanism of apple tissue under repeated compressive load was investigated. More refined failure characteristics may lead to improved processing and logistics aspects of the given fruits. For our study, the following failure-related factors are considered during the cyclic measurements of Golden Delicious apples: the viscoelastic parameters, the dissipated energy, and the rupture point of the cell-structure, which is described with the time to failure parameter (TTF). For the determination of viscoelastic components, the three element Poynting-Thomson body was applied, and a closed-loop control system is identified with the measured creep data. From the hysteresis loop – in each cycle of the force-deformation parametric curve – the dissipated energy can be calculated with a numeric integration method. The rupture point of the fruit tissue – where the measuring pin is breaking through the peel and the cortex – is observed with a high-framerate video analysis, so that the time index of the failure point can be evaluated. The focus is to define the influence of the mentioned factors to the TTF parameter of the examined fruit material. During the statistical evaluation of the resulted data, the failure of time can be successfully determined with a multiple lienar regression model of the determined viscoelastic and dissipated energy variables. With the resulted equation, the failure time of Golden Delicious apples can be predicted based on the measured failure-related parameters obtained during the compressive load tests.

Keywords: repeated load; fruit damage; analysis of variance; time to failure; mechanical fatigue

INTRODUCTION

Studying the damage resistance and failure susceptibility of fruits are one of the most important topics in the area of food processing and logistics. A very significant amount of the agricultural and horticultural crops never gets to the costumer, because the mechanical effects are often leading to a failure in the cell structure and consequential spoiling of the product, decreasing market value. Failure characteristics of fruit materials can be described with many different approaches. During destructive compressive tests, the stress-deformation curves of biological materials have been investigated from several aspects.

Fruits and vegetables are usually described with viscoelastic models **(Mohsenin, 1986)**, where the creeping and stress relaxation phenomenon have an important role; as **Sitkei (1986)** pointed, the creeping behaviour occurs in more technological and manipulating process (e.g. the settling of silage and granular piles, or the deformation of fruits by their dead weight), but the relaxation time is also an important factor in the area of food industry, e.g. in the case of juice industry **(Gorji Chakespari et al., 2010)**. The fruit firmness is also determined with certain parts of the relaxation curve **(Blahovec, 1996)**. The destructive fruit and vegetable researches are based on the evaluation of the force-deformation characteristics, that resulted during creep or stress relaxation tests **(Tscheuschner and Doan, 1988; Zhao et al., 2017; Miraei Ashtiani et al., 2019)**. These examinations are usually performed with different universal or custom loading devices.

Since most of the postharvest operations are affecting the crops with impact or repeated compressive mechanical forces, the laboratory tests aim to reproduce these circumstances, and observe the fruit material during its colliding or its fatigue process. Fruit-to-fruit damage or collusion with rigid materials can be examined with a pendulum impacting device **(Ferreira et al., 2008; Wang et al., 2018)**, or firmness measurements can be performed with drop tests **(Wang et al., 2009; Vursavus, Kesilmis and Oztekin, 2017)**. In other cases, the dropping of pear fruits **(Yousefi, Farsi and Kheiralipour, 2016)**, the mechanical interaction between two apples **(Ahmadi, Barikloo and Kashfi, 2016)**, or the collusion against a rigid flat plate is simulated with finite element analysis **(Dintwa et al., 2008)**.

For the reproduction of the repeated mechanical effects from various manipulations, some research already

experimented with cyclic load: **McLaughlin and Pitt (1984)** investigated a cell-rupture model during their fatigue examinations, while **Lee, Tan and Waluyo (2012)** and **Bohdziewicz and Czachor, (2016)** studied the dissipated energy during cyclic load conditions.

Mohsenin (1986) and **Sitkei (1986)** specified two typical point in the deformation characteristics of the biological materials, which are related to the mechanical failure: the biological yield, and the rupture point. The biological yield point is an initial fracture inside the microstructure of the cell system, which can lead to a more extent damaged volume, and ultimately, to spoiling. However, the biological systems are capable to regenerate, it can be a reasonable limit for the mechanical effects to stay under this value. The rupture point means a significant damage in the biostructure, which indicates the mechanical failure. It often comes with a clearly visible breaking point at the deformation graph, but in some cases – when the loading force is fast – the determination of this time instant can be more difficult.

The applied and absorbed mechanical energy is greatly responsible for the volume of the resulting fruit damage during an impact, a compression, or a vibration process **(Hussein, Fawole and Opara, 2018).** The dissipated energy is strongly related to the failure mechanism of the biological materials, which can be calculated from the hysteresis loop of the stress-strain characteristics; for the investigation of the energy indicators, the area between the loading and unloading curve is usually observed **(Ciupak and Gładyszewska, 2011; Lee, Tan and Waluyo, 2012; Diels et al., 2016)**. In case of certain apple types, the hysteresis parameters are showing a significant relationship with viscoelastic properties **(Lee, Tan and Waluyo, 2016)**. The momentum-formula is also applied for the determination of energy-balance, when the fruits are exposed to colliding during different drop tests **(Lien and Ting, 2014; Stropek and Gołacki, 2013)**.

The energy dissipation related to fatigue degradation and cracking is an active research area in other fields as well; although, the dissipated energy under the hysteresis loop can be determined in each cycles of a repeated mechanical load, it is often questioned, that it is fully or partially reversible, and whether can be linked to the damage or not **(Kim, Roque and Birgisson, 2006; Kahirdeh and Khonsari, 2015)**.

From the aspect of fruit bin and package design, the effect of transportation to the fruit piles is usually inspected during simulated examinations with vibration-pads. The most dangerous frequencies – that causing the highest volume of fruit damage – are determined with accelerometer sensors. According to the consilient report of different studies, the frequency range under 10 Hz is the most dominant, and it is responsible for the most extensive losses **(Fischer et al., 1992; Hinsch et al., 1993; Vursavuş and Özgüven, 2004)**. In order to keep the quality of piles as good as possible – besides the bin and package optimizing – an appropriate transport-planning can be a viable solution as well, if the poorly maintained road segments can be avoided **(Springael, Paternoster and Braet, 2018)**.

In the case of biological or other viscoelastic materials, the creeping curve is divided into different phases, where the last section is beginning with a crack initiation point, which is indicating the failure of the material. However, the literature is not specifying the precise determination of this occurrence, the accurate identification can be very important. Since the stress-strain characteristics of fruits are not always indicating the rupture point clearly and accurately (especially in the case of fast loading speeds, which cause a steep rising in the deformation curve), a image-processing based observation with high framerate can be set for the determination of the failure time – when the measuring pin is getting through the damaged fruit peel and cortex during cyclic compressive testing. Obtaining this parameter, our study aims to explore the influence of the dissipated energy and the viscoelastic parameters to the fatigue mechanism of the selected Golden Delicious apple texture.

Scientific hypothesis

Hypothesis H1: The time to failure (TTF) parameter of pome fruits can be described with the multivariate linear regression of viscoelastic properties and an indicator of dissipated energy.

Hypothesis H2: Our previous study reported **(Farkas, Fenyvesi, and Petróczki, 2019b)**, that the elastic modulus parameters from the viscoelastic model are not showing any dependence of the frequency in case of Golden Delicious apples. The viscous part relates to the applied frequency settings in a non-linear way, so we assume, that the frequency variables will not have any role on the determination of the failure time either.

MATERIAL AND METHODOLOGY

To perform the cyclic load measurements, the custom developed DyMaTest device is applied **(Fenyvesi, 2007; Petróczki and Fenyvesi, 2014)**. The examined apple is placed into a sand bed, where a measuring pin of 4 mm diameter is loading the peel surface in a 10 mm deformation range. The fruits were measured and modelled as a structure of peel and cortex.

Before the investigations, the creeping response of the prepared sand is tested with control measurements. In order to perform this inspection, a solid ball bearing of 32 mm diameter is loaded. The deformation graph is not showing any creeping behaviour in the measuring range of the photoelectric sensor. The sand is dried and filtered with a mesh layer-by-layer **(Pillinger et al., 2018)**, then it is compacted with a metal tamper.

For the current examinations, a cosinusoidal force-function was adjusted in the software environment of the computer-controlled instrument:

$$F_m(t)=F_{max}(1-cos(\omega t)), \quad (1)$$

where F_m is the measured cosinusoidal load function, F_{max} is the amplitude of the force (N), and ω is the angular velocity of the loading (s^{-1}).The deformation response is also cosinusoidal, and with the consideration of the creeping process, it is described with the following equation before the rupture point:

$$w_m(t)=jt+k+w_{max}(1-cos(\omega t-\delta)), \quad (2)$$

where w_m is the measured deformation function, w_{max} is the amplitude of the deformation (mm), j and k are the coefficients of the linear part of creeping (j denotes the slope and k is the y-intercept), ω is the angular velocity (s^{-1}), while δ is the phase angle difference between force and deformation.

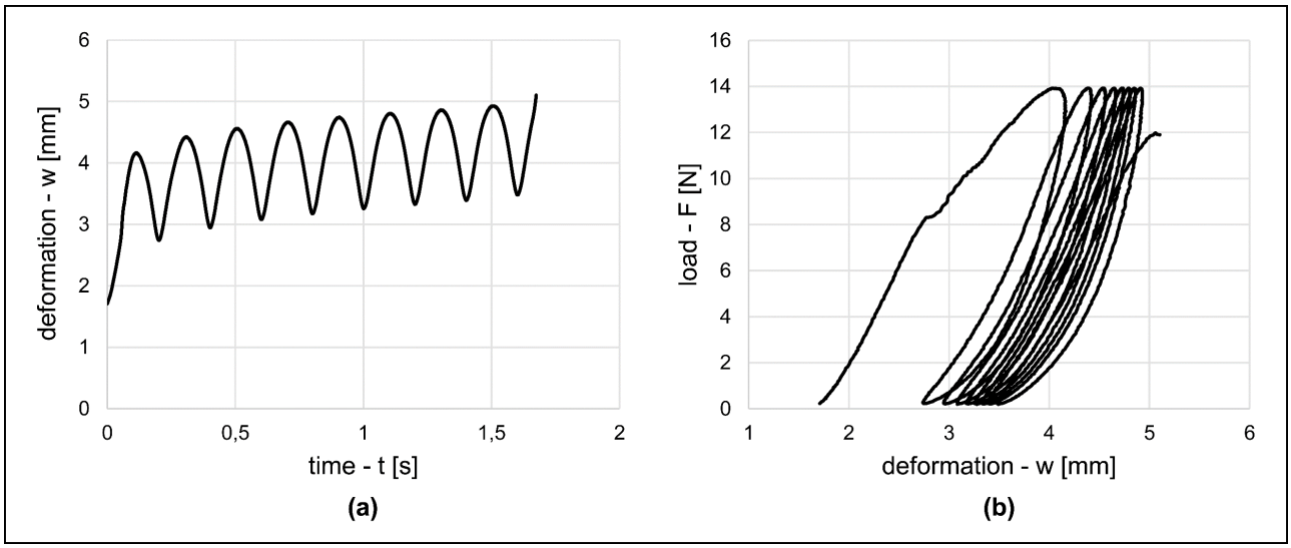

Figure 1 Typical deformation-time (a) and force-deformation (b) characteristics of Golden Delicious apple tissue.

Because of the repeated compressive examinations, the tested biomaterial is showing a dynamic creeping behaviour: while the mean value of the cosinusoidal load is constant, the curves - that envelope the maximum and minimum points of the deformation – have a similar character to the creeping during a constant load. This dynamic creeping can be seen at Figure 1 (a), where the graph of deformation as a dependent of time is presented.

The resulted deformation curves are investigated before the rupture point, where the irreversible failure of the fruit texture is certainly occurring. Because of the relatively fast rising nature of the loading curve, the breaking point is not always showing itself, so this specific occasion is determined with a camera, which is capable of a recording with 240 frames per second. The rupture moment - when the measuring pin is getting through the peel – is registered during the frame analysis (shown in Figure 2) as a time parameter and referred as 'time to failure' in the present study (TTF - **Gnedenko et al., 1999**).

Figure 1 (b) is illustrating the force-deformation characteristic of the examined apples, where the areas between the loading and unloading curves are representing the dissipated energy of each cycle. The calculation of this internal loop area is based on the following general formula:

Figure 2 Frame from the video analysis during the registration of the time to failure (TTF) parameter.

$$E_D = \int_0^T F_m \frac{dw}{dt} dt, \quad (3)$$

where E_D denotes the dissipated energy (N.mm), while T is the time period of the given load cycle (s). For theoretical calculation, inserting equation (1) and (2) into our previous formula:

$$E_D = \int_0^T F_{max}(1 - cos(\omega t))(j + \omega w_{max} sin(\omega t - \delta))dt. \quad (4)$$

For the numeric integration of equation (4), the resulted load and deformation data from the cyclic compressive test are available as lookup-table inputs in a Simulink block diagram. During the evaluation of the measurements, the ratio of dissipated energy **(Delgadillo and Bahia, 2005)** is calculated:

$$E_{DR} = \frac{\sum_{i=0}^{n} E_{Di}}{E_{Dn}}, \quad (5)$$

where E_{DR} is the ratio of dissipated energy (-), $\sum_{i=0}^{n} E_{Di}$ denotes the sum of dissipated energy at the given load cycle (N.mm), and E_{Dn} is the dissipated energy of the last calculated cycle (N.mm).

The peak value of this ratio is used to mark the inner rupture of the fruit material (Figure 3): since this maximum value is clearly connected to the failure mechanism of the crops **(Farkas, Fenyvesi and Petróczki, 2019a)**, it must be included during the correlation analysis.

The viscoelastic behaviour of the examined Golden Delicious apples is investigated with a Simulink-based control loop system. For the identification, the presented measurement data before the rupture point of the fruit material is applied. The measured deformation (wm) and the calculated (w) output of the mathematical model are displayed and compared in one scope (Figure 4).

The Poynting-Thomson body contains two elastic components (E1, E2 – $N.mm^{-1}$), and one viscous part (η – $Ns.mm^{-2}$). The three-element visoelastic structures are the simplest models, that can handle the creep phenomenon of the given material – where the constant stress is causing an

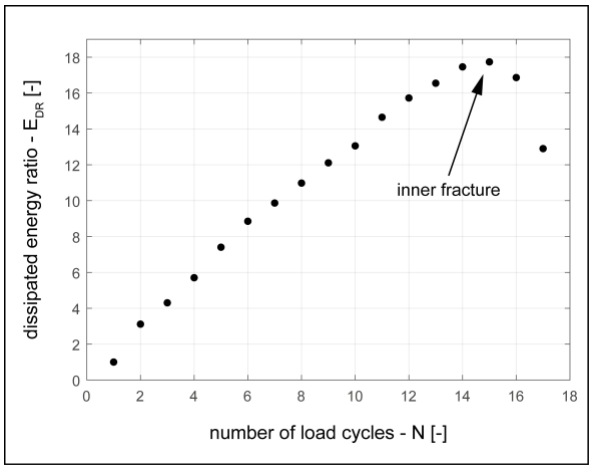

Figure 3 Energy peak related to inner fracture during the fatigue process of Golden apples **(Farkas, Fenyvesi and Petróczki 2019a)**.

increasing trend in the deformation process. With the control loop system and a minimum search method, the three viscoelastic parameters can be determined with the best fit of the calculated deformation to the actual deformation curve **(Farkas, Fenyvesi and Petróczki, 2019b)**.

For our study, Golden Delicious apples were chosen for the experiments, which is representative at the Hungarian food producing and the consumer market.

The measurements were performed on 25 Golden Delicious specimens. Each fruit was loaded in 6 different spots, using different frequency settings, so the described viscoelastic parameters and dissipated energy values were evaluated in 150 cases overall.

The samples were stored in an ambient laboratory environment (~25 °C, with the relative humidity of ~60 RH%). The fruit sample parameters were: ~160 g (±30 g), density: ~0.875 g.cm^{-3} (±0.03 g.cm^{-3}). The measurements were performed within the confines of few hours, so degradation and further ripening could be neglected during the investigations.

According to the literature, the most dangerous frequency values were reported under 10 Hz during the transportation process **(Fischer et al., 1992; Hinsch et al, 1993; Vursavuş and Özgüven, 2004)**, so the frequency values were adjusted in this range: the steps were divided for nearly identical intervals (2.5, 3.7, 5, 7.5, 10, and 11.6 Hz, respectively) set by practical options on the measuring device.

Statistic analysis

For the statistical analysis of the measurement data, we used the SPSS statisctics 25 software, where the linear regression tool is applied. To describe the failure time as a function of the measured dissipated energy parameters and the components of the Poynting-Thomson model, multiple linear regression models were examined. For the verification of these models, we used the analysis of variance (ANOVA) method.

RESULTS AND DISCUSSION

Besides the resulted parameters from the compressive load tests, the applied frequency vales are also considered during our investigations.

From the viscoelastic properties, the viscous part is clearly the most important parameter in the characterization of the failure mechanism. Our previous study showed, that only these values are standing in strong correlation with the frequency settings, but this trend has a non-linear nature **(Farkas, Fenyvesi and Petróczki, 2019b)**. From the aspect of linear description of failure time, the frequency parameters did not appear in any model suggestions (presented in Table 1).

The relationship between the E_{DRmax} values and the TTF parameter is representing the connection of the inner damage of cell structure and the rupture point. This relation could add another important aspect to the model to improve the approximation of the failure behavior in case of pome fruits.

From these variables, there are four regression model is resulted with different coefficients of determination (shown in Table 1). The elastic response of the fruit tissue must determine the failure process as well: in model 3, E_1 modulus is improving the fit considerably, so this elastic parameter is reasonable to include into the characterization. However, E_2 is also increasing the R_2 in model no. 4, the difference between the two options is so small, that this variable just means an unnecessary to describe the elastic behavior – the E_1 is representing it for the given material

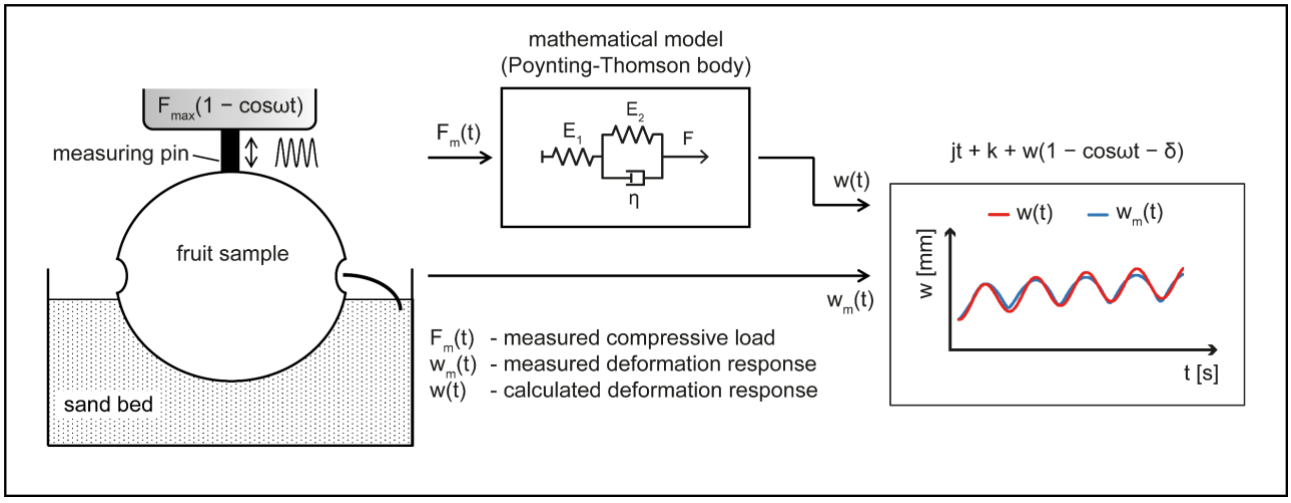

Figure 4 Identification of the Poynting-Thomson viscoelastic model **(Farkas, Fenyvesi and Petróczki, 2019b)**.

already. Considering these standpoints, model no. 3 is chosen with the following coefficients:

$$TTF = 0.533 + 2.736\eta + 0.141E_{DRmax} - 0.261E_1 \quad (6)$$

We verified the validity of our model with the analysis of variance: the significance level is $p < 0.05$, so the resulted model is applicable for the quantitative description of the investigated phenomena (shown in Table 2).

Figure 5 (a) is showing the relationship between the measured time to failure parameters and the predicted values from the applied regression model, while (b) is representing the categories of relative difference, where all the 25 specimens were divided. Most of the examined samples fall into the 5 – 10% range of error, the average of difference is 6.20% in the case of 13 apples. This group is followed by the category of error under 5%, where the average value is 2.61%, the data were obtained from 6 samples. 5 of the investigated apples are showing 12.81% relative difference, and 1 sample resulted 20.22% during the comparison of the dependent variable. This consequence about the relative error is composed of three different aspects:

1. The coefficients of the Poynting-Thomson model were also determined with a regression method during the parameter identification **(Farkas, Fenyvesi and Petróczki, 2019b)**. Although the R_2 values were fell into the 0.967 – 0.998 range when the measured creep data was compared with the Simulink-based visoelastic model, the relaitve error of this approximation cannot be calculated.
2. The registration of the time to failure parameter was performed in a dynamically changing section during the rupture process: the frame analysis also carrying the possibility of error, since the sample rate has its own limitations.
3. The dissipated energy calculations were pointing to the calculation of the E_{DRmax} value, which is indicating the inner rupture of some level, but the accurate background of this

Table 1 Linear regression model summary.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.902(a)	.814	.812	1.03413	.814	641.502	1	147	.000
2	.963(b)	.927	.926	.64922	.113	226.984	1	146	.000
3	.971(c)	.943	.941	.57824	.015	39.039	1	145	.000
4	.972(d)	.945	.943	.56840	.002	6.064	1	144	.015

Note: a Predictors: (Constant), η; b Predictors: (Constant), η, EDRmax; c Predictors: (Constant), η, E_{DRmax}, E_1; d Predictors: (Constant), η, E_{DRmax}, E_1, E_2.

Table 2 ANOVA summary of the linear regression model.

Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	794.762	3	264.921	792.307	0.000
Residual	48.483	145	0.334		
Total	843.245	148			

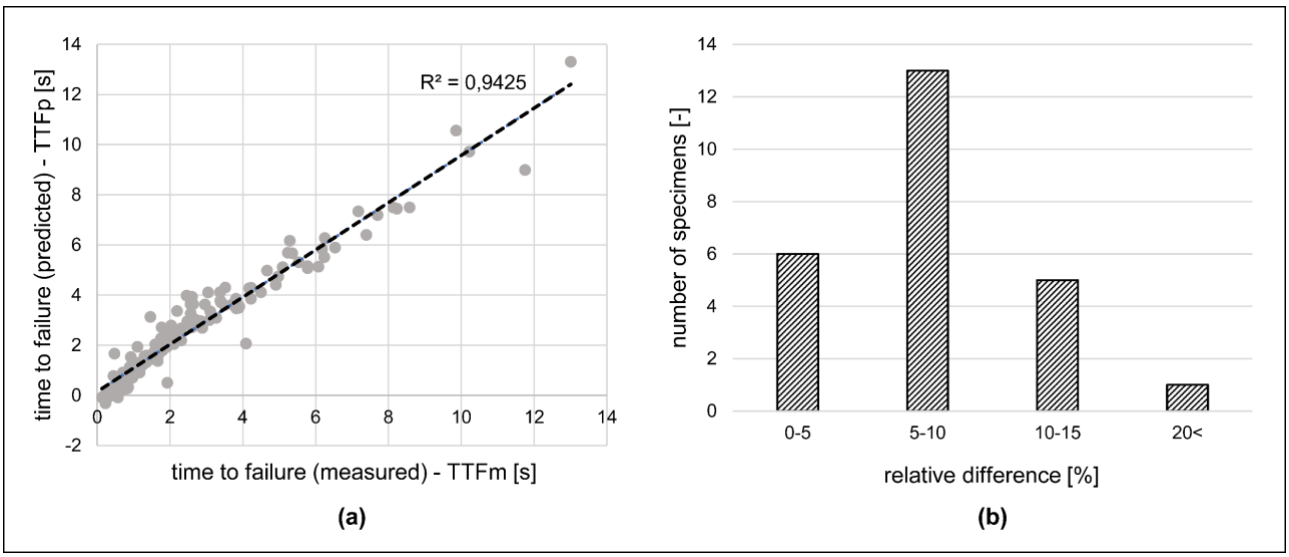

Figure 5 Relationship betweeen the measured and calculated TTF parameter in case of 150 tests (a). Number of specimens in different relative error categories in case of 25 examined apples (b).

relationship is still waiting for more investigations. While the circumstances of the cracking propagation e.g. in the area of asphalt pavement is more explored **(Sangpetngam, 2003)**, the role of the dissipated energy ratio in the cellular level is a future plan to investigate in case of different fruit materials.

In the case of our study, the following answers were formed for our hypotheses:

1. A multiple linear regression model with certain visoelastic parameters and a dissipated energy indicator can describe the failure time of Golden Delicious apples with an overall relative difference of 7.22%
2. The frequency settings in the range of 2.5 – 11.6 Hz are not influencing the failure time in the resulted linear regression model.

The resulted equation is a simple linear description of the Golden apples' failure mechanism, which can be applied for further transportation studies or cyclic load simulations.

CONCLUSION

Viscoelastic modelling and dissipated energy calculations are commonly applied methods in the studies about damage susceptibility of different fruits: the parameters from these approaches can be related to the failure mechanism separatel. In our research, we have extended on the previous approaches to supply variables for a multiple regression model at the same time.

The visoelastic coefficients were determined with a custom developed control-loop method, while the dissipated energy calculation was successfully implemented from another research area to the present investigations of fruit materials.

The novel frame-by-frame image analysis allows to gather direct information from a rapidly changing process, supplementing the mathematical approach for registering the rupture phenomenon. Based on the measurement results from the developed methods, our novel, multiple linear regression model can describe the time to failure occasion in case of the examined Golden Delicious apples in the most important frequency range. Also, the presented model can describe the failure characteristics with an acceptable relative difference. It is suggested, that the model can be used for further fruit types. The paper presents a simple three-element material description, but the failure mechanism can be characterized in further experiments, where the visoelastic approximation can be extended with more complex variables.

The current focus of the compressive load settings (which is aimed to reproduce the most dangerous frequencies during the transportation) can also be extended with other load circumstances from other manipulating stages of the food processing chain.

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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR RAPID DETECTION OF *L. MONOCYTOGENES* IN MEAT

Yuliya Yushina, Anzhelika Makhova, Elena Zayko, Dagmara Bataeva

ABSTRACT

There is a continued need to develop improved rapid methods for detection of foodborne pathogens. Rapid and sensitive methods for enumeration of *Listeria monocytogenes* are important for microbiological food safety testing purpose. The aim of this project was to evaluate a commercial loop-mediated isothermal amplification (LAMP) based system with bioluminescence, named as 3M™ Molecular Detection Assay (MDA), was validated for the detection of *L. monocytogenes* in food products with a standard GOST 32031-2012 method as reference. The results of this study revealed that a commercial LAMP-based method performed equally effective compared with method, showing from 94% to 100% specificity and sensitivity, respectively. The LAMP-based method was shown to be rapid and reliable detection technique for *L. monocytogenes* present at low numbers (10 CFU.g^{-1}) on raw meat and meat products and can be applicable in meat industry. Thus, compared with the microbiological method based GOST 32031-2012, the LAMP assay is a relatively rapid and highly sensitive method for detecting *L. monocytogenes* and will facilitate the surveillance for contamination of *L. monocytogenes* in food. The 3M MDS result and culture-based detection (GOST 32031-2012) did not differ significantly (p >0.05) regarding the number of positive samples.

Keywords: meat; *L. monocytogenes* detection; LAMP-method

INTRODUCTION

Listeria monocytogenes is a pathogen that causes the severe foodborne disease such as listeriosis **(Swaminathan and Gerner-Smidt, 2007; Warriner and Namvar, 2009)**. There is approximately 1600 illnesses and 260 deaths each year are due to listeriosis in USA **(Centers for Disease Control and Prevention, 2014)**. European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) reported that in 2008 – 2016 in Europe an increasing trend of human listeriosis cases was observed with 2536 cases of which 97.7% were hospitalized and 16.2% were with case fatality **(EFSA and ECDC, 2017)**. A major concern for processors of risk food products is a survive, multiply; persist under harsh conditions in food processing environments **(Gandhi and Chikindas, 2007; Carpentier and Cerf, 2011)**. *L. monocytogenes* can occur in raw or processed foods that are contaminated during processing. **(Koreňová and Oravcová, 2011; Bogdanovičová et al., 2015)**. It is estimated that more than 99% of human listeriosis results from consumption of contaminated food, particularly ready-to-eat (RTE) foods, such as dairy products, smoked fish **(Allerberger and Wagner, 2010; Koch et al., 2010)**.

Indrawattana et al. (2011) reported that 15.4% from 104 of the raw meat samples collected from supermarkets and open markets in the Bangkok metropolitan area were contaminated with *L. monocytogenes*. In Morocco, for instance, *L. monocytogenes* was present in 2.3% of 426 poultry and red meat samples collected in 2008 **(Ennaji et al., 2008)**.

The official methods for the detection of this pathogen in foods, are based on culture techniques **(Law et al., 2015)** are reliable but present disadvantages, such as time-consuming and lengthy. This is a major drawback of particular importance for food products with short shelf-life, for performing outbreak analysis, and for self-monitoring in production plants **(Garrido-Maestu et al., 2017; Shan et al., 2012)**. The appearance of molecular methods, such as the Polymerase Chain Reaction (PCR), and real-time PCR (qPCR), has allowed to overcome these limitations. More recently, isothermal DNA amplification approaches are gaining interest, being among the most popular loop-mediated isothermal amplification (LAMP). It presents several advantages over PCR/qPCR, such as being performed at constant temperature or having higher specificity due to the present of a several number of primers **(Abdulmawjood et al., 2016; Wang et al., 2015)**. LAMP

can be monitored in real-time by measuring the increase in fluorescence of DNA binding dyes **(Seyrig et al., 2015)**.

The objective of this study was to evaluate the performance of a commercial loop-mediated isothermal DNA amplification (LAMP) based method with bioluminescence named as 3M™ Molecular Detection System (MDS) for the detection of *L. monocytogenes* in raw meat and ready-to-cook (RTC) meat products using the 3M™ Molecular Detection Assay (MDA). The study was conducted for the detection of low inoculum levels of *L. monocytogenes* in comparison to the GOST 32031 method to validate LAMP-based method.

Scientific hypothesis

LAMP-based system provides rapid and reliable results for the detection *L. monocytogenes* in raw meat and meat products and can be applicable in meat industry. Sensitivity and specificity shold be more than 90%. Kappa-value should be more than 0.85.

MATERIAL AND METHODOLOGY

Raw meat (beef, pork) and meat products (RTE, RTC) were selected as objects of study. Pork ground and beef ground samples were used for artificial contamination and further detection of method sensitivity threshold. All samples were purchased at a local supermarket in central region of Russian Federation from September 2018 through March 2019.

Cultures preparation

L. monocytogenes ATCC 35152 NCTC 7973 and ATCC 13932 serovars 4b (from American Type Culture Collection (Manassas, VA, USA), were activated in 10 mL of tryptone soya broth (TSB, Oxoid, England) for 24 h at 37 °C. The cultures were centrifuged (Eppendorf, Germany) at 3000 g for 10 min, washed twice with 0.1% (w/v) peptone water (Oxoid, England), and resuspended in 1 mL of 0.1% (w/v) peptone water (Oxoid, England), and then mixed (1:1, v/v) to prepare a 2-strain cocktail (10_1 and 10_2 CFU.mL^{-1}). Before inoculation, the counts of prepared 2-strain cocktail of *L. monocytogenes* diluted in 0.1% (w/v) PW (Oxoid, England) were enumerated by spread plating an aliquot of 100 µL on tryptone soya agar (TSA, Oxoid, England) in duplicate and incubating TSA plates at 37 °C for 48 h to estimate the inoculum levels.

Inoculation procedure

There were two inoculation levels for matrix: a high inoculation level of approximately 100 CFU.g^{-1} and a low inoculation level of approximately 10 CFU.g^{-1}. Also was used uninoculated samples as negatives controls.

L. monocytogenes detection with a commercial lamp-based system

The detection of *L. monocytogenes* cells by the commercial LAMP-based kit (3M Molecular Detection Assay *Listeria monocytogenes*; 3M) was performed according to the manufacturer's manual. Briefly, 25 g of sample were mixed with 225 ml Demi-Fraser broth (3M, USA). Then 20 µL of UVM enrichment was added to a tube with lysis solution. The mixture was warmed in a heat block (Germany, IKA) at 100 °C for 15 min, followed by immediate cooling at room temperature in a chilling block (3M, USA) for 10 min. After mixing by inversion, 20 µL of this lysate was mixed with the pellet in the reagent tube from the assay kit. The reagent tube was placed in a molecular detection system (3M, U.S.A.) for the detection of *L. monocytogenes* cells via isothermal amplification and bioluminescence for 75 min. All analyses included negative and reagent controls to validate the performance of the molecular detection system.

Detection *L. monocytogenes* by GOST 32031

The samples were examined for the presence of *L. monocytogenes* bacteria in accordance with GOST 32031-2012. 25 g of the meat was homogenized in 225 cm^3 of Demi-Fraser broth (Merck, Germany) and incubated at 30 °C for 24 hours. Then 0.1 cm^3 the enriched culture was added to 10 cm^3 of Fraser's broth (Merck, Germany) and cultured at 37 °C for 48 hours. From each broth after the end of incubation with a 3 mm loop, the enriched material was streaked onto a chromogenic agar for *Listeria* (Agar *Listeria* according to Ottaviani and Agost (Merck, Germany)) and selective nutrient agar for *Listeria* PAL (FBUN GNC PMB, Russia) and incubated at 37 °C within 24 – 48 hours. On chromogenic agar for *Listeria*, *L. monocytogenes* grows in the form of blue colonies with an area around; on PAL, brown colonies with black halos. Colonies typical of the genus *Listeria* and *L. monocytogenes* were seeded on tryptone soya agar with yeast extract (TSAYE) and incubated at 37 °C for 18 – 24 hours, which was then confirmed using biochemical tests (Oxoid, England).

Statistic analysis

Sensitivity and specificity of the commercial LAMP-based kit for the detection of *L. monocytogenes* were defined as the number of samples truly positive (T_{pos}) and truly negative (T_{neg}), respectively, compared with the GOST method. The sensitivity, specificity, and accuracy of the commercial LAMP based kit were calculated as follows:

$$\text{Sensitivity} = [T_{pos} / (T_{pos} + F_{pos})]$$

$$\text{Specificity} = [T_{neg} / (T_{neg} + F_{neg})]$$

where T_{Pos} and T_{Neg} are the number of positive and negative samples, respectively, confirmed by both the GOST and commercial LAMP-based kit, and F_{pos} and F_{neg} are the number of positive and negative samples, respectively, confirmed by the commercial LAMP-based kit. Kappa value of concordance, describing the statistical agreement between the two detection methods was calculated, as described. Kappa values were classified as follows: 0.01 indicated no concordance; 0.1 to 0.4 indicated weak concordance; 0.41 to 0.60 indicated clear concordance; 0.61 to 0.80 indicated strong concordance; and 0.81 to 1.00 indicated nearly complete agreement.

A chi-square test (AOAC, Official Methods of Analysis Program Manual) for significant difference was used to determine whether the proportion of positive samples was different between the 3M MDS and the GOST-method.

RESULTS AND DISCUSSION

L. monocytogenes can multiply over a wide range of pH and osmolarity, at low temperatures, and both under aerobic and anaerobic conditions, this is a particular concern and necessitates control along the food chain.

A wide variety of culture and alternative methods have been developed in order to detect or quantify this pathogen in food. In this study, the effectiveness of the commercial LAMP-based kit was evaluated in comparison to the standard culture GOST method for quickly detection of *L. monocytogenes* on different food matrices as artificially contained so and naturally contained.

In this study, a 2-strain cocktail of *Listeria monocytogenes* was used for the detection sensitivity threshold.

L. monocytogenes detection in artificial contamination samples

At the inoculum levels of 10_1 and 10_2 CFU.g^{-1} both methods abled the detection of *L. monocytogenes* in all samples (Table 1), resulting in 100% specificity and sensitivity (kappa value 1). At the inoculum level of 10_0 CFU.g^{-1} (not uninoculated *L. monocytogenes*), both methods were unable to detect *L. monocytogenes*. False negative results were not obtained.

L. monocytogenes detection in samples with native microflora (raw meat and meat products, purchased in local supermarkets)

The results samples show high specificity of the LAMP-method (not less than 90%) (Table 2). The 3M MDS result and culture-based detection (GOST 32031-2012 method) did not differ significantly (p >0.05) regarding the number of positive samples.

In similar studies, the high specificity and sensitivity of the method on artificially infected matrices has also been proven. *L. monocytogenes* was detected in 11 samples of pork by LAMP – method and in 10 samples by GOST 32031. One sample was not confirmed according to the reference method and was identified as false-positive (F_{pos}). The sensitivity of the method in the study of pork samples was 90% (Kappa value 0.93), specificity – 100%.

In another study the LAMP-GNP/DNA probe assay was applied to the detection of 200 raw chicken meat samples and compared to routine standard methods. The data revealed that the specificity, sensitivity, and accuracy were 100, 90.20, and 97.50%, respectively **(Wachiralurpan et al., 2018)**.

Also, in the study of 32 samples of beef ready-to-cook products, 1 false positive result was found by the LAMP method. The sensitivity and specificity of the method were 88% and 100%, respectively, with a Kappa-value of 0.92.

Table 1 Comparison of LAMP-based method and GOST-method for the detection *L. monocytogenes* in artificial contamination samples.

Level Inoculated (CFU.g^{-1})	Matrix	LAMP and GOST		LAMP		Sensitivity (%)	Specificity (%)	Kappa
		T-pos	T-neg	F-pos	F-neg			
10_0	Raw ground beef	0/5	0/5	0/5	0/5	100	100	1
	Raw groud pork	0/5	0/5	0/5	0/5	100	100	1
10_1	Raw ground beef	5/5	5/5	5/5	5/5	100	100	1
	Raw groud pork	5/5	5/5	5/5	5/5	100	100	1
10_2	Raw ground beef	5/5	5/5	5/5	5/5	100	100	1
	Raw groud pork	5/5	5/5	5/5	5/5	100	100	1

Note: T-pos, T-neg are true positive and negative samples, confirmed by both GOST and LAMP-based methods; F-pos and F-neg are false positive and negative samples, confirmed only by LAMP-based technique or GOST method, respectively.

Table 2 Comparison of LAMP-based method and GOST-method for the detection *L. monocytogenes* in native contamination samples.

Food matrix	LAMP and GOST		LAMP		Sensitivity (%)	Specificity (%)	Kappa
	T-pos	T-neg	F-pos	F-neg			
pork	10	25	1	0	90	100	0.93
beef	5	18	0	1	100	94	0.88
RTC pork	3	16	0	0	100	100	1
RTC beef	8	24	1	0	88	100	0.92
RTE	1	16	0	0	100	100	1

Note: T-pos, T-neg are true positive and negative samples, confirmed by both GOST and LAMP-based methods; F-pos and F-neg are false positive and negative samples, confirmed only by LAMP-based technique or GOST method, respectively.

In this case a false positive result could be caused by the DNA amplification on injured or sub lethally cells that cannot be detected by ISO **(Lim et al., 2015)**. Another explanation for false positive results is the using of 4 to 6 primers with a much higher concentration in the LAMP method than in the classical methods based on PCR. In terms of efficiency, the PCR and real-time PCR assays could detect *L. monocytogenes* based on the listeriolysin O gene (*hly*) with a detection limit of 8 – 10 CFU **(Rip and Gouws, 2009)**. However, these assays required sophisticated equipment and post-amplification manipulations that took more time to obtain results **(Gianfranceschi et al., 2014)**.

This could lead to an increase in the possibility of non-specific amplification caused by forming primer dimers **(Wang et al., 2015)**. 23 samples of beef were analysed. 5 positive and 18 negative results were detected and confirmed by both research methods (GOST and LAMP). However, 1 sample was false negative by LAMP-based method compared with GOST 32031-2012. The sensitivity of the method in the study of beef in this case was 100% (Kappa value 0.88), and the specificity – 94%. In other studies, false negative results were also obtained **(Lim et al., 2015)**. These authors showed that 1 naturally contaminated sample of duck wings was presented as false-negative. The validation study also showed 91% sensitivity and 95% specificity, Kappa-value 1.

In the study of 19 ready-to-cook pork samples and 17 meat ready-to-eat products, no significant (p >0.05) differences in the results obtained by the LAMP and GOST 32031-2012 methods were found. The sensitivity and specificity of both methods was 100% with Kappa-value 1. Such a convergence of the two methods can be associated with an enough viable cells of *L. monocytogenes* in the sample to identify them.

Several false-positive and false-negative results were obtained at low levels of inoculum (10_1 CFU/10 cm_2), for the LAMP method have been reported **(Mikš-Krajnik et al., 2015)**. It also reports at a 10_2 CFU/100 cm_2 microbial cell level, both methods were suitable for detection of *L. monocytogenes* and had 100% specificity and sensitivity.

In another study the LAMP method was employed to test 94 retail food samples effectively. Sensitivity in detection of *L. monocytogenes* by the LAMP was higher than that of PCR and none of the conventional method positive samples was missed by the LAMP method **(Shan et al., 2012)**.

Listeriosis outbreaks were seen in many countries including Japan, the United States and countries of Europe **(EFSA, 2011; Miya et al., 2015; Self et al., 2019)**. Human infections caused by *L. monocytogenes* have become a global health concern. The presence of *L. monocytogenes* in processing environment at slaughterhouses, deli meat factories or in retail may be a reason of cross-contamination. *Listeria monocytogenes* can contaminate various foods via food processing environments and contamination of raw materials. Hence, there is a necessity a variety of methods for rapid detection of foodborne pathogens as it is required in many food analyses.

In this study rapid LAMP method of *L. monocytogenes* detection was performed. Despite the having of false-negative and false-positive results, LAMP-based method was effective and easier to perform than some of standardized assays and has the advantage to reduce analysis time (less 2 days comparing with 5 days GOST method). Furthermore, this technique is 10 times more sensitive than the conventional PCR assay as reported **(Wachiralurpan et al., 2017)**. The occurrence of false-positive results can be reduced by preventing cross-contamination, high humidity and temperature when working with reaction mixture tubes **(Bird et. al.,2013, Wang et al., 2015)**.

CONCLUSION

The results of this study revealed that a commercial LAMP-based method performed equally effective compared with method, showing from 94% to 100% specificity and sensitivity, respectively. The LAMP-based method was shown to be rapid and reliable detection technique for *L. monocytogenes* present at low numbers (10 CFU.g_{-1}) on raw meat and meat products and can be applicable in meat industry. Loop-mediated isothermal amplification (LAMP) has become a powerful alternative to polymerase chain reaction (PCR) for pathogen detection in food matrices.

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INVESTIGATION OF THE PROCESS OF PRODUCTION OF CRAFTED BEER WITH SPICY AND AROMATIC RAW MATERIALS

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ABSTRACT

This scientific work demonstrates the stages of the process of inspiring the spicy aromatic raw materials of Badian, which is added to the craft beer in the process of its digestion. In addition, the work shows an analysis of the composition of spicy aromatic raw materials which will be used as an additive. The research proves the rational quantity and concentration of alcoholic spiro-aromatic raw materials for beer and determines the effect of alcoholic spiro-aromatic raw materials on beer indices. We have clarified the organoleptic and physicochemical parameters of beer with spicy aromatic raw materials Badian and composition based on infusions of Badian and cinnamon. As a result, we received water-alcohol infusions of spicy aromatic raw materials and developed new types of beer on their basis. On the basis of the conducted studies, the regression equation of the dependence of the content of actual dry substances and the volumetric fraction of alcohol from the change in the amount of spray-aromatic raw material and alcohol concentration in the alcohol-alcoholic infusion of spin-aromatic raw materials was obtained. In addition, we conducted calculations on the cost-effectiveness of adding these types of spiced aromatic raw materials to beer.

Keywords: crafted beer; Badian; cinnamon; spicy aromatic raw material; infusions

INTRODUCTION

Beer is a popular drink in many countries of the world due to its taste and aroma, especially among young people. Today, the technology of beer production is aimed at the development of new varieties with the addition of non-traditional vegetable raw materials, which give the drink specific characteristics of taste and increase the demand for products **(Basařová et al., 2010; Punčochářová et al., 2015)**.

In addition, beer, made with the addition of plant material, has improved organoleptic and physicochemical characteristics and a longer expiration date.

By adding antioxidants of plant material to beer, we can reduce the oxidative and toxic effects of alcohol on the human body. Non-harmful antimicrobial and antifungal substances of natural origin, in the case of adding to beer, may improve its qualitative parameters and prevent contamination by microorganisms and the use of preservatives in the production of beverage **(Hucker et al., 2016; Chaya et al., 2015; Ganbaatar et al., 2015)**. Beer with added spices has been known for a long time. In the past, adding spices helped to preserve the freshness of the beer, disguised any odd flavours and even prevented the beer from spoiling. Herbs served as a replacement for hops, adding bitterness and a specific astringency to beer. Now, on the contrary, spices, and herbs are used to produce beer with an unusual taste and aroma. They are added in the form of aqueous-alcoholic infusions during the boiling mash, during fermentation, in the period of digestion or just before bottling. In the production of such beer, both light and dark wheat malt can be used. To achieve the best result in the production of beer with herbs and spices it is necessary to withstand finished products for 2 – 3 months. During this time, beer acquires a richer aroma of added ingredients, it increases the content of alcohol and disappears sharp flavor **(Mascia et al., 2016; Salailenbi Mangang, Das and Deka, 2017)**.

Cinnamon, coriander, ginger, nutmeg, pepper, vanilla, various herbs, flowers, coniferous plants, as well as mixed compositions of berries and fruits are the most widely used in the production of beer **(Kábelová-Ficová et al., 2017; Sukhenko et al., 2019a)**.

In recent years, the importance of creating alcoholic beverages with the use of medicinal infusions of essential oils has increased: lemon, mint, sage, and others. Such infusions not only improve the organoleptic characteristics of products, but also enrich them with biologically active substances, which makes them useful for human health **(Omelchuk and Golovchenko, 2011; Baert et al., 2012)**.

This research seeks to explore the recent development of the craft beer industry, using brewing industry as a prism for development elsewhere in Ukraine. The research is

conceptual in nature. A conceptual approach has been taken for three reasons, firstly, it allows engagement with the research in order to introduce new concepts. Secondly, engagement with the research through conceptual frameworks enables people to see issues in a way they had not previously, and thirdly, findings from such research can help broaden our understandings about the kinds of solutions that should be considered and are most appropriate to pursue to enhance development of the craft brewing industry.

Scientific hypothesis

The scientific hypothesis is to create a new product – craft beer with the addition of infusion of spicy-aromatic raw material of almonds, which will lead to a drink with pronounced organoleptic characteristics. Scientific work involves the possibility of adding and composition of several spicy-aromatic additives in the form of infusions. This will allow us to get a harmonic drink with natural ingredients with a long shelf life.

MATERIAL AND METHODOLOGY

We chose Badian and cinnamon as an additive to beer. These spices are rich in essential oils, flavonoids, micro, and macro elements and have a positive effect on the human body **(Romanova, Pribylsky and Tertytsia, 2008)**.

Extraction of spicy-aromatic raw material is recommended by the method of infusion (maceration) since it does not require complicated and expensive technological equipment, skilled personnel, easy to carry out for mini-breweries **(Sukhenko et al., 2019b)**. For the extraction of components of spicy aromatic raw materials, we chose water-alcohol solutions, as they are the best solvents of essential oil compared to water **(Sukhenko et al., 2017)**. Spicy aromatic infusions are recommended to be introduced into beer before digestion **(Lazarov and Zheplinska, 2017; Zhosan and Zheplinska, 2018)**. Determination of the organoleptic parameters and the content of dry matter in the spices and aromatic raw materials were determined according to the method described in state standard of Ukraine 4705:2006 **(DSTU 4705:2006)**, which is based on the determination of the refractive index of the test solution after alcohol distillation.

Determination of the alcohol content in the prepared water-alcohol solutions was carried out by an aerometric method based on measuring the concentration of ethyl alcohol by the hydrometer in the distillate obtained after distillation of alcohol from the investigated infusion **(DSTU 4705:2006)**.

Determination of organoleptic parameters of the finished beer was carried out in accordance with state standard of Ukraine **(DSTU 7103:2009)**.

The transparency of beer was determined in a glass in which it is pre-poured and viewed in passing light.

The aroma and taste of beer were determined organoleptically, immediately after the sample was placed at a temperature 12.0 ±2.0 °C in the tasting glass.

Determination of physicochemical indices of infusions and prepared beer was carried out on the beer analyzer PBA-B generation M (Figure 1) **(Shakhvorostova and Zheplinska, 2018)**.

Statistic analysis

Mathematical and statistical processing of the experimental data was carried out in the determination of Kohren, Fisher and Student's criteria using full-factor experiment and using the least squares method. The accuracy of the data obtained was determined using the Kokhren criterion, and the adequacy of the mathematical model was verified using the Fisher and Student criteria. The values were evaluated through mean and standard deviation by Microsoft Excel 2013.

RESULTS AND DISCUSSION

First of all, we have prepared aqueous-alcohol solutions with concentrations of 45, 50 and 55% for the research. To do this, we took distilled water and alcohol 96.3%. We added 10 g of the chopped pelican in 0.9 m_3 of aqueous-alcoholic solutions. So, we prepared 9 samples for infusion, which was carried out within 14 days.

The organoleptic parameters of infusions are determined according to **DSTU 4705:2006**. In our case, the infusion of spicy-aromatic raw materials which had the parameters presented in Table 1.

According to **DSTU 4705:2006** the appearance of the infusion should be transparent, without external inclusions; color, taste, and aroma are inherent in the plant material from which they are made, without any taste and smell.

Figure 2 shows a change in the content of dry matter in bulk with the use of spicy and aromatic raw materials for 14 days.

As can be seen from the graphs presented in Figure 2, on the seventh day of infusion there is a noticeable increase in the content of dry matter in each of the infusions. Looking at the results obtained on the content of dry matter in virtually different concentrations with Badian, we can talk about their maximum values when reaching 8 days of insistence. However, after seven days of infusion, the dry matter content is also large and, compared with the first day, it increases by 45% and 50% of infusions by 1.5% by weight and for a 55% infusion of 2.0% by weight. Further tightening is inappropriate, because there is no increase in the content of dry matter, and sometimes such an action leads to a decrease in its value.

For conducting experimental studies on a mini brewery, a young beer that wandered for 7 days at a temperature of 8 – 10 °C was selected for this purpose: for this, a beer was made of 100% light malt and prepared beer wort with a concentration of dry matter of 11.0%, for the digestion of which was used the yeast S-23, which belongs to the germicidal yeast. For the chilling of the wort, bitter hops Tetnanger and aromatic honey Salaz were used.

In experimental samples of young beer, various volumes of water-alcohol infusions of Badian were added. Drinking was carried out in the laboratory for 7 days at +6 ºC, and then another 7 days at +2 ºC.

Ready-made samples of beer were compared among themselves by organoleptic and physicochemical indicators.

Table 2 shows the concentration and amount of alcohol – alcoholic spices and aromatic raw materials for different

beer samples, including the control sample. The organoleptic and physicochemical indices of beer with the addition of water-alcoholic tincture of Badian are given. In Table 4 can be observed a similar decrease in the pH value. The low pH value contributes to the diacetyl digestion, which positively affects the quality of the finished beer. Regarding the results of the actual extract, the content of dry matter in the initial wort and the content of alcohol, we see an increase in their values compared to the control due to the addition of a certain amount of water-alcohol infusion, so that alcohol is added to the samples and their concentration increases.

Compared to the control sample, the color of beer becomes more intense due to the transition of the colorants of Badian to beer. The actual degree of fermentation increases, the maximum value of which is 65.21% for sample 17 when added 55% water-alcoholic infusion. All samples of beer, except for one, have a higher actual degree of fermentation, which satisfies the requirements for beer.

The actual digestibility values obtained for 17 samples are larger, and therefore they all show better results than the control sample. However, sample 14 with a score of 23.3 can be considered the best because it feels a pleasant aroma and harmonious taste with an excellent tasting score and with a small amount of added extract.

The nature of the change in the pH value can be seen in Figure 3, the minimum value of which we have at 50% water-alcohol infusion.

In Figure 3 it is possible to see a change in the value of the actual extract, respectively, for infusions of Badian, from which it is evident that the addition of 0.002 m^3 of water-alcohol infusion of Badian to beer leads to an increase in the actual extract observed for all three concentrations of infusion. Further increase in the amount of infusion is not feasible due to a slight increase or even a decrease in the value of the actual extract.

In Figure 3 and Figure 4 depicts the graphs of alcohol content in beer depending on the amount of added alcoholic beverages of spices and aromatic substances – for ginger, parsley and nutmeg.

As can be seen from Figure 4, when adding from 0.001 to 0.003 m^3 of 55% alcoholic water-alcohol ginger to beer gives the same value of alcohol content, which is 4.69% vol. And when you add 45 and 50% of water-alcoholic infusions, the alcohol content is lower, but is in the area of the required values for beer in accordance with state standard of Ukraine 3888:2015 **(DSTU 3888:2015)**. Increasing the amount of infusion from 0.0015 m^3 is not feasible, as it leads to sharp jumps in alcohol content.

In Figure 5 it can be seen that the addition of Bayard extracts in different amounts and concentrations leads to an increase in the total alcohol content, and the tendency to increase this value at a higher concentration of aqueous-alcohol solution is clear. The content of alcohol with the addition of infusions in the amount of 0.0035 m^3 in all cases is the largest and is: at a strength infusion of 45% vol. – 4.5%; 50 and 55% vol. – 4.58% and 4.66% respectively; in the control sample – 4.14% by volume. The increase in the alcohol content is due to the addition of alcohol from alcoholic beverages to Badian.

After a detailed analysis of the organoleptic and physico-chemical parameters of the beer samples obtained during the use of infusions, it was found that the best are: infusion of Badian with a strength of 50% by volume added to the beer in the amount of 0.002 m^3.

The consumer, choosing one or another beverage (and especially beer), increasingly focuses on certain criteria, the main of which are organoleptic qualities, the content of natural ingredients, the health effect and the convenience of packaging. Raw materials of Ukraine are rich in natural, environmentally friendly vegetable spices and aromatic raw materials, which may be an alternative substitute for hazardous food additives of synthetic origin. Therefore, the issue of studying the chemical composition of plant spices and aromatic raw materials and technological aspects of its use in beverage technology is relevant **(Techakriengkrai et al., 2012; Sheiko et al., 2019)**.

To create a composition of beer, we selected a spicy aromatic infusion of Badian and cinnamon. The conducted researches allowed to establish rational amounts of data added to 0.002 m^3 of young beer substances, namely: Badian in the amount of 0.002 m^3 50% water-alcohol solution and cinnamon in the amount of 0.0025 m^3 45% water-alcohol solution.

Table 5 shows the organoleptic characteristics of beer, with the addition of the abovementioned mix. It demonstrates that the beers look the same, whilst the aromatic characteristics contribute to give the beer a clear taste and freshness, with notes of the anise tree. By taste, this composition differs from the control sample with the harmony and flavor of cinnamon. According to the tasting assessment, the composition of Badian and Cinnamon received
23.5 points, in contrast to the control sample, which is lower by one point.

According to the physical and chemical parameters of the finished beer, the sample obtained at 0.11 units showed less value of the pH value, which is indicated by an increase in the shelf life of such beer. The value of the actual extract decreased by 20% (from 5.82 to 4.65% by weight), which suggests the best of beer fermentation. The content of dry matter and alcohol has increased due to the addition of extracts of water and alcohol solutions. The color in the sample of beer with the composition of badyan and cinnamon extracts increased by 4.7% due to the transition of colorants in the infusion. The actual degree of fermentation has increased by 25%.

Consequently, on the basis of the results obtained from the organoleptic and physico-chemical parameters, it is possible to draw conclusions about the expediency of using the composition of spiced aromatic raw materials (Badian and cinnamon) in beer technology. Ready-made drink has pleasant organoleptic properties, necessary physical, chemical and functional qualities. Expanding the assortment of beer using a composition of spicy aromatic vegetable raw materials will saturate the modern market with healthy food.

Figure 1 Beer Analyzer PBA-B Generation M.

Table 1 Organoleptic parameters of infusion of Badian.

Parameter	Ginger		
	45% of alc.	50% of alc.	55% of alc.
Appearance	Transparent with brilliance		
Color	Dark brown		
Taste	Spicy, without any additional tastes		
Aroma	Spicy, without any additional tastes		

Table 2 Concentration and amount of alcohol-alcoholic infusion of spin-aromatic raw materials in beer samples.

Sample	The concentration of the aqueous-alcoholic solution of spin-aromatic raw materials, %	Amount of water-alcohol solution of spiced-aromatic raw materials, m3
1	45	1.0
2		1.5
3		2.0
4		2.5
5		3.0
6		3.5
7	50	1.0
8		1.5
9		2.0
10		2.5
11		3.0
12		3.5
13	55	1.0
14		1.5
15		2.0
16		2.5
17		3.0
18		3.5

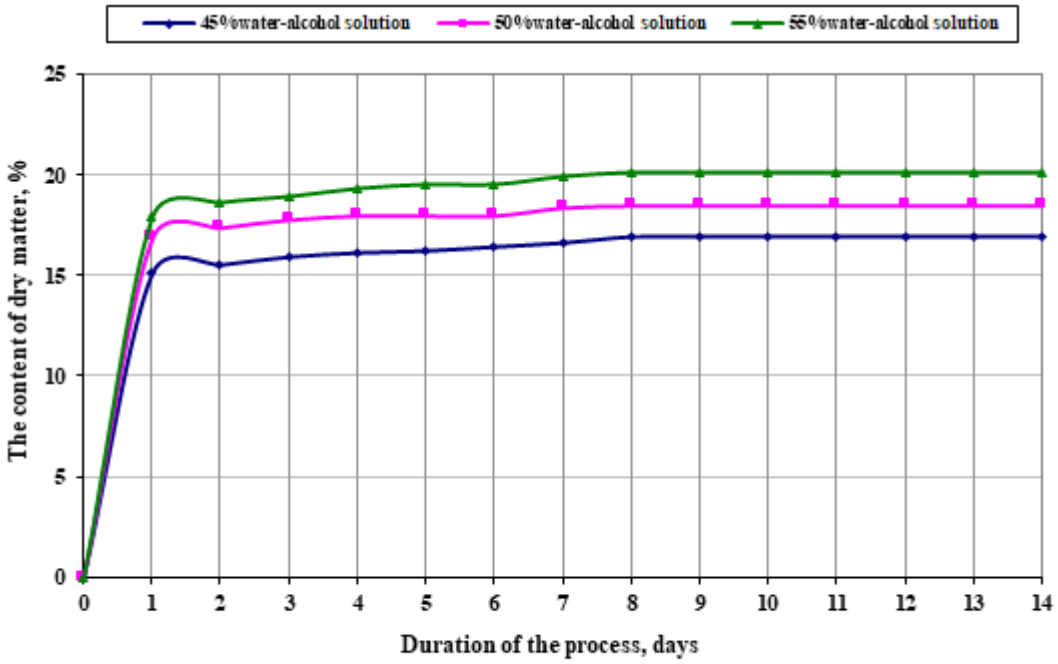

Figure 2 Change in the content of dry matter from the duration of infusion for Badian respectively in Table 2 and Table 3.

Table 3 Organoleptic parameters of beer samples with the addition of water-alcohol tincture of Badian.

Sample	Appearance	Aroma	Taste	Tasting, score
Controlling	Opaque foamy liquid	As in a regular light beer	Hop flavor, soft bitter bitterness, no scent of Badian	22.8
1	Same	Slight Badian aroma	Same	22.8
2	-«»-	Same	-«»-	22.8
3	-«»-	-«»-	-«»-	22.5
4	-«»-	-«»-	-«»-	22.8
5	-«»-	-«»-	-«»-	22.7
6	-«»-	-«»-	Harmonious, tangible taste of Badian, soft hops' bitterness	22.8
7	-«»-	The clear fragrance of Badian	Same	22.0
8	-«»-	Same	-«»-	21.3
9	-«»-	-«»-	-«»-	23.5
10	-«»-	-«»-	-«»-	23.5
11	-«»-	The clear fragrance of Badian	-«»-	22.5
12	-«»-	-«»-	-«»-	22.0
13	-«»-	-«»-	-«»-	21.7
14	-«»-	-«»-	-«»-	21.7
15	-«»-	-«»-	-«»-	21.7
16	-«»-	The vigorous scent of Badian	The vigorous scent of Badian	21.5
17	-«»-	Same	Same	21.5
18	-«»-	-«»-	-«»-	21.5

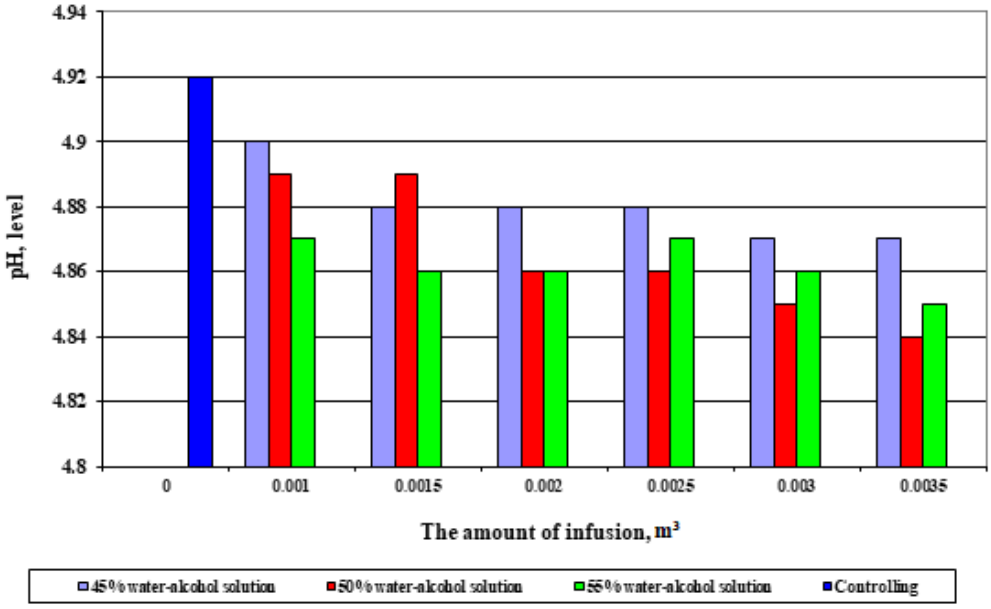

Figure 3 Changing the pH value from the amount of added water-alcohol infusion for Badian.

Table 4 Physico-chemical parameters of beer samples with the addition of water-alcohol infusion of Badian.

Sample	pH	Extractive, %		The content of dry matter in the initial wort, %	Alcohol		Color, EBC	The actual degree of digestion, %
		Visible	Actual		% of the mass	%		
K	4.92	2.53	4.04	10.39	3.24	4.14	18.9	62.37
1	4.90	2.61	4.15	10.56	3.28	4.19	20.8	62.06
2	4.88	2.58	4.15	10.68	3.35	4.28	18.9	62.52
3	4.88	2.68	4.24	10.75	3.34	4.27	26.9	61.96
4	4.88	2.65	4.21	10.75	3.35	4.28	19.0	62.16
5	4.87	2.48	4.11	10.97	3.51	4.49	20.8	63.84
6	4.87	2.51	4.16	11.02	3.52	4.50	21.7	63.66
7	4.89	2.57	4.11	10.56	3.30	4.21	19.5	62.37
8	4.89	2.49	4.07	10.69	3.39	4.33	19.0	63.24
9	4.86	2.46	4.07	10.78	3.44	4.39	21.9	63.58
10	4.86	2.43	4.08	10.97	3.53	4.51	20.7	64.16
11	4.85	2.46	4.13	11.12	3.59	4.58	34.5	64.20
12	4.84	2.39	4.08	11.19	3.65	4.66	26.5	64.88
13	4.87	2.56	4.12	10.64	3.34	4.26	19.1	62.61
14	4.86	2.58	4.15	10.74	3.37	4.31	20.3	62.68
15	4.86	2.56	4.17	10.89	3.44	4.40	23.9	63.06
16	4.87	2.44	4.09	10.96	3.53	4.50	20.8	64.08
17	4.86	2.37	4.09	11.31	3.71	4.74	18.9	65.21
18	4.85	2.52	4.19	11.18	3.59	4.58	19.2	63.87

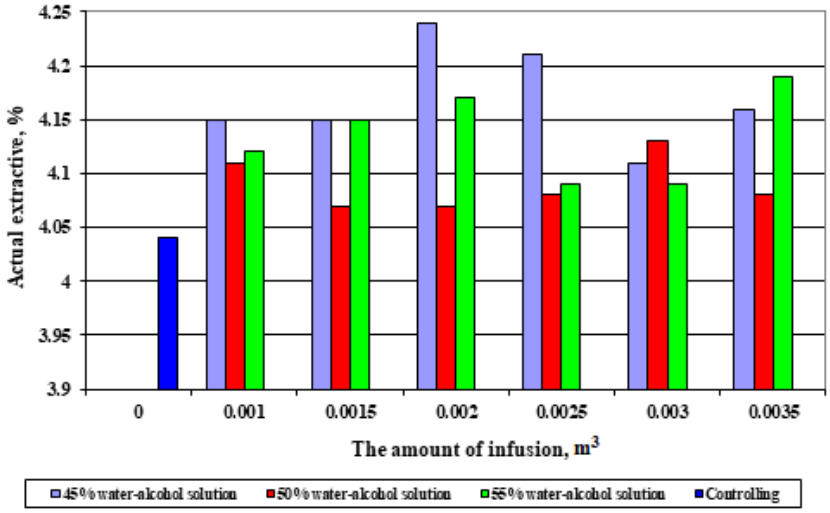

Figure 4 Dependence of the actual extract on the amount of added water-alcohol infusion for Badian.

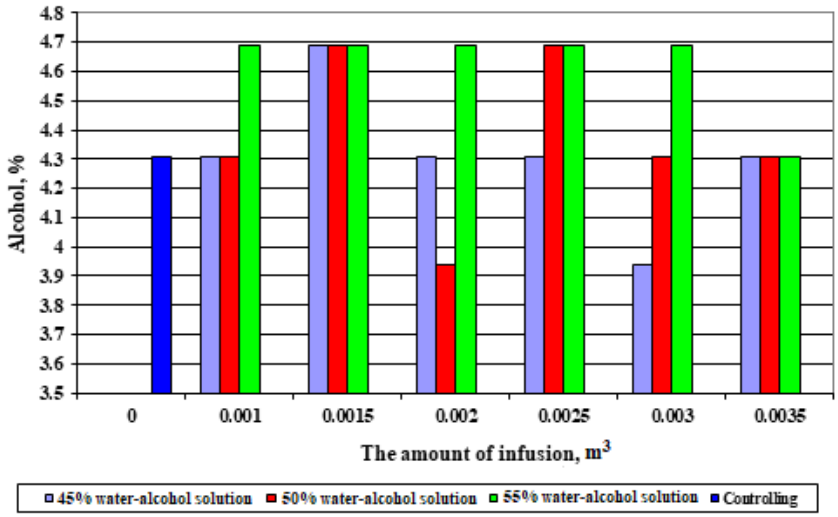

Figure 5 Dependence of alcohol content on the amount of added water-alcoholic infusion for Badian.

Table 5 Organoleptic parameters of beer with the addition of composition.

Sample	Appearance	Aroma	Taste	Tasting, score
Controlling	Opaque foamy liquid	Weak yeast flavor	Saturated, sweet, residual rough hops' bitterness	22.8
Composition	Same	Clear fresh taste with Badian aroma	Full, harmonious smell of cinnamon	23.5

Simulation of the infusion process was carried out using a complete two-factorial experiment **(Kolyanovska et al., 2019)**. On the basis of these experiments, the equation of the dependence of the content of the actual dry substances in the beer within the limits of the amount of the amount of Badian infusion from 0.001 to 0.0035 m3, the concentration of alcohol in the water-alcohol solution of Badian from 45 to 55% with an average relative error of 0.36%.

We got the alcohol dependence equation in beer within the limits of changing the amount of infusion Badian from 0.001 to 0.0035 m3, the concentration of alcohol in a water-alcohol solution Badian from 45 to 55% with an average relative error of 0.06%.

The economic effect of the sale of beer with the present in comparison with beer without them will be for beer with:

- Badyan – 0.08 $ for 10L;
- the composition of infusions of Badian and cinnamon – 0.1 $ for 10L.

CONCLUSION

It is established that the addition of tincture of Badian positively affects the organoleptic characteristics of beer, gives it a pleasant taste and aroma. Adding infusions of spices and aromatic substances leads to a decrease in the pH, increase the content of alcohol and color. Adding spicy aromatic raw materials to beer allows you to get the finished product, which will contain biologically active substances such as essential oils, vitamins, essential amino acids, micro-and macro elements, colorants that provide a pleasant taste and aroma, have a prophylactic act and increase the shelf life of beer. The conducted researches allow giving the following recommendations for the addition of tincture of spices and aromatic raw materials to beer:

- 50% water-alcohol infusion of banyan in the amount of 0.002 m3 to 2 m3 of young beer after 8 days of insistence;
- Badian – in the amount of 0.002 m3 prepared in 50% aqueous alcohol solution (10 g/dal) and cinnamon – in the amount of 0.002 m3, prepared in 45% water-alcohol solution (12.5 g/gave) – for the composition.

The obtained regression equations will allow to determine in beer the content of actual dry substances and the volume fraction of alcohol in the amount of changes in the amount of spices and aromatic raw materials intakes from 0.001 to 0.0035 m3 and the concentration of alcohol in them from 45 to 55% by volume. The economic effect of adding spices and aromatic raw materials to beer and the expediency of using such infusions for obtaining craft beer is established.

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ORYCTES RHINOCEROS LARVA OIL SUPPLEMENTATION IMPROVES TISSUE ANTIOXIDANT STATUS IN CHOLESTEROL-FED RATS

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ABSTRACT

Experimental evidence from previous study has demonstrated the hypolipidemic effects of *Oryctes rhinoceros* oil (ORO) when fed as a supplement to a cholesterol-based diet. Due to renew interest in the consumption of insect derived oil, the present study was designed to elucidate the effect of *Oryctes rhinoceros* oil (ORO) supplementation in comparison to vitamin E on oxidative status in some tissues of rats fed a cholesterol-based diet. Forty (40) Swiss albino rats were divided into 4 groups (n = 10) and maintained on a basal diet (cholesterol free as control), a cholesterol-based diet (5% cholesterol as cholesterol), a cholesterol-based diet supplemented with ORO (cholesterol + ORO) and a cholesterol-based diet supplemented with vitamin E (Cholesterol + vit E) for 10 weeks. Animals in the cholesterol group had a significantly (p <0.05) higher malondialdehyde (MDA), conjugated diene and nitric oxide concentrations in the serum, liver, heart, kidney and lung compared to control, cholesterol + ORO and cholesterol + vit E groups. Tissue glutathione (GSH) concentration was significantly (p <0.05) higher in rats fed cholesterol-based diet supplemented with ORO and vitamin E compared to those fed cholesterol-based diet alone. Xanthine oxidase activity was significantly (p <0.05) reduced in tissues of rats fed ORO and vitamin E supplemented diets compared to cholesterol rat group. In addition, catalase and superoxide dismutase activities in the various tissues examined were significantly (p <0.05) higher in both ORO and vitamin E supplemented groups compared to the cholesterol group. No significant difference was observed between animals fed ORO and vitamin E supplemented diets. These results showed that *Oryctes rhinoceros* larva oil exhibited similar protective effects to vitamin E against diet-induced oxidative stress in rats. In addition, data from this study showed that *Oryctes rhinoceros* larva oil possessed antioxidant property. Overall, the potential nutritional benefit of *Oryctes rhincoceros* larva oil consumption on cardiovascular health could possibly involve its ability to upregulation of cellular antioxidant defense mechanisms.

Keywords: edible insect; *Oryctes rhinoceros* larva oil; cholesterol-based diet; oxidative stress; antioxidant

INTRODUCTION

Palm oil remains the most traded vegetable oil globally as it constitutes over 50% packaged products in supermarkets. However, in order to sustain the increase demand for palm oil or palm oil-based products several hectares of land are cleared yearly for palm plantations. The world population is estimated to exceed 9 billion by 2050 **(Teoh, 2010)**. This no doubt portends a serious challenge in terms of food security and environmental issues. Specifically, an astronomical increase in the demand for palm oil or palm-based product is expected. Thus, there is an urgent need to evaluate other nutrient sources for feed production. The use of edible insects for feed is widely viewed as a healthier and more sustainable potential solutions to overcoming the challenge of food security **(Van Huis, 2013)**. Insects are rich sources of valuable nutrients such proteins, fat, vitamins, minerals and energy. Compared to oil palm trees insects have better yield per hectare due to their high rate of proliferation and short life cycle, requires less space and can be reared on agro-waste stream.

Thus far, research on the utilization of edible insects for animal feed production has focused on their protein content. Nevertheless, insects contain between 5% to 40% (% dry matter, DM) oil with a better fatty acid profile compared to palm oil **(Womeni et al., 2009)**. In Europe, the use of animal-based protein with the exception of fish for animal feed production is restricted. However, there exist no prohibition on the use insect oil. Research on the use of insect oil in animal feeding trial is scanty. A recent study by **Belghit et al. (2019)** demonstrated that liver triacylglycerol level was reduced in freshwater Atlantic salmon fed insect meal and insect oil compared fish fed the control diet. Similarly, **Oluba et al. (2008a)** reported that the supplementation of *O. rhinoceros* oil with a cholesterol-based diet in rats resulted in improvement in serum lipid profile and reduced susceptibility to atherosclerosis.

The highly lipid soluble radical, nitric oxide ($NO^{\cdot}$) diffuses readily through cellular membranes, interacting with other radicals including superoxide, peroxide etc thus potentiating their actions **(Pacher, Beckman and Liaudet, 2007; Birben et al., 2012)**. Nitric oxide radical is also capable of simultaneously reacting with superoxide radical to form peroxinitrite radical **(Pacher, Beckman and Liaudet, 2007)**. Peroxinitrite radical is a very reactive 1thiolesters group of cysteine and methionine residues in peptides and proteins are potential oxidizing targets for peroxinitrite. Unregulated generation of free radicals in the body has been implicated in the pathogenesis of tissue damage in several diseased conditions such as ageing, diabetes, cardiovascular diseases, etc **(Uttara et al., 2009; Valko et al., 2007)**. Dietary fats in the form of cholesterol are considered to be important in the initiation of free radical production in these clinical disorders **(Nevin and Rajamohan, 2006)**. These free radicals subsequently attack and breakdown membrane phospholipids and thus trigger lipid peroxidation **(Sevanian and Hochstein, 1985; Thomas et al., 1990)**. Several dietary modifications including the inclusion of antioxidant such as vitamin E have been shown to ameliorate the attendant susceptibility of biological molecules to lipid peroxidation in humans and laboratory animals **(Farombi and Nwaokeafor, 2005; Adefegha et al., 2014)**. Reports from several studies have demonstrated that dietary oils improve plasma lipid profile as well as affect lipid peroxidation and antioxidant parameters in rats **(Oluba et al., 2008a; Oluba et al., 2008b; Celebi and Utlu, 2006)**.

The consumption of *Oryctes rhinoceros* (palm beetle) larva as a delicacy is a common practice in Nigeria especially in the Southern part of the country where palms are cultivated on a commercial scale. *O. rhinoceros* larvae feeds on decaying organic matter (palm logs, manure and rubbish dumps). These larvae are either eaten raw, boiled, fried or roasted and are sometimes used as meat substitute in the preparation of stews and soups. Reports on the proximate composition of O. *rhinoceros* larva from Nigeria by several investigators have shown that it contains as much as 38% oil (by dry weight). with high level (65%) of unsaturated fatty acids. The oil being majorly composed of unsaturated fatty acids has been shown to be hypercholestrolemic in action **(Oluba, Josiah and Fagbohunka, 2014)**. Presently, insect oil is gaining prominence in the scientific field as well as increased acceptability among consumers. Hence, there is urgent need to intensify scientific efforts on the possible nutritional and health benefits of insect oil. Therefore, this study was carried out to investigate the effects of *Oryctes rhinoceros* oil (ORO) supplementation in comparison to vitamin E on tissue oxidative status in rats fed a cholesterol-based diet.

Scientific hypothesis

Oryctes rhinoceros oil protect rat tissue against lipid peroxidation.

Oryctes rhinoceros oil possesses antioxidant activity.

MATERIAL AND METHODOLOGY

Insect material

Live *Oryctes rhinoceros* larvae were collected from decaying palm trees at Igoba village near Akure (Nigeria). The larvae were transported to the laboratory in an open plate within 2 h of collection. They were authenticated and identified at the Department of by a zoologist at the Department of Biology, University of Benin (Nigeria). The larvae were rinsed with distilled water before being anaesthetized by freezing. The frozen larvae were thaw at 37 °C and oven dried at 50 °C for 72 h. The dried larvae sample was powdered using a mechanical grinder and the powdered sample kept in an air-tight container at 4 °C for further analysis.

Oil extraction

Oil from the powdered larvae sample (100 g) was extracted using Soxhlet apparatus using hexane as solvent. The extracted oil was dried and stored in a dried dark air-tight container and refrigerated until required for further analysis.

Animal care and ethical consideration

This study was approved by the Animal Ethics Committee of the Department of Chemical Sciences, Joseph Ayo Babalola University, Ikeji-Arakeji, Nigeria and was conducted in compliance to NIH guidelines for the care and use of laboratory animals **(ILAR, 1985)**. Forty (40) male Swiss albino rats (weighing between 50.5 – 55.1 g) aged 6 weeks were purchased from the Department of Biochemistry, University of Ibadan (Nigeria) and used for the study. The animals were housed in wooden cages with raised wire-gauze floors at a temperature of 25.7 ±2.3 °C and a relative humidity of 45% – 60%, with 12 h light/dark cycles.

Experimental diet

Diet formulation and preparation followed the prescription of the American Institute of Nutrition. The formulated diets were designated: control, cholesterol, cholesterol + ORO and cholesterol + vit E. The composition of the respective diet is as shown in Table 1. Prior to the feeding experiment animals were conditioned to the laboratory environment for a period of 2 weeks. All through the 10 weeks feeding trial, animals in each group were given food and water *ad libitum.* Food intake and body weight were recorded daily. At the end of the 10-week feeding experiment, the animals were fasted overnight, weighed and euthanized with chloroform and sacrificed by cervical dislocation. The blood from the rats was rapidly collected by direct heart puncture into plain sample bottles, and the serum was prepared and stored at -4 °C until it was required for analysis. The liver, heart, kidneys and lung were quickly excised, washed with ice-cold phosphate buffered saline, freed of fat, weighed and stored separately at -4 °C until required for further analysis.

Biochemical analyses

Weighed portions of liver, heart, kidney and lung were separately minced with scissors and homogenized in solution (1:2 w/v) containing 0.15 M KCl and 3 mM EDTA, pH 7.4 in ice. The homogenates were diluted 4-folds and centrifuged at 10,000 x *g* at 4 °C for 15 min. The supernatant was decanted and used for the various analyses. Lipid peroxidation was determined by the method described by **Buege and Aust (1978)** while conjugated diene level was determined spectrophotometrically following the method of **Recknagel and Glende Jr. (1984)**. Nitric oxide (NO) concentration was estimated in terms of its stable metabolic product, nitrite, using Griess reaction as described by **Kang, Bansal and Mehta (1998)**. Xanthine oxidase (XOD) was determined according to the method described by **Litwack et al. (1953)**. Reduced glutathione (GSH) level was estimated according to the method of **Moron, Depierre and Mannervik (1979)**. Catalase (CAT) activity was assayed according to the methods described by **Aebi (1984)**. Superoxide dismutase (SOD) activity was determined following the method of **McCord and Fridovich (1969)**. The method of **Gornall, Bardawill and David (1949)** was adopted in protein determination.

Statistic analysis

Results are mean ± SEM of 10 determinations. Statistical comparison of means was by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using SPSS version 20. p <0.05 was considered significant.

RESULTS AND DISCUSSION

Food intake and body weight

Rats fed cholesterol-based diet consumed significantly (p <0.05) higher amount of food compared to control rats fed normal rat diet (Table 2). Mean weekly body weight gain was significantly (p <0.05) in rats fed cholesterol-based diet only compared to control and those fed cholesterol-based diet supplemented with ORO and vit E. No observable significant (p >0.05) difference was seen in rats fed ORO supplemented diet and vit E supplemented diet in terms of their mean weekly body weight gain (Table 2).

Lipid peroxidation parameters

Lipid peroxide concentration as determined by tissue malondialdehyde concentration was significantly (p <0.05) elevated following feeding with cholesterol. However, when rats were fed cholesterol + ORO and cholesterol + Vit E diets, serum, liver, heart, kidney and lung MDA concentrations were significantly (p <0.05) reduced compared to its level in rats fed cholesterol diet only. Serum, heart, kidney and lung MDA concentrations in cholesterol + ORO was not significantly (p >0.05) different from that in cholesterol + vit E fed rats (Figure 1a). Conjugated dienes concentration in the serum, liver, heart, kidney and lung in rats fed cholesterol diet was significantly (p <0.05) higher compared to rats fed cholesterol + ORO and cholesterol + vitamin E diets (Figure 1b). Nitric oxide (NO) level in the serum and lung of rats fed cholesterol diet supplemented with ORO and vitamin E was significantly (p <0.05) lower compared to the observed level in rats fed cholesterol diet alone (Figure 1c).

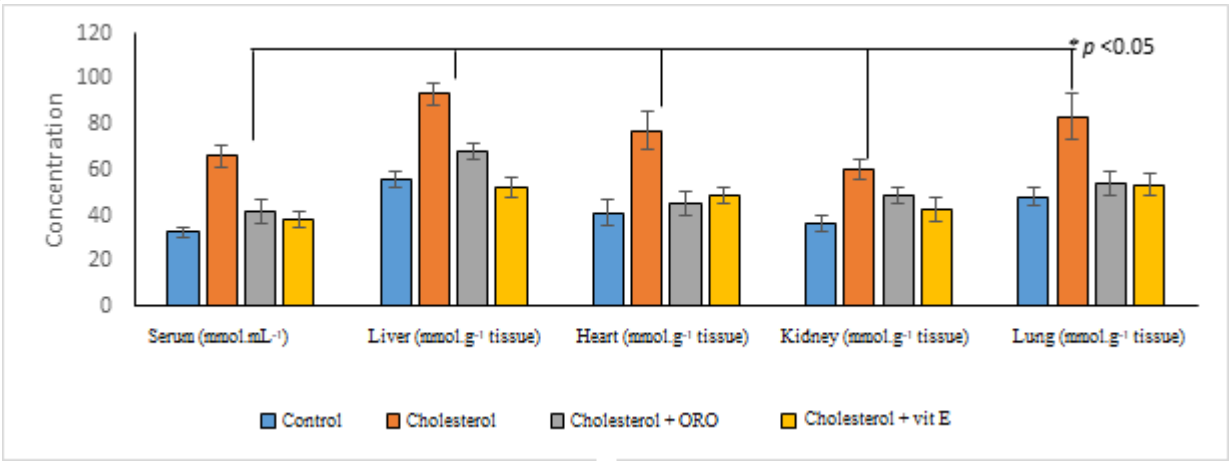
Antioxidant parameters

Xanthine oxidase activity was significantly (p <0.05) lower in rats fed ORO and vitamin E supplemented diets compared to those fed cholesterol diet without supplementation (Figure 2a). Reduced glutathione (GSH) concentration reduced significantly (p <0.05) in tissues of rats fed cholesterol diet compared to rats fed normal diet. However, in rats fed cholesterol diet supplemented with *Oryctes rhinoceros* oil (Cholesterol + ORO) and vitamin E (Cholesterol + Vit E) significant improvement was observed in serum, liver, heart, kidney and lung GSH concentrations compared rats fed cholesterol diet only (Figure 2b). Catalase activity was observed to be significantly (p <0.05) lower in serum, liver, heart, kidney and lung of rats fed cholesterol diet compared to rats fed normal diet. On the other hand, in rats fed cholesterol diet supplemented with *Oryctes rhinoceros* oil (Cholesterol + ORO) and vitamin E (Cholesterol + Vit E) CAT activity was significant (p <0.05) higher in serum, liver, heart, kidney and lung compared rats fed cholesterol diet only (Figure 2c). Tissues (serum, liver, heart, kidney and lung) SOD activities were significantly (p <0.05) lower in rats fed cholesterol diet compared to rats fed normal diet. However, in rats fed cholesterol diet supplemented with *Oryctes rhinoceros* oil (Cholesterol + ORO) and vitamin E (Cholesterol + Vit E) SOD activity was significant higher in these tissues compared rats fed cholesterol diet only (Figure 2d).

Cholesterol-based diets have been implicated to be particularly damaging to the vascular membrane. Thus, hypercholesterolemia and related cardiovascular syndrome are predominant in regions of the world, where fat-rich foods constitute the bulk of their daily meals. A recent report from a study by **Oluba, Josiah and Fagbohunka (2014)** showed that consumption of a cholesterol-based diet led to imbalance in serum lipid profile and release of proinflammatory cytokines. The inflammation resulting from tissue damage that most often accompany high fat consumption is viewed as a consequence of oxidative stress.

In the present study, inclusion of ORO as a supplement to a cholesterol-based diet led to normalization of body weight. Rats fed cholesterol-based diet tend to be overweight which could have been a consequence of increased fat deposit in the body. The observed higher relative organ (liver, heart, kidney and lung) weight further gave credence to this assumption. The development of fatty liver has been reported in high-fat fed rats.

(a)



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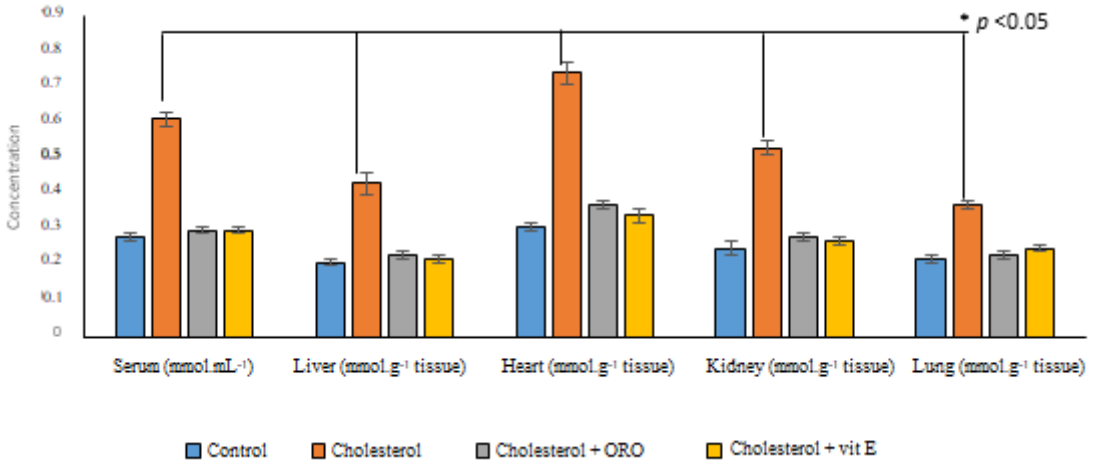

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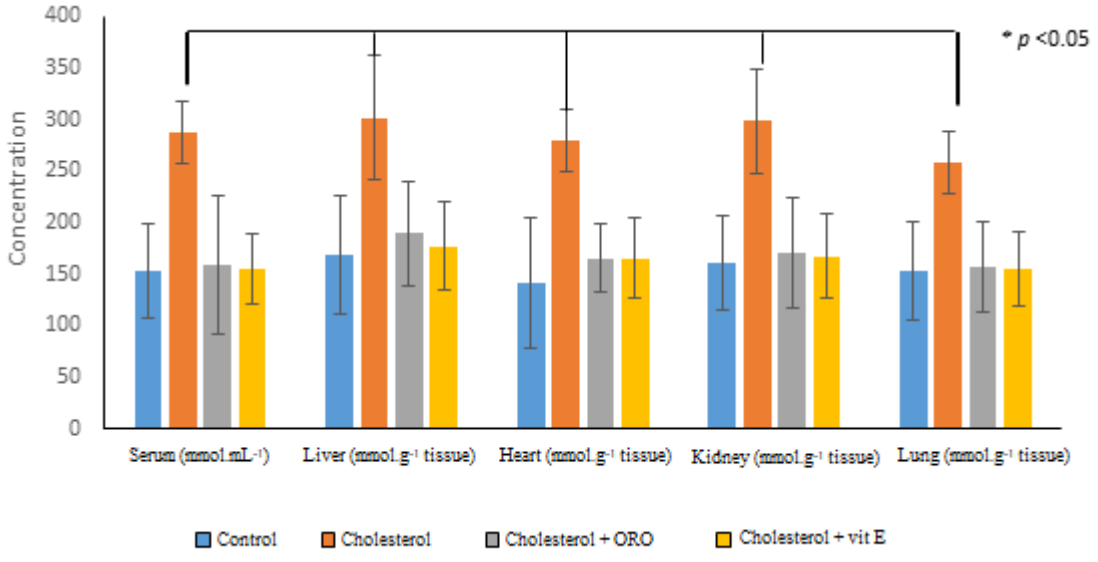
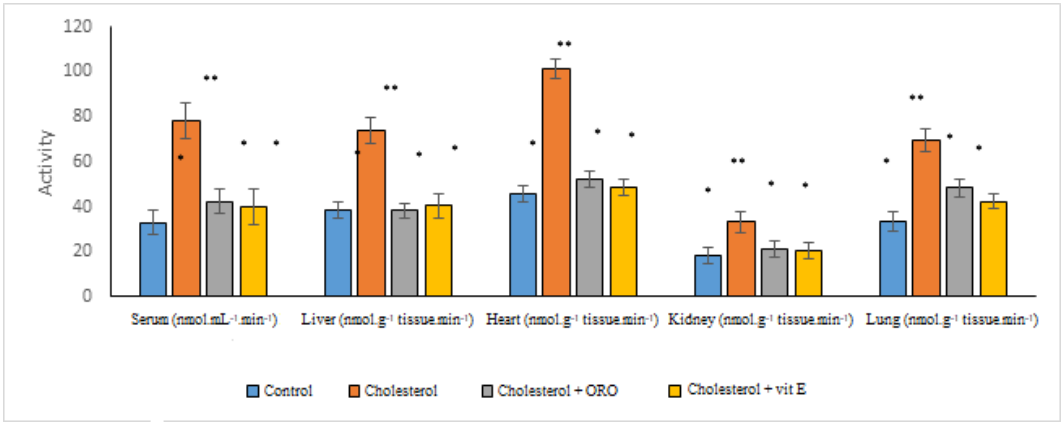


Figure 1 Tissue (a) MDA and (b) conjugated dienes and (c) nitric oxide (NO) concentrations in rats fed *Oryctes rhinoceros* oil supplemented diet over a period of 10 weeks. Note: Values are means ± SEM of 10 determinations. * Significant (p <0.05) different from control. ORO: *Oryctes rhinoceros* oil; vit.: vitamin.

(a)



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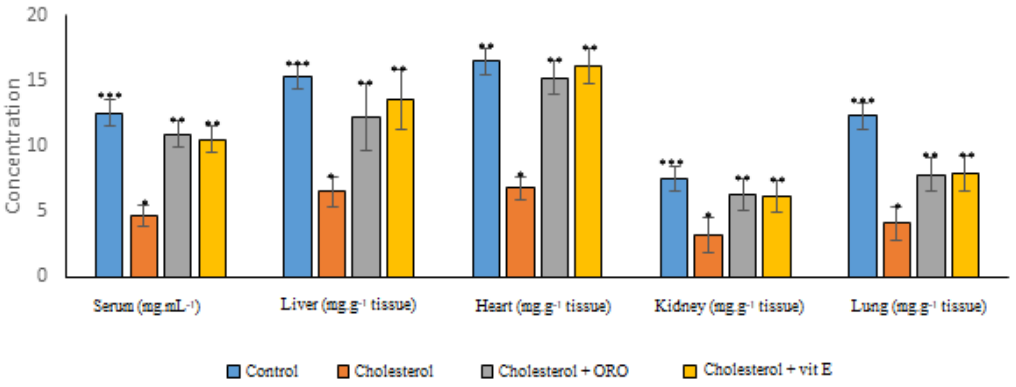

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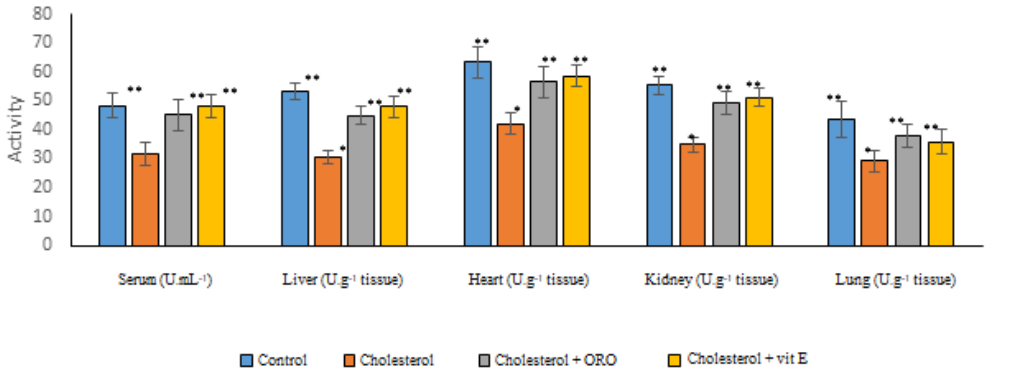

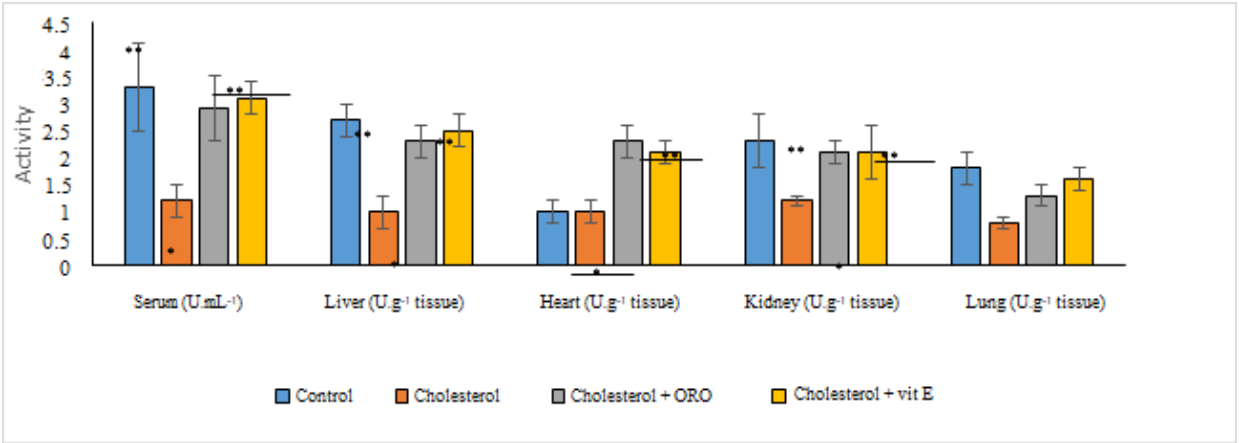

(d)

Figure 2 Tissue (a) Xanthine oxidase (b) GSH concentration (c) catalase activity (CAT) and (d) superoxide dismutase activity (SOD) in rats fed Oryctes rhinoceros oil supplemented diet over a period of 10 weeks. Note: Values are means ± SEM of 10 determinations. Bars carrying different alphabets are significant ($p < 0.05$). ORO: Oryctes rhinoceros oil; vit.: vitamin.

Table 1 Animal grouping and dietary regimen.

Feed composition (g.kg^{-1}, DM)	Control	Cholesterol	Cholesterol + ORO	Cholesterol + Vit E
Corn flour	600.0	600.0	600.0	600.0
Fish meal	200.0	200.0	200.0	200.0
Cholesterol	-	5.0	5.0	5.0
Mineral premix (AIN-76)[a]	30.0	30.0	30.0	30.0
Vitamin premix (AIN-76)[b]	10.0	10.0	10.0	10.0
Fiber	100.0	100.0	100.0	100.0
Groundnut cake	60.0	60.0	60.0	60.0
ORO	-	-	5.0	-
Vit E	-	-	-	5.0

Note: ORO, *Oryctes rhinoceros* larva oil.

[a] Mineral premix (AIN-76) composed of Ca as $CaSO_4$ (5.2 g.kg^{-1}), Ka as KCl (3.8 g.kg^{-1}), Na as NaCl (1.1 g.kg^{-1}), Mg as $MgSO_4$ (0.5 g.kg^{-1}), Fe as $FeSO_4$ (34.25 mg.kg^{-1}), Zn as $ZnSO_4$ (36.75 mg.kg^{-1}), Mn as $MnSO_4$ (59.34 mg.kg^{-1}), Cu as $CuSO_4$ (6.73 mg.kg^{-1}), Co as $CoCl_2$ (0.03 mg.kg^{-1}), and I as KI (0.21 mg.kg^{-1}).

[b] Vitamin premix (AIN-76) composed of vitamin A (4.0 IU.g^{-1}), vitamin D_3 (1.0 IU.g^{-1}), α-tocopherol (64.24 IU.kg^{-1}), thiamine (5.90 mg.kg^{-1}), riboflavin (6.29 mg.kg^{-1}), niacin (30.15 mg.kg^{-1}), pantothenic acid (15.26 mg.kg^{-1}), choline (1040.0 mg.kg^{-1}), pyridoxine (7.12 mg.kg^{-1}), folic acid (2.10 mg.kg^{-1}), biotin (0.21 mg.kg^{-1}), vitamin B_{12} (10.10 mg.kg^{-1}), and vitamin K (0.50 mg.kg^{-1}).

Table 2 Food intake and body weight of rats fed a cholesterol-based diet supplemented with *Oryctes rhinoceros* larva oil for 10 weeks.

Treatment group	Food intake (grat^{-1}.day^{-1})	Initial body weight (g)	Final body weight (g)	Mean body weight gain (grat^{-1}.week^{-1})
Control	10.9 ± 2.5^{a}	52.6 ± 5.8^{a}	158.5 ± 12.1^{a}	13.2 ± 2.1^{a}
Cholesterol	15.5 ± 1.7^{b}	50.5 ± 3.7^{a}	228.9 ± 15.3^{c}	22.3 ± 2.8^{b}
Cholesterol + ORO	15.5 ± 2.1^{b}	55.1 ± 2.5^{a}	171.3 ± 15.3^{b}	14.5 ± 1.0^{a}
Cholesterol + Vit E	14.8 ± 2.8^{b}	53.8 ± 3.2^{a}	168.8 ± 11.9$^{a.b}$	14.4 ± 1.3^{a}

Note: Values are means ± SEM of 10 determinations. Values in the same column carrying different alphabets are significant (p <0.05). ORO: *Oryctes rhinoceros* oil; vit.: vitamin.

The hyperlipidemia that develops following the consumption of a high-fat diet has been shown to substantially contributes to the depletion of both non-enzymic (GSH) and enzymic (SOD, catalase and GPx) **(Oluba et al., 2008b; Ojieh et al., 2009; Oluba et al., 2011)**. Findings from this study showed that increased dietary consumption of cholesterol could create of a pro-oxidant state and a depletion of cellular redox status. This observation agrees concur with the report of **Oluba et al. (2008b)** which showed a significant increase in plasma MDA concentration with a concomitant decrease in cellular antioxidant level in rats fed a high cholesterol diet.

The increased tissue MDA, conjugated dienes and nitric oxide concentrations in animals fed cholesterol-based diet alone in this study could be attributed to augmentation in the rate of cellular lipid peroxidation due to high fat (cholesterol) in the diet. This assertion is further reimbursed by the fact that xanthine oxidase activity was higher in rats fed cholesterol diet without supplementation compared to rats fed ORO and vitamin E supplemented diets. Xanthine oxidase has been demonstrated to be a primary source of superoxide radical **(Gomez-Cabrera et al., 2005)**. MDA and conjugated dienes are established markers of lipid peroxidation while nitric oxide through its free radical activity is capable of interacting with cellular components such proteins, DNA, and lipids within the cell in a process known as nitrosative stress leading to cytotoxicity **(Chang, Liao and Kuo, 2001)**. Thus, the reduction in tissue MDA, conjugated diene and nitric oxide levels in rats fed ORO and vitamin E supplemented diets in this study is beneficial and could give an indication of an anti-oxidative potential of ORO. The antioxidant activity of vitamin E is well established in literature **(Niki et al., 1985; Traber and Atkinson, 2007)**. In this study, ORO demonstrated a similar effect on tissue MDA, conjugated diene and nitric oxide concentrations to vitamin E showing that its potential antioxidant effect could be comparable to that of vitamin E.

An important mechanism of action of oxidative stress is via enhanced generation of ROS and/or RNS, which most often form conjugates or adducts with cellular components such as DNA, membrane lipids, proteins and carbohydrates. Thus, the increased serum and lung xanthine oxidase activities observed in rats fed cholesterol diet without supplementation could provide a justification that the pro-oxidant effect of dietary cholesterol could involve its effect on xanthine oxidase activity. In the coronary artery of hypercholesterolemic individuals, NADPH oxidases and xanthine oxidase have been reported to be the major sources of superoxide radicals. However, these reactive oxygen species in the cells are neutralized by cellular antioxidant defense system including GSH,

CAT, and SOD. Thus, oxidative stress is the resultant effect of a disequilibrium between cellular oxidants versus antioxidants **(Oluba, 2019)**. Several studies have shown that alteration of antioxidant enzyme activities in different kinds of stress was associated with a depletion of GSH, CAT and SOD and an increase of lipid peroxidation, all of which can lead to oxidative stress and finally cell death **(Bouayed and Bohn, 2010)**. Overexpression of the peroxisomal enzyme, catalase which catalyzes the reduction of hydrogen peroxide to water and molecular oxygen have been shown to reduce atherosclerosis in high-fat fed rats **(Yang et al., 2009)**. Results from the present study showed that ORO could act to normalize the attendant depletion of cellular antioxidant molecules when fed as a supplement to a cholesterol-based diet.

CONCLUSION

In conclusion, this study which was carried out to evaluate the effect of *Oryctes rhinoceros* larva oil in comparison with vitamin E on tissue lipid peroxidation and antioxidant defense systems in rats fed a cholesterol-based diet showed that *Oryctes rhinoceros* larva oil supplementation decreased tissue lipid peroxidation and increased the antioxidant defense molecules in rats. These results showed that consumption of *Oryctes rhinoceros* larva oil extracted from *Oryctes rhinoceros* larva, exhibited similar protective effects to vitamin E against diet induced oxidative stress in rats. Overall, the potential nutritional benefit of *Oryctes rhinoceros* larva oil on cardiovascular health could possibly involve its ability to upregulation of cellular antioxidant defense mechanisms.

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DYNAMICS OF CHANGES IN TOTAL CAROTENOIDS AND ANTIOIXDANT ACTIVITY IN FRUITS OF SELECTED VARIETIES OF *CUCURBITA MOSCHATA* DUCH. DURING STORAGE

Adriána Maťová, Alžbeta Hegedűsová, Alena Andrejiová, Ondrej Hegedűs, Magdaléna Hugyivárová

ABSTRACT

Cucurbita moschata Duch. is a vegetable, native to the Central America and the northern parts of South America, not very well known in Slovak republic. It is a seasonal crop which is appreciated for its nutrimental and bioactive components providing human health benefits and its option of relatively long period of storage. The aim of this study was to assess the dynamics of changes of total carotenoids content and antioxidant activity in the pulp of the fruit of *Cucurbita moschata* Duch. after the harvest and during the storage, as well as the effect of the variety on total carotenoids content and antioxidant activity. The experiment was realised in 2018 in the experimental fields of the Botanical Garden of Slovak University of Agriculture (SUA) in Nitra. Six different varieties of *Cucurbita moschata Duch.* – Liscia, Matilda, Orange, Serpentine, UG 205 F1 and Waltham were examined. The harvest was was held in the second week of September 2018. The storage took place in the hall of Departmet of Vegetable. The analysis were realised after the harvest (day 0), after the storage (day 60 and day 120). Total carotenoids content after the harvest ranged from 3.80 to 8.42 mg.100g^{-1} FM. In the DM the content ranged from 49.66 to 91.32 mg.100g^{-1} .The period of 60 days of storage had positive influence on total carotenoids content in FM, as we have recorded an increase of TCC in the case of all observed varieties. After the period of 120 days of storage we have recorded both increase and decrease, depending on the variety. The increase of the total carotenoids content during the whole period of storage was by 15%. The values of the antioxidant activity after the harvest ranged from 2.76% to 10.31%. After the 60 days of storage, we have recorded both increase in ´Liscia, Serpentine, Waltham' and decrease in the ´Matilda, Orange, UG 205 F1' variety. During the following 60 days of storage significant differences were found for all the varieties in all observed variants (storage period), except for the´ Matilda' variety. Antioxidant activity significantly decreased after 60 days of storage (by 15%), but it was followed by statstically significant increase (by 25%) after 120 days of storage. The increase of the antioxidant activity during the whole period of storage was by 6.5%, but this change was not statistically significant. The variety of *Cucurbita moschata* Duch. had stastically proven effect both on the total carotenoids content and the antioxidant actvity.

Keywords: *Cucurbita moschata* Duch.; storage; total carotenoids content; antioxidant activity; variety

INTRODUCTION

Cucurbita moschata Duch. (pumpkin) is an annual plant representing the family of *Cucurbitaceae*. Alongside with *Cucurbita pepo* L. and *Cucurbita maxima* Duch. it belongs to the most economically important species in the genus of *Cucurbita*. This species have different climatic adaptations and are widely distributed in agricultural regions worldwide **(Darrudi et al., 2018)**.

Pumpkin is a very popular vegetable species, especially in tropical and subtropical countries. *Cucurbita moschata* is originating in Central America and northern part of South America. However cultivation of *Cucurbita moschata*, also known as butternut squash, in Slovakia is only at its development stage being cultivated in small areas of the southern parts of Slovak territory **(Andrejiová et al., 2016)**.

Butternut squash is grown mainly for its fruits, but edible parts of the plant include the flowers, leaves, roots and seeds **(Kaur, 2017)**. There are many results showing the beneficial effects of the consumption of butternut squash fruits. This vegetable is considered as a rich source of nutrients and phytochemicals, such as vitamins (vitamin A, vitamin B2, vitamin C and vitamin E), minerals (potassium and calcium), carbohydrates, aminoacids, fiber and the most abundant bioactive compounds – carotenoids and polyphenols **(Bouamar et al., 2017)**. *Cucurbita moschata* is also popular for its low energetic value and an option of relatively long time of storage. The recent increase in the popularity of *Cucurbita* species has stimulated the

researches in the area of their nutritional composition. Therefore, the main component of the pumpkin pulp is its levels of carotenoids **(Provesi and Amante, 2015)**.

Carotenoids are pigments, which naturally occurre in plants, fungi, algae and also in bacteria. There are identified more than 650 different types of these molecules in nature, including around 100 types present in the human diet. Humans can not synthesize carotenoids, we are forced to take it via supplementation **(Eggersdorfer and Wyss, 2018)**. These natural pigments are ususally C40 tetraterpenoids with a long conjugated chain of double bonds, characterized with a range of functions in human health. This particular feature is responsible for both their pigmenting properties and the ability of many of these molecules to interact with free radicals and singlet oxygen and therefore act as an effective antioxidants **(Young and Lowe, 2018)**.

Although many types of carotenoids have been identified, research focuses on those that are the most prominent in the human diet. *Cucurbita moschata* is considered to be high in carotenoids, especially β-carotene and lutein. Other carotenoids indentified in this vegetable are α-carotene and minor carotenoids as ζ-carotene, zeaxanthin, violaxanthin, β-carotene-5,6-epoxide, β-cryptoxanthin, taraxanthin, auroxanthin, phytofluene, neurosporene and neoxanthin **(Jacobo-Valenzuela et al., 2011)**. **Jaswir et al. (2014)** add, that the most important carotenoids in human diet are α-carotene, β-carotene, lutein, zeaxanthin and β -cryptoxanthine. Consumption of carotenoids has been associated with various health benefits, including their great antioxidant activity, reduced risk of age-related macular degeneration, cataract and coronary heart disease. There are further epidemiological evidences about their role in immune response enhancement and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases and atherosclerosis **(Andrejiová et al., 2016; Jacobo-Valenzuela et al., 2011)**.

Bouamar et al. (2017) reports, that it is well known that carotenoids have antioxidant activity and protect against oxidative stress, which can lead to the diseases mentioned above. *Cucurbita moschata* also contains a large heterogenous group of secondary metabolites called polyphenols, which are as well known to decrease the risk of this defects. Carotenoids and polyphenols are well-known of capability in cellular redox imbalance modulation, as well as the endothelial and metabolic processes regarding the pathogenesis of inflammatory. One of the causes of neurodegenerative diseases formation is the increased presence of free radicals, which are undesirable for the body. Therefore, antioxidants are important, they can delay the process of oxidation of vital compounds and inhibit the formation of free radicals in the early stages. Natural antioxidants, originating from plants are highly recommended in drug and food forms. It has been proven that, they had therapeutic effects, great nutrition and higher safety, while synthetic antioxidants can cause organ damages , as they can accumulate in the human body.

Cucurbita moschata is showing to be a promising plant in terms of its nutritional composition and invites several research teams to examine its premises **(Indrianingsih et al., 2019)**. However, there are many factors which can affect the level of the total carotenoids and its antioixdant activity. The content of these substances in the fruit of the pumpkin differ from one variety to another, and can also be influenced by external factors – climate, nutrition, water availability, habitat, storage conditions, etc.

Moreover, the stability of carotenoids is influenced by several factors, such as the storage time and temperature, the availability of oxygen and light, and the type of carotenoid involved **(Wibowo et al., 2015)**. The aim of the work was to determine the content of total carotenoids and antioxidant activity in fruits of 6 selected varieties of *Cucurbita moschata* Duch. and to record the changes in their dynamics.

Scientific hypothesis

Storage is one of the main factors affecting the level of the bioactive substances such as carotenoids. During the storage, the process of maturation occurs and physiological changes take place in the fruits, which are reflected in the examined values of total carotenoids and antioxidant activity. We expect, that the storage and variety of *Cucurbita moschata* Duch. have a significant impact on the dynamics of changes of total carotenoids content and antioxidant activity.

MATERIAL AND METHODOLOGY

This field experiment was founded in 2018 in the experimental fields of the Botanical Garden of Slovak University of Agriculture (SUA) in Nitra. Six different varieties of *Cucurbita moschata Duch.* – Liscia, Matilda, Orange, Serpentine, UG 205 F1 and Waltham were examined. The growing cycle at the experiment location was initiated on April 2018 and was followed by cultivating routine season. The cultivation area is located in a very warm agro-climatic region, characterized with a very dry subregion, the average annual temperature is 10 °C and the average annual rainfall is 584.5 mm. The soil is characterized as a glue fluvisol, formed on alluvial sediments.

Experiment organisation

The total experimental area was 202.5 m^2. This area was fertilized on the basis of the agrochemical soil analysis, which was carried out at the Department of Agrochemistry and Plant Nutrition of SUA. Ammonium nitrate (27%) have been applied before the sowing and then short before the blooming. For all cultivars 3 seeds were sowed to the nest to the dept of 3 cm. After the plant growth, unification took place. Nine plants were cultivated within one cultivar. The crop management was carried out in accordance with the usual agrotechnical procedures. Studied varieties belong to the group of medium early to medium-late with maturing from mid-September. The harvest was held in the second week of September 2018. Fruits were botanically mature, having a typical skin and pulp color, showing the best qualities in terms of growing conditions. They were primarily intended for storage and analysis. They were harvested manually and placed unwashed in the storage hall at the Department of Vegetable Production. This hall is not primarily intended for vegetable storing. It is covered, spacious, airy, without the possibility of storage conditions regulation (temperature, humidity). Storage was

free, airy, on concrete floor according to varieties. Absenting regulation of storage conditions and uninsulated space caused storage conditions to be influenced by the development of outdoor weather. The average temeperature in the hall during the storage was 20 °C. By lowering the outside air temperature, the temperature inside the hall also decreased, also the relative humidity of the air increased in the hall, as evidenced by a certain percentage of rotting fruit found at the beginning of December. The fruits were covered with a white nonwoven fabric to prevent the surface wetting of the fruits, its freezing and cold.

Figure 1 Field experiment with pumpkins.

Average sample preparation

The average sample for each variety was prepared from 5 fruits. The size of the fruits was more or less identical and of the same stage of maturity. We washed the fruits and thoroughly cut them into 4 parts. Two opposing parts were stripped of peel, seeds and cut into the cubes of the same size. The average samples were prepared by homogenizing an mixing the prearranged material.

Figure 1 *Cucurbita moschata* Duch. – Serpentine variety during ripening.

Total carotenoid content estimation (TCC)

The extraction of samples have been done at the Laboratory of Beverages, AgroBioTech Research Center of SUA in Nitra. The estimation of total carotenoid content was realised in the laboratory of Department of Vegetable Production of SUA in Nitra. The content of total carotenoids was estimated by spectrophotometric measurement of substances absorbance in petroleum ether extract in three repeatings on spectrophotometer PHARO 100 at 445 nm wavelengths **(Hegedűsová, Mezeyová, Andrejiová, 2015)**. Total carotenoid content was recalculated according to the relationship reported by **Biehler et al. (2010)**.

Figure 2 Homogenized average sample.

Antioxidant activity estimation (AOA)

Determination of antioxidant activity was realised in the laboratory of Department of Chemistry, Janos Selye University in Komárno by DPPH method (2,2-diphenyl-1-picrylhydrazyl, Merck, Darmstadt, Germany). Determination of AOA was performed with a spectrophotometer Jenway 6301, Bibby Scientific Ltd., UK. 10 g of homogenized mixture of the used material (*Cucurbita moschata* Duch.) and 40 mL of methanol (70%, V/V, Fisher Scientific UK, Loughborough, UK) were added into 250 mL extraction flasks. They were standing at room temperature for 20 hours and then extracted with horizontal shaker for 4 hours. DPPH inhibition and spectrophotometric measurements were performed after a constant time of 30 min 0.2 mL of the extract was pipetted into the spectrophotometer cuvette, supplemented with 70% methanol to 2.0 mL, and 4 mL of DPPH solution about 25 $mg.L^{-1}$ concentration was added. Immediately after the DPPH solution was added, the absorbance of the mixture was measured at 517 nm (At_0). After 30 min the absorbance of each sample was measured at 517 nm (At_{30}). The AOA was calculated based on this following relationship **(Hegedűs et al., 2019)**.

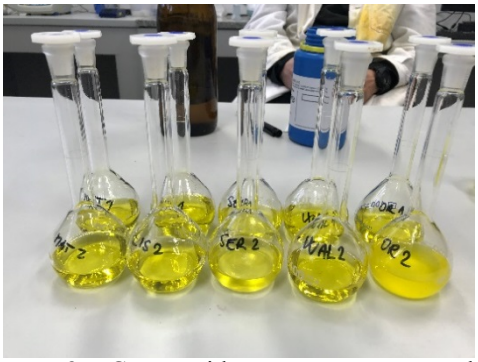

Figure 3 Carotenoids extracts prepared for measuring.

$$\% ARA = \left(1 - \frac{At_{30}}{At_0}\right) \times 100 \times V_2 / (n \times V_1)$$

At_{30} – absorbance of the sample after 30 min; n – weigh of the sample in g; V_1 – pipetted volume of the sample (0.2 mL); V_2 – supplemented volume of the extract by methanol (according to the stated method always 2.0 mL); At_0 – the initial sample absorbance value.

Statistic analysis

The obtained data were processed into tables in Microsoft Office Excel 2007. Statgraphics Centurion was used XVII (StatPoint, USA) using ANOVA (Multivariate Analysis of Variance) analysis and testing LSD differences at significance level α = 0.05. Uncertainty of the analytical method for AOA determination was expressed as an expanded uncertainty and was calculated in program Metro2003.

RESULTS AND DISCUSSION

The total carotenoids content in the pulp of the fresh fruits of selected varieties of *Cucurbita moschata* Duch. after the harvest and after the storage is given in the Table 1. The values ranged from 3.80 to 8.42 mg.100g^{-1}. In the dry matter the content ranged from 49.66 to 91.32 mg.100g^{-1}. The highest content was determined in ˈOrangeˈ variety, which is known for its intensive orange colour of the pulp. Our results are comparable with those, which determined **Andrejiová et al. (2016)** in the range from 9.33 to 15.10 mg.100g^{-1}. Other authors examined various genotypes of *Cucurbita moschata* Duch., while the total carotenoid content in the fresh fruits ranged from 12.46 mg.100g^{-1} to 69.9 mg.100g^{-1} **(Carvalho et al., 2015)**. **Priori et al. (2017)** reports the total carotenoid content ranging from 10.8 mg.100g^{-1} to 36.7 mg.100g^{-1}. The stability of carotenoids differs depending on many factors. One of the main factors which has a great impact on the stability and the associated total carotenoids content is the storage. It is important to study the factors related to the loss of colour of foods based on pumpkin since colour retention during storage is one of the parameters of food quality **(Gliemmo et al., 2009)**. On the basis of our results, we can generalize that the period of 60 days of storage had positive influence on total carotenoids content (TCC) in fresh matter. We have recorded an increase of TCC in the case of all observed varieties. A similar result has been reported by **Andrejiová et al. (2016)**, who stated that the 52 days of storage had positive impact on TCC with the exception of one variety. After the period of 120 days of storage we have recorded interesting results. In the case of five observed varieties the TCC decreased, while the values were still slightly higher than after the harvest (Liscia, Matilda, UG 205 F1) and for the ˈOrangeˈ and ˈSerpentineˈ lower. On the contrary in case of ˈWaltham'variety the TCC increased by 35%. Statistically significant differences were found in this variety, both between the 0. day – 60. day and the 0.day – 120. day.

Conti et al. (2015) indicate in his research an intensifying in colour in the pulp of the fruit of *Cucurbita moschata* Duch. after 60 days of storage and similary a very slight decrease after 180 days of storage. The incongruitties of these results suggest that the post harvest dynamics of TCC in the fruits of *Cucurbita moschata* Duch. may result from the interaction of various factors, affecting the metabolism of these important compounds. Biosynthesis of carotenoids continue in fruits even during the postharvest period, until the plant material is not treated in the way, which could inactive the carotenogenesis responsible enzymes. Obviously, the high storage temperature and conditions supporting wilting may cause carotenoids degradation as well **(Rodriguez-Amaya, 1997)**. Antioxidants are a heterogeneous category of molecules, which play an important role in human health such as preventing cancer and cardiovascular diseases, and lowering the incidence of many different diseases. The beneficial influence of many foodstuffs and beverages, including fruits, vegetables, tea, coffee and cacao, on human health has been recently recognized to originate from their antioxidant activity. Antioxidants are compounds or systems that can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. They can use several mechanisms: (I) scavenging species that initiate peroxidation, (II) chelating metal ions so that they are unable to generate reactive species or decompose peroxides, (III) quenching superoxide, preventing formation of peroxides, (IV) breaking the auto-oxidative chain reaction, or (V) reducing localized oxygen concentrations **(Oroian and Escriche, 2015; Gülçin, 2012)**. The evolution of antioxidant activity (AOA) in the fruits of Cucurbita moschata Duch. is presented in the Table 2. The values of AOA in fresh pulp of the fruits ranged from 2.76% to 10.31%. The highest rate was recorded for the ˈMatildaˈ variety. After the 60 days of storage changes have occurred. In the case of ˈLiscia, Serpentine, Waltham' variety we have recorded an increase, while in the ˈMatilda, Orange, UG 205 F1ˈ we have observed an decrement of AOA. On the basis of our results, we can declare during the following 60 days of storage an increase of AOA in all the observed varieties.

Table 1 Total carotenoids content in the pulp of the fruit of *Cucurbita moschata* Duch. after the harvest and after the storage.

Variety	TCC after the harvest (mg.100g^{-1} ±*SD*)	TCC after 60 days of storage (mg.100g^{-1} ±*SD*)	TCC after 120 days of storage (mg.100g^{-1} ±*SD*)
	FM	FM	FM
Liscia	6.78 ±3.85^{a}	7.95 ±1.82^{ab}	6.92 ±5.07^{b}
Matilda	5.69 ±1.43^{a}	7.37 ±5.27^{a}	5.81 ±3.85^{a}
Orange	8.42 ±6.08^{a}	8.58 ±1.22^{a}	5.34 ±0.81^{a}
Serpentine	3.80 ±1.22^{a}	3.98 ±2.23^{a}	3.22 ±11.54^{a}
UG 205 F1	6.25 ±2.63^{a}	7.61 ±1.22^{b}	6.64 ±1.22^{ab}
Waltham	6.39 ±0.41^{a}	8.28 ±2.23^{b}	8.66 ±1.02^{b}

Note: TCC – total carotenoids, FM – fresh matter, *SD* – standard deviation. Values with different italics letters are significantly different at p <0.05 by LSD in ANOVA.

Table 2 Antioxidant activity in the pulp of fresh fruit of *Cucurbita moschata* Duch. after the harvest and after the storage.

Variety	AOA after the harvest converted to 1 g (% ±*SD*)	AOA after 60 days of storage converted to 1 g (% ±*SD*)	AOA after 120 days of storage converted to 1 g (% ±*SD*)
Liscia	3.15 ±0.35^{a}	5.36 ±0.59^{b}	6.95 ±0.76^{c}
Matilda	10.31 ±1.13^{a}	7.08 ±0.78^{b}	7.19 ±0.79^{b}
Orange	8.34 ±0.92^{a}	5.58 ±0.61^{c}	7.43 ±0.82^{b}
Serpentine	2.76 ±0.30^{a}	4.73 ±0.52^{b}	5.77 ±0.64^{c}
UG 205 F1	6.98 ±0.77^{a}	3.00 ±0.33^{c}	5.33 ±0.59^{b}
Waltham	5.65 ±0.62^{a}	6.04 ±0.66^{b}	7.33 ±0.81^{c}

Note: AOA – antioxidant activity, *SD* – standard deviation. Values with different italics letters are significantly different at p <0.05 by LSD in ANOVA.

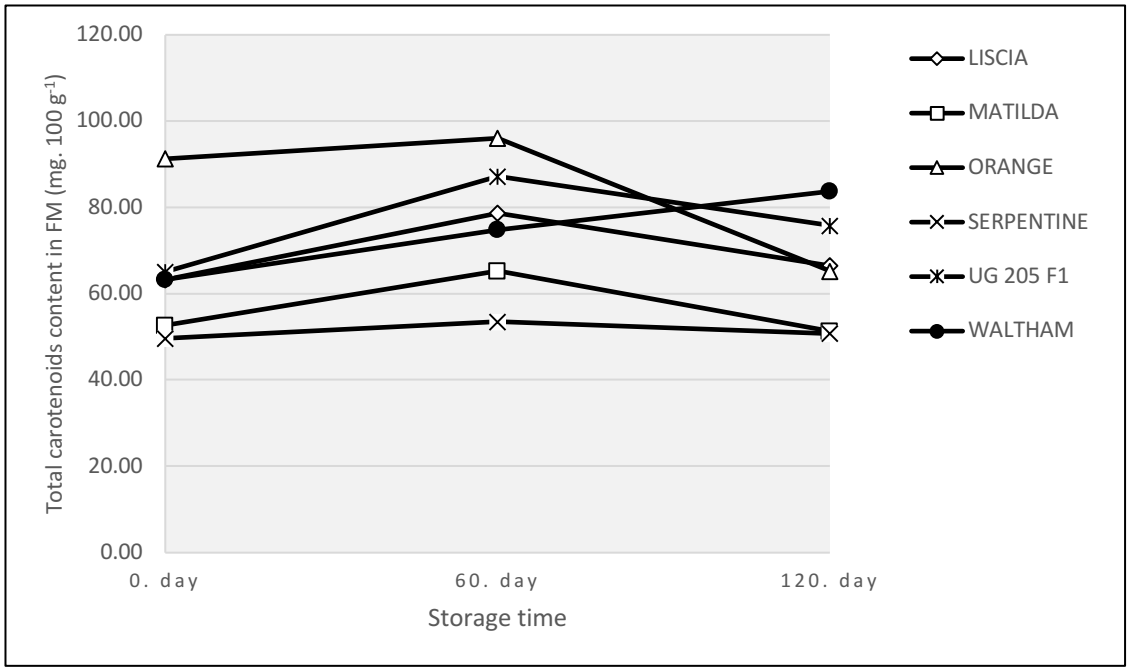

Figure 5 Dynamics of changes of total carotenoids content in the dry matter of the fruit of *Cucurbita moschata* Duch.

Significant differences were found for all the varieties in all observed variants (storage period), except for the'

The evolution of antioxidant activity (AOA) in the fruits of Cucurbita moschata Duch. is presented in the Table 2. The values of AOA in fresh pulp of the fruits ranged from 2.76% to 10.31%. The highest rate was recorded for the 'Matilda' variety. After the 60 days of storage changes have occurred. In the case of ' Liscia, Serpentine, Waltham ' variety we have recorded an increase, while in the' Matilda, Orange, UG 205 F1 ' we have observed an decrement of AOA. On the basis of our results, we can declare during the following 60 days of storage an increase of AOA in all the observed varieties.

Significant differences were found for all the varieties in all observed variants (storage period), except for the 'Matilda' variety for the storage period variant 60 to 120 days of storage. The antioxidant activitiy in general is affected both by physical and chemical factors.

Li et al. (2012) reports, that the postharvest storage may affect the composition of some phytochemicals in plants; however, the degree of the effect depends more on the storage conditions. Metabolism of the phytochemicals begins right after harvest, and it can involve complex biochemical reactions during transportation and postharvest storage. These reactions can lead to significant changes in plant attributes (taste, smell, appearance and texture), and the health promoting phytochemicals, such as those with strong antioxidant activities. Storage temperature, atmosphere gas composition and use of chemicals are major factors that influence the quantity and quality of phytochemicals and so AOA.

The Figure 5 is showing the dynamics of changes in the dry matter of the fruit for each variety after the harvest and the storage period. As we can see, in each case there is an increasing tendency in TCC after the 60 days of storage. However, after this period a decrement appears. Despite, we recorded an exception as well at the 'Waltham ' variety, characterized by 12% increment during the next 60 days of storage. The biggest abundance of TCC in DM was recorded in the 'Orange ' variety, by 28% during the 120 days of storage.

Bonina-Noseworthy et al. (2016) also indicate an increment of TCC in DM after 60 days of storage in the ranges from 4.2 mg.100g^{-1} to 14.5 mg.100g^{-1} (harvest) to 8.4 mg.100g^{-1} to 23.9 mg.100g^{-1} (60 days of storage).

Based on the data from our research displayed in the Figure 6, we can conclude that there are significant differences between individual varieties of the observed

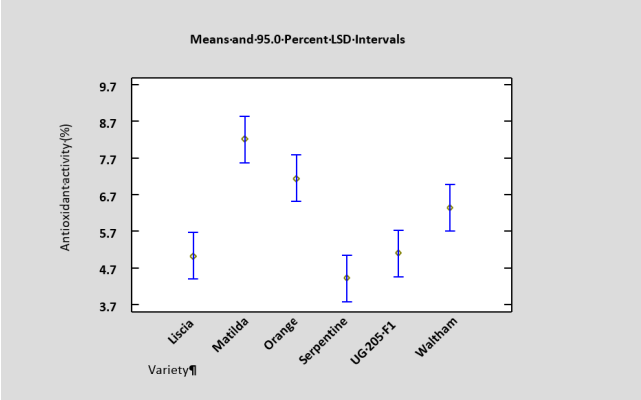

Figure 6 Antioxidant activity of *Cucurbita moschata* Duch. in dependency on selected variety (LSD test, p >0.05).

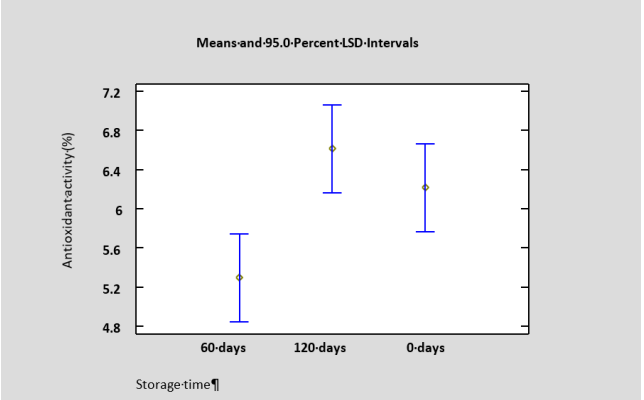

Figure 7 Total antioxidant activity of *Cucurbita moschata* Duch. during the storage (LSD test, p >0.05).

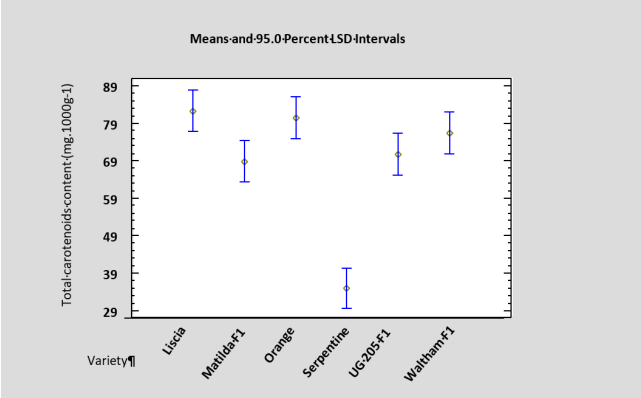

Figure 8 Total carotenoids content of *Cucurbita moschata* Duch. in dependency on selected variety (LSD test, p >0.05).

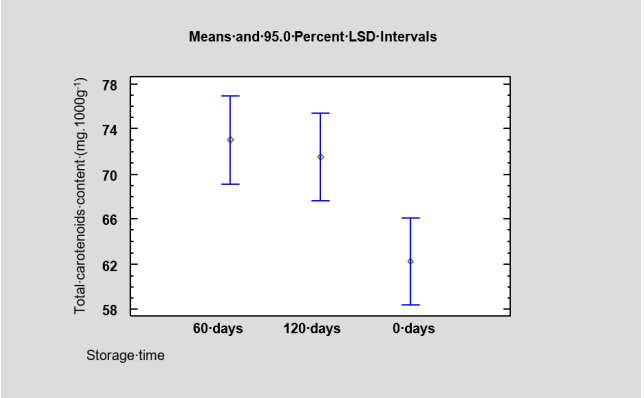

Figure 9 Total carotenoids content of *Cucurbita moschata* Duch. during the storage (LSD test, p >0.05).

varieties. Significant differences were found between 'Liscia', 'Matilda' variety and other evaluated varieties. The greatest contrast was shown between the 'Matilda' and 'Serpentine' variety. The effect of the variety on the antioxidant activity was statistically proven.

Šlosár et al. (2018) also report that, the variety of the used plant material is a major factor, which can affect the antioxidant activity.

Antioxidant activity significantly decreased after 60 days of storage (by 15%), but it was followed by statstically significant increase (by 25%) after 120 days of storage (Figure 7). The increase of the antioxidant activity during the whole period of storage was by 6.5%, but this change was not statistically significant.

Conti et al. (2015) report the data of the single antioxidants content in the fruit of *Cucurbita moschata* Duch., separately for ascorbic acid, α-carotene, β-carotene and lutein. The results of their study indicate a significant intital increase in α-carotene after 120 days of storage, but this was followed by its gradual reduction until the end of the storage period (another 120 days). Lutein dropped below the limit detection within the first 120 days, both ascorbic acid and β-carotene increased during the storage.

On the basis of our results showed in the Figure 8, we can state that there are significant differences between the examined varieties of *Cucurbita moschata* Duch. in terms of TCC. These differences were found mainly between the Serpentine' variety and the other varieties. The highest contrast was examined between the 'Liscia' and Serpentine variety.

The effect of the variety on the total carotenoids content was statistically proven. **Andrejiová et al. (2016)** also confirm the influence of the variety on the TCC.

Total carotenoids content significantly increased after 60 days of storage (by 17%). This increment was followed by 2% decrease after the next 120 days of storage (Figure 9). The increase of the total carotenoids content during the whole period of storage was by 15% and was statistically significant. **Provesi et al. (2011)** declare that storage period of 180 days did not significantly influenced the TCC in the brazil variety of *Cucurbita moschata* Duch. the 'Menina Brasileira' variety.

CONCLUSION

Cucurbita moschata Duch. is a less-known vegetable in Slovak republic, which is consumed here just a little. This cultivar is more spread in the northern part of South America and the Central America. Among the cucurbitaceous vegetables, *Cucurbita moschata Duch.* has always been very appreciated for its high yield, good storage period, longer periods of consumption, high nutritive value, and has numerous traditional medicinal uses. The aim of this study was to assess the dynamics of changes of total carotenoids content and antioxidant activity in the pulp of the fruit of *Cucurbita moschata* Duch. after the harvest and during the storage, as well as the effect of the variety on total carotenoids content and

antioxidant activity. Total carotenoids content after the harvest ranged from 3.80 to 8.42 mg.100g^{-1} FM. In the DM the content ranged from 49.66 to 91.32 mg.100g^{-1} , while the highest content was determined in ˊ Orange ' variety. The period of 60 days of storage had positive influence on total carotenoids content in FM, as we have recorded an increase of TCC in the case of all observed varieties. After the period of 120 days of storage we have recorded both increase and decrease, depending on the variety. The increase of the total carotenoids content during the whole period of storage was by 15% and was statistically significant. The values of the antioxidant activity after the harvest ranged from 2.76% to 10.31%, while the highest rate was recorded for the 'Matilda ' variety. After the 60 days of storage, we have recorded both increase in ˊ Liscia, Serpentine, Waltham ' and decrease in the 'Matilda', Orange, UG 205 F1 ' variety. During the following 60 days of storage an increase occurred in all of the observed varieties. Significant differences were found for all the varieties in all observed variants (storage period), except for the ˊ Matilda ' variety for the storage period variant 60 to 120 days of storage. Antioxidant activity significantly decreased after 60 days of storage (by 15%), but it was followed by statstically significant increase (by 25%) after 120 days of storage. The increase of the antioxidant activity during the whole period of storage was by 6.5%, but this change was not statistically significant. On the basis of our results, .we can equally state that variety of *Cucurbita moschata* Duch. had stastically proven effect both on the total carotenoids content and the antioxidant actvity.

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IMPROVEMENT OF GROWTH, PRODUCTIVITY AND SOME CHEMICAL PROPERTIES OF HOT PEPPER BY FOLIAR APPLICATION OF AMINO ACIDS AND YEAST EXTRACT

Amina Aly, Noha Eliwa, Mohamed Hassan Abd El Megid

ABSTRACT

A greenhouse experiment was conducted during the seasons of 2016 – 2017 to compare the impact of foliar amino acids binding (0.5, 1 and 2 g.L^{-1}) and yeast extract (2.5, 5 and 10 g.L^{-1}) on certain development and physiological parameters of hot pepper (*Capsicum annuum* L.). The results cleared that foliar application of amino acid (2 g.L^{-1}) or yeast (10 g.L^{-1}) increased development parameters of hot pepper compared to control in both first and second seasons. Amino acids foliar implementation with (2 g.L^{-1}) gave higher content of anthocyanins, ascorbic acid, lycopene and ß- carotene contents as compared with the control. Also, 10 g.L^{-1} foliar application of yeast extract showed the best results as compared to control in both first and second seasons. Foliar application of amino acids contents increased phenol and flavonoid contents of hot pepper fruits. Maximum increase was observed at 2 g.L^{-1} amino acids in both seasons. While 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and lipid peroxidation contents increased with 2 g.L^{-1} amino acids and 10 g.L^{-1} yeast foliar application. The HPLC analysis of ethanolic extract of hot pepper fruits has shown fifteen phenolic compounds. Phenolic compounds were increased by increasing the concentration of amino acid and yeast extract foliar application in the both two seasons. In conclusion it is recommended to use amino acid (2 g.L^{-1}) and yeast extract (10 g.L^{-1}) foliar application as they play a key role in productivity, also in protecting the environment as eco-friendly and cost-effective inputs for the farmers.

Keywords: hot pepper plant; amino acids; yeast extract; active compounds; phenolic compounds; HPLC

INTRODUCTION

One of the traditional plants that has so many pharmacology effects is chilli fruit (*Capsicum* sp.) which belong to the family *Solanaceae*. Chilli pepper and their isolated constituents including capsaicinoids have shown also beneficial therapeutic effects, including antioxidant, anti-inflammatory, anticancer, antimicrobial and anti-immune modulator effects **(Popelka et al. 2017)**. Hot pepper (*Capsicum annum* L.) is a significant global horticultural plant and gives food taste, flavour and colour **(Caporaso et al., 2013)**. It is well recognized for its elevated bioactive content, powerful antioxidant ability and is one of world's most famous new foods owing to its mixture of colour, aroma and dietary importance. Also, it is considered a good source of bioactive compounds, such as vitamins, pro-vitamins, and antioxidant compounds **(Martínez et al., 2007)**. The plant is indigenous to North and South America, is most efficient in hot, dry environment and is used in Africa and other nations of the globe for medicinal purposes **(Igbokwe, Aniakor and Anagonye, 2013)**. Pepper fruit includes various compounds that may contribute total antioxidant capacity (TAC), including flavonoids, phenolics, ascorbates, carotenoids, and capsaicinoids **(Palma et al., 2015)**. In defence against biotic and abiotic stress the significant functions of crop phenolics emerged. They demonstrated broad variety of biological operations, including antithrombotic, antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antiviral, vasodilatory and anticarcinogenic **(Soobrattee et al., 2005)**. The option of using natural secure agents to stimulate development and output of vegetable plants has been a major problem nowadays. During crop development phases, the application of bio-fertilization to plants was thought to be a good option to chemical fertilization. Bio-fertilizers are microbial structures comprising living bodies of separate microorganisms that have the capacity to mobilize crop nutrients in the land from unusable to usable shape. It is regarded eco-friendly, plays a major part in the manufacturing of crops, helps to construct the missed microorganisms and improves oil health **(Zhang et al., 2013)**. In the same regard **Mahmoud and Hafez (2010)**

disclosed that, bio-fertilizers increased crop yields by 20% – 30%, improved plant development and optimized ground biological activity environment. Biostimulants use as amino acids and yeast extract boost crop development overcoming the damaging impact of some environmental pressure. The significance of amino acids is primarily due to their wide spread use as pigments, vitamins, coenzymes, purine and pyrimidine bases for the biosynthesis of a wide range of development and non-protein nitrogenous products. Many researches have verified that amino acids can have a direct or indirect impact on crop development and yield physiological operations **(Mohamed, 2006)**. Yeast extract is a wealthy source of many development products (riboflavin, thiamine, pyridoxine, niacin, and vitamins B1, B2, B3 and B12), cytokinin's and many of the natural compounds such as protein, carbohydrates, nucleic acid and lipids. Dry yeast foliar application had a positive impact on plant development, output and chemical structure **(Mohamed, 2005)**.

Scientific hypothesis

Hot pepper is well known for its high bioactive content and strong antioxidant capacity and considers as one of most popular fresh products in the world due to its combination of colour, flavour and dietary importance. This research aims to explore the effect of bio-fertilizer (amino acids and yeast extract) on certain hot pepper (*Capsicum annuum* L.) physiological and biochemical parameters.

MATERIAL AND METHODOLOGY

Chemicals

All reagents from Sigma–Aldrich S.P.A. (Milan, Italy) were acquired and had an elevated degree of hardness (purity >97%).

This experiment was conducted in the greenhouse belonging to the Natural Products Dept., National Center for Radiation Research and Technology. Nasr- city, Cairo-Egypt, from April to August 2016 – 2017 to assess the impact of amino acids and yeast extract foliar implementation on some physiological and chemical parameters of hot pepper (*Capsicum annuum L.)*. Hot pepper seeds of (variety: Muria, Batch: 6800) planted with peat moss in small pot (5 cm). When the seedlings had (2 – 3) real leaves, they were transported to the greenhouse where they placed 70 cm apart and 40 cm between plants on both ends of the rows. All environmental requirements have been fulfilled and agricultural needs were done. The experimental site's soil mechanical and chemical assessment is displayed in Table 1.

The levels of amino acids (0.5, 1.0 and 2.0 $g.L^{-1}$) or yeast extract (2.5, 5.0 and 10.0 $g.L^{-1}$) were tested, the first was released one month after the plants transferred to the greenhouse then sprinkle early in the morning frequently every week until the fruiting phase. After 70 days vegetative characters of plants such as: plant height, fruit size, fruit diameter, complete amount of fruit/plant, fresh weight of one fruit (g) and dry weight of fruits (g) were evaluated after 70 days.

The fruits of both two seasons were cut and homogenized using liquid nitrogen and weighed at a part of (25 g) and then lyophilized for 48 hours (Virtis model 10–324). The samples were ground to pass a 0.5-mm sieve and stored at -20 °C until the analysing the bioactive materials.

Table 1 Soil mechanical and chemical analysis of the experimental site.

Soil properties	Experimental year	
	Soil properties 2016	Soil properties 2017
pH(1:1)		
EC (1:1) $dS.m^{-1}$	7.32	7.31
Soluble anions ($meq.L^{-1}$)	2.2	1.8
CO_3		
HCO_3	1.5	1.3
Cl	4.5	3.6
SO_4	8.0	5.9
Soluble cations ($meq.L^{-1}$)	11.7	9.8
Ca^{++}	10.5	9
Mg^{++}	3.5	2
K^+	0.50	0.4
Na^+	11.2	9.2
Sand (%)	57.3	58.4
Silt (%)	21.2	18.2
Clay (%)	21.5	23.4
Texture class	Sandy clay loam	Sandy clay loam

Total phenolics content

Phenolic content was analysed using the Folin-Denis reagent in accordance with **Shahidi and Naczk (1995)** technique. A one mL of sample extract was mixed with 0.5 mL of Folin-Denis and 1.0 mL of concentrated Na_2CO_3 solution, adding 3.0 mL of distilled water. The absorbance was evaluated at 725 nm (Jasco V530, Japan) against the blank after an hour. The outcome results were displayed as gallic acid equivalents, in $mg.100\ g^{-1}$ D.W.

Flavonoids content

In the extracts the content of flavonoids were determined by aluminium chloride colorimetric assay as mentioned by **Marinova, Ribarova and Tanassova, (2005)**. The absorbance was measured against the blank at 510 nm. Total flavonoids were shown as gallic acid equivalents, in $mg.100\ g^{-1}$ D.W.

Analysis of phenolic compounds by HPLC

The methanolic extract was conducted by re-dissolving 100 mg of sample in 1.0 mL of methanol (80 percent) and filtering through a 0.2 μm filter sterilized membrane prior to HPLC assessment. Injection with 50 pJ set circuit using Rheodyne injection values (Model 7125). Tow portable phases used a steady stream frequency of 1 $mL.min^{-1}$: (A) 0.5% acetic acid in distilled fluid at pH (2.65) and 0.5% acetic acid in 99.5% acetonitrile liquid (B). The elution gradient was linear from (A) to (B) over 50 min, using a 254 nm wavelength UV detector. Phenolic compound identification was performed by comparing retention times and spectral information with authentic standards

Lipid peroxidation content

As outlined by **Buege and Aust (1978)**, the amount of lipid peroxidation in the samples was determined as reactive metabolites 2-thiobarbituric acid (TBA) mainly malondialdehyde (MDA). Absorbance of pink colour was assessed at 532 nm and adjusted by subtracting the absorbance at 600 nm ((Jasco V530, Japan) for non-specific turbidity. The concentration was calculated based on A532 – A 600 (Σ =155 $mM^{-1}cm^{-1}$), the MDA level was calculated. The obtained results have been displayed as $\mu mol.g^{-1}$ FW.

Scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The radical Scavenging activity on radical DPPH extracts against 2,2- Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity was determined as outlined by **Gulluce et al. (2004)**. Add 0.5 mL of each sample to 1.0 mL of DPPH (2 mM) ethanolic solution. The absorbance was plotted at 517 nm (Jasco V530, Japan) against the background after 30 min of the incubation.

Anthocyanins content

The content of anthocyanins was determined using acidified methanol (1% HCl). As mentioned by **Fuleki and Francis (1968)** and modified by **Du and Francis (1973)**, the samples were evaluated spectrophotometrically at 535 nm (Jasco V-530, Japan) where the molecular weight of cyaniding-3-Oglucoside 449.2 $g.mol^{-1}$ and the molar extinction coefficient index (26.900 $L.mol^{-1}.cm^{-1}$) the results are expressed as $mg.100g^{-1}$ F.W.

Ascorbic acid content

The content of ascorbic acid was measured using the method of titration of 2,6-dichlorophenol indophenols **(A.O.A.C., 2000)**. The results are expressed as $mg.100g^{-1}$ F.W.

Hot pepper fruits- extraction

Ethanol was used to prepare the sample extracts as described by **(Nuutila et al., 2003)**. A ground sample (2.0 g) was extracted with ethanol (25 mL) by stirring for one night at room temperature. The blend was centrifuged for 20 minutes at 3000 rpm, with an extra 25 mL ethanol and the supernatant decanted. The mixed supernatant of the two extractions was used for assessment as outlined below.

β- Carotene and lycopene content

For determining β- Carotene and lycopene the technique of **(Nagata and Yamashita, 1992)** was used. The dried ethanol sample (100 mg) was stirred for 1 min with 10 mL of acetone: n-hexane mixture (4:6) for 1 min and filtered by Whatman filter paper No.4. The filtrate absorption was evaluated at 453, 505, 645 and 663 nm. The β-carotene and lycopene contents were calculated as follow:

Lycopene ($mg.100mL^{-1}$) = $-0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$

β-Carotene ($mg.100mL^{-1}$) = $0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$

The outcomes were given as extract ($\mu g.g^{-1}$ D.W.)

Statistic analysis

Data were evaluated using difference statistical software evaluation (ANOVA) and multiple range technique used by Duncan to compare any important variations between samples. Mean scores for Duncan's multi-range experiment using IBM SPSS software 24 as a statistical resource was compared to p <0.05 important point **(Duncan, 1955)**.

RESULTS AND DISCUSSION

Growth parameters

Regarding to the role of the foliar implementation of bio-fertilizer (amino acids and yeast extract) on growth parameter of hot pepper, Table 2 presented that there was gradual increase in plant height occurred by increasing the amino acids concentration and reached to the maximum increase in the concentration (2 $g.L^{-1}$) in the first and second seasons 62.8 and 64.4 cm, respectively. Same trend was observed when yeast extract was applied and the highest increase was recorded in the highest concentration of yeast extract (10 $g.L^{-1}$) for the both seasons (59.6 and 62.1 cm), respectively. Otherwise other vegetative growth parameters were significantly influenced by the all used amino acids concentrations and the most stimulatory effect happen in the concentration (2 $g.L^{-1}$) for fruit length (5.9 cm), fruit diameters (1.8 cm), fruit number/plant (71), fresh weight of fruit (2.9 g) and dry weight of fruits (1.8 g), respectively in the first season and same trend was observed in the second season. Yeast extract treatment caused a positive effect on the fruit length, fruit diameters, fruit number/plant, fresh weight of fruit and dry weight of fruits of hot pepper, comparable to control treatments in the two periods. Because of the immediate function of amino acids in enhancing the tissue materials of proteins and vital enzymes for managing cellular activities or activating antioxidants, the use of foliar implementation of amino acids on crop shooting is one of the contemporary techniques used to enhance crop development and production. These findings may be due to the reality that yeast extract as a natural source of protein, cytokinins stimulates cell division and enlargement as well as the synthesis of protein, nucleic acid and chlorophyll. All of these promoting substances by yeast extract were produced highly improvement of different growth parameters which exhibited on high values of fruit length, fruit diameters, fruit number/plant, fresh weight of fruit and dry weight of fruits of hot pepper. **Marhoon and Abbas (2015)** outlined that the use of amino acids has resulted in a clear rise in plant height and number of branches of sweet pepper stems as their concentration rises. Because of their immediate function in enhancing the tissue materials of proteins and vital enzymes for managing cellular activities or activating antioxidants, the use of foliar implementation of amino acids on crop shooting is one of the contemporary techniques used to enhance crop development and production **(Cerdán et al., 2009)**. Meanwhile, **Serna et al. (2012)** have been discovered that spraying of pepper fruits with a combination of amino acids has resulted in an increased photosynthesis effectiveness, giving the greatest development in vegetative. Furthermore, **Korkmaz et al. (2012)** investigated that the use of amino acids contributed to a

marked rise in fruit height, amount of leaves and dry shoot weight relative to untreated plants. Otherwise, **Shafeek, Ellaithy and Helmy (2014)** stated that, all hot pepper parameters were gradually and substantially improved in the first and second seasons by raising the yeast extract concentration in spraying solution from 2 g.L^{-1} up to 4 g.L^{-1}. Furthermore, enhancing the vegetative development of hot pepper crop by raising the yeast extract level may result in yeast residues being an element of nature containing cytokinins and many secure, safe and non-pollutant nutrient components. In the same concern, **Maraei, Eliwa and Aly (2019)** confirmed that the use of plant biostimulants has positive effects on growth and bioactive compounds in sweet pepper plants, especially when used at appropriate concentrations. The influence was evaluated through the response of vegetative growth, and some physical and chemical characteristics of sweet pepper fruits.

Phenols and flavonoids contents

As shown in Figures 1 and 2, there were significant increases caused by amino acids foliar implementation and the best values were observed at 2 g.L^{-1} amino acids (448 and 237 mg.100g^{-1} D.W.) for phenol and flavonoids, respectively in first season and same trend was noticed in the second season. Whereas, 10 g.L^{-1} foliar application of yeast extract resulted in the highest content of phenols and flavonoids in the both two seasons.

Profile of phenolic compounds by HPLC

Fifteen phenolic compounds have been shown in the HPLC profile of methanolic extract of hot pepper fruits (Table 3). Foliar application of amino acid (2 g.L^{-1}) and yeast extract (10 g.L^{-1}) enhanced most of the phenolic compounds. The most abundant phenolic compounds were; *P*-Hydroxy benzoic acid, Caffeine, Ferulic acid, *P*-Coumaric acid, Salicylic acid, Ellagic acid, Benzoic acid and *O*-Coumaric acid.

Table 2 Effect of foliar application of amino acids and yeast extract on morphological character of hot pepper plant.

Treatment		Plant height (cm ±*SD*)	Fruit length (cm ±*SD*)	Fruit diameter (cm ±*SD*)	Total no. of fruit/plant ±*SD*	Fresh weight of one fruit (g ±*SD*)	Dry weight of one fruit (g ±*SD*)
First season							
Amino acid	Control	50.1 ±0.90 E	5.0 ±0.49 C	1.1 ±0.11 E	55 ±1.8944 F	2.1 ±0.089 D	0.42 ±0.08 E
	0.5 g.L^{-1}	52.3 ±1.89 E	5.2 ±0.89 CB	1.3 ±0.19 CD	58 ±1.894 E	2.4 ±0.187 C	0.48 ±0.08 D
	1 g.L^{-1}	53.4 ±1.97 D	5.3 ±0.78 B	1.4 ±0.15 C	59 ±1.897 DE	2.6 ±0.178 BC	0.50 ±0.067 CD
	2 g.L^{-1}	62.8 ±1.7 A	5.9 ±0.78 A	1.8 ±0.18 A	71 ±1.788 A	2.9 ±0.167 A	0.58 ±0.04 A
Yeast extract	2.5 g.L^{-1}	54.9 ±1.5 CD	5.3 ±0.516 B	1.4 ±0.17 C	60 ±2.516 D	2.6 ±0.189 BC	0.52 ±0.08 C
	5 g.L^{-1}	56.0 ±1.87 C	5.4 ±0.51 B	1.6 ±0.18 AB	65 ±1.894 C	2.7 ±0.109 BA	0.54 ±0.04 B
	10 g.L^{-1}	59.6 ±1.89 B	5.6 ±0.894 B	1.7 ±0.18 A	68 ±1.890 B	2.8 ±0.183 BA	0.56 ±0.09 AB
LSD		1.9027	0.2178	0.1499	1.9672	0.229	0.0223
Second season							
Amino acid	Control	53.1 ±1.89 E	5.2 ±0.83 D	1.5 ±0.18 E	59 ±1.894 E	2.3 ±0.178 C	0.46 ±0.016 D
	0.5 g.L^{-1}	55.3 ±1.51 D	5.4 ±0.78 D	1.7 ±0.15 D	60 ±2.516 DE	2.4 ±0.144 C	0.50 ±0.053 C
	1 g.L^{-1}	57.7 ±1.89 C	5.7 ±0.89 C	1.8 ±0.17 CD	62 ±1.788 DC	2.8 ±0.189 B	0.56 ±0.197 B
	2 g.L^{-1}	64.4 ±1.51 A	6.37 ±0.51 A	2.1 ±0.16 A	73 ±1.576 A	3.2 ±0.178 A	0.66 ±0.067 A
Yeast extract	2.5 g.L^{-1}	57.2 ±1.98 C	5.7 ±0.89 C	1.90 ±0.18 CB	63 ±2.5163 C	2.7 ±0.151 B	0.58 ±0.04 4 B
	5 g.L^{-1}	58.7 ±1.89 C	6.0 ± 0.89 B	1.95 ±0.15 B	69 ±1.894 B	2.8 ±0.1894 B	0.59 ±0.055 B
	10 g.L^{-1}	62.1 ±1.87 B	6.1 ±0.268 B	2.02 ±0.13 A	70 ±1.890 B	2.9 ±0.157 B	0.60 ±0.048 B
LSD		1.5801	0.262	0.158	1.8359	0.2422	0.0242

Note: Values aremean of three replications ±standard deviation. Different letters indicate statistically significant differences at $p \leq 0.05$.

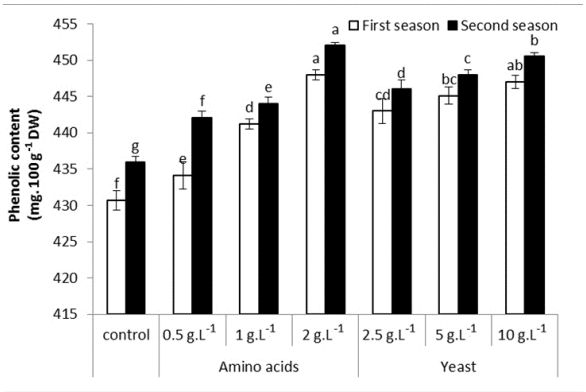

Figure 1 Effect of foliar application of amino acid and yeast extract on phenol contents of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

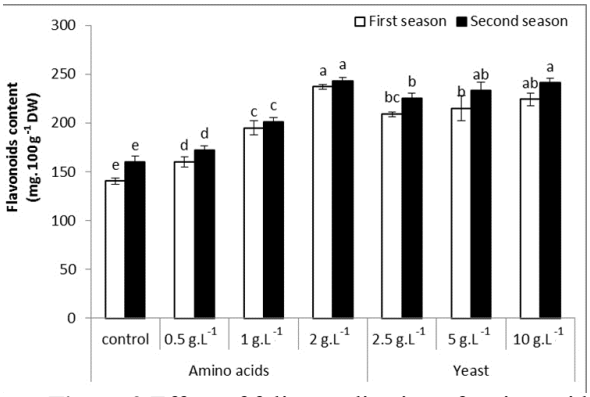

Figure 2 Effect of foliar application of amino acid and yeast extract on flavonoid contents of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

These compounds were increased by increasing the concentration of amino acid and yeast extract and this tendency was clear in the both two seasons. In general pepper fruits can provide the types of nutritional and health benefits with consumption of fresh pepper fruit.
Pepper fruits contain complex phenolic compounds binding with sugars and glycosides, also its phenolics content and antioxidant activity have been researched **(Materska and Perucka, 2005)**. The largest concentration of total soluble phenols and flavonoids were discovered with dry yeast extract of 10, 15 and 20 percent in neem plants. **(Taha, Ibrahim and Aziz, 2016)**. The phenolics content of wheat seeds treated by yeast and germinated for four days were increased according to **Gawlik-Dziki et al. (2016)**. It was found that the application of biostimulants resulted in a significant increase in the total phenolic content, sinapic acid content, as well as quercetin content, in the treated plants **(Kałużewicz, Gąsecka and Spiżewski, 2017)**. As far as phenolic compounds are concerned, plant extract increases the phenylpropanoid synthesis enzyme activity which increases phenolic compounds **(Ramachandra and Ravishankar, 2002)**. As well as the derivatives of ferulic and sinapic acids have been determined in pericarp of red pepper fruit cv. Bronowicka Ostra **(Materska et al., 2003)**. Because the profile of phenolic compounds is equally important for biological activity **(Anantharaju et al., 2016)**. Syringic acid is well known for its anti-cancer, anti-proliferative, sedative, decongestant and hepato-protective conduct **(Kampa et al., 2004)**.

Table 3 Profile of phenolic compounds content of hot pepper fruits using HPLC chromatogram as affected by amino acids and yeast extract.

Phenolic compounds	First seasons							Second season						
		Amino acids			Yeast extract				Amino acids			Yeast extract		
	Control	0.5 g.L^{-1}	1.0 g.L^{-1}	2.0 g.L^{-1}	2.5 g.L^{-1}	5.0 g.L^{-1}	10.0 g.L^{-1}	Control	0.5 g.L^{-1}	1.0 g.L^{-1}	2.0 g.L^{-1}	2.5 g.L^{-1}	5.0 g.L^{-1}	10.0 g.L^{-1}
Gallic acid	2.06	2.98	3.24	3.49	3.56	3.98	4.20	1.69	1.82	2.41	3.97	3.05	4.31	4.80
Catechol	2.83	2.89	2.96	3.62	3.04	3.16	3.22	2.56	2.73	2.97	3.59	3.07	3.43	3.75
p-hydroxy benzoic acid	11.45	20.56	26.90	32.65	15.58	19.78	27.97	13.67	16.20	22.71	29.46	16.85	20.77	25.93
Caffeine	11.31	12.95	15.20	20.18	14.83	17.39	21.95	10.31	13.10	18.20	23.27	16.46	19.47	23.89
Vanillic acid	0.91	1.56	2.31	2.90	1.44	1.85	2.17	0.61	1.03	1.66	1.81	0.89	1.62	1.96
Caffeic acid	0.72	0.89	0.93	2.40	1.03	2.46	3.61	0.98	1.45	1.80	2.69	0.88	1.01	1.47
Syringic acid	2.89	3.56	4.27	5.92	2.93	3.81	4.59	2.21	3.11	3.33	3.75	2.40	3.81	4.26
Vanillin	1.27	2.10	2.89	3.50	1.95	2.33	2.82	1.40	2.47	3.30	3.36	3.89	3.26	3.72
p-Coumaric acid	4.58	3.39	1.85	2.52	3.94	3.32	2.07	3.76	3.94	4.25	4.65	3.49	4.85	4.15
Ferulic acid	6.88	4.61	3.34	3.90	6.04	4.88	3.59	6.86	4.41	4.63	6.38	4.10	5.53	5.48
Ellagic acid	2.25	2.96	1.13	1.13	1.48	ND	2.58	1.91	2.20	1.84	2.49	2.56	2.77	2.98
Benzoic acid	1.29	1.11	0.93	0.09	0.12	1.57	0.79	1.06	1.85	1.32	1.45	1.23	1.68	1.98
o-Coumaric acid	1.08	0.89	1.64	0.40	0.63	0.08	0.82	1.46	1.42	1.97	2.49	1.56	1.14	0.95
Salicylic acid	2.79	1.23	3.79	1.87	ND	2.57	2.78	1.89	1.95	2.91	3.27	2.13	2.63	2.81
Cinnamic acid	0.41	0.43	0.47	0.52	0.44	0.46	1.64	0.54	0.53	0.60	1.38	0.97	0.81	1.45

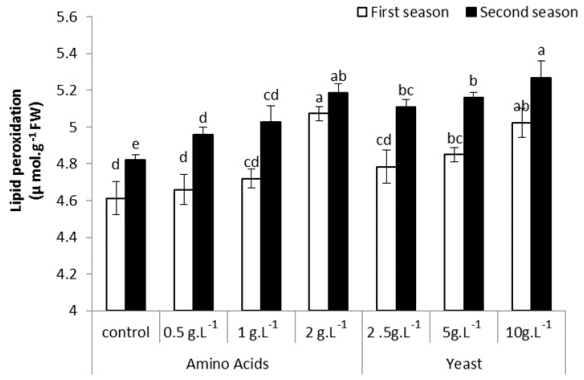

Figure 3 Effect of foliar application of amino acids and yeast extract on lipid peroxidation of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p<0.05$

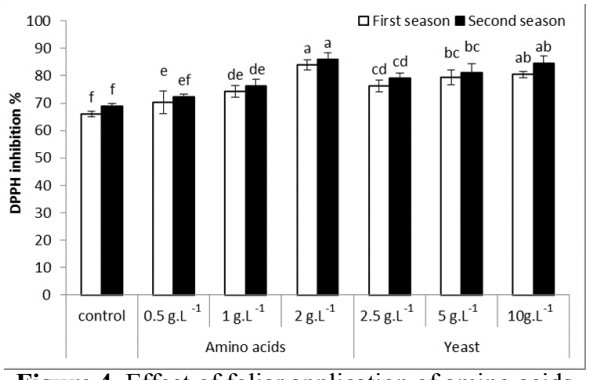

Figure 4 Effect of foliar application of amino acids and yeast extract on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p<0.05$

One of the most important phenolics, ferulic acid, is known for its physiological activities, such as, anti-microbial, anti-inflammatory, anti-cancer etc. It also reduces` serum cholesterol improves the viability of sperm **(Mussatto, Dragone and Roberto, 2007)**. Also, *P*-Coumaric acid, is frequently found in foods such as barley, marine beans, tomatoes carrots, etc. and is well-documented for its antioxidant behaviour. Antioxidant conduct is thought to reduce cancer nitrosamine formation in the abdomen **(Ramadoss Karthikeyan, Chapala and Puttagunta, 2015)**. It is well known that phenolic compounds possess many biological activities; the pro-health impact of polyphenols is related mainly to their commonly reported antioxidant properties resulting from their ability to neutralize free radicals, disrupt autooxidation chain reactions, chelate transition metal ions, and inhibit the activity of pro-oxidant enzymes **(Carocho and Ferreira, 2013)**. While, Caffeic acid is one of the main compounds of hydroxyl Cinnamic acid present and is a well-known antioxidant that encourages immunity, controls blood lipid and anti-mutagenic levels.

Lipid peroxidation content and free radical scavenging activity (DPPH)

With respect to the content of lipid peroxidation, Figure 3 showed that 2 g. L^{-1} foliar amino acids application had the highest content (5.073 µmol MDA g^{-1} F.W.) in the first season. Whereas, 10 $g.L^{-1}$ of yeast foliar application had the highest content in the second season (5.27 µmol MDA g^{-1} F.W.). Results obtained in Figure 4 also, showed that in the first and second seasons foliar application 2 $g.L^{-1}$ amino acids had the largest inhibition percentage of 1,1-Diphenyl-2-picrylhydrazyl (DPPH). Additionally, exogenous use of crop development regulators has a positive effect on MDA accumulation relative to controls. Plant development regulators play an efficient part in preserving fruit cell membranes fluidity and integrity. The equilibrium between the production and detoxification of ROS is sustained by enzymatic and nonenzymatic antioxidants. The highest antioxidant activities estimated as free radical scavenging activity against DPPH and reducing power were increased of treated quinoa seeds by yeast extract **(Abdallah, Habbasha and El Sebai, 2016)**.

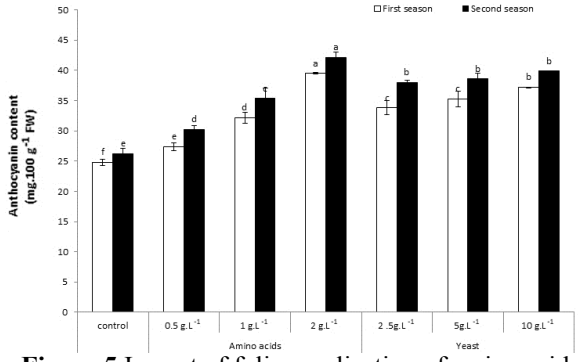

Figure 5 Impact of foliar application of amino acids and yeast extract on anthocyanins content of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

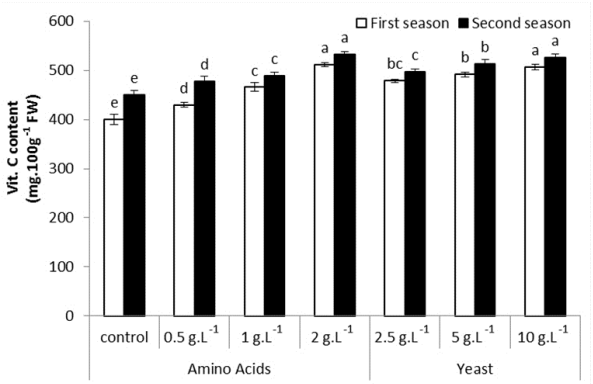

Figure 6 Impact of foliar application of amino acids and yeast extract on vitamin C content of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

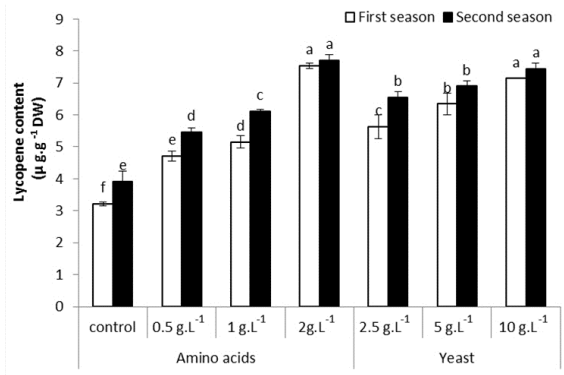

Figure 7 Impact of foliar application of amino acids and yeast extract on lycopene content of hot pepper fruits. Vertical bars show standard deviation (n = 3). letters indicate statistically significant differences at $p \leq 0.05$.

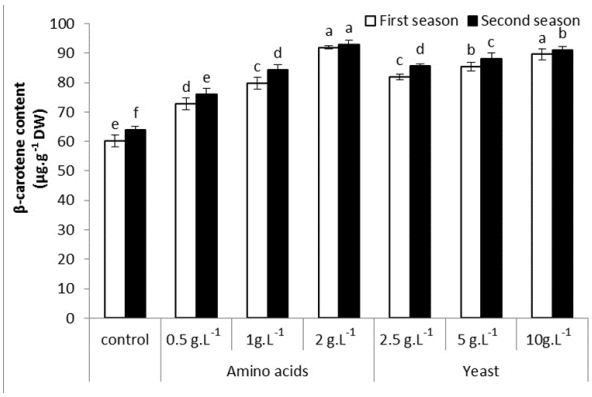

Figure 8 Impact of foliar application of amino acids and yeast extract on β- carotene of hot pepper fruits. Vertical bars show standard deviation (n = 3). Different letters indicate statistically significant differences at $p \leq 0.05$.

Anthocyanins, Vitamin C, lycopene and β-carotene.

Differences in anthocyanins content among hot pepper were noted as influenced by amino acids foliar application and yeast extract (Figure 5). The results showed that in the first and the second seasons (39.49 and 42.14 mg.100g^{-1} F.W.) the anthocyanins content of hot pepper fruits increased with foliar implementation of amino acids (2 g.L^{-1}). As well as 10 g.L^{-1} yeast extract foliar application showed the highest anthocyanins content in the first and second seasons, (37.20 and 39.87 mg.100g^{-1} F.W.) respectively. The results in (Figure 6) also showed that in first and second seasons, foliar application with (2 g.L^{-1}) amino acid had the highest ascorbic acid content (512 and 533 mg.100g^{-1} F.W.), respectively compared to the control. The content of lycopene improved with foliar implementation of amino acids, and the highest rise was noticed in both seasons with 2 g.L^{-1} amino acid (7.53 and 7.70 µg.g^{-1} D.W.). Otherwise, 10 g.L^{-1} foliar yeast extract application yielded the highest lycopene content in the first and second seasons (7.14 and 7.44 µg.g^{-1} D.W.), respectively as declared in (Figure 7). In the first and second seasons, β-carotene content increased with 2 g.L^{-1} foliar amino acid application (91.9 and 93 µg.g^{-1} D.W.), respectively. While, for the first and second seasons the content of 10 g.L^{-1} of yeast extract reduced to (89.6 and 91.1 µg.g^{-1} D.W.), respectively (Figure 8). **Belal, El-Kenawy and Uwakiem (2016)** stated that spraying flameless grapevines three times with single or combined methionine, glutamic acid and argentine, considerably enhanced complete anthocyanins and total phenols. The promotional impact of amino acids on the development and fruiting of flameless grapevines could be ascribed to their beneficial action to protect crops from oxidative stress, improving proteins biosynthesis by polymerizing amino acids, ethylene, GA_3, IAA, cyokinins, pigments of crops, and organic foods **(Davies, 1982)**. Meanwhile, **Abou-Zeed et al. (2014)** found that the use of yeast extract has been helpful in enhancing the vegetative vigour and nutritional status of Balady Mandarin trees leading to increase yield and improv the fruit quality and contents of vitamin C. As indicated in other studies, the use of yeast extract resulted in increased production of ascorbic acid **(Zlotek, 2017)**. Otherwise, **Abo Sedera et al. (2010)** found that spraying strawberry crops by amino acids (peptone) considerably improved fruit vitamin C relative to control treatment at 0.5 and 1.0 g.L^{-1}. **Shafeek, Ellaithy and Helmy (2014)** discovered in the same concern that hot pepper vitamin C was promoted by increased the amount of yeast extract from 2 g.L^{-1} to 4 g.L^{-1} but this rise did not achieve the important amount.

CONCLUSION

The use of biostimulants as amino acids and yeast extracts stimulate the growth of crops to overcome the harmful effect of some environmental stress. The findings revealed that foliar of amino acid or yeast extract application improved the growth parameters of hot pepper compared to control in both the first and second seasons, particularly foliar amino acid (2 g.L^{-1}) or yeast (10 g.L^{-1}) implementation. With foliar application of amino acid or yeast extract improved phenol, flavonoid, anthocyanins, ascorbic acid, lycopene and ß-carotene relative to control in the first and second seasons. Foliar application 2 g.L^{-1} amino acids had the greatest 1,1-Diphenyl-2-picrylhydrazyl (DPPH) inhibition proportion and the highest lipid peroxidation content. The HPLC techniques that represent the impact of foliar application of amino acid or yeast have elucidated the derivatives of phenolic compounds. Phenolic compounds were increased by increasing the concentration of amino acid and yeast extract foliar application in the both two seasons.

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APPLICATION OF ULTRA-HIGH-TEMPERATURE PROCESSING OF RAW MILK TO IMPROVE CHEESE QUALITY

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ABSTRACT

The increase in natural cheese production has brought issues related to ensuring the production of high-quality competitive products to the fore. The development of the cheese market requires constant improvement of the existing methods of production and the search for new technological solutions, which will allow us to counterbalance the low quality of raw materials, which is currently a serious problem for domestic cheese production. A promising method of realising the benefits of high-temperature (HT) and ultra-high-temperature (UHT) milk processing in cheese making is the development of new types of cheese with a high moisture content; however, there are very few publications that discuss these approaches. The development of advanced technologies for the production of low-temperature second-degree solid cheeses with the use of HT and UHT processing, related to the improvement of the technological process and the equipment and technological scheme of production of solid cheeses. The main direction of the development of cheese production at the present stage is the improvement of existing technological processes, the development of resource-saving technologies and the improvement of the natural solid rennet cheese quality. The results of our research, related to the study of the composition and safety of milk raw materials, the impact of various technological factors on the cheese production process and the quality of the products obtained, are the basis for our resource-saving technology for the production of solid rennet cheese.

Keywords: milk; cheese; UHT processing; cheeseability of milk; cheese ripening

INTRODUCTION

Most dairies, including cheese-making companies, today experience some difficulties in ensuring the production of sufficient raw milk, which meets certain requirements, such as safety, nutritional and biological value, and technological properties. Fresh cow's milk contains all the nutrients and biologically valuable substances necessary for the human body in a well-balanced ratio and in easily digestible form **(Kuznytsov and Shyler, 2003)**. When selecting milk for cheese making, it is necessary to take into account the quality and safety of milk, as well as the specific requirements for cheese. Milk for cheese making is considered to be according to the accepted technology, in compliance with the rules of sanitation, a high yield of a product of guaranteed quality can be obtained. Raw milk should not contain chemical or microbiological contaminants. “Cheeseability” is a complex characteristic of milk. One of the major requirements of cheese making is its ability to quickly deal with the formation of a dense clot that gives off serum and retains fat. A second fundamental requirement is that milk should be a good environment for the development of the required microflora for the formation of organoleptic indicators of cheese **(Kuznytsov and Shyler, 2003; Skott, Robynson and Uylby, 2005)** (Table 1). The protein content in milk determines the yield of cheese. When it comes to protein and its role in cheese production, protein-casein, the amount of which in milk is proportional to the total protein content, first of all is used in practice. The total protein content of milk is most often used as a criterion for milk cheese. If the degree of protein usage and consequently casein in the production of cheese decreases, it decreases the transition of fat to cheese and as a consequence increases the loss of fat with serum. This is confirmed by the data provided by **Hudkov (2003)**, according to which the decrease in casein content in milk by 0.23% in the production of Parmesan cheese is accompanied by an increase in fat loss with serum by 2.04% and, as a consequence, a decrease in cheese output by 0.62%.

Table 1 Comparative characteristics of milk and cheese in the composition of essential amino acids.

Amino acids	Amino acid content in proteins, mg.100g^{-1}	
	Standard	Cheese
Tryptophan	1.0	1.4
Phenylalanine + tyrosine	6.0	10.5
Leucine	7.0	10.4
Isoleucine	4.0	5.8
Threonine	4.0	4.8
Methionine + cystine	3.5	3.2
Lysine	5.5	8.3
Valine	5.0	6.8
Total	36.0	51.6

Table 2 Indicators of cheese-making capacity of milk raw materials in cheese-making.

Value	Indicators
Number of spores of lactose-fermenting microorganisms in 1 cc, no more	13
Sort, no lower	I
Class on rennet-fermentation test, no lower	II
Class on reductase test, no lower	II Class
KMAFANM, CFU in 1 cc	1 x 10^6
Number of somatic cells in 1 cc	500000
Acidity T, no more	18
Mass fraction of protein, no lower	2.8

Milk protein in essential amino acids is very close to the "ideal or reference protein" proposed by the World Health Organisation FAO/WHO **(Skott, Robynson and Uylby, 2005; Hudkov, 2003)**. Comparative characteristics of the most important essential amino acids of "reference protein", milk and cheese according to **Hudkov (2003)** are given in Table 2.

Any thermal action affects the components of milk and its physical and chemical properties. The degree of exposure mainly depends on the temperature regime and the duration of the temperature. The protein system has high heat resistance due to casein, which refers to heat-resistant proteins. Without coagulation, it withstands heating at a temperature of 140 °C for 10 – 20 min. The main mineral components involved in the rennet coagulation, as well as in the structure and texture of the cheese, are calcium and phosphorus. The last ones are in milk both in true solution and in colloidal form. In fresh raw milk, ~33% of calcium and 53% of phosphorus account for the true solution. In milk thermal treatment, a considerable part of soluble forms of phosphorus and in particular calcium becomes colloidal **(Tverdokhleb and Ramanauskas, 2006)**. The heat treatment changes the salt composition of milk and, first of all, the composition of calcium salts. These changes are due to the transition from the monomolecular form of hydrophosphates and calcium dehydrophosphates to poorly soluble calcium phosphate, which is aggregated and colloid deposited on casein micelles. In this case, there is irreversible mineralisation of the caseinate-calcium-phosphate complex, which causes a disruption of the structure of micelles and a decrease in the heat resistance of milk. Part of the calcium phosphate together with the denatured whey proteins form a milk stone.

A promising method of realising the benefits of HT and UHT milk processing in cheese making is the development of new types of cheese with a high moisture content; however, there are very few publications on this approach regarding the usage of HT and especially UHT milk processing.

Scientific hypothesis

This article proposes a way to increase the cheesiness of milk, increase the cheese yield and intensify the cheese ripening process by using HT and UHT processing of raw milk at temperatures above 100 °C. During HT processing, there is a high bactericidal effect, the transition of the whey proteins to milk curd, while improving the moisture-holding capacity of the curd and reducing the ripening time of the cheese. This will solve the problem associated with process improvements, increased yields and an expanded range of high-quality cheese with shortened maturation.

MATERIAL AND METHODOLOGY

We used a set of conventional and special physical, chemical, biochemical, physicochemical, microbiological, mathematical methods in this work, with graphic processing of the results corrected for work with milk raw materials and solid rennet cheeses. Industrial testing of the advanced equipment and technological scheme and developed technology of solid rennet cheese production from milk, which was HT processing, was carried out at the experimental unit, which was installed by the staff at the UMMU. The production of experimental batches, tasting and the sale of hard cheese using HT milk processing was carried out in the production conditions of the LLC “Litinskyi Dairy Factory”, Litin City in the Vinnytsia Region of Ukraine. Substantiation of the selection of starters for the process of making rennet cheese with low temperature of the second heating from milk, which has been pre-treated and UHT treated, was considered using concentrates BK-Uglich-5A produced by the Experimental Biofabrication of Uglich, Russia and

ALU production TIMM Kiev, Ukraine. As the main bacterial starter, we used mesophilic lactic acid bacteria, which is used for cheeses with a low temperature of second heat. In addition, we also used ferments of thermophilic lactic acid sticks. The starter was used in various combinations. The peculiarity of the bacterial starter culture is that after HT treatment at 81 ±1 °C with a holding time of 25 s and UHT treatment at 120 ±1 °C with a holding time of 5 s, the milk was cooled to a temperature of 66 °C for 10 s and further cooled up to 10 °C, that is, until the milk matures. The cooled milk was stirred for 5 min to distribute the constituent components of the milk evenly and 0.1% of the main bacterial leaven made from a concentrate comprising *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetilactis*, *Leuconostoc lactis* was added and left for 12 h for maturation (Table 3). After 12 h, the milk was heated to the fermentation temperature of 34 °C to make a different ratio of basic and additional fermentation: 0.7% basic and 0.1% additional, 1.5% basic and 0.3% additional, 2.0% basic and 0, 5% extra. As a result of the conducted research, it is established that with the increase of the total amount of bacterial starter culture, there is an increase in the acidity of the milk before settling, a decrease in the duration of the grain mixing after the second heating and an increase in the acidity of the serum at all stages of the technological process. For the fermentation of milk that has undergone HT processing, we conducted with some differences in comparison with the existing technological process, which is widely used in the production of natural rennet cheeses with a low second heat. The peculiarity of introducing bacterial starter cultures is that after HT treatment at 81 ±1 °C with a holding time of 25 s and UHT treatment at 120 ±1 °C with a holding time of 5 s. The milk was cooled to 66 °C for 10 s and further cooled up to 10 °C, that is, until the milk ripens. The cooled milk was stirred for 5 min to distribute the components of the milk evenly and 0.1% of the main bacterial starter made from concentrate was added, left for 12 h for maturation. After 12 h, the milk was heated to the fermentation temperature of 34 °C and made a different ratio of basic and additional fermentation: 0.7% basic and 0.1% additional, 1.5% basic and 0.3% additional, 2.0% basic and 0.5% extra.

In the technological process of cheese production, we used an experimental unit for HT steam contact processing of milk, mounted by the staff at TIMM UAAN, according to the agreement. The principle of operation of the pilot plant is shown in Figure 1.

Normalised milk was fed into the collection for normalised milk 2 and pump 7 was sent to the section 1-2 of the plate heat exchangers, where the milk is heated by the secondary steam coming from vacuum chamber 3, to a temperature of 35 – 40 °C. The heated milk from section 1-2 enters section 1-3, where it is heated by hot water, which has a temperature of 90 – 95 °C and is fed through automatic valve 9 from the water heating system to a temperature of 60 – 65 °C. In injection device 4, the heated milk is mixed with purified water vapor at a temperature of 140 – 160 °C.

The water vapor was purified before being mixed with milk, passing through a cyclone filter to separate condensed moisture droplets and mechanical impurities and metal-ceramic titanium filter inserts for microfiltration.

After HT heating with a holding time of not more than 3 s, the milk enters vacuum chamber 3, where it is instantly cooled to a temperature of 75 – 78 °C, with the vacuum depth in chamber 3 being 0.06 – 0.08 MPa. To ensure the rational use of the vacuum chamber heat, secondary steam pump M2 is supplied in sections 1-2 and 1-4 of the plate heat exchanger, where it is condensed by cooling upon contact with cold milk and water. The milk cooled in the vacuum chamber enters section 1-1 of the plate heat exchanger, where it is cooled to the fermentation temperature of 32 – 34 °C and goes to the cheese-making bath for fermentation. For the following technological operations, we used traditional equipment typically used for making cheese.

Statistic analysis

The experiments were performed in triplicate. The mathematical description of the technological process of cheese aging is a kind of regression equation found by statistical methods on the basis of experimental data. In the processing of experimental data for the significance level $p = 0.05$, such statistical criteria as the Cochran criterion were used to assess the homogeneity of the variances, the Student's test to evaluate the significance of the calculated coefficients, and the Fisher criterion to evaluate the adequacy of the obtained equation. As a result of the statistical processing of the experimental data, when determined the influence of the above technological factors on the moisture content of cheese by the Kohren criterion, it is determined that $0.2_p < 0.48_t$. This indicates that the resulting variance is homogeneous and there are no gross errors.After deriving the equation and determining the significance of the calculated coefficients according to the student's test, a regression equation was obtained, which describes the dependence of cheese moisture on such technological factors as the temperature of the second heating, the amount of added water for deoxidation and the mass fraction of salt in the product. Checking the adequacy of the coefficients of the equation by the Fisher criterion showed that $0.07_p < 9.3_t$. That is, the obtained regression equation adequately describes the process of cheese aging. Data processing, calculations and graphing were performed in MathCAD 2015.

RESULTS AND DISCUSSION

The main type of heat treatment of raw milk in the raw production industry is pasteurisation, which results in the reduction of pathogenic and technically harmful microorganisms to a safe level. The pasteurisation regimes of used raw milk in the production of hard rennet cheeses do not destroy all the microflora. Even milk pasteurisation at a temperature of 75 – 76 °C for 20 – 25 s, which corresponds to the upper limit of heat treatment of raw milk in the production of solid rennet cheeses, ensures the efficiency of neutralisation of heat-resistant bacteria by only 94.6%. The best results are obtained with high-temperaature (HT) and ultra-high (UHT) processing of milk with a holding time of 20 – 24 s **(Zolotyn, 1979)**.

Table 3 Characteristics of yeast lactobacilli used in this work.

Name of yeast	Manufacturer	Yeast stock LB	The Kind of yeast
Basic complex	Experimental biofactory. Uglich, Russia	*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *Lactococcus lactis* subsp. *diacetilactis, Leuconostoc lactis*	BC-Uglich-5A - There are diplococci, chains of cocci of different lengths
Additional	State experimental enterprise of bacterial fermentations TIMM	*Lactobacillus acidophilus* (residual race)	BZ "ANV" cocci present with sticks of different lengths

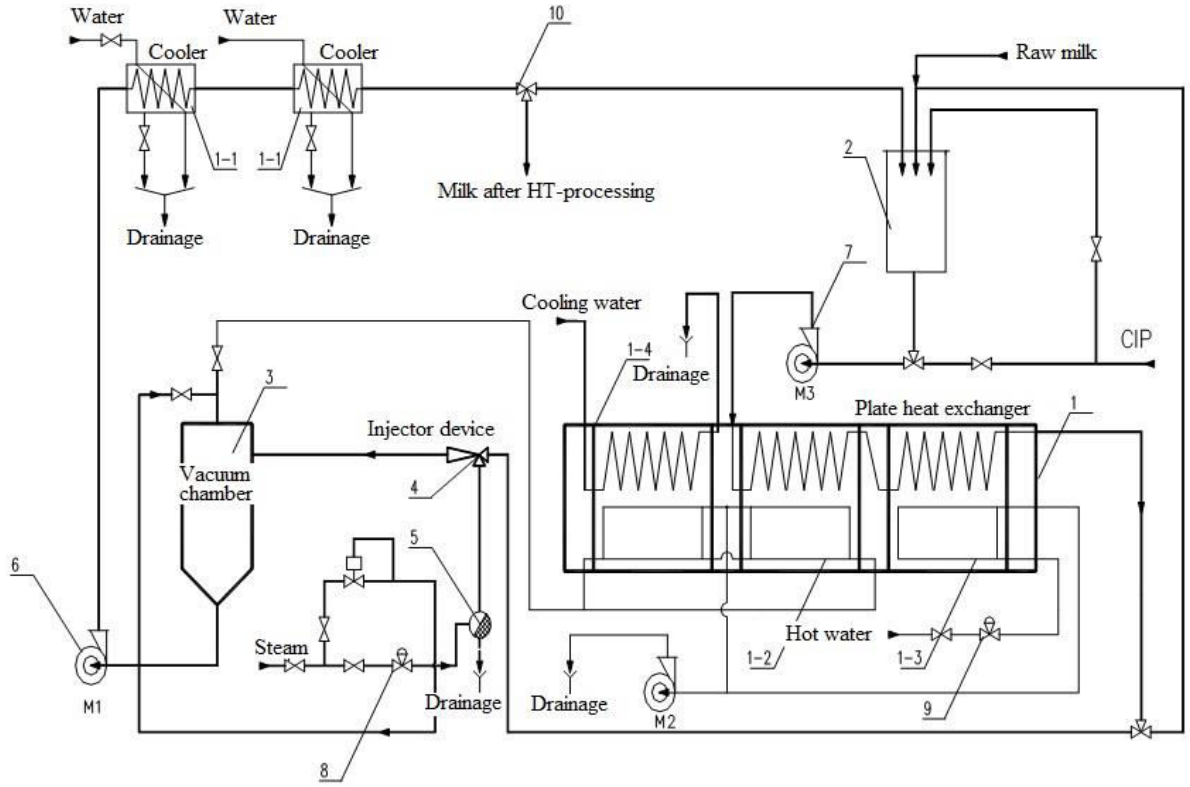

Figure 1 Scheme of sterilisation chamber for direct (steam contact) heating of milk by injection (injection) of steam into milk. Notes: 1-1 – coolers; 2 – collection of normalised milk; 1-2 – heat exchanger; 1-3 – heater; 3 – vacuum chamber; 1-4 – plate heat exchanger; 4 – injection device; 5 – valve; 6 and 7 – centrifugal pumps; 8 and 9 are valves; M1, M2 and M3 are pumps for pumping milk.

Table 4 Costing of 1 ton of cheese by cost items, UAH.

	Name of cost items	Cheese «Bravo»		Cheese «Litinskyi»
		HT	UHT	
1	Raw materials and basic materials	27196.60	26702.5	27974.10
2	Transportation and procurement costs	5229.89	5229.89	5229.89
3	Cost of packaging and packaging	930.26	930.26	930.26
4	Cost of auxiliary materials	39.50	39.50	39.50
5	Cost of energy costs	1080.90	1080.90	638.19
6	Basic and additional wages of production workers	377.63	377.63	377.63
7	Deductions on social insurance	140.40	140.40	140.40
8	Equipment maintenance and operating costs	453.16	453.16	453.16
9	Common production costs	736.38	736.38	736.38
10	Other production costs	180.69	178.45	182.60
	Production cost	36365.71	35869.07	36702.11
11	Administrative expenses	1454.63	1434.76	1467.85
12	Selling expenses	363.66	358.69	367.02
	Total cost	38184.0	37662.52	38536.98

The possibility of using short-term high temperatures is explained by the fact that living cells of microorganisms respond more quickly to high temperatures than components of milk. In other words, the rate of destruction of microorganisms in the short-term action of high temperatures is higher than the rate of chemical reactions and the destruction of the constituent components of milk over this period of time.

To conclude, HT and especially UHT treatment is a very effective way of destroying bacterial microflora and improving the quality of raw milk by such an indicator as a "contamination tank", which improves the raw material milk. However, it should be noted that under the influence of high pasteurisation temperatures, changes occur in the salt composition of milk raw materials, as well as in the structure and properties of proteins **(Tepel, 1979; Singh and Waungana, 2001; Kazumoto and Tetsuo, 1988; Bashaeva and Khaerdynov, 2008; Adámek et al., 2016)**. The usage of high pasteurisation temperatures of milk in cheese making is mainly limited to soft cheese technologies and is almost not used in the production of hard cheese **(Tverdokhleb and Ramanauskas, 2006; Horbatova, 1984; Khramtsov, Emelianov and Evdokymov, 2006)**. This leads to a deterioration of rennet coagulation and dehydration of the curd grain **(Khramtsov, Emelianov and Evdokymov, 2006)**.

Work on this topic was carried out at a working enterprise on the order of the same company for the development of technology for the production of new types of rennet cheeses with short maturation (20 days).

To improve the cheese making and sedimentation of the milk, which has undergone HT treatment, the application of a double dose of calcium chloride, rennet enzyme and 0.1%, the introduction of yeast in the amount of mesophilic lactobacilli 1.5% and thermophilic lactobacilli *L. acidophilus* – 0.3% for 12 h at 10 – 12 °C. It is proved that cheese is made of milk, which according to the rennet-fermentation test corresponds to three classes, after HT processing and provides the formation of high-quality indicators. The effect of bacterial starter composition on the maturation process and the quality of the cheese was investigated. It is established that the increase in the total amount of bacterial fermentation leads to an increase in the acidity of the milk before sedimentation, a reduction in the duration of grain mixing after the second heating and an increase in the acidity of the serum. The influence of technological factors on the process of ripening of rennet cheese with the low temperature of the second heat is investigated. Optimal technological parameters of ripening of rennet cheeses for obtaining a product with high organoleptic properties are established. The use of HT milk processing allows us to increase the yield of cheese, because part of the whey proteins goes into the clot, and then into the cheese, which increases its output and moisture-holding capacity. The use of highly effective bacterial starters, high moisture content in the product and the maturation of the product at elevated temperatures allow us to obtain cheese with a short maturation period of 25 – 30 days.

The economic efficiency from the implementation of the developed technology is 352.98 UAH for HT processing per 1 ton of Bravo cheese at UHT processing 869.6 UAH per 1 ton of Bravo cheese, which is achieved by reducing the cost of materials, by maximising the use of whey protein and increasing the mass fraction of moisture in the finished product and reducing the duration of maturation (Table 4). However, it is necessary to take into account the social effect of the introduction of the developed technology, which is that it allows the use of raw materials of personal peasant farms at the excess content of microorganisms of the second and third temperature groups in milk. This is while obtaining cheeses of guaranteed quality with high microbiological and topical in the modern period of development of the agrosphere of Ukraine in the period of market economy.

CONCLUSION

As a result of our research, the technological parameters of the production of solid cheese are substantiated and the influence of HT processing on the physical and chemical properties of raw milk is investigated.

1. For the first time in Ukraine the effect of HT and UHT processing on the process of coagulation of protein fractions of milk in cheese production was investigated.
2. It is established that at temperatures of heat treatment of milk (115 – 125 °C) and holding (20 – 24 s), 0.2% of whey proteins additionally pass into cheese curd and cheese (8 – 9%) increases.
3. It is established that the use of UHT milk treatment compared to traditional pasteurization regimens adopted in cheese making increases the antibacterial effect by more than 200 times.
4. Mathematical model of dependence of quality of firm cheese on technological factors is obtained.
5. Provision has been extended for data that affect the formation of quality indicators and the ability to store solid cheese from milk that has undergone HT and UHT.

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THE IMPACT OF EU SUPPORT RESOURCES ON BUSINESS SUCCESS OF FAMILY-OWNED BUSINESSES

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ABSTRACT
Nowadays, we live in an accelerated, complex, globalized world, where expectations are high for everyone. The child of today has to train a lot to be successful. The enlargement of the European Union and the expansion of the Schengen zone opened gates to society and economy that were not dreamed before by the countries of Central and Easter Europe. Many businesses were able to develop and grow, and they could achieve the goals they set until the end of the ´90s. The situation has changed since the turn of the millennium. The global markets, the easily accessible products and services, the convenience of the World Wide Web, the growing competition, the multinational companies and foreign chains, the high consumer expectations and the requirements and standards of EU have resulted the end of many businesses. The aim of the paper was to evaluate the impact of European Union support funds on the business of family enterprises in the southern districts of the Slovak Republic. The research material was obtained from primary sources. Data were subject of deeper analysis by statistical methods. Subsequently hypotheses were formulated and verified by use quantitative methods. According to results, in a group of businesses not supported by EU programs more than half of the respondents could not develop in the last 3 years, they had negative results. It can be stated that if external support and consultancy are present in family business life, the younger generation will find the family business more dynamic, innovative and attractive and therefore they will continue to run the family business.

Keywords: family business; small and medium-sized enterprises; agribusiness; financial support; European Union

INTRODUCTION
Entrepreneurship of small and medium-sized enterprises accounts for up to 98% of all business activities. A relatively large proportion of these are family enterprises particularly common in the food processing sector. Agrisector and food processing sector are integral part of Slovak land regions. There are some differences between regions in Slovakia and in another EU regions, enterprises and their strategies or concepts **(Švec and Madleňák, 2017; Antošová et al., 2017; Selivanova-Fyodorova et al., 2019; Horská et al., 2019)**. However, when examining family enterprises, we encounter a problem of their definition. The family businesses do not have a precise definition either in Slovakia or in another member state of the EU. There is also a lack of legal frameworks that would specifically support this type of business, e.g. the employment of family members or the quality of products and services **(Mura, 2017)**. But there are specificities or conditions that can be used to determine whether a business is a family business **(Hudáková et al., 2014)**. The large part of family businesses belongs to the SME sector, but in the absence of a formal definition, their measurability is low. An exact number cannot be given for their proportion **(Vágány et al., 2015; Androniceanu et al., 2017)**. Some estimates and research reports show that the proportion of family businesses within the SME sector in Europe is about 70% and 80% **(Ivanová, 2018)**. Some estimates also indicate a higher ration. Compared to this, the proportion of family businesses within the SME sector on the North American continent can reach 90% **(Hnátek, 2015)**. The family businesses have 80% share in creating new jobs and 60% share in full employment. In the Middle East, this figure is over 90%. In Japan 99% of businesses are family-owned businesses **(Baassiri, 2018; Hammadeh, 2018)**. For a more accurate classification, many authors and researchers tried to create a definition, but most of them only listed factors specific to family businesses, generally based on the following four aspects **(Csákné Filep, 2012)**:
- the ownership of the business can be linked to members of a particular family or to members of 2 – 3 families,
- the majority of the family´s ownership and thus the influence of its decisions **(Strážovská and Jančíková, 2016)**,
- cooperation and active participation of not only one, but two or even three generations in regular and extraordinary

activities and decision-making situations affecting family business,
- members of the owner family intend to transfer ownership and management of the business to the next generation (child, grandchild).

In the late 1990s, the American Massachusetts Mutual Life Company conducted a research on American family businesses. The aim of the research was to define an acceptable definition or criteria for better measurability and investigation of family businesses. In 1997, the results of their research were published. According to them, if at least one of the following three criteria is met, we are talking about a family business. These three criteria are **(Strážovská et al., 2008; Vilčeková et al., 2018)**:
- the owner of the business treats his own business as a family business and sees it as a family business,
- the owner of the business is going to give the ownership of the family business to the young generation in the future,
- within the owner of the family business, at least one (or more) family members are work in leadership; in addition, other family members are working in the business, who are involved in management and everyday operational tasks.

In order to look at the characteristics and definition criteria of family businesses from more sides, let´s look at a list of the definitions of the **European Commission (2018b)** and **Pricewaterhouse Coopers (2015)**:
- an enterprise can be treated and examined as a family business if the opinion of the family or one of its members has decisive weight in making decisions, regardless of the size of the business size (micro-enterprise, small and medium-sized enterprise, large enterprise). These members are natural persons who established the business and who invested the assets and capital of the business at the time of its foundation. The assets of the business are owned by them or their relatives,
- members of the family use majority power in decision-making, either directly or indirectly, so their opinion plays a crucial role,
- minimum one member of the family is actively and permanently involved in the management and operational activities of the business,
- enterprises whose shares are listed on a stock exchange may be considered as family business if the owner and the members of his family hold at least 25% of the decision-making rights.

It can be concluded that the common interest in family businesses is one of the most effective driving forces, thus in general it can be concluded that corporate goals and family goals overlap.

By interpreting **The Treaty of Lisbon (2007)**, it can be clear to everyone that the main purpose of the treaty is to make the European Union the most dynamic and competitive economy in the world. This statement carries a huge responsibility for the SME sector, since the SME sector is the main driver of every economy, including the European Union. That is why it is very important that the SME sector receive the appropriate support at both state and EU level. The primary goal is to give all opportunities to the SME sector to increase its competitive advantage. Managing all this, by enabling businesses to effectively and professionally exploit the intellectual capital of their employees, their abilities and affinity. The combination of these two factors will determine the future of the SME sector, including the economic growth of the EU and the achievement of short- and long-term goals. It is no secret that the EU places great emphasis on innovation activity of the SME sector. It is well known that this sector is best suited for implementing certain innovation activities, as they are more responsive (due to their size) to changes in market needs and circumstances **(Todericiu and Stăniț, 2015; Čulková et al., 2015; Duma, 2015; Martyniuk, 2016; Huňady et al., 2017; Dvorský et al., 2018; Oláh et al., 2019)**.

It must be mentioned that the development **(Grabara, 2019)** and competitiveness of the SME sector was hit by the 2008 global economic crisis (like large enterprises and governments). According to **Hágen and Holló (2017)**, the most important player in recovering from similar economic crisis is the SME sector. This must be recognized by all governments, and they should take appropriate steps to reduce the negative effects of the crisis in the SME sector. Thus, both parties (the SME sector and the government) can make a positive return.

Over the last few years, the EU has recognized that the SME sector needs serious support. That is why the EU´s economic policy decision-making bodies have decided to spend more than 450 billion € on support member states through the European Structural and Investment funds (there are more of them) between 2014 and 2020. The purpose of this support is to encourage small and medium-sized enterprises to create jobs and innovate **(Andrejovská et al., 2016)**. The aim of the EU is therefore to continue to play a leading economic role in the world market. In order to achieve this goal, citizens of the member states must have jobs and enterprises must be able to develop and innovate continuously. Thanks to this, the enterprises can respond effectively to changes in environmental nuisance **(Musová et al., 2018)**.

Another purpose of the EU´s economic decision-making bodies is to improve the internationalization of the SME sector. In the past, there was a serious deficiency that the SME sector did not export products or services to foreign markets **(Andrejovská et al., 2019; Androniceanu et al., 2019)**. Although it is also a fact that nearly 50% of the SME sector´s businesses (mainly medium-sized enterprises) already does engagement in productive activities **(Muafi Grabara and Sudiyarto Siswanti, 2019; Mustafin et al., 2018)**, so they produce products (predominantly in the agriculture or construction sector; **Supeková, 2015; Ubrežiová et al., 2008; Peráček et al., 2018)**. Although the willingness of the SME sector to export has slightly improved by 2018, there is still plenty to develop in this regard **(Dupcsák and Marsalek, 2016; Milošovičová et al., 2018)**.

Table 1 shows the five European Structural and Investment Funds and shows which areas are supported by each fund. It is important to mention that these five funds are managed jointly by the **European Commission (2018a)** and the member states of EU. The aim of these funds is to support investments which has primary purpose is to create jobs and increase the EU´s economy, keeping in mind sustainability and environmental awareness **(Buzás et al., 2003)**. The five funds primarily support five main areas, which are the following:
- innovation and research,
- the digital agenda,

- the low-carbon economy,
- help to protect and preserve natural assets
- support for small and medium-sized enterprises.

Table 1 The European structural and investments funds.

Name of fund	Support area
European Regional Development Fund	Contribute to reducing disparities between the levels of development of European regions and to reduce the backwardness of the least favoured regions.
European Social Fund	Promoting employment and social inclusion – helping people to get a job (or a better job); integrating disadvantaged people into society and ensuring fairer life opportunities for all.
Cohesion Fund	Supports infrastructure projects and projects related to energy or transport, as long as they clearly benefit the environment.
European Agricultural Fund for Rural Development European Maritime and Fisheries Fund	Supports European policy on rural development. Helping fishermen in the transition to sustainable fishing: support coastal communities in diversifying their economies.

Note: Source - own editing based on https://ec.europa.eu, online.

Scientific hypothesis

Hypothesis 1: There is a significant relationship between the subjective sense of success of family business leader and the active use of EU funds.

Hypothesis 2: There is a significant relationship between the presence of the support systems and external consultancy and the successfulness of the generation change.

By verification of hypothesis we used the level of probability $\alpha = 0.05$. This value we compared with the level of significance (*p*-value).

MATERIAL AND METHODOLOGY

The study includes primary and secondary data collection. Secondary data collection took place during the compilation of the theoretical part of the study, while the primary research was carried out in the empirical part of the study. Primary research was realized in first half of 2019 in Slovak south regions.

Statistic analysis

The most important part of the primary research was the statistical analysis of hypotheses by IBM SPSS Statistics 25. The statistical methods were used to test the hypotheses include crosstabs, chi-square test (Cramer´s V, Phi coefficient and Lambda coefficient) and frequency. Cross-tabulation is one of the most useful analytical tools and is therefore the most commonly used. Cross-tabulation tables provide a wealth of information about the relationship between the variables (nominal or ordinal). The chi-square test was used to determine whether is a significant difference between the expected frequencies and the observed frequencies in one or more categories. The chi-square statistic was used to show a relationship between two categorical variables. In statistics, Cramer´s V was used to determine strengths of association after chi-square test has determined significance. The phi coefficient is a measure of the degree of association between two variables. The phi coefficient is the quotient weighted number of the value of chi-square test and of observation units. Lambda is defined as an asymmetrical measure of association between two nominal variables, by assessing the proportional reduction of error by considering the independent variable when compared to ignoring the independent variable in the prediction of the dependent variable. Frequency analysis is a descriptive statistical method that shows the number of occurrences of each data in the sample. The relative frequencies are often shown as percentages of proportions by relation to all data as 100%.

RESULTS AND DISCUSSION

Testing of hypotheses

This part of the study deals with the testing of the hypotheses. The research question for the first hypothesis was the following:

Research question 1: Is there a relationship between the subjective sense of success of family business leader and the European Union support systems involved in family business?

It should be mentioned that instead of the success of the family business we have examined the subjective sense of success, because we cannot measure the "success" variable. Due to the nature of the research, there was no data on turnover, profit or wealth.

According the first research question, our first hypothesis was:

Hypothesis 1: There is a significant relationship between the subjective sense of success of family business leader and the active use of EU funds.

The cross-tabulation was used to test the hypothesis. The following two questions were used:

- Does the family business apply for EU support in the last 5 years?
- In your opinion, the family business in the last 3 years:

o developed dramatically (1)
o developed fast and steadily (2)
o developed slowly (3)
o stagnated (4)
o declined slowly (5)
o declined rapidly (6)
o close to bankruptcy (7)

The reader may notice that while we examined a 5-year period in the first question, we examined a 3-year period in the second question. This can be explained by the fact that in most cases the time of tender submission can be several months, even 1 – 2 years.

Table 2 The cross-table analysis between the subjective sense of success and the EU funds.

			The family business in the last 3 years							Total
			1	2	3	4	5	6	7	
EU supports in the last 5 years	Yes	Count	10	42	22	2	4	0	0	80
		% within EU supports in the last 5 years	12.5%	52.5%	27.5%	2.5%	5.0%	0.0%	0.0%	100.0%
	No	Count	2	20	42	24	58	24	36	206
		% within EU supports in the last 5 years	1.0%	9.7%	20.4%	11.7%	28.2%	11.7%	17.5%	100.0%
Total		Count	12	62	64	26	62	24	36	286
		% within EU supports in the last 5 years	4,2%	21.7%	22.4%	9.1%	21.7%	8.4%	12.6%	100.0%

Note: Source – data from primary research, own editing based on SPSS.

Therefore, the impact of an application submitted 5 years ago can only be felt and reflected in the performance of the business in the last 3 years. In addition, we considered the raking of respondents about the supports. For this reason, we thought that they would be able to evaluate the supports from the beginning of the project up to the project closure and final performance reporting in a 5-year run. Table 2 shows the results of the cross-table analysis.

Cross-table analysis shows that fewer family businesses applied for EU supports than for state supports. Of the 286 respondents, 80 family businesses applied for EU support. If we look at the affirmative answers, it can be seen that 52.5% of respondents said that their family business developed fast and steadily in the last 3 years. According to 27.5% of respondents their family business developed slowly and according to 12.5% of respondents their family business developed dramatically. This positive development was represented by 92.5% of respondents, so it can be assumed that there is a relationship between the two variables. In the next step, we observed the negative answers, where we got different results. According to 42.7% of respondents the development of their family business showed a negative trend in the last 3 years. 11.7% of respondents said that the development of their family business stagnated, so it did not begin to decline, but it could not develop. Summarizing the negative answers, it can be seen that more than half of the respondents could not develop in the last 3 years, they had negative results. Compared to the results of the above positive effect, we considered it important to examine whether our assumption is relevant for the two variables, so we subsequently carried out a chi-square test for the variables examined. In Table 3 we can see the results of statistical analysis. Based on the results of chi-square test, it can be concluded that it is worth to investigate these two variables, since the significance level is below the excepted 0.05, namely 0.000. The asymptotic significance of Likelihood Ratio is 0.000. The statistics in the Table 3 are at 6 degrees of freedom. It could be mentioned that in the measurements lambda coefficient

Table 3 Chi-square tests for EU grants.

Chi-Square Tests			
	Value	**df**	**Asymptotic Significance (2-sided)**
Pearson Chi-Square	111.088[a]	6	0.000
Likelihood Ratio	124.102	6	0.000
N of Valid Cases	286		

Note*:* Source – data from primary research, own editing based on SPSS.

Table 4 The strength of the relationship between the examined variables.

Symmetric Measures		
	Value	**Approximate Significance**
Nominal by Nominal	Phi	0.623
	Cramer's V	0.623
	Contingency Coefficient	0.529
N of Valid Cases	286	

Note: Source – data from primary research, own editing based on SPSS.

was 0.219 at a significance level of 0.000, which clearly shows a strong relationship. The following table shows the values of other indicators of the chi-square test. In Table 4 we can see the results of statistical analysis about the strength of the relationship between the examined variables. Both the Phi coefficient and the Cramer´s V are 0.623 and their significance level is 0.000. On this basis, it can be concluded that there is a positive and medium relationship

between the subjective sense of success of family business leader and the active use of EU funds/grants.

Therefore, on the basis of the above statistical calculations, it can be concluded that there is a positive and medium relationship between the variables examined. These two variables were the subjective sense of success of family business leader, that is, the ability of the family business to develop in the last 3 years depending on whether it has benefited from EU funds/grants in the last 5 years. In the light of these facts, the hypothesis 1 was accepted.

The second hypothesis examines the relationship between the presence of the European Union funds/grants and the successfulness of the generation change. The research question for the second hypothesis was the following:
Research question 2: Does the presence of the support systems and external consultancy in family businesses have a positive impact on generation change?

According to literature review and our own experiences and the information gathered at the beginning of the research, we have investigated whether there is a significant relationship between the external (EU or state) supports and external consultancy in the family businesses and the generation change in family business. We thought that if these factors are present in the family business, young people will find the family business more dynamic, innovative and attractive and therefore they will continue to run the family business. We assume that the younger generation would be reluctant to run the family business if the family business is less dynamic and is without external consultancy and support. According the first research question, our first hypothesis was:

Hypothesis 2: There is a significant relationship between the presence of the support systems and external consultancy and the successfulness of the generation change. The statistical data examining of frequency was used to test the hypothesis, since the questions and the data obtained from the questionnaires used in our research allowed to use this method. The statistical data examining of frequency was used in connection with EU grants, which included the answers of external consultancy and the generation change. The Table 5 shows the results of statistical data examining. The table was evaluated from top to bottom. The combined presence of the European Union supports and external consultancy in family businesses resulted a 68% probability of successful generation change. It can be stated that there is a strong relationship between the factors examined. In the opposite, in the case of a negative answer to both external factors, only 26.3% of the respondents expect the successful generation change. 73.7% of respondents answered that the generation change in the family business will not be or may not be successful. According the results, it can also be stated that there is a relationship between the variables. Analysing the combined answers, not specific conclusion can be draw, as in both cases (thus, either in the case of a negative answer to one or the other external factor) respondents predicted the success of the generation change with a 66.7% ratio. In conclusion, examining the second hypothesis, we can conclude that the presence of the European Union supports, and external consultancy has an impact on the successfulness of the generation change, but it should be examined in a deeper, more targeted way. However, this research, especially the composition of the questionnaire does not make it possible to investigate this.

Table 5 Frequency table.

			Do you see an opportunity to generation change?				
EU supports in the last 5 years	Did you use help by an external organization/ specialist?			Frequency	Percent	Valid Percent	Cumulative Percent
Yes	Yes	Valid	Surely yes Maybe yes	34	68.0	68.0	68.0
			Not at all May not	16	32.0	32.0	100.0
			Total	50	100.0	100.0	
	No	Valid	Surely yes Maybe yes	8	66.7	66.7	66.7
			Not at all May not	4	33.3	33.3	100.0
			Total	12	100.0	100.0	
No	Yes	Valid	Surely yes Maybe yes	16	66.7	66.7	66.7
			Not at all May not	8	33.3	33.3	100.0
			Total	24	100.0	100.0	
	No	Valid	Surely yes Maybe yes	52	26.3	26.3	26.3
			Not at all May not	146	73.7	73.7	100.0
			Total	198	100.0	100.0	

Note: Source – data from primary research, own editing based on SPSS.

Thus, we leave this hypothesis open, and we plan a future research that we intend to carry out in a larger sample, geographically extending, using different methodology.

From the future research, we except to answer the significance of the relationships examined in the second hypothesis. We will be able to prove these through statistical methods and draw relevant conclusions. Based on currently available data, the second hypothesis was accepted, but we consider it necessary to clarify it in more detail and to examine it in depths in the future.

Observing the family business factors is the focus of many authors. They have analysed several factors **(Hudáková et al., 2014)**. According to particular research results, support mechanisms of European Union funds play an important role in business activities **(Dupcsák and Marsalek, 2016; Andrejovská et al., 2019; Strážovská and Jančíková, 2016; Duma, 2015)**. The sustainable development of regions **(Horská et al., 2019)**, countries and businesses **(Grabara, 2019)** should be the interests of the Union itself as well as the EU Member States. A similar research was carried out by **Huňady et al. (2017)** with conclusion that the economic development of regions depends in many ways on innovation and support mechanisms. In this context, **Ivanová (2018)** identified barriers in the development of small and medium-sized enterprises, which include family businesses. In addition to bureaucracy, the most burdensome for them are financial difficulties in ensuring development. In this respect, EU support mechanisms are an important aid for businesses. Since the entrepreneurial success of family businesses **(De Alwis, 2016)** is determined by many factors, the special attention should be given to them **(Hnátek, 2015)**.

CONCLUSION

Results of the research shows that that less family businesses applied for EU support than for state supports. Looking at businesses supported by EU programs, majority of respondents said that their family business developed fast and steadily in the last 3 years. In a group of businesses not supported by EU programs more than half of the respondents could not develop in the last 3 years, they had negative results. Subsequently we concentrated to answer some important questions about the development constraints of this type of business. These include inappropriately exploited external assistance, such as public tender or support programs, European Union development and support programs, or even external consultancy by an organization or specialist. We have come to realize that these factors are not only important for businesses to be able to develop and preserve financial stability, to improve their competitiveness or market position, but also have another impact. This is nothing else than a generation change that is often mentioned as the biggest problem source of family businesses. It can be stated that if external support and consultancy are present in family business life, the younger generation will find the family business more dynamic, innovative and attractive and therefore they will continue to run the family business. Leaving this issue open, we have decided to look forward to future research, in which we want to investigate this issue more specifically, with a larger number of elements and different methodologies.

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CHARACTERIZATION OF COMPISOTE EDIBLE FILMS FROM ALOE VERA GEL, BEESWAX AND CHITOSAN

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ABSTRACT
Environmental consciousness as well as individual's demand for ready to eat food, recently, has changed the trends in food packaging leading to the development of biodegradable and edible packaging. Emulsified edible films have better transparency, superior mechanical properties and provide barriers to water and other atmospheric gases. Edible films if not consumed, biodegrad chemically. In present study, edible films were, initially, prepared using Chitosan and *Aloe vera* at different concentrations. Films were then subjected to physical and mechanical testing. Films with 20% *Aloe vera* had low thickness as compared to films with no *Aloe vera.* These films also had superior mechanical properties and lower water vapor permeability. Films with 20% *Aloe vera* were, then, selected and beeswax was dispersed in Chitosan-*Aloe vera* solution at concentration upto 2.0% followed by film preparation through casting technique. Thickness and water vapor permeability were observed to be improved with increase in concentration of beeswax. Tensile strength of edible films was also improved 1.3 times when concentration of beeswax increased from 0.5 to 2.0%. Percentage elongation decreased with increase in beeswax concentration in the emulsified films. No change in particle size was observed with change in concentration of beeswax. Emulsions were also stable at room temperatures. Decrease in transparency of emulsified edible films was observed with increase in beeswax content in the emulsified films. In addition, cost analysis of the films proved them reasonable to be used as an alternate of synthetic packaging materials.

Keywords: edible film; chitosan; beeswax; *Aloe vera* gel; physiochemical properties

INTRODUCTION

Today, increasing the awareness of consumers about natural based food products with no chemical preservatives has led to explore innovative methods in preserving food to extend shelf life. The number of papers devoted to the developing of storage methodologies are on an increased and amongst them, edible coating has drawn considerable attention in recent years due to accentuated application of edible material in packaging over chemical types. In the technology of edible coating, the surface of food commodity is commonly covered by a thin layer of edible material; acting as a barrier to confine the gaseous movements and moisture transfer, control respiratory rate, and thereby reduce weight loss during storage **(Sogvar, Koushesh Saba and Emamifar, 2016)**. Moreover, a coat forming agent such as *Aloe vera* might have natural antioxidant and antimicrobial properties **(Vieira et al., 2016)** or be the carrier of those **(Koushesh Saba and Sogvar, 2016)**.

Aloe vera is an evergreen perennial plant belonging to the family *Liliaceae* and indigenous in countries with arid climate like Arabian countries. The reason to survive in tropical areas can be referred to its stems with high capacity of retaining moisture and its fleshy leaves **(Misir, Brishti and Hoque, 2014)**. Being an herbal medicator extensively consumed across the globe, *Aloe vera* has represented a long history of functional and remedial activities since ancient times through its broad spectrum of bioactive components **(Guo and Mei, 2016; Pothuraju, et al., 2015)**.

Besides myriad health benefits of *Aloe vera*, recently, it is being valorized by different pharmacy and food industries **(Soltanizadeh and Ghiasi-Esfahani, 2015)**. Technically, *Aloe vera* creates a gel which is found to be an antifungal agent, keeping effectively quality and inhibiting microbial spoilage through its antimicrobial activities **(Ahmed, Singh and Khan, 2009)**. In addition, *Aloe vera* gel has been shown to postpone oxidative browning, maintain moisture and control respiratory rate either in pre or post-harvest stages in a group of agro-food products like table grape **(Valverde et al., 2005)**, strawberry **(Vahdat, Ghazvini and Ghasemnezhad, 2010)**.

On the other hand, application of other natural bio-polymers like chitosan along with *Aloe vera* gel in coating surface of agricultural and crop products can enhance the gel functionality and synergize its above stated physiochemical properties.

Chitosan itself is renown to be used separately as edible coating in terms of its intrinsic features like being non-detrimental to human body, being environmentally friendly, possessing cationic nature and antimicrobial potency **(Alves and Mano, 2008; Pillai, Paul and Sharma, 2009)**. Beeswax, another natural compound, is also applied as coating agent. Beeswax consist of a long chain of aliphatic alcohols (oleate esters) with a possible chemical formula of $C_{15}H_{31}COOC_{30}H_{61}$. Beeswax in combination with chitosan, provides barrier to moisture and strength to the film. Food products have high moisture content and readily lose moisture after coming into contact with ecological conditions **(Velickova et al., 2013a)**. Nowadays, combination of different edible biopolymers to make a new edible coating is of much interest. Therefore, the aim of this study is to evaluate physio-chemically the edible film made from the combination of beeswax, chitosan and *Aloe vera* gel.

Scientific hypothesis

Edible films were developed using Aloe vera and chitosan and emulsified with beeswax using tween20 as an emulsifier.

The following hypothesis were tested: either beeswax had any effect on the the film properties or not. Increasing the concetration of beeswax in the films forming solution of Aloe vera and chitosan may improve the physical properties (thickness, moisture) of edible films. Increasing the concetration of beeswax in the films forming solution of Aloe vera and chitosan may improve the optical properties (transparency) of edible films. Increasing the concetration of beeswax in the films forming solution of Aloe vera and chitosan may improve the mechanical properties (tensile strength and % elongation) of edible films. Increasing the concetration of beeswax in the films forming solution of Aloe vera and chitosan may improve the barrier properties (water vapor permeability) of edible films.

MATERIALS AND METHODS

Materials

The research was conducted in different laboratories of Department of Food Engineering, University of Agriculture, Faisalabad. Chitosan, beeswax, glycerol and Tween20 was purchased from a registered scientific store at Faisalabad. *Aloe vera* was obtained from Department of Forestry and Range Management (FRM).

Preparation of *Aloe vera* gel

Aloe vera leaves, obtained from Department of Forestry and Range Management was stored at 8 °C from the time of harvest until utilization. Aloe vera then, washed using distilled water at 40 °C to remove impurities such as dust and dirt **(Pinzon, Garcia and Villa, 2018)**. After this, *Aloe vera* was peeled using ordinary knife in such a way that essential *Aloe vera* gel could be easily extracted. Then, *Aloe vera* gel was extracted in a glass beaker and blended using domestic blender resulting in a uniform solution. The solution was further screened by passing through sieves (0.1mm) to remove impurities. Pure *Aloe vera* gel solution was stored at 5 °C in a refrigerator to avoid the oxidation of phenol contents till further use.

Development of film forming solution

Acetic acid solution (2% v/v) was prepared by dissolving 2 mL of acetic acid in distilled water to make its volume up to 100 mL. Chitosan (2 g) was dissolved in 100 mL of (2% v/v) acetic acid solution to form 2% (w/v) chitosan solution. Films were prepared by varying the concentration of *Aloe vera* in chitosan solution from 0 – 20% as shown in Figure 1and Table 1.

Glycerol was added in the solution as a plasticizer. Then, films were developed by casting method by pouting the 20 mL of sample solution in the petri dishes (9 cm diameter). These petri dishes were placed in the desiccators at 40 °C for drying.

Development of emulsified edible films

After mechanically and physically testing the films, a suitable concentration of film forming solution was selected and emulsified with beeswax. For complete dissolution of beeswax, 0.25 mL of glycerol was added as a plasticizer and Tween20 (25% of oil) was added as an emulsifier. For preparation of emulsified films, selected Chitosan-*Aloe vera* solution was homogenized with beeswax (melted at 65 °C) at concentrations shown in Table 2.

To develop films, chitosan blended *Aloe vera* solution was heated up to 75 °C so that it can be mixed easily with beeswax. These films were homogenized at 13500 rmp for 1 min to develop the stable emulsions. This resulted in bubble formation in the film forming solution. These solutions were placed in the sonicator (ELMA E60H) to remove bubbles for 20 min. The film forming solutions were poured into petri dishes (9 cm diameter) in such a way to form a film of uniform thickness. These Petri dishes were shifted to the hot air oven/desiccators with the temperature of about 40 °C for the time until emulsified edible films were easily peeled off from the surface of petri dishes.

Evaluation of physic-chemical properties of film

Stability of emulsions

Stability of emulsions was determined according to **Purwanti et al. (2018)** with slight modifications. This was done by keeping the emulsion sample for 3 days to check the separation of beeswax due to Ostwald ripening or coalescence. A sample of 6 mL was taken and poured in a test tube. Tubes were placed in test tube rack at room temperature for 3 days. The initial height and final height was checked after 3 days to measure any oil separation. The emulsion stability was determined using following formula:

$$S = \frac{h0 - ht}{h0} \times 100 \quad (1)$$

Where, S is the stability of emulsion, h_0 is the initial height of emulsion in the glass vial (cm), and h_t is the height of emulsion at the measuring time (cm).

Moisture content (MC)

The moisture content of edible films was calculated using oven drying method by drying in an oven (BOHA-102 Canada) at 100 ±2 °C till constant weight is reached. Initially, all the films were standardized at room conditions

(25 °C and 70% RH). Then, films were weighed and Moisture content was determined as "the percentage of weight loss in the initial weight, using:

$$MC(\%) = \frac{Mi-Md}{Mi-Mp} \times 100 \qquad (2)$$

Where, M_i is the mass of the film specimen and petri dish before drying (g), M_p is the mass of petri dish (g) and M_d is the mass of film specimen and petri dish after drying (g).

Thickness measurements

Film thickness was measured using Digital Micrometer (Model: Mitutoyo LC = 0.001 and Range: 0 – 25 mm, Japan). Firstly, the instrument was calibrated to check the zero error. Films were, then, peeled off from the surface of petri dish and thickness of the films was measured at four random positions along rectangular strip and at center. Average of all five values was taken as the film thickness in milimeters.

Transparency

Transparency of the films was measured using UV Visible Spectrophotometer (T80, UK). Firstly, films were cut down into uniform strips of cuvette size. These films then placed on one side of the cuvette with film side facing the lense. Wavelength of UV Visible was adjusted to 600 nm and calibrated with distilled water. Cuvettes were, then, placed into the jacket of UV Visible Spectrophotometer. Film's transparency was compared with transparency of distilled water. As the light passed through the films, % transmittance was displayed on software display panel.

Water vapor permeability

The water vapor permeability was determined by gravimetric analysis with ASTM E96-95 standard usually known as water vapor transmission rate (WVTR). Films were cut into a uniform size of 7x7 cm and standardized with ambient environment (25 °C and 70% RH). 10 mL of water was taken into the test tubes of 1.5 cm in diameter and 12 cm in height. Further, strength at the mouth of test tubes was provided with gum so that water cannot evaporate through openings. Then, these test tubes were placed in the desiccators at room temperature and 0% RH for 12 h. Relative humidity was maintained at 0% by using silica gel placed at different locations in the desiccators. Atmospheric pressure was calculated using barometer at 0% RH and at 100% RH. Mass lost during 12 h was measured during weight balance.

$$\text{WVP}(\text{mgm}^{-2}.\text{h}^{-1}.\text{Pa}^{-1}.\text{mm}) = \frac{\Delta m \times X}{A \times t \times \Delta P} \qquad (3)$$

Where Δm is the mass change over time (mg), X is the thickness (mm) t is the time (h), A is film area (m^2) and ΔP is the partial vapor pressure (Pa) difference of the atmosphere with silica gel and pure water (3179 Pa, at 22 °C).

Particle size analysis

Size of the particles of the emulsion was determined by Particle size analyzer (Better Size ST A8311, China). It describes the distribution of beeswax particles in the film forming solution of chitosan with minimum and maximum size. The sample cup was first auto-cleaned to remove all the contamination and impurities in the distilled. Emulsified beeswax in film forming Chitosan-Aloe vera blend was poured in the sample cup when all the sample the bubbles were removed from the water. The sample was poured in the cup until a constant absolute value reached between 5 and 10. After few minutes, machine showed the results in cumulatively as well as differentially. The particle size of 50% beeswax was checked at D50 which represents maximum particle size of 50% grains in the solution.

Evaluation of mechanical properties of films

Ultimate tensile strength (UTS) and percent elongation (% E) of the films was determined using a universal testing machine. Films were cut into a uniform size of 8 cm length and 3 cm in thickness was placed between the jaws of the machine.

Statistic analysis

Analysis of Varience (ANOVA) was applied using SAS 2.0 software and significance of means was tested at $p < 0.05$.

RESULTS AND DISCUSSION

Films thickness

Thickness of chitosan-based and emulsified films was observed to be significantly affected by the concentration of *aloe vera* and beeswax respectively at 5% probability ($p < 0.05$). Thickness of chitosan-based films was observed to be decreasing 74.1 μm at S1 to 52 μm at S5 with the increase in Aloe vera concentration in chitosan-based film forming solution. Reduction in film thickness was similar to the studies of **Khoshgozaran-Abras et al. (2012)** and emulsification of selected concentration of *Aloe vera* **(Abugoch et al., 2011)**. *Aloe vera* and chitosan with beeswax from 0% to 2.0% resulted in a slight increase in the thickness of the films at S52 but continuous reduction in the film thickness was observed from 131 μm at S52 to 100 μm at S54 (Figure 2). Film thickness reduced to 23.6% as concentration was changed from S52 to S55 which is opposite to the studies of **Velickova (2013b)** and **Indriyati (2018).**

Moisture content

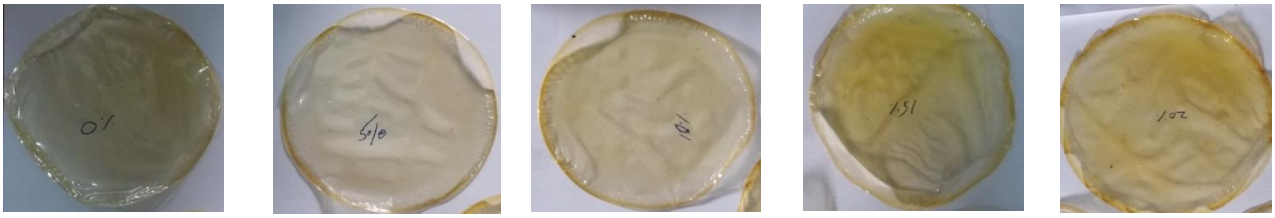
Films were subjected to environmental condition to check how much water these films can absorb. After standardization with environmental conditions, the moisture content was determined by oven drying method. Films of *Aloe vera* and chitosan observed to be significantly affected the moisture in the films ($p < 0.05$). Moisture content was continuously decreasing with increase in *Aloe vera* concentration. Maximum moisture content was observed at S1 (30.633%) which was observed to be continuously decreasing with increase in *Aloe vera* concentration at S5 (13.8667%). Emulsified *Aloe vera*-chitosan composite films were also treated with same protocol. The same effect was observed with increase in beeswax concentration 21.67% at S52 to11.86% at S55 in the emulsified films (Figure 3).

Table 1 Formulations of primary films.

Treatments	2% Chitosan Solution (%)	*Aloe vera* Gel (%)
S1	100	0
S2	95	5
S3	90	10
S4	85	15
S5	80	20

Table 2 Concentrations of emulsion using Beeswax.

Treatments	2% Chitosan Solution and *Aloe vera* (%)	Beeswax (%)
S51	100	0
S52	95	5
S53	90	10
S54	85	15
S55	80	20



S1 **S2** **S3** **S4** **S5**

Figure 1 Films of different concentration of *Aloe vera* (%).

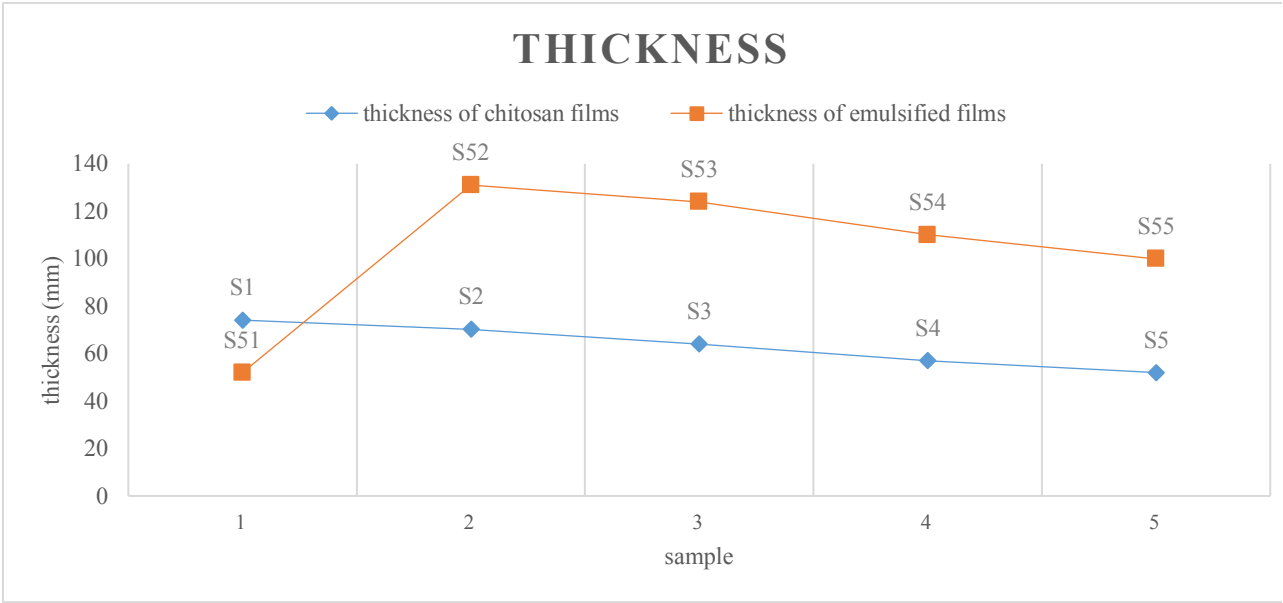


Figure 2 Comparison of thickness of chitosan and emulsified films. Note: *Si = Aloe vera concentration; S5i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52= 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

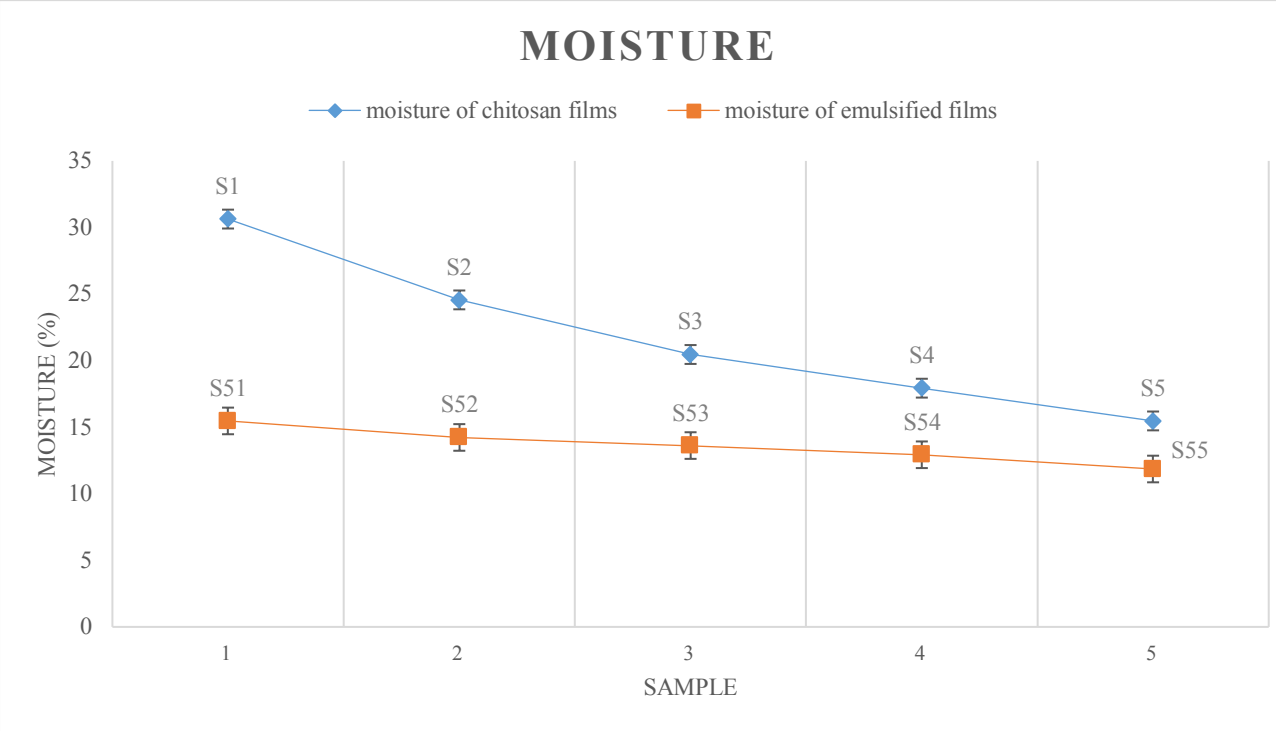


Figure 3 Comparison of moisture in chitosan and emulsified edible films. Note: *Si = Aloe vera concentration; S5i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52 = 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

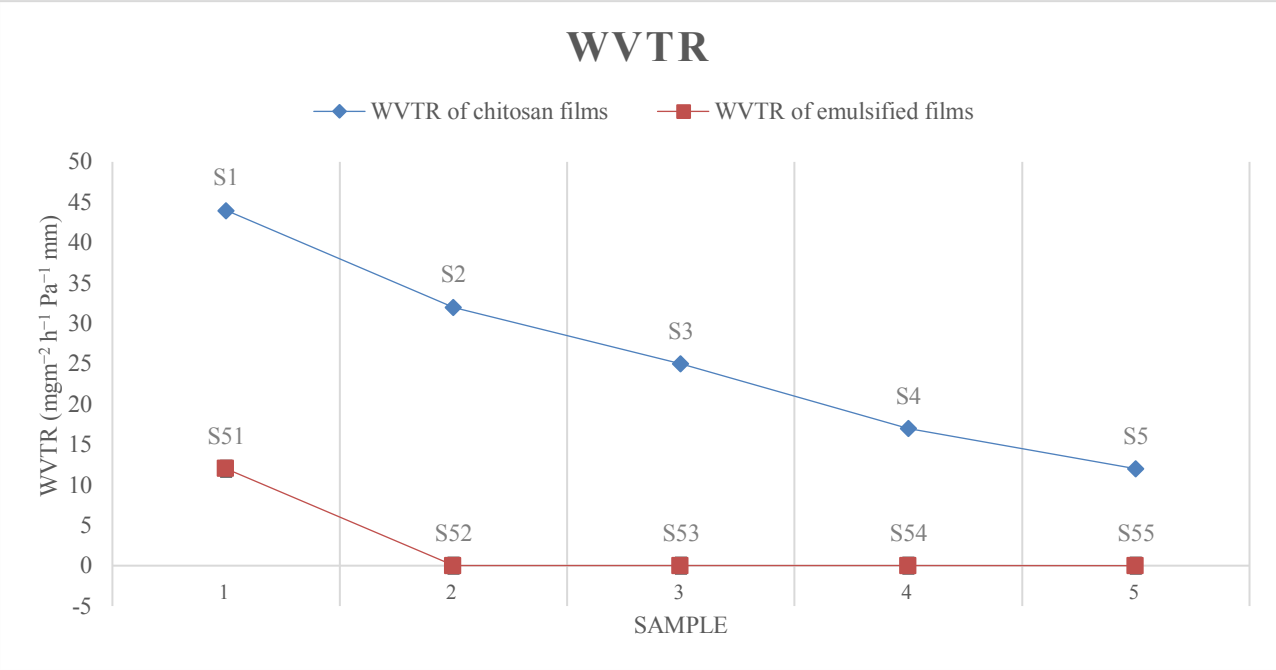


Figure 4 Comparison of water vapor permeability of chitosan and emulsified edible films. Note: *Si = Aloe vera concentration; S5i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52 = 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

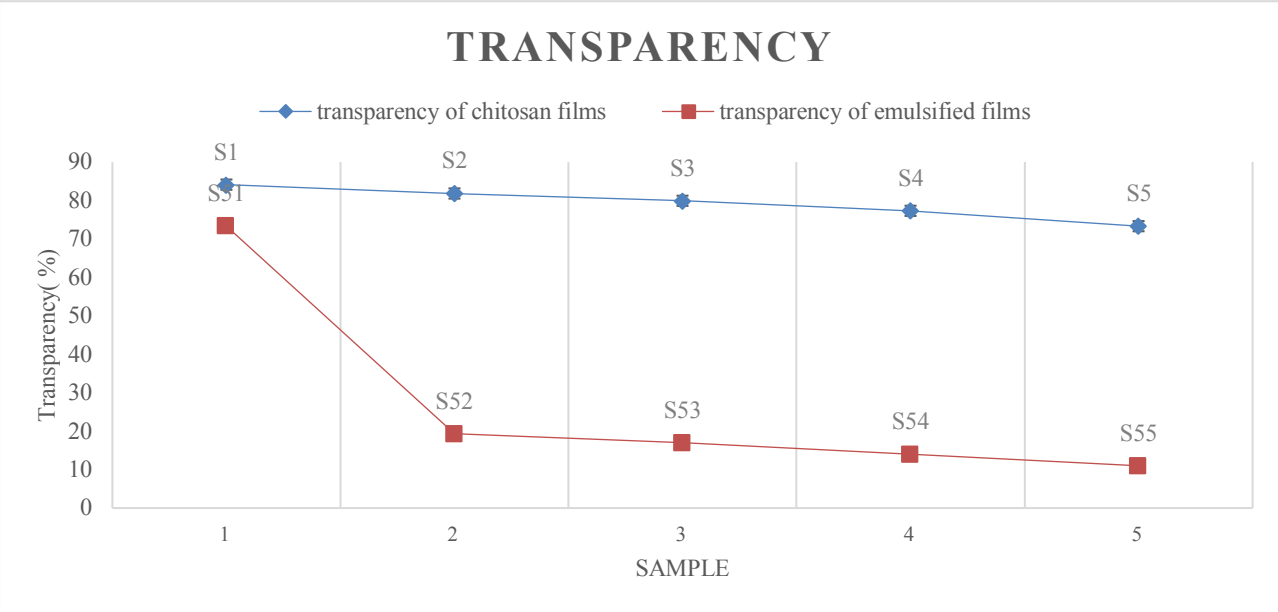


Figure 5 Comparison of transparency of chitosan and emulsified edible films. Note: *Si = Aloe vera concentration; S1i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52 = 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

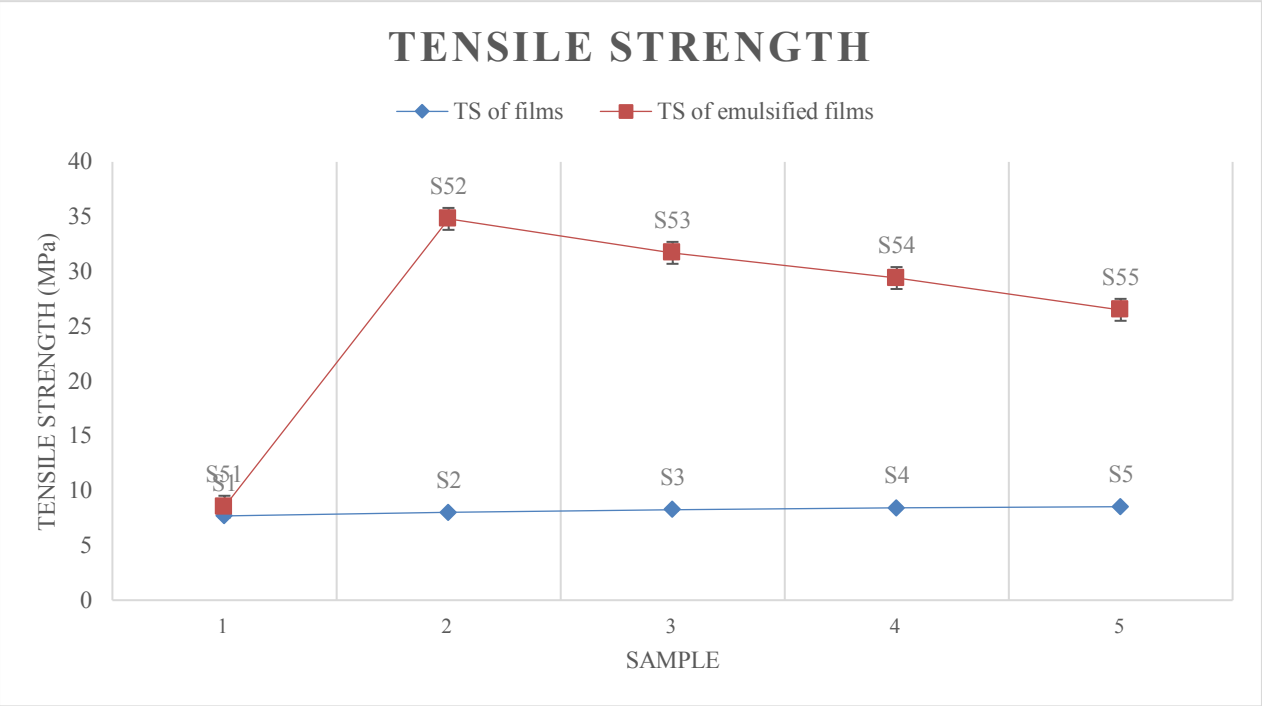


Figure 6 Comparison of tensile strength (TS) of chitosan and emulsified edible films. Note: *Si = Aloe vera concentration; S1i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52 = 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

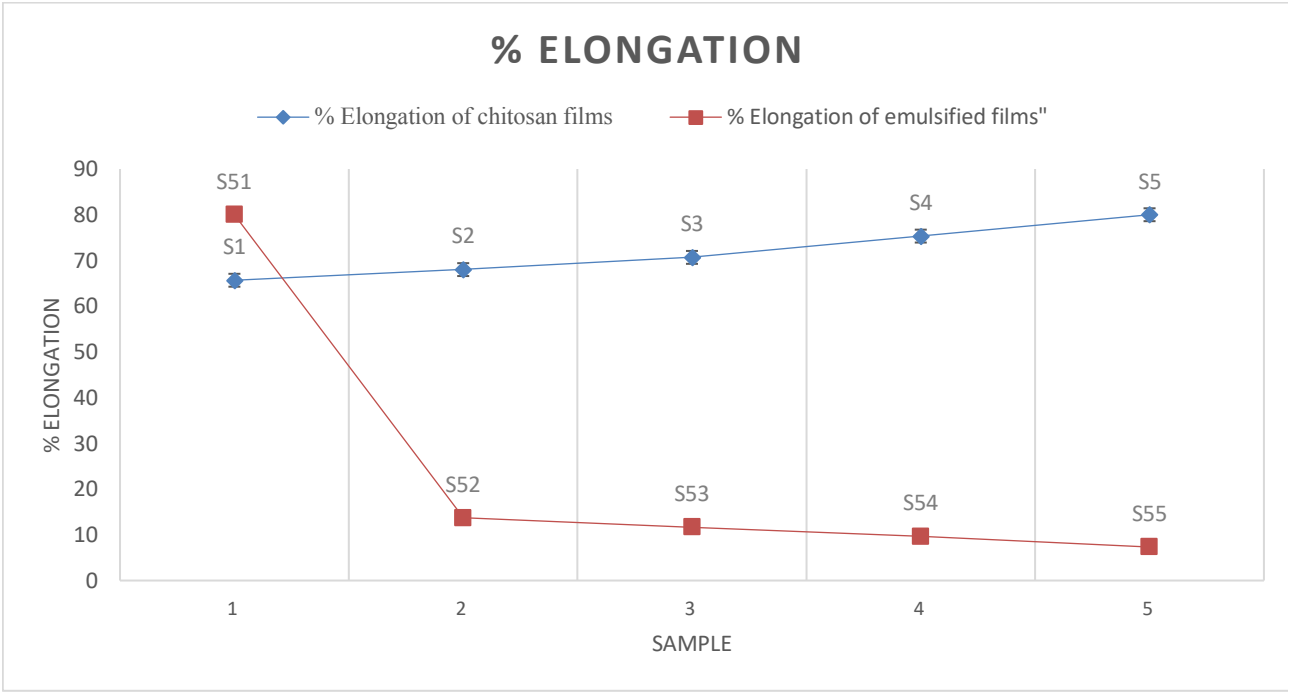


Figure 7 Comparison of % elongation of chitosan and emulsified edible films. Note: *Si = Aloe vera concentration; S5i = Beeswax concentration; S1 = 0%; S2 = 5%; S3 = 10%; S4 = 15%; S = 20%; S51 = 0%; S52 = 0.5%; S53 = 1.0%; S54 = 1.5%; S55 = 2.0%.

Water vapor permeability

Water vapor permeability chitosan films and beeswax films depended upon the *Aloe vera* and beeswax concentration in film forming chitosan respectively (Figure 4). Water vapor permeability was observed to be continuously decreasing as 0.044 $mgm^{-2}.h^{-1}.Pa^{-1}.mm$ at S1 to 0.012 $gm^{-2}.h^{-1}.Pa^{-1}.mm$ at S5 with increase in *Aloe vera* concentration in solution which was explained by **Ortega-Toro et al. (2017)**, **Khoshgozaran-Abras et al. (2012)** and **Elsabee and Abdou (2013)**. The higher water vapor permeability of the chitosan film could be due to its hydrophilic nature. It allows the water to come in interaction with matrix and results in increasing their rate of permeation **(Khoshgozaran-Abras et al., 2012)**.

Statistical analysis also proved that water vapor permeability significantly (p <0.05) reduced when the concentration of beeswax increased in the film forming solution. Chitosan-*Aloe vera* blend when mixed with beeswax produced hydrophobic effect in the films as lipid phase repels water **(Miranda et al., 2004)**. Water vapor permeability was observed to be reduced up to 81.12% when concentration of beeswax was increased from 0.5% to 2%.

Transparency

Transparency of edible films with respect to concentration of *Aloe vera* was statically analyzed and edible films was observed to be reduced 84.1% at S1 to 73.3% at S5 with incorporation of *Aloe vera* gel blend in film forming chitosan which was in agreement with **Pereira et al. (2011)**, **Khoshgozaran-Abras et al. (2012)**, and **Elsabee and Abdou (2013)**. Chitosan was observed to be transparent and decrease in transparency was due to incorporation of *Aloe vera* which contains phenols. Oxidation of phenols may be resulted in decrease in transparency of *Aloe vera*. Transparency of emulsified edible films with beeswax was also significantly affected (p <0.05). 43.1% reduction in transparency of emulsified films was observed from S52 to S55 **(Gomes de Santos et al., 2017)**. Chitosan films were observed to be transparent and decrease in transparency may be due to incorporation of *Aloe vera* and beeswax. Oxidation of phenols in *Aloe vera* as well as yellowish appearance of *Aloe vera* may be resulted in decrease in transparency of emulsified films (Figure 5).

Mechanical Properties

Means of tensile strength and percentage elongation at break of films was observed to be increasing with increase in *Aloe vera* concentration which was shown in the studies of **Pereira et al. (2011)**.

As the concertation of Aloe vera increased from S1 to S5, tensile strength and percent elongation at break was observed to be increased about 10.92% and 21.82% respectively. However, *Aloe vera* at 0% and 5% concentration was observed to have no significant effect (p < 0.05) on tensile strength as they are bearing same superscripts (Figure 6).

It was observed that tensile strength of emulsified films observed to be decreasing from 34.8 MPa at S52 to 26.5 MPa at S55 and percentage elongation was also reduced from 13.67% at S52 to 7.33% at S55. Comparing S51 with S52, S53, S54 and S55, a significant (p < 0.05) reduction in the % elongation was observed as 80% at S52 to 7.33% at S55 (Figure 7). The same trend was shown in the studes of **Velickova et al. (2013b)**.

Particle Size Distribution

Particle size of beeswax in *Aloe vera*-chitosan emulsion was tested with varying concentration of beeswax. These

emulsions were homogenized at 13,500 rpm for 1 min. No difference in particle size was observed when these films were subjected to particle size analysis. The films were tested considering D50 as standard which describe the particle size of 50% of the solution. The particle size of 50% film forming solution was observed to be 8.764 microns in all films.

Stability of emulsified edible films

Emulsified film forming solution prepared from *Aloe vera*-chitosan blend incorporating beeswax was tested for stability. 7 mL sample was measured and poured into the test tubes placed in test tube rack. These samples were kept for 3 days to check any separation in the emulsified film forming solution under ambient conditions. After 3 days, no change in the height and phase separation was observed in emulsified film forming solution.

CONCLUSION

A thin film of edible material is consumed and stops the transfer of oxygen, moisture, and the movement of solute from food. The edible films enrich the organoleptic characteristics of packaged foods as these films have several desired components (flavors, colors, sweeteners etc.). *Aloe vera* imparts antioxidant and antimicrobial effect in the biodegradable films. In order to control the diffusion rate of preserving agents from the surface to the interior of the food, these films are used on the food surface. Though, the permeability and mechanical characteristics of the edible films are usually inferior than synthetic films but, edible films reduce pollution and waste. In present study, edible films were, first, prepared using Chitosan and *Aloe vera* at different concentrations. The prepared edible films were then subjected to physical and mechanical analysis. The data obtained from various treatments was then analyzed using SAS 9.0 package. It was concluded that films with 20% *Aloe vera* had lower thickness, lower transparency, better mechanical properties and better water vapor permeability as compared to those with lower concentration of *Aloe vera*. Films with 20% *Aloe vera* were, then, selected and emulsified with beeswax in dispersed phase in Chitosan-*Aloe vera* solution at concentration upto 2.0% followed by film preparation through casting technique. The data obtained was again analyzed using SAS 9.0 package. The results revealed that thickness, tensile strength and water vapor permeability were increased when the concentration of beeswax increased from 0.5 to 2.0%. However, elongation at break and transparency was found to be decreasing with increase in beeswax concentration in the emulsified films. Further, no change in particle size was observed with change in concentration of beeswax. Insignificant difference ($p < 0.05$) was observed in the stability of emulsified blends after 24 hrs when stored at room temperature (25 °C, RH 70%). In addition, feasibility of these films was checked by calculating the cost on their development. Development cost of edible films found to be reasonable for high value and perishable products as an alternative of synthetic packaging.

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THE STUDY CORRELATION BETWEEN PHYSICOCHEMICAL PROPERTIES, BOTANICAL ORIGIN AND MICROBIAL CONTAMINATION OF HONEY FROM THE SOUTH OF UKRAINE

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ABSTRACT

The honey production is one of the most important agriculture branches of Ukraine. Since the beginning of 2000 year, honey becomes one of the first products of agriculture production that was allowed to export from Ukraine to the EU countries. The present study aims to evaluate microbial composition of honey of higher and first grades from south of Ukraine, which differ in water, sugar content and acidity. The correlation beetween physicochemical properties, botanical origin and microbial composition of honey were investigated. Total aerobic count (TAC), total count of fungi (TFC) and yeast (TYC), number of sporeforming anaerobes (SFA), Bacillus cereus and Clostridium botulinus were determined. Plate dilution method with specific cultural mediums and incubation conditions was used for indication and identification of microorganisms as well for quantitative CFU count determination in 1g of honey. The results indicated that the value of TAC was from 1.4 ±0.1 x 10^2 to 3.7 ±0.5 x 10^2 CFU.g^{-1} in higher grade honey and from 2.1 ±0.3 x 10^2 to 4.6 ±0.4 x 10^2 CFU.g^{-1} in first grade honey. Average amounts of fungi and yest in the first grade honey were more than in higher grade honey and amounted 15.±0.9 CFU.g^{-1}, 6.48 ±0.7 CFU.g^{-1} and 17.98 ±1.2 CFU.g^{-1}, 8.04 ±0.8 CFU.g^{-1} respectively. Also it was found that, 25.8% and 37.1% of higher and first grade honey samples had sporeforming anaerobes. The more of samples with Bacillus cereus and Clostridium botulinum were among samples of first-grade honey samples. In addition, we have determined that, samples of buckwheat honey and honey from fodder crops of both grades were more contaminated with all microorganisms than samples of sunflower honey and honey from fruit and non-fruit trees.Results of this work has revealed the relationship between the number of different groups and species of microorganisms in higher and first grade honey from south of Ukraine and its physicochemical properties and botanical origin. Our research could help to substantiate the sources of contamination of honey and confirm the existing findings of scientists about the direct proportional dependence of the content of microorganisms in honey, depending on its moisture content, acidity and sugars.

Keywords: *honey; total aerobic count; fungi; yeast; sporeforming anaerobes; Bacillus cereus; Clostridium botulinum*

INTRODUCTION

At the thought of experts from the World Health Organization, microbiological risks, related to the quality of raw materials and food products are one of the most important biological threats at the present stage. Outbreaks of diseases caused by the consumption of hazardous foods are reported worldwide. In the case of honey, scientists are reporting that it can be dangerous for children because of botulism, which is caused by *Clostridium botulinum*. The presence of *Clostridium botulinum* in honey is a rare phenomenon, but contamination with this microorganism depends on total microbial contamination of honey. If the total number of microorganisms in honey is higher, that the more likely the presence in the honey of this dangerous microorganism **(Grenda et al., 2018; Grabowski and Klein, 2017)**.

Honey is natural product, produced by honeybees and has viscid fluid state, sweet flavor and specific aroma. Well known, that due to contain of significant amounts of minerals, vitamins, simple sugars, organic acids, antioxidants and enzymes, honey is considered as good product with nutritional and therapeutic properties **(Da Silva et al., 2016; Alvarez-Suarez et al., 2018)**. Therefore, this product is very popular among people, including children due to its consumptional properties. In addition, much is known about antiinflammatory and antimicrobial characteristics of honey and effective using it for increase the state of immunity, wound healing, gastrointestinal disorders, skin diseases and even cancer **(Rao et al., 2016; Kateel et al., 2018; Mabrouka, Ayed and Grara, 2018; Pasias et al., 2018)**.

The honey production is one of the most important agriculture branches of Ukraine. Since the beginning of

2000 year, honey becomes one of the first goods of agriculture production that was allowed to export from Ukraine to the EU countries. According to official data, Ukraine is the largest in Europe and the third largest in world producer of honey **(Dankevych, Dankevych and Pyvovar, 2018)**.

Council Directive 2001/110/EC and **Regulation (EC) No. 470/2009** specify key common criteria as quality norms for honey of all EU countries and those countries that export this product. According to these regulations, honey must be natural and free from foreign and harmful components.

However, honey can be associated with some adverse health effects of human, especially when contaminated with hazardous chemicals or microorganisms **(Laredj and Waffa, 2017; Silva et al., 2017; Kostić et al., 2019)**.

In recent years there has been growing interest in studying the presence of microorganisms in honey and bee products and bee products as potential sources of pathogenic microorganisms. A number of studies have been carried out and reported about bacterial species found in honey samples produced in different country. Among the isolated microorganisms were the following: *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Streptococcus epidermidis*, *Corynebacterium sp.*, *Klebsiella pneumonia*, *Escherichia coli*, *Salmonella sp.* and *Proteus spp.* **(Saba, Shakeel and Kamran, 2015; Adadi and Obeng, 2017)**.

In contrast to the physicochemical properties, microbial contamination of honey is not regulated because of the lack of legislation in the European Union and other countries.

Microorganisms get in the honey from digestive tract of honeybees, which have natural microorganisms, from nectar, pollen and flowers, from environment of beehive, and during processing **(Sinacori et al., 2014; Bentabol Manzanares et al., 2017)**.

It was found that moisture content, low acidity, presence of sugars (glucose, fructose and sucrose) and natural antimicrobial substances are influenced on microbial composition of the honey **(Fernández et al., 2017; Alvarez-Suarez et al., 2018; Pomastowski et al., 2019)**. The microbial spectrum of honey can include pathogenic bacteria especially those which capable to sporoforming, fungi and yeasts **(Kunová et al., 2015; Silva et al., 2017)**. These microorganisms can survive in honey and causing of undesirable changes by reducing the shelf-life of the product. But more important is that such honey can be harmful to the health of the consumer. Therefore, it is very important to know the diversity of microorganisms, which can be present in honey, especially due to disseminate pathogenic for human microorganisms in the international traded marketing. In addition, to the best of our knowledge, this is the first study that discribe the microbiological quality of honey from Ukraine.

Scientific hypothesis

The main scientific hypothesis of this study was to examine the differences in microbiological composition of two grades of honey of different botanical origin from south of Ukraine. In this context we tried to study correlation beetween physicochemical properties, botanical origin of honey and presence of microorganisms. We believe that quantitive and qualitive presence of microorganisms in honey depends on its geographical and botanical origin.

MATERIAL AND METHODOLOGY

Samples collection

The work was performed in the microbiology laboratory of Public Helth Department of Sumy State University and Odessa Regional State Laboratory of the Civil Service of Ukraine on the quality of food safety and consumers` protection. A total of 124 samples of honey were investigated. Samples were gathered from 9 apiaries located in Odessa region (southern part of Ukraine) and their studies were conducted during years 2016 – 2018.

Fresh honey samples were examined – no more than one month after selection, and kept in good hygienic conditions on apiaries. By origin, honey was from the following honey melliferous plants: sunflower, buckwheat, fruit trees (cherry, apple, pear, cherry, peach, cherry-plum), non-fruit trees (forest trees: false acacia, linden), fodder crops (alfalfa, clover, rape, sweet clover).

Before conducting microbiological studies of honey, we tested it for physicochemical indicators in an accredited according to the requirements of the international standard **ISO 17025:2005** Odessa Regional State Laboratory.

Physicochemical studies of honey were conducted to determine the honey grade according to the requirements of the state standard of Ukraine **(DSTU 4497:2005)**. Requirements for physicochemical indicators of honey according to **DSTU 4497:2005** are shown in Table 1.

The honey samples were categorized on the basis of their physicochemical indicators into two groups for further microbiological investigation. In order to better comparison of microbiological research results, we selected an equal number of honey samples; 62 samples of higher grade honey and 62 samples of first grade honey.

Methods of microbiological analysis of honey samples

Total aerobic count (TAC), total count of fungi (TFC) and yeast (TYC), number of sporeforming anaerobes (SFA), *Bacillus cereus* and *Clostridium botulinus* were determined. Plate dilution method with specific cultural mediums and incubation conditions was used for indication and identification of microorganisms as well for quantitative CFU count determination in 1 g of honey. Serial dilutions were prepared using peptone water as diluent by weighing 10 g of honey into 90 mL of sterile diluents. The 1.0 mL of the dilution was inoculated into sterile plates with specific cultural mediums using the surface spread plate technique and incubated.

Determination of TAC was carried out with using nutrient agar (Oxoid, UK) and surface spread plate technique acording to **DSTU ISO 7218:2007** and aerobicaly incubated at 37 ±1 °C for 48 h. After incubation period colonies were calculated.

For determination of TFC and TYC all dilution of honey samples were inoculated onto Dicloran-Glicerol selective medium (Himedia, India) acording to the standard method of **ISO 21527-2:2008**. The plates were incubated at 25 ±1 ºC for 5 – 7 days, to growth of moulds and yeasts colonies. For identification of fungi from yeasts we used

macroscopic examination and studied cultural characteristics (colony size, form, consistency, surface and pigmentation) and microscopic examination of morphological characters under microscope (observation of vegetative cells).

The number of sporeforming anaerobes was determined by classical method. Seeding on iron sulfite agar (Oxoid, UK) was performed according to **ISO 15213:2003**. After anaerobicaly incubation of samples at 37 ±1 °C for 48 h cultural, microscopic and biochemical identification of anaerobs was made.

Statistic analysis

All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations. Student t-test was used for the statistical analysis of the obtained results. Data are presented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Common knowledge that, honey is a natural product with a large amount of sugars (fructose, glucose and sucrose), enzymes, macro- and microelements. The presence of these components in honey causes a low content of water in it. This all makes honey an unfavorable environment for most microorganisms. In addition, honey also has antimicrobial compounds that fall into it from the flowers or through the process of their transformation into a hive **(Pasias et al., 2018)**. Given these conditions, few types of microorganisms have the ability to develop or remain in honey. Due to this honey is product, that has less number of microorganisms and even demonstrate antimicrobial characteristics against some pathogenic bacteria **(Rao et al., 2016; Kateel et al., 2018; Mabrouka, Ayed and Grara, 2018)**. Despite all these numerous inhibiting factors of honey, some types of microorganisms which enter the honey during its production and storage they still remain viable and can even multiply, causing its spoiling and can be harmful for consumers health **(Silva et al., 2017; Pasias et al., 2018)**. Microorganisms in the honey may arise from the nectar and different parts of plant flower, as well as during of processing **(Adadi and Obeng, 2017)**.

In recent literature, there are no information about microbiological contamination honey from Ukraine. Thereby, in this work, we report results of microbiological analysis of two grades of honey from south of Ukraine. The honey is an important source of income for farmers in the area. The Odessa region lies in the steppe zone of Ukraine, where the main agricultural plants are sunflower, buckwheat, rape, white and yellow sweet clovers, alfalfa, clover. Other plants as dandelion, linden and false acacia also play important role as melliferous plants. Of particular importance for beekeeping in the Odessa region are plantations of fruit trees (apple, pear, cherry-plum, cherry, peach, cherry).

According to the requirements of the State Standard of Ukraine **(DSTU 4497:2005)** and in accordance with the physico-chemical parameters of honey determine its grade – higher or first grade (Table 1). Higher grade honey is characterized by low water content (up to 18.5%), while for first grade honey this prameter should not exceed 21%. According to the **Council Directive 2001/110/EC**, the water content in honey should be no more than 20%. Water content of honey is a major indicator that affects the survival and reproduction of bacteria in honey.

Other indicators of honey that affect on quantitative and qualitative microbial composition are its acidity and sugar content. Both of these indicators are higher for first-grade honey. Therefore, based on the results of physico-chemical indicators of honey, we have formed two groups of honey samples (1st group – the highest grade honey and 2nd group – the first grade honey) for microbiological research and examined the total number of microorganisms, the number of molds and yeast, and the spore-forming microorganisms. The results of these studies are shown in Table 2.

Our results in Table 2 show, that the maximum number of microorganisms (total bacterial counter) in the honey samples was not more than 3.7 ±0.5 x 10^2 CFU.g^{-1} for the higher grade honey and not more than 4.6 ±0.4 x 10^2 CFU.g^{-1} for the first grade honey. And it averaged 2.62 ±0.1 x 10^2 CFU.g^{-1} and 3.67 ±0.3 x 10^2 CFU.g^{-1} in each grade respectively.

According to **Kunová et al. (2015)** these parameters was lower than our data in most samples of honey samples from Slovakia and ranged from 8.9 x 10^1 to 2.18 x 10^1 CFU.g^{-1}. In honey samples from the Czech Republic – 2.0 x 10^2 – 1.38 x 10^3 CFU.g^{-1} and Germany – 7.55 x 10^1 – 4.77 x 10^2 CFU.g^{-1}.

At the same time **Adjaloo et al. (2017)** assessed of Honey Quality in Ghana and noticed that Total aerobic count (TAC) was maximum 7.9 × 10^3 CFU.g^{-1}, which was much more than the maximum value of this indicator for Ukrainian higher grade honey and did not exceed the mean value of the both grades.

Adadi and Obeng (2017) assessed of bacterial quality of honey producedin Tamale metropolis (Ghana) and recorded the least mean bacterial count of 6.0 x 10^4 CFU.g^{-1} in location B and the highest, 1.1 x 10^5 CFU.g^{-1}, samples taken from location D. At the same time, these authors did not found bacteria in samples from location E and F. The variation in bacterial species at the various production location and the absence of bacteria growth in two locations is an indication of the differences in production practices, as well as hygienic conditions.

Also we found, that the most amount of microorganisms were in buckwheat honey and honey from fodder crops, the less was in honey obtained from sunflower, non-fruit and fruit trees.

Although our results on the total number of microorganisms in honey differ slightly from others researchers, it can nevertheless be argued that there is a relationship between bacteria counts in honey and its physicochemical components and geographical origin **(Kunová et al., 2015; Saba, Shakeel and Kamran, 2015; Pasias et al., 2018; Pomastowski et al., 2019)**.

In addition, our data is consistent with result of **Sinacori et al. (2014)**, who suggested that the highest microbial diversity was found in multifloral honeys. However, the same author belived that no correlation among the microbial species and the botanical/geographical origin of honey, but some strains of microorganisms were highly adapted as they were found in several samples of different origin honey.

Table 1 Physicochemical indicators of higher and first grades of honey in Ukraine according to National Standard of Ukraine **(DSTU 4497:2005)**.

Name of indicators	Higher grade	First grade
Water Content (moisture),%, not more	18.5	21.0
Mass fraction of reducing sugars (to anhydrous substance),%, not less	80.0	70.0
Mass fraction of sucrose (to anhydrous substance),% not more	3.5	6.0
Diastase number (to anhydrous substance), units Goth, not less	15.0	10.0
Hydroxymethyl-furfural (HMF) content, mg per kg, not more	10.0	25.0
Acidity, milliequivalents of hydroxide (0.1 mol.dm^{-1}) per kg, not more than	40.0	50.0
Proline content, mg per 1 kg, not less	300	300

Table 2 The level of contamination two grades of honey by anaerobic bacteria, fungi, yeast, (n = 62 samples of honey of higher grade and n = 62 samples of honey of first grade).

Honey produced from different melliferous plants species	The level of contamination honey samples, CFU.g^{-1}±SD					
	Honey of higher grade			Honey of first grade		
	APC x 10^2	TFC	TYC	APC x 10^2	TFC	TYC
Sunflower honey	2.1 ±0.3	14.1 ±0.9	5.4 ±0.5	3.9 ±0.2	17.2 ±1.3	7.1 ±0.5
Honey from buckwheat	3.1 ±0.2	16.4 ±1.4	6.8 ±0.4	4.4 ±0.4	18.9 ±1.9	8.6 ±0.2
Honey from fruit trees*	2.8 ±0.3	14.2 ±1.1	6.5 ±0.6	3.2 ±0.6	17.6 ±0.9	7.9 ±0.3
Honey from non-fruit trees**	1.4 ±0.1	15.4 ±0.7	6.1 ±0.2	2.1 ±0.3	17.1 ±0.8	7.4 ±0.5
Honey from fodder crops***	3.7 ±0.5	17.2 ±0.9	7.6 ±0.4	4.6 ±0.4	19.1 ±1.1	9.2 ±0.8
Average value	**2.62 ±0.1**	**15.46 ±0.9**	**6.48 ±0.7**	**3.64 ±0.3**	**17.98 ±1.2**	**8.04 ±0.8**

Note: APC – Aerobic Plate Count, TFC – total fungal count, TYC – total yeast count; * Fruit trees: cherry, apple, pear, cherry, peach, cherry-plum; **Non- fruit trees (forest trees): false acacia, linden; ***Fodder crops: alfalfa, clover, rape, sweet clover.

Table 3 Results of honey sample studies by contamination of sporeforming anaerobes, include *Bacillus cereus* and *Clostridium botulinum*.

Honey produced from different melliferous plants species	Honey of higher grade				Honey of first grade			
	No. investigated samples	No. positive samples/percentage			No. investigated samples	No. positive samples/percentage		
		SFA	Bacillus cereus	Clostridium botulinum		SFA	Bacillus cereus	Clostridium botulinum
Sunflower honey	13	3/23.1	3/23.1	0	13	5/38.5	4/30.8	1/7.7
Honey from buckwheat	12	5/41.7	4/33.3	1/8.3	12	6/50.0	4/33.3	2/16.7
Honey from fruit trees*	14	2/14.3	2/14.3	0	14	4/28.6	4/28.6	0
Honey from non-fruit trees**	11	2/18.2	2/18.2	0	11	3/27.3	3/27.3	0
Honey from fodder crops***	12	4/33.3	3/25.0	1/8.3	12	5/41.7	4/33.3	1/8.3
Total	62	16/25.8	14/22.6	2/3.2	62	23/37.1	19/30.6	4/6.5

Note: SFA – Total number of sporeforming anaerobes; * Fruit trees: cherry, apple, pear, cherry, peach, cherry-plum; **Non-fruit trees (forest trees): false acacia, linden; ***Fodder crops: alfalfa, clover, rape, sweet clover.

The next important groups of microorganisms in honey that maintain its physicochemical parameters are microscopic fungi and yeast. We found some correlation between of number of these groups of microorganisms with grade of honey and physicochemical components respectively. It was found that the higher grade of honey had the lower the number of microorganisms than first grade in 1.12 – 1.2 for fungi and in 1.2 – 1.3 times for yeast.

Besides, in the first grade honey amount of yeast and fungi was the more than in higher grade honey. Thus, the number of fungi in the first grade honey was on average 17.98 ±1.2 CFU.g^{-1}, which is on average 1.2 – 1.5 times higher than in the higher grade honey. Yeast in samples of first grade honey was within 7.1 ±0.5 and 9.2 ±0.8 CFU.g^{-1}, and in samples of higher grade honey – 5.4 ±0,5 – 7.6 ±0.4 CFU.g^{-1}. In the first grade honey, the increase in the amount of yeast was in 1.2 – 1.3 times.

The least amount of fungi and yeast was in honey samples from sunflower, fruit and non-fruit trees comparatively to honey samples from buckwheat and fodder crops. This can be explained by the fact that flowers of buckwheat and fodder crops are more contaminated by microorganisms from soil as these plants are undersized than trees and sunflowers.

In contrast to our study **Sinacori et al. (2014)** reported wide range in quantity of yeasts or moulds with maximum concentrations of 18,600 and 27 CFU.g^{-1}, respectively. The author attributes the high concentration of fungi in honey samples to the high moisture content.

As reported **Kunová et al. (2015)**, four samples of honey from 3 different EC country were negative for microscopic fungi count. The maximum value of microscopic fungi in one sample of honey was 1.5 x 10^2 CFU.g^{-1} (from Slovakia). In research performed by **Sinacori et al. (2014)**, with honey samples of different types of melliferous plants, 17 of filamentous fungi of different species were isolated.

The next part of our study was to assess presence of total count of sporeforming anaerobes and in particular *Bacillus cereus* and *Clostridium botulinum*. The results of the studies are shown in Table 3.

The data in Table 3 indicate the intensity of contamination of honey with sporeforming microorganisms, and also characterize the incidence of honey samples detection which were contaminated with this type of microorganisms. As shown in Table 3, the higher grade honey samples had less anaerobic bacteria than first-grade honey samples. It was found that, 25.8% of the 62 studied samples of hight grade honey had sporeforming anaerobes, in 22.6% and 3.2% of cases had *Bacillus cereus* and *Clostridium botulinum* respectively. In the first-grade honey samples, these microorganisms were slightly higher at 37.1% for sporoform bacteria, 30.6% for *Bacillus cereus*, and 6.5% for *Clostridium botulinum*.

As mentioned by **Maikanov et al. (2019)** among 197 honey samples from Kazakhstan, *C. botulinum* was noticed in only one (0.5%). This result is lower than our result and results which were obtained in the Europe countries **(Matovic et al. 2015; Wojtacka et al., 2017; Grenda et al., 2018)**.

C. botulinum spores in 5 honey samples (8.47%) originating from different regions of the Republic of Serbia were found by **Matovic et al. (2015)**. The highest number of anaerobic isolates were detected in the samples of buckwheat Polish honey, at 10/26 (38%) **(Rózanska, 2011)**.

It is well known, that the main sources of sporeforming anaerobes in honey are the conditions of honey harvest and hygienic practices on the apiaries, type of melliferous plants and soil, climate.

Sporeforming microorganisms were found with higher frequency in samples of buckwheat honey and in honey from fodder crops in both grades – in higher grade honey and in first grade honey.

In samples of higher grade honey from buckwheat and fodder crops *Clostridium botulinum* were found in 8.3% of cases. In the first grade honey samples, *Clostridium botulinum* was found in the 1 sample of sunflower honey, in the 1 sample of honey from fodder crops and in two buckwheat honey samples. But more often than *Clostridium botulinum*, *Bacillus cereus* was found in honey samples. *Bacillus cereus* were isolated in 14 samples of higher grade honey and 19 samples of the first grade honey, 22.6% and 30.6%, respectively. In contrast of it, the presence of *Clostridium botulinum* spores in honey has been reported extensively **(Abdulla et al., 2012; Grant, McLauchlin and Amar, 2013)**. **Saba, Shakeel and Kamran (2015)** could not find spors of *C. botulism* during their study. **Sinacori et al. (2014)** reported that the highest microbial diversity was found in multifloral honeys. But this author also suggest, that no correlation among the microbial species and the botanical/geographical origin.

The results of this study clearly showed that the honey samples collected from apiaries of from south of Ukraine contained different amounts of microorganisms, that depended on the botanical origin of the honey as well as on the honey grade.

CONCLUSION

By our research we established that honey from the south of Ukraine, which according to the state standard and its physicochemical indicators belongs to the higher and first grades, has low microbial contamination.

In addition to the above, our research found that there was a relationship between microbial contamination of honey and its botanical origin. Samples of buckwheat honey and honey from fodder crops of both grades were more contaminated with all microorganisms than samples of sunflower honey and honey from fruit and non-fruit trees.

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THE CORRELATION OF INTAKE PHYTATE AND TANNIN ON SERUM TRANSFERRIN RECEPTOR AND HEMOGLOBIN IN STUNTED OVERWEIGHT ADOLESCENTS

La Mani, Sitti Fatimah-Muis, Apoina Kartini

ABSTRACT

Stunted overweight teenagers are at risk of having iron deficiency. Iron deficiency is caused by various factors including the high food absorption inhibitors of iron such as phytate and tannins. Phytate and tannin contain polyphenol compounds which have a strong ability to bind iron so that it inhibits iron absorption in the intestine. This study aims to analyze the correlation between phytate, tannin intake and serum transferrin receptor (sTfR) and hemoglobin in stunted overweight adolescents. The research method was a cross-sectional study of 64 stunted overweight adolescents selected by consecutive sampling in four high schools/vocational high schools in Banyumanik District, Semarang City. Phytate and tannin intake data using SQ-FFQ method. The serum transferrin receptor examination uses the ELISA method and the hemoglobin level uses the Cyanomethemoglobin method. The results of the study, most of the respondents had high phytate and tannin intake of 96.9% and 89.1%. Respondents with low serum transferrin receptor were 7.8% and low hemoglobin levels were 7.8%. There was no correlation between phytate intake with serum transferrin receptor or hemoglobin (p_1 = 0.937 r_1 = -0.010, p_2 = 0.192 r_2 = 0.165). Tannins were significantly correlated with serum transferrin receptor and hemoglobin (p_1 = 0.005 r_1 = 0.344, p_2 = 0.002 r_2 = -0.374). Based on multivariate analysis, tannin is a determinant of hemoglobin (R^2 = 0.257). Conclusion is that tannin is positively correlated with serum transferrin receptor and hemoglobin in stunted overweight adolescents. Excessive tannin intake can cause deficiency in stunted overweight adolescents.

Keywords: Stunted overweight; sTfR; Hemoglobin; Fitat; Tannin

INTRODUCTION

Iron deficiency and stunted overweight are two nutritional problems that still occur in Indonesia. Iron deficiency and stunted overweight are known to be interconnected and often occur simultaneously **(Balitbang, 2013)**. Stunted overweigt is the nutritional state of a person who has a short body and is overweight. Malnutrition in early life marked by stunted, the risk of overweight and obesity in adolescence. The mechanism of obesity in stunted children due to low energy intake during growth causes high cortisol levels and low IGF-1. These hormonal changes are related to body fat storage while the low IGF-1 hormone allows interference with lipolysis in breaking down fat, therefore that, long-term adaptation in stunted children causes impaired fat oxidation **(Stanojevic, Kain and Uauy, 2007)**.

The prevalence of short and very short adolescents aged 16 – 18 years in Semarang City reached 18.3% and 3.7%. The prevalence of obese and obese adolescents is 7.6% and 2.7%. Semarang City is one of 16 districts/cities with obesity prevalence above the provincial prevalence of 5.4% obese and 1.7% obese **(Santoso et al., 2013)**. Research in several countries proves that stunted children have a risk of overweight and obesity as teenagers. In a Brazilian study, the prevalence of stunted adolescents was 11%, and 30% of them were obese **(Hoffman et al., 2000)**. Research in South Africa, amounting to 14.8% of secondary school students stunted and there was a tendency to overweight **(Mukuddem-Petersen and Kruger, 2014)**. Research in Indonesia in Bangsri Subdistrict, Jepara Regency, and stunted incidence in young women was 23.3% and 11.1% classified as obese **(Saraswati and Sulchan, 2016)**.

The increasing prevalence of stunted overweight and obesity adolescents is caused by changes in lifestyle and eating patterns to sedentary lifestyle and consumption of energy-dense foods. Energy-dense food consumption is a shift in traditional and high consumption patterns of eating vegetables and fruits and is shifting to high-energy and low micronutrient food consumption habits **(Feeley et al., 2012)**. Habits of physical activity in adolescents have decreased by an average of 7% per year **(Dumith et al., 2011)**.

Stunted overweight and obesity are body conditions where there is excess fat accumulation with an unproportionally increased characteristic of adipose tissue **(Kruger, Pretorius and Schutte, 2010)**. High fat accumulation will cause inflammation which is at risk of iron deficiency **(Aigner, Feldman and Datz, 2014)**. Iron deficiency occurs when an imbalance between erythropoiesis and the amount of body iron stores **(Urrechaga, Borque and Escanero, 2013)**. Iron status in the body is influenced by factors that inhibit iron absorption. Absorption of iron is inhibited by phytate and tannins. Fats and tannins can bind mineral elements in the form of zinc, iron, calcium, protein to form water-insoluble complexes making iron, protein difficult to be absorbed so that it can cause iron deficiency **(Hurrel and Engli, 2010)**.

Iron deficiency in adolescents will have a negative impact on health, namely the occurrence of growth and development disorders, fatigue, increased body susceptibility to infections, decreased physical ability and endurance as well as academic ability **(Hassan et al., 2016)**. This study aims to analyze the correlation of intake phytate, tannins against serum transferrin receptors and hemoglobin in stunted overweight adolescents.

Scientific hypothesis

The hypothesis of this study is that phytate and tannin intake correlates with serum transferrin receptor and hemoglobin in stunted overweight adolescents.

MATERIAL AND METHODOLOGY

Statement of Ethics

This study was approved by the Health Research Ethics Commission (KEPK) of the Faculty of Medicine, Diponegoro University as stipulated in Ethical Clreance No. 67/EC/FK UNDIP/III/2019 dated March 11, 2019. Written agreement was obtained from all participants and participating parents.

Study Design

A cross-sectional study was conducted at SMAN 9 Semarang, Hidayatullah Islamic High School, SMKN 11 Semarang, Hidayah Vocational School. Research respondents were 64 people selected by consecutive sampling according to inclusion and exclusion criteria **(Sastroasmoro and Ismael, 2014)**. Inclusion criteria are stunted overweight and obese adolescents aged 15 – 18 years and classified as stunted overweight and stunted obesity if the TB/U indicator with Z-Score < -2 SD and overweight based on BMI/U with Z-Score > 1 SD 2 SD and obesity based on BMI/U with Z-Score > 2 elementary school in the same age group **(Cashin and Oot, 2018)**. Willing to be a research respondent by filling out informed consent. Exclusion criteria were suffering from chronic illness in the last 1 month, suffering from infectious disease in the last 2 – 3 weeks, having menstruation in the last 1 week, consuming iron tablets in the last 3 months.

Data collection

Measurements of body weight using a digital Camry EB9003 scale with an accuracy of 0.1 kg, placed on a flat floor surface. Respondents took off their footwear, dressed as seminally as possible, took off their hats and took off their cell phones, watches, wallets and other objects that could affect the outcome of the weighing. Respondents are welcome to rise on the digital scale, both feet are in the middle of the scale and look straight ahead. The height is measured using Seka's microtoise with the accuracy of 0.1 cm, taped to a wall as high as at least 2 meters with a flat surface. Respondents were asked to take off their footwear and then stand upright against the wall. The heel, calf, buttocks, shoulders, head stick well to the wall and look straight ahead. The anthropometric measurements of the respondents were carried out to determine nutritional status based on TB/U < -2 SD, BMI/U overweight z-score 1 SD 2 SD and obesity z-score ≥2 SD. Nutrition status data using WHO-Antro Plus Software. Phytate intake and tannin respondents were obtained through interviews using the SQ-FFQ (Semi Quantitative-Food Frequency Questionnaire) form. Phytate and tannin intake data were processed using Nutrisoft sofware

Biochemical Analysis

Blood sampling was conducted in the morning between 8 and 10 am by experienced analysts from the Diponegoro University GAKY laboratory. Blood was taken as much as 3 cc through the antecubital vein and analysed at the GAKY Laboratories at Diponegoro University. The serum transferrin receptor level of the respondents was measured using the ELISA method. Serum transferrin receptor levels were categorized as normal (1.8 – 4.6 $mg.L^{-1}$), low (<1.8 $mg.L^{-1}$) and high (> 4.6 $mg.L^{-1}$) **(Sumarmi et al, 2016)**. Hemoglobin levels were measured using the Cyanmethomoglobin method. Categorizes hemoglobin levels which are normal in men (13.2 – 17.3 $g.dL^{-1}$), low (<13.2 $g.dL^{-1}$) and high (> 17.3 $g.dL^{-1}$) and in women categorized as normal (11.7 – 15.2 $g.dL^{-1}$), low (<11.7 $g.dL^{-1}$) and high (> 15.2 $g.dL^{-1}$) **(Association of Clinical Pathologists, 1996)**.

Statistic analysis

Statistical analysis using SPSS Version 21. Test the normality of the data using Kolmogorov-smirnov. Data are presented in the form of percentages, medians and maximum-minimum values. Spearman correlation test to see the correlation between phytate, tannin intake and serum transferrin receptor and hemoglobin with significant at *p*-value <0.05 and 95% confidence intervals. The strength of correlations was determined by *r*-value. Multivariate analysis using linear regression test **(Dahlan, 2011)**.

RESULTS AND DISCUSSION

Subject Characteristics

Table 1 shows that the number of research respondents was 64 people and the majority were women (65.6%). The age of research respondents is mostly 16 years, which is 46.9%. This result is in line with the study in Semarang City High School where most of the stunted overweight are adolescent girls by 88.46% **(Afifah, Sulchan and Nissa, 2017)**.

Table 1 Subject characteristics.

Characteristics	n	%	Average ±*SD*	Median (min – max)
Gender				
Male	22	34.4		
Female	42	65.6		
Age (Years)			15.97 ±0.73	16 (15 – 17)
15	18	28.1		
16	30	46.9		
17	16	25.0		
Nutritional Status(TB/U)			-2.14 ±0.22	-2.04 (-3.3 – (-2.01))
Very Short	1	1.6		
Short	63	98.4		
Nutritional Status (IMT/U)			2.02 ±0.71	1.86 (1.03 – 5.15)
Overweight	42	65.6		
Obesity	22	34.4		

Table 2 Fitat intake, Tannin, sTfR levels and Hemoglobin.

Characteristics	n	%	Average ± *SD*	Median (min – max)
Fitat intake			1193.7 ±334.01	1103 (674 – 2173)
High ≥718 mg	62	96.9		
Low ≤718 mg	2	3.1		
Tannin intake			0.38 ±0.25	0.29 (0 – 1.16)
High >0.29 mg	57	89.1		
Low <0.29 mg	7	10.1		
Iron Status (sTfR)			2.69 ±0.60	2.77 (1.04 – 3.53)
High (1.8 – 4.6 mg.L^{-1})	59	92.2		
Low (< 1.8 mg.L^{-1})	5	7.8		
High (> 4.6 mg.L^{-1})	0	0		
Iron Status (Hemoglobin)			14.10 ±1.78	13.9 (9.4 – 17.8)
Male			15.88 ±1.08	15.8 (13.9 – 17.8)
Normal (13.2 – 7.3 g.dL^{-1})	19	29.7		
Low(<13.2 g.dL^{-1})	0	0		
High (>17.3 g.dL^{-1})	3	4.7		
Female			13.17 ±1.30	13.25 (9.4 – 15.6)
Normal (11.7-15.2 g.dL^{-1})	35	54.7		
Low (<11.7 g.dL^{-1})	5	7.8		
High (>15.2 g.dL^{-1})	2	3.1		

The accelerated linear growth in adolescent girls takes place at the age of 9.5 – 14.5 years and slows down at the age 16 years and stopped at the age of 19 years, while men started at around 14.4 years and stopped at 21 years. Age 14 years is the average maximum age for women experiencing first menstruation, where menstrual conditions are associated with changes in the hormones estrogen and progesterone which increase lipoprotein lipase activity and fat stores in the body. Adolescent girls have more fat around 22 – 26% than men around 18 – 23% **(Brown, 2011; Habánová et al., 2010)**. This makes women easier to overweight than men, especially in stunted conditions **(Kruger, Margetts and Vorster, 2004)**.

Table 2 shows that the majority of respondents had a high phytate intake of 96.9% with an average of 1193.7 ±334.01 mg. The high intake of phytate respondents came from daily foods such as rice, tempeh and tofu. Most of the respondents' tannin intake in the high category was 89.1%. The high intake of respondent tannins came from tea consumption. Respondents with low serum transferrin receptor were 7.8% and low hemoglobin levels were 7.8%.

Correlation of Intake of Fitat, Tannin with sTfR and Hemoglobin

Bivariate analysis was performed to see the correlation between independent variables including phytate and tannin intake on serum transferrin receptor and hemoglobin dependent variables. Bivariate analysis can be seen in Table 3 below.

Table 3 Correlation of intake of Fitat, Tannin to sTfR and Hemoglobin.

Variabel	sTfR		Hemoglobin	
	p	*r*	*p*	*r*
Phytate	0.937	-0.010	0.192	0.165
Tannin	0.005*	0.344	0.002*	-0.374

Note: * Significant (p <0.05).

Multivariate Analysis

Table 4 multivariate analysis shows that tannin is a weak determinant of hemoglobin. The R^2 value of the results of the multiple linear regression test was 25.7% which means

tannin was able to affect hemoglobin by 25.7% while 74.3% was influenced by other variables not examined in this study.

Table 4 Double Linear Regression Test for Hemoglobin.

Variabel	Hemoglobin	
	B	***P***
Phytate	-0.000099	0.869
Tannin	-3.674	0.000*

Note: *Adjusted *R* Square = 0.257.

Phytate acid is the main form of phosphorus storage in cereals and legumes **(Kumar et al., 2010)**. The average daily intake of phytate a vegetarian is 2000 – 2600 mg per day, while non-vegetarians daily intake is 150 – 1400 mg phytate per day **(Reddy and Shridhar, 2002)**.

The high intake of phytate subjects came from everyday foods such as white rice, tempeh and tofu. In 100 grams of white rice there are 126 mg phytate, in 25 grams tempeh there are 99 mg phytate and in 25 grams tofu is 94 mg phytate. Fitat is an iron absorption inhibitor, its effect is influenced by the dose. 2 mg intake of phytate inhibits iron absorption by 18%, and 25 mg of phytate inhibits iron absorption by 64%, and 250 mg of phytate inhibits iron absorption by 82% **(Hallberg, Brune and Rossander, 1989)**.

Phytate intake did not correlate with serum transferrin receptor (p = 0.937) or hemoglobin (p = 0.192). This is due to the phytate intake of respondents such as rice having gone through various processes starting with the process of grinding, washing repeatedly so that the phytate content can be lost. Other sources of phytate intake such as tempeh and tofu have gone through a fermentation process so as to reduce phytate content and increase iron absorption. Research of, feeding high phytate does not affect the status of ferritin iron and serum transferrin receptor **(Armah et al.,2015)**.

Fitat has a stable tendency towards heat, the inhibitory effect of phytate can be reduced by boiling the boiled water is discarded **(Sotelo, Gonzales-Osnaya and, Sanchesz-Chinchilla, 2010)**.The process of grinding, heating, fermentation can also degrade phytate content. The fermentation process in grains can increase the bioaccessibility of iron **(Hurrel and Engli, 2010)**. Fermentation can induce phytic hydrolysis, besides the fermentation process also produces organic acids that have the potential to increase iron absorption **(Hotz and Gibson, 2007)**. Meat consumption and ascorbic acid can also overcome the inhibitory effect on phytate **(Hurrel and Engli, 2010)**.

Most respondents had tannin intake in the high category of 89.1% with a mean tannin intake of 0.29 (0 – 1.16 mg). The results of the Spearman correlation test analysis showed that tannin was significantly correlated with serum transferrin receptor (p = 0.005), positive correlation was weak (r = 0.344). This shows that the higher the tannin intake the higher serum transferrin receptor levels. Whereas tannin with hemoglobin showed a significant correlation (p = 0.002), negative correlation was weak (r = -0.374). This shows that the higher the tannin intake, the lower the hemoglobin level. This result is in line with research in high school adolescents in Makassar City which shows that there is a significant correlation between tannin intake and hemoglobin p = 0.013, **(Indriasari and Jafar, 2015)**.

Research in India, respondents who were given a full meal consumed together with 1 cup of tea decreased iron absorption by 59% (p = 0.001) in the anemia group and 49% (p = 0.01) in the control group **(Thankachan et al, 2008)**. Absorption of non-heme iron in food consumed together with water is 10 – 13% but if the same food is consumed with 200 ml of tea will reduce Fe absorption by 2 – 3% **(Nelson and Poulter, 2004)**.

The results of multiple linear regression analysis, tannin is a weak determinant factor for hemoglobin. The R^2 value of the multiple linear regression test results is 25.7% meaning tannins are able to influence hemoglobin by 25.7% while 74.3% is influenced by other variables not examined in this study.

Tea contains tannins that can bind minerals (including iron) and in some teas (especially black tea) polyphenol compounds which act as antioxidants have been oxidized, so they can bind minerals such as Fe, Zn, and Ca so that iron absorption is reduced **(Thankachan et al., 2008)**, If the body's iron needs are not met through food intake, the iron reserves in the body will decrease. If this negative balance lasts a long time then the availability of iron in the body will be compensated so that erythropoesis occurs with iron deficiency. This will cause an initial increase in serum transferrin receptor concentration progressively and if it continues there will be a decrease in hemoglobin levels **(Zimmermann and Hurrell, 2007)**.

CONCLUSION

Tannins are positively correlated with serum transferrin receptor and negative correlation with hemoglobin. Excessive tannin intake can cause iron deficiency in stunted overweight adolescents.

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STUDY OF CHEMICAL STRUCTURE, ANTIMICROBIAL, CYTOTOXIC AND MECHANISM OF ACTION OF *SYZYGIUM AROMATICUM* ESSENTIAL OIL ON FOODBORNE PATHOGENS

Behrooz Alizadeh Behbahani, Mohammad Noshad, Fereshteh Falah

ABSTRACT

In this study, chemical composition (gas chromatography-mass spectroscopy), chemical structure (fourier transform infrared spectroscopy) and antioxidant potential (β-carotene bleaching assay and DPPH/ABTS-radical scavenging activity tests) of *Syzygium aromaticum* essential oil (SAEO) were evaluated. Eugenol (75.11%) was found to be the major compound of SAEO. Eugenol, as the main chemical constituent of SAEO, showed its signature peaks in the wavenumber range of 720 – 1250 cm^{-1}, ascribing to the C=C region. The antimicrobial activity of SAEO on *Escherichia coli*, *Staphylococcus aureus*, *Listeria innocua* and *Pseudomonas aeruginosa* were evaluated. The scanning electron microscopy (SEM) was then applied to unravel the antibacterial mechanism of SAEO on *E. coli* as the most resistant strain and *L. innocua* as the most sensitive strain. The MTT assay was also used to investigate the cytotoxicity effect of SAEO on human colonic cancer cell lines (HT29 cell line) and the highest cytotoxic effect was observed at 200 $mg.mL^{-1}$ concentration of SAEO. The SEM micrographs revealed that the SAEO treatment was able to manifestly increase the cell permeabilization and membrane integrity disruption. This means that the entirety of the cell membranes was remarkably affected by the essential oil, which could lead to cytoplasm secretion and subsequent cell death. The data strongly suggest that SAEO had a potential antioxidant, antimicrobial and cytotoxicity activity.

Keywords: *Syzygium aromaticum*; Scanning electron microscopy; Cytotoxic effect; Antimicrobial activity; HT29 cell line

INTRODUCTION

In recent years, foodborne pathogenic and spoilage bacteria have led to the emergence of one of the important food safety challenges, i.e. new foodborne disease outbreaks **(Zhang et al., 2016)**. It is also worthwhile to note that the lipid oxidation reaction could lead to the formation of potentially toxic side-reaction products capable of threating human health **(Zhong et al., 2015)**. In these contexts, chemical synthetic preservatives have been frequently used in the food industry to suppress microbial growth and lipid oxidation reaction; however, their usages have been a controversial topic, owing to their potential to create health problems **(López-Malo et al., 2006; Spickett and Forman, 2015)**. Therefore, there is a necessity to seek new and safe food-grade antioxidant and antimicrobial agents to amend food shelf-lives. Essential oils (EO) derived from aromatic plants have gained a lot of attention in the food industry not only for their natural origin, but also due to their documented benefits in food and human health. These biologically active compounds confer versatile biological characteristics including antimicrobial, insecticidal, antioxidant, analgesic, anti-tumor, anti-inflammatory, and anti-diabetic effects **(Ribeiro-Santos et al., 2017)**. *Syzygium aromaticum* L. (clove) is known as an evergreen tree and its commercial products are cloves, clove oil, and oleoresin. The essential oil of *S. aromaticum* is normally extracted from its stem, unopened buds, and leaves. Clove oil is used in dental formulations, toothpaste, soaps, mouth washes, breath freshner, insect repellent, and cosmetic items owing to different kind of biological properties, such as antibacterial, antifungal, anthelmintic, analgesic, and anti-carcinogenic activities **(Srivastava, Srivastava and Syamsundar, 2005)**. Eugenol is the major chemical compound of *S. aromaticum* essential oil (SAEO) which its antibacterial and antifungal activities have been reported in the literature **(Shokeen et al., 2008; Braga et al., 2007)**. However, as seen in the literature and to the best of authors' knowledge, there is no data available indicating the mechanism of antimicrobial activity of SAEO towards pathogenic and spoilage bacteria. This study was therefore aimed to unravel the antibacterial effect of SAEO through mechanistic approaches to provide more insights into the mode of

antibacterial action. Moreover, the antioxidant activity of the bioactive essential oil was investigated in this study.

Scientific hypothesis

The essential oil of *Syzygium aromaticum* has outstanding antibacterial effect against food-borne spoilage and pathogenic bacteria, in conjugation with superb radical scavenging activity.

MATERIAL AND METHODOLOGY

Materials

S. aromaticum were procured from a local market (Ahwaz, Iran). β-carotene, linoleic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) (ABTS), gallic acid, and quercetin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Other chemical reagents and materials were of analytical grade and purchased from Sigma-Aldrich Co. (St Louis, MO, USA) or Merck Co. (Germany).

Essential oil extraction

The plant was firstly verified and then dried at room temperature in a dark place, followed by grinding to obtain powdered forms *via* a laboratory grinder. The powdered plant (20 g) was dispersed in distilled water (750 mL) and the resulting dispersion was then subjected to a hydrodistillation-based Clevenger apparatus for 3.0 h, according to a method introduced by **Behbahani and Shahidi (2019**) with some modification. The extracted essential oil (SAEO) was dried using anhydrous sodium sulfate followed by storing at 4 ºC in glass vials until analyses.

Essential oil analysis

Gas chromatography-mass spectroscopy (GC-MS)

GC-MS technique was applied to identify the main chemical compounds of SAEO, based on the method of **Behbahani et al., (2017a)**. For this purpose, 0.2 µL of SAEO was injected to the GC column and the separation of chemical compounds of the essential oil was performed at the heating rate of 5 °C.min^{-1} and the ionization energy of 70 eV that was provided by the carrier gas helium with a rate of 1.1 mL.min^{-1}. A sequential process consisted of the comparison of normal spectra of chemical compounds with those of alkenes (C_8-C_{28}), the calculation of Kovats retention index, and the referring of the resulting indices to the natural compounds library was then used to identify the main chemical constituent of SAEO.

Fourier transform infrared spectroscopy (FTIR)

The essential oil was mixed with potassium bromide followed by compressing the mixture to obtain an appropriate pellet. The FTIR spectrum of the essential oil was then recorded in the wavenumber range of 500 – 4000 cm^{-1} with 4 cm^{-1} resolution using a Perkin Elmer model FTIR spectrometer.

Total phenol content

The procedure of **Do et al. (2014)** with minor changes was employed to measure the total phenol content of the SAEO. Briefly, 0.10 mL of the sample was added to 1.0 mL of 10% v/v Folin-Ciocalteu reagent. The resulting solution was vortexed for 5.0 min and it was then charged with 0.30 mL of 10% Na_2CO_3 solution. After incubation of the solution at ambient temperature for 2.0 h, its absorbance was read at 760 nm. Gallic acid was used as standard (0 – 0.50 mg.mL^{-1}) and the total phenol content of the SAEO was expressed as mg gallic acid equivalent per g dried essential oil.

Total flavonoid content

The total flavonoid content of the sample was determined utilizing the method of **Tohidi, Rahimmalek and Arzani (2017)**, with some modification. The SAEO (0.10 mL; 0.10 mg.mL^{-1}) was charged and mixed with 0.30 mL of 5.0% $NaNO_2$ solution. $AlCl_3$ (0.30 mL; 10% w/v) was added and the solution was mixed for 6.0 min, followed by adding 2.0 mL of NaOH solution (1.0 M). The absorbance of the obtained solution was recorded at 510 nm and quercetin was applied as standard (0 – 0.5 mg.mL^{-1}). The total flavonoid content was then calculated and recorded as mg quercetin per g dried essential oil.

Antioxidant assays

DPPH-radical scavenging (DPPH-RS) activity: SAEO (100 µL) was charged and mixed with ethanolic solution of DPPH radicals (0.12 mM; 3.90 mL). The resulting solution was kept at ambient temperature in a dark place for 30 min and its absorbance was read at 517 nm. DPPH-RS activity of SAEO was then measured as below **(Behbahani, Noshad, and Falah, 2019)**.

$$\text{DPPH-RS activity (\%)} = [1\text{-A sample/A control}] \times 100$$

Where: A sample and A control are the absorbance of the sample and control (distilled water instead of sample), respectively.

ABTS-radical scavenging (ABTS-RS) activity: The method of **Shan et al. (2005)** was used to evaluate the ABTS-RS activity of the essential oil. ABTS$^{\bullet}$ cations were firstly generated by reacting the same volumes of ABTS (7.0 mM) and potassium persulfate (2.45 mM) solutions following keeping the obtained solution at room temperature, under dark conditions for 16 h. The ABTS$^{\bullet}$ solution was then diluted with methanol to reach 0.70 ±0.20 absorbance at 734 nm. Thereafter, 3.90 mL of the diluted ABTS$^{\bullet}$ solution was mixed with SAEO (0.10 mL) or methanol (0.10 mL; as control sample). The solution was stored for 6.0 min at ambient temperature and the absorbance was measured at 734 nm. The ABTS-RS activity of the essential oil was calculated according to the following equation:

$$\text{ABTS-RS activity (\%)} = [1\text{-As/Ac}] \times 100$$

Where: As indicates the absorbance of the samples and Ac stands for the absorbance of the control.

β-carotene-linoleic acid assay: The inhibitory effect of SAEO against the bleaching of β-carotene-linoleate solution was evaluated *via* a spectrophotometric method based on the below equation **(Dapkevicius et al., 1998)**:

Inhibitory effect (%) = $[(AA_{(120)} - AC_{(120)})/(AC_{(0)} - AC_{(120)})] \times 100$

Where: $AA_{(120)}$ is the absorbance (at 490 nm) of sample after 120 min incubation time, and $AC_{(0)}$ and $AC_{(120)}$ are the absorbance of control sample at time zero and after 120 min incubation time, respectively.

Antibacterial assays

The antibacterial effect of the essential oil (SAEO) was evaluated towards *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Listeria innocua* ATCC 33090, according to the following antimicrobial tests.

Disk diffusion agar (DDA) assay: This antimicrobial test was done based on the method of **Noshad, Hojjati, and Behbahani (2018)** with minor changes. The blank discs were firstly impregnated with 20 µL of SAEO for 15 min at ambient temperature, followed by placing them on the culture media that were previously contaminated with bacterial strains. The media were incubated at 37 °C for 24 h and the inhibition zone areas around discs were determined by a ruler (mm) and expressed as antibacterial activity.

Well diffusion agar (WDA) assay: The WDA test was performed utilizing a procedure introduced by **Behbahani and Fooladi (2018)**. In this antibacterial assay, several holes with 6.0 mm in diameter were firstly created on the surface of Mueller Hinton agar medium (contaminated with bacteria) and then charged with 20 µL of SAEO. The inhibition zones around the holes were measured after incubation of the medium at 37 °C for 24 h, and reported as antibacterial effect.

Minimum inhibitory concentration (MIC) assay: The MIC of SAEO was evaluated using the microdilution assay in 96 well plates, according to the modified method of **Yeganegi et al. (2018)**. Sequential concentrations of SAEO (0.39, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50, 100, 200 $mg.mL^{-1}$) were prepared in Mueller Hinton broth medium and then sterilized through syringe filters (0.45 µm). In the next step, 200 µL of each concentration was added to the wells containing 20 µL of microbial suspensions with 0.5 McFarland equivalent. The plates were stored at a constant temperature of 37 °C for 24 h and they were incorporated with 20 µL of 5.0% w/v 2,3,5-triphenyltetrazolium chloride solution. The plates were re-incubated and the lowest concentration of SAEO that inhibited the growth of bacterial strains (confirmed by the lack of an amethystine or dark red color in the wells), was considered as the MIC of the essential oil.

Minimum bactericidal concentration (MBC) assay: In this antibacterial test, 100 µL of the solution in microbial growth-free wells (based on the MIC results) was cultured on the surface of Mueller Hinton agar medium, followed by incubation at 37 °C for 24 h. The lowest SAEO concentration that killed the bacterial strains (no visible colony formation) was reported as the MBC of the essential oil **(Alghooneh et al., 2015)**.

Antibacterial mechanism

Scanning electron microscopy (SEM) was performed to unravel the mechanism of action of SAEO on cell membranes of the most sensitive and resistant bacteria to the essential oil (i.e., *L. innocua* and *E. coli*, respectively), following the method of **Lv et al. (2011)** and **Moghayedi et al. (2017)**, with minor changes. For this aim, the microorganisms were cultured in a broth medium at their MIC values and incubated at 37 °C under shaking conditions. Next, the microbial suspension was centrifuged for 5.0 min at 5000 g, and the precipitate (contains microorganisms) was washed twice with phosphate buffered solution (0.10 M, pH 7.0). The suspension was filtered using a polycarbonate filter and the filtrate was fixed in a glutaraldehyde solution (2.50% v/v), which was followed by keeping the solution at 4 °C for 2.0 h. The sample was washed several times with double distilled water and it was then dehydrated successively with ethanol solution of various concentrations (30%, 50%, 70%, 80%, 90%, and 100%) for 10 min. In the final step, the samples were dried to completely evaporate the ethanol and coated with gold for SEM analysis. The morphology of *E. coli* and *L. innocua* was checked before and after treatment with SAEO, using a LEO 1450 VP model SEM apparatus.

Cytotoxicity effect of SAEO

The cytotoxic effect of SAEO was measured against colon cancer cell line (HT29 cell line) by MTT assay. The cells (Bu Ali Research Institute of Mashhad, Iran) were cultured in DMEM (Dulbecco's Modified Eagle Medium) high glucose medium supplemented with fetal calf serum (10% v/v) and penicillin/streptomycin, and incubated at 37 °C under constant humidity 95% and 5.0% CO_2 pressure. HT29 cells were seeded in 96-well flat-bottom plates (approximately 100,000 per well) until 50-60% confluence was achieved. The medium was then replaced with a complete culture medium containing DMEM and fetal bovine serum (200 µL) and various concentrations of SAEO (0.39, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50, 100, 200 $mg.mL^{-1}$) were added to each well. The blank medium was regarded as control medium. The cell proliferation was quantified by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 24 h incubation time as follows. The MTT solution (30 µL; 5.0 $mg.mL^{-1}$) was added to each well and the plates were incubated for 3.0 h in a CO_2-equiped incubator. After removing the medium gently and adding DMSO (200 µL) into the wells, an ELISA/microplate reader at 570 nm reference filter was used to record the absorbance of the mixture. The SAEO concentration ($mg.mL^{-1}$) that was able to inhibit the cell growth by 50%, was calculated and defined as IC_{50}. The cell viability curves were plotted with regard to the control cells.

Statistic analysis

All the experiments were done at three replicates. Data were analyzed through one-was ANOVA by SPSS software at 95% confidence level ($p < 0.05$), and recorded as means ±standard deviation.

RESULTS AND DISCUSSION

Chemical composition of SAEO

Six compounds were confirmed in SAEO according to the GC-MS analysis, with eugenol being the main detected constituent among others (75.11%). Caryophyllene (14.05%) was the second bioactive compound identified in SAEO, followed by phenol, 2-methoxy-4-(2-propenyl) (6.09%) and humulene (3.35%). Other minor constituents were delta-cadinene (0.71%) and caryophyllene oxide (0.69%). The results are in congruent with findings of other researchers, who reported that eugenol is the main biologically active compound of the essential oil of *S. aromaticum* **(Cortés-Rojas, de Souza and Oliveira, 2014; Ranasinghe, Jayawardena and Abeywickrama, 2002)**.

Structural analysis of SAEO

The FTIR spectrum of SAEO obtained *via* the hydro-distillation method is depicted in Figure 1. The region of 3000 – 3 500 cm^{-1} is due to the hydroxyl (OH) groups of phenolic and alcoholic compounds of SAEO. The peaks at around 2847 cm^{-1} and 2937 cm^{-1} are attributed to the frequency asymmetrical patterns of CH_2- and CH_3- groups of alcoholic compounds in the essential oil **(Mohammed, Abdulkadhim and Noori, 2016)**. Eugenol, as the main chemical constituent of SAEO, showed its signature peaks in the wavenumber range of 720 – 1250 cm^{-1}, ascribing to the C=C region. Moreover, the sharp peaks at 1642.22, 1607.06, and 1513.75 cm^{-1} are assigned to the C=C stretching vibration of aromatic moiety of eugenol, in well agreement with the eugenol spectrum reported by **Pramod et al. (2015)**. The peak at 1268 cm^{-1} could be ascribed to the characteristic bands of =C-H in-plane bending absorption of the aromatic rings and $–CH_2$ swing in alkanes; whilst, the peak at 1234 cm^{-1} is likely due to the C-O-C symmetric expansion of aromatic acid esters and C-OH vibrational stretching of phenolic compounds, which the latter peak is often attributed to the absorption of esters and eugenol in essential oils **(Jeyaratnam et al., 2016**. The peaks located at the wavenumbers of 1122 cm^{-1} and 1035 cm^{-1} are probably ascribed to the C-O stretching vibrations and deformation vibration of C-OH groups. In addition, the peaks at 992 cm^{-1} and 743 cm^{-1} could be due to the bending vibration absorption of C-H groups and =CH vibration absorption of benzene rings, respectively. Likewise, the vibration absorption of alkenes could be observed at the wavenumber of 645 cm^{-1} **(Li, Kong and Wu, 2013)**.

The content of total phenolic and flavonoid compounds

S. aromaticum is one of the main vegetal sources of phenolic compounds, such as flavonoids, hydroxyphenyl propens, hydroxycinamic acids, and hydroxybenzoic acids. The total phenolic and flavonoid compounds of the essential oil were found to be 48.14 ±0.12 mg gallic acid.g^{-1} and 23.26 ±0.51 mg quercetin.g^{-1} dry weight of the SAEO. Phenolic acids such as gallic acid and its derivatives (e.g., hydrolysable tannins) are found in high concentration in clove oil. Flavonoid compounds like kaempferol, quercetin, and its glycosylated derivatives are also presented in clove in lower contents **(Cortés-Rojas et al., 2014)**. Total phenolic content of 0.0896 mg gallic acid.g^{-1} dry weight of *S. aromaticum* fruit and 114.41 – 519.33 mg gallic acid.g^{-1} *S. aromaticum* extracts have been reported by **Wojdyło, Oszmiański and Czemerys (2007) and Neaz (2019)**, respectively. These differences could be ascribed to the various extraction techniques and plant parts used for essential oil extraction. Indeed, the oils from the bud, stem, and leaves of clove differ considerably in quality and yield. In addition, the origin, variety, maturity at harvest, and quality of raw materials in conjugation with pre-treatments and extraction modes can influence both yield and composition of the resulting essential oil **(Neaz, 2019)**. Phenolic compounds are strongly contributed to the antioxidant and antimicrobial capacity of bioactive EO.

Antioxidant activity

DPPH-RS, ABTS-RS, and β–carotene bleaching assays were applied to evaluate the antioxidant activity of SAEO. The relatively stable organic DPPH radical has been extensively used to determine the antioxidative potential of plant extracts and single compounds **(Goupy et al., 2003; Zhang et al., 2019)**.

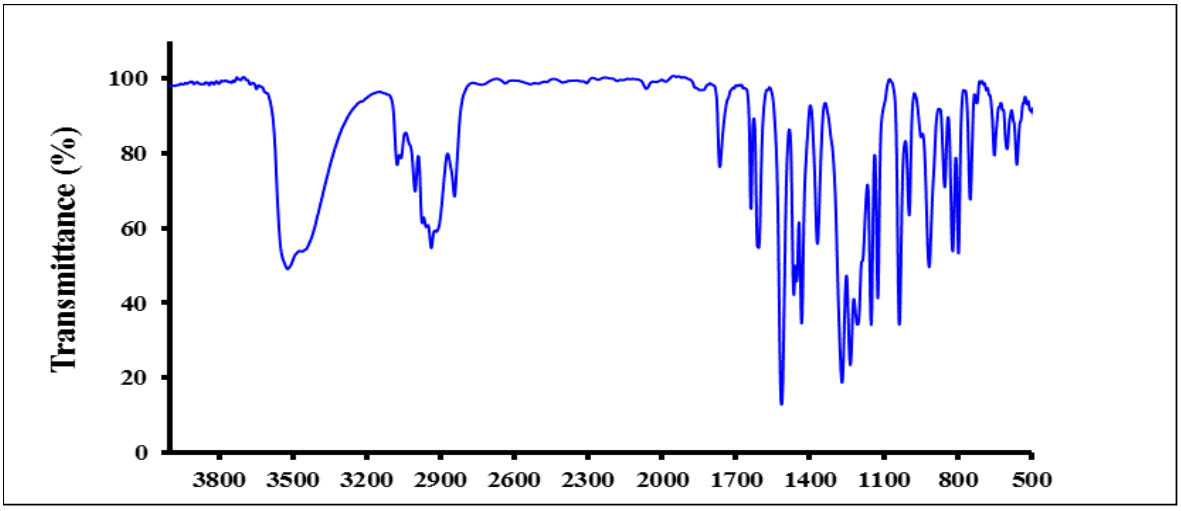

Figure 1 FTIR spectrum of *S. aromaticum* essential oil.

As can be seen from Table 1, the DPPH-RS activity was increased as the concentration of SAEO increased from 50 to 600 μg.mL^{-1}. This means that the SAEO contains bioactive compounds with the ability to neutral DPPH radicals through hydrogen atoms and electron donation **(Wang et al., 2016; Jabbari and Jabbari, 2016)**. The antioxidant capacity measured by ABTS radical test indicated the same trends and relationships as did DPPH-RS assay, and the ABTS-RS activity of the essential oil increased as a function of SAEO concentration. This might be due to the same principle of two DPPH and ABTS assays upon reacting with antioxidant compounds **(Ćavar et al., 2012)**. The SAEO was also able to remarkably suppress the rapid discoloration of β–carotene, and it showed a strong hydroperoxide scavenging power of 66.62%, likely *via* trapping and neutralizing the linoleate free radicals capable of deterioration of β–carotene **(Barros et al., 2007)**. This manifestly high antioxidant activity of SAEO is mainly due to its polyphenol compounds, such as phenolic acids and flavonoids.

Antibacterial activity

The antibacterial effect of SAEO was evaluated by the qualitative and quantitative assays, such as DDA, WDA, MIC, and MBC. As can be seen from Table 2, SAEO showed strong inhibitory effects against *L. innocua*, *S. aureus*, *P. aeruginosa*, and *E. coli* with the inhibition zone values of 27.10, 29.15, 22.30, and 20.50 mm in DDA test and 32.00, 35.15, 24.40, and 22.65 mm in WDA test, respectively. It is clear that the inhibition zone (i.e. higher antibacterial effect) is higher in WDA assay than that in DDA one, mainly due to the direct contact of SAEO and bacteria in the former; however, in the DDA antimicrobial test, the essential oil should be diffused from the discs into culture medium to exert its inhibitory effect **(Behbahani, et al., 2017b)**. It is also worthwhile to note that the gram positive bacteria (*L. innocua* and *S. aureus*) were inhibited to a more extent by
the essential oil compared to the gram negative bacteria. In addition, the MIC and MBC results revealed that the SAEO was more active against the gram positive bacteria and lower concentration of SAEO was required to inhibit the growth of *L. innocua* and *S. aureus* or kill them. This could be probably ascribed to the presence of a single diffusible mucopeptidic laver in gram positive bacteria that makes them more susceptible to antimicrobial agents; whilst, the complex lipopolysaccharide layer on the outer cell membrane of gram negative bacteria have the potential to remarkably reduce the diffusion rate of lipophilic antibacterial compounds across the cell membrane (**Behbahani and Imani Fooladi, 2018**).

Antibacterial mechanism

The morphological changes in bacterial cells treated with SAEO were investigated by SEM micrographs. It is clear that the untreated bacteria *E. coli* and *L. innocua* had their typical striated wall structures (Figure 2A, B); however, the morphology of cell membranes of the bacteria underwent severe detrimental changes upon treating with SAEO for 12 h at the MIC values of 1.56 mg.mL^{-1} for *L. innocua* (Figure 2A) and 6.25 mg.mL^{-1} for *E. coli* (Figure 2B). The SEM micrographs revealed that the SAEO treatment was able to manifestly increase the cell permeabilization and membrane integrity disruption. Treated *E. coli* had a malformed and incomplete/sunken shape in concomitant with the lack of cell walls (Figure 2B). This means that the entirety of the cell membranes was remarkably affected by the essential oil, which could lead to cytoplasm secretion and subsequent cell death. Similar findings were reported by **Behbahani, Noshad, and Falah (2019)**, who worked on the antibacterial mechanisms of cumin essential oil against some pathogenic and spoilage bacteria.

Cytotoxic effect of SAEO

MTT assay is used to evaluate the cytotoxic effects of essential oils owing to its simplicity. Despite the fact that the assay could not always be the best choice, the activation level of cells could be quantified usefully by this method, through an independent mode with bacteria and eukaryotes' proliferation **(Ramak and Talei, 2018)**.

Figure 3 indicates the cytotoxic effects of different concentrations of SAEO on HT29 cell lines after 24 h reaction period. The cytotoxicity effect was dependent on the essential oil concentration; the higher SAEO concentration, the higher was cytotoxicity.

Table 1 *In vitro* antioxidant activity of *S. aromaticum* essential oil.

SAEO concentration (μg/mL)	DPPH-RS activity (%)	ABTS-RS activity (%)
50	29.64 1.10^{e}	27.18 0.56^{e}
100	39.44 0.78^{d}	38.44 0.50^{d}
200	47.62 0.92^{c}	49.81 0.10^{c}
400	57.20 0.95^{b}	61.14 0.19^{b}
600	77.10 1.05^{a}	79.50 0.17^{a}

Note: Means with different letters in each column differ significantly (p<0.05).

Table 2 *In vitro* antibacterial activity of *S. aromaticum* essential oil against some pathogenic and spoilage bacteria.

Microbial strains	**Antimicrobial assays**			
	Disk diffusion agar (mm)	**Well diffusion agar (mm)**	**MIC (mg/mL)**	**MBC (mg/mL)**
E. coli	20.50 ±0.55	22.65 ±0.28	6.25	50
P. aeruginosa	22.30 ±0.56	24.40 ±0.44	3.125	25
L. innocua	27.10 ±0.40	32.00 ±0.23	1.56	6.25
S. aureus	29.15 ±0.50	35.15 ±0.19	0.78	6.25

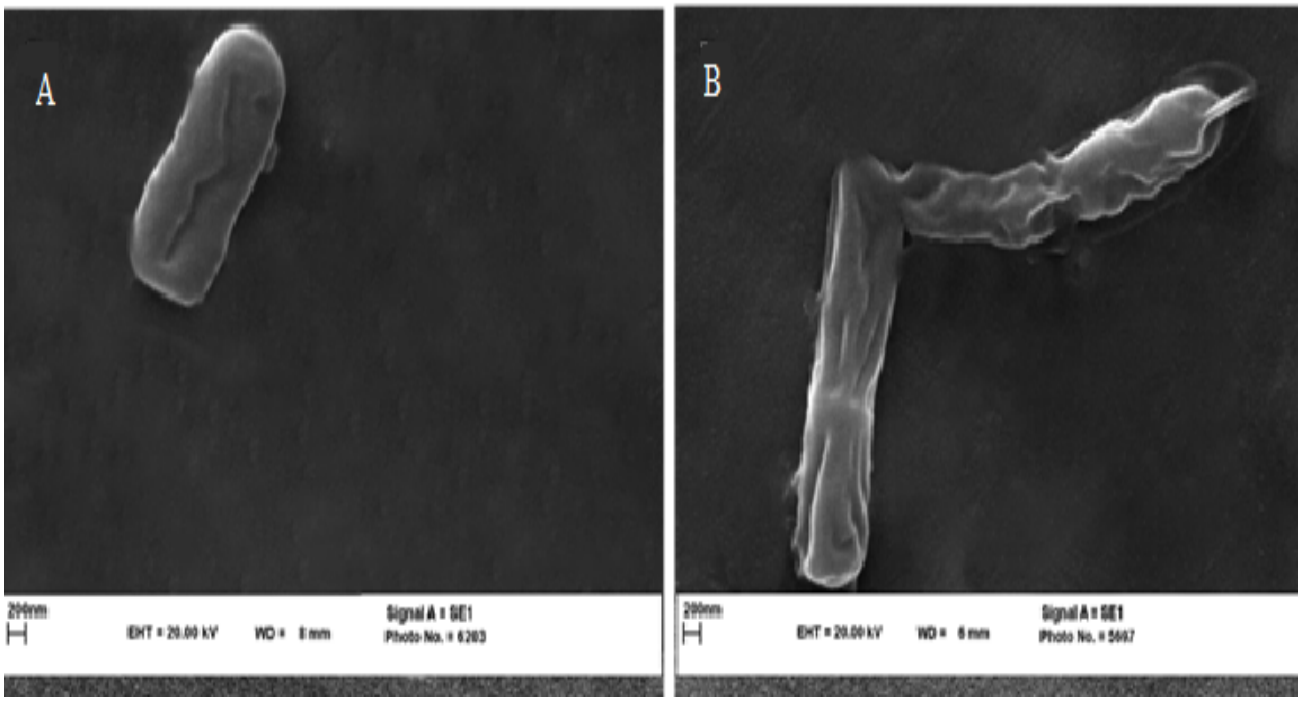

Figure 2 SEM images of *L. innocua* (A) and *E. coli* (B) cells after treatment with *S. aromaticum* essential oil for 12 h.

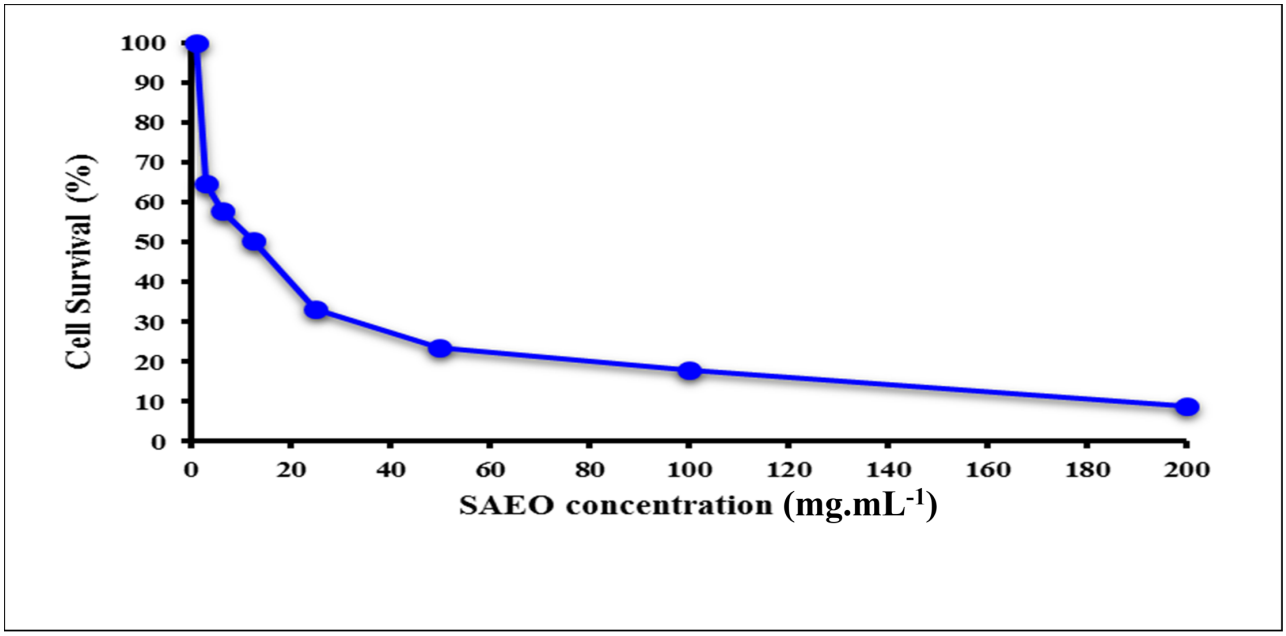

Figure 3 Cytotoxic effect of various concentrations of *S. aromaticum* essential oil on colon cancer cell line (HT29 cell line) after 24 h reaction period.

It can be seen from Figure 3 that the highest percentage of cell viability was observed at 3.25 mg/mL SAEO concentration, and a relatively low cell survivability was found when essential oil concentration was increased up to 200 mg.mL^{-1}. It is noteworthy that the IC_{50} value of the purified active compound was also calculated to check its maximum permissible concentration, and IC_{50} value was observed to be 13.51 mg.mL^{-1}. It can be confirmed by the MTT data that low concentrations of SAEO could stimulate cell proliferation substantially (p<0.05).

Indeed, essential oils have a lipophilic nature and high affinity for cell membranes. In this way, they could result in manifest changes in the polarization of cancer cell and particularly mitochondrial membranes along with ionic channels and disturbing membrane potential, thereby inhibiting proton pumps and ATP production **(Lesgards et al., 2014; Frolova, et al., 2019)**. It was also reported that essential oils could disrupt the membrane ionic pumps and lead to ion (calcium) and membrane proteins leakage **(Bakkali et al., 2008)**. The cytotoxicity of SAEO and its major components to human skin cell and other herbal extracts through MTT assay have been reported in the literature **(Prashar, Locke, and Evans, 2006; Turan et al., 2018; Ogbole, Segun, and Adeniji, 2017)**.

CONCLUSION

A sequential procedure consisted of hydrodistillation-based extraction of *S. aromaticum* essential oil followed by GC-MS analysis of the resultant oily solution yielded a bioactive essential oil rich in eugenol. The essential oil of *S. aromaticum* had outstanding antibacterial effect against food-borne spoilage and pathogenic bacteria, in conjugation with superb radical scavenging activity. The antibacterial mechanism of *S. aromaticum* essential oil on the most sensitive (i.e. *L. innocua*) and resistant (i.e. *E. coli*) bacteria was then evaluated by the SEM micrographs, and it was observed that the essential oil caused an increase in cell permeabilization and membrane integrity disruption. In addition, the essential oil showed a dose-dependent cytotoxic effect on the colon cancer cell line (HT29 cell line) and higher essential oil concentration resulted in a higher cytotoxicity effect. *S. aromaticum* essential oil could be therefore used to develop functional food products to possibly suppress radical attacks in human body and treat colonic cancers.

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THE AMINO ACID PROFILE AFTER ADDITION OF HUMIC ACIDS AND PHYTOBIOTICS INTO DIET OF BROILER CHICKEN

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ABSTRACT

The aim of the study was analysed the effect of humic acids separately and humic acids in combination with phytobiotic as garlic and oregano powder on amino acid (AA) profile of the most valuable parts of Ross 308 chicken. A total of 200 pcs Ross 308 broiler chickens of mixed sex were randomly divided into 4 groups (n=50): control group (C) without supplementation, experiment group E1 (2% humic acids), E2 (80% humic acids and 20% garlic powder) and E3 (90% humic acids and 10% oregano powder). Fattening period lasted for 42 days and all groups were kept under the same conditions. After slaughter, the AA profiles of breast and thigh samples were determined. In comparison with control group, 6 out of 10 AA was significantly affected ($p \leq 0.05$) by used dietary supplementation – Met, Cys and His in thigh and Leu, Phe, His and Arg in breast muscle. AA composition of breast muscle was positively affected mainly by humic acids and 10% oregano powder supplementation (E3), while thigh muscle by humic acids and 20% garlic powder (E2). The highest obtained AA in breast muscle was Leu (2.02 g.100 g^{-1}) in E3 group and thigh muscle His (1.15 g.100 g^{-1}) in E2 group ($p \leq 0.05$). In conclusion, humic acids and 10% oregano powder supplementation (E3) elicited to the best AA profile of chicken breast muscle but also the worst AA profile in thigh muscle so the effect of such a supplementation is disputable. On the other hand, humic acids and 20% garlic powder supplementation resulted into slight increase of AA in both breast and thigh muscle (E2).

Keywords: broiler chicken; amino acid; humic acid; garlic; oregano

INTRODUCTION

Meat and meat products are generally an important source of protein. Proteins are naturally occurring complex nitrogenous compounds and the percentage of the meat protein component varies considerably between different types of meat **(Marangoni et al., 2015)**. Average protein content in chicken meat is about 20 % and more than 40 % of AAs are essential ones **(Kim et al., 2017)**. Chicken meat offers all the EAAs and is characterized by a high content of lysine, leucine, aspartic acid and glutamic acid **(Sales and Hayes 1996; Soriano-Santos 2010; Ramane et al., 2011)**.

Although commercial feed additive containing antibiotics can increase productivity, antibiotic residues in meats might have negative effects on human health when meats were consumed **(Barton and Hart 2001; Khaksefidi and Rahimi, 2005)**. Thus, it needs alternative feed additives, which are safer, drug-free residue and to meet consumer demand. The possibility of using new natural materials including oregano, garlic and humic asids in diet of poultry is being researched **(Haščík et al., 2017; Kalafova et al., 2018)**. Many natural substances have been shown to express positive effects on growth performance and different health parameters in animals **(Hong et al., 2012; Thiamhirunsopit et al., 2014)**.

Many of plants and herbs can be used to improve the meat quality **(Liu et al., 2008)**, because of development of antibiotic resistance and their banned in the European Union since 2006. In addition, they consider the other alternative feed supplements, replacing the use of antibiotics in chicken nutrition. Probiotics and bee products **(Trembecká et al., 2017)**, organic acids, prebiotics, symbiotics, enzymes, organic minerals **(Fulton et al., 2002)** and plant essential oils **(Cross et al., 2007)** have been tested as suitable substitutes.

Plants and plant products, commonly referred to as phytobiotics, are natural, nontoxic, residue free, and readily available. Therefore, they are a suitable alternative to growth antibiotics. They have several effects on animals, such as appetite stimulation, increase digestive secretion, have immunostimulatory, bactericidal, antiviral and antioxidant effects **(Hashemi and Davoodi, 2011; Giannenas et al., 2013)**. Specially the medicinal activities of some natural plants as garlic (*Allium sativum* L.), thyme

(*Thymus vulgaris*), oregano (*Origanum vulgare*) and basil (*Ocimum basilicum*) are well known and documented **(Kostadinović, 2013)**.

Humates, one of the potential substances alternative to antibiotics in the diet of poultry, are formed from decayed plant matter with the aid of living bacteria in the soil. Humates include humus, humic acid, fulvic acid, ulmic acid, and trace minerals. Humic acid is a natural organic acid and has been shown to influence digestion, immune response and general performance of broilers **(Ozturk et al., 2012)**. The humic acids inclusion in broiler diets may stimulate changes in digestion dynamics, assimilation of nutrients and meat metabolite profiling, resulting in desirable meat compositional and organoleptic physiognomic quality **(Ozbey and Esen, 2007)**.

In chicken and pork, humic acid inclusion in diets was observed to desirably modify meat colour mainly due to accelerated myoglobin synthesis **(Wang et al., 2008; Ozturk et al., 2012)**. Moreover, in pork, humic acid was observed to have an effect of increasing the fat marbling values and to reduce back fat thickness, probably due its influence on protein and lipid distribution **(Wang et al., 2008)**. From the results of **Disetlhe et al. (2019)**, it can be concluded that the inclusion of enzymes and potassium humate in canola-based broiler diets had beneficial effects on the carcass and meat quality parameters in terms of breast weights, water holding capacity and colour.

Oregano (*Origanum vulgare* L.) is generaly an aromatic herb used to improve organoleptic characteristics of foods. Also it is a natural, less toxic, residue free feed supplement for poultry when compared to other synthetic ingredients. Its oil contains key bioactive components, including as thymol and carvacrol **(Figiel et al., 2010; Alagawany, et al., 2018)**. These phenolic compounds have an antimicrobial, antioxidant, antiviral, immunomodulatory and antiparasitic effect. The potential advantages of utilising oregano extract, in poultry diets include improved feed intake and feed conversion, enhanced digestion, expanded productive performance, down-regulated disease incidence and economic losses **(Arcila-Lozano et al., 2004; Fasseas et al., 2007)** and reduce conventional antibiotics use **(Symeon et al., 2010)**. From the available literature, average inclusions of oregano essential oil up to 600 mg/kg in broiler diets increased body weight gain. Using 1% oregano oil in broiler diets improved feed conversion ratio and feed utilisation **(Alagawany, et al., 2018)**. But, chemical composition of oregano oil may vary due to weather, season of year, harvest cycle, process of extraction and crop location **(Baydar et al., 2004; Ortega-Nieblas et al., 2011)**.

Garlic (*Allium sativum* L.) is widely used in all parts of the world as a spice and herbal medicine for the prevention and treatment of a variety of diseases, ranging from infections to heart diseases **(Javandel et al., 2008)**. Garlic is today use for a variety of reasons, it has anti-microbial, anti-bacterial, anti-inflammatory effects etc. **(Mansoub, 2011)**. Garlic was reported as a natural feed additive, it has improved broiler chickens growth and feed conversion **(Stanaćev et al., 2012)**. The major active ingredients in garlic are allicin, ajoene, diakyl polysulphides, s-allylcysteine etc which may be responsible for the various properties of garlic **(Canogullari et al., 2010)**. Garlic has been found to lower serum, liver and tissue cholesterol **(Stanaćev et al., 2012)**. In broilers, it was reported that garlic as a natural feed additive, improved broiler growth and feed conversion ratio, and decreased mortality rate **(Tollba and Hassan, 2003; Puvača et al., 2013)**. **Suriya et al. (2012)** suggested that inclusion of 0.5% garlic may have the potential to be an alternative to antibiotic growth promoter for broiler chicken.

The aim of the present study was analysed the effect of supplying humic acids separately and humic acids in combination with phytobiotic as garlic and oregano powder on amino acid (AA) profile of the most valuable carcass parts of Ross 308 chickens.

Scientific hypothesis

We are expecting the significant rise of AA content in breast and thigh muscle of broiler chicken after addition of humic acids, garlic and oregano into their diet.

MATERIAL AND METHODOLOGY

Animals and experimental design

The experiment was realized in the experimental poultry station of Slovak University of Agriculture (SUA) in Nitra. Chickens were randomized into four groups, each containing 50 birds. In control group we used complete feed mixture without any additives. Group of chickens E1 was fed a diet containing 2 kg of preparation Humac Natur per 100 kg feed mixture. The group marked as E2 was fed a diet containing 1.6 kg of preparation Humac Natur per 100 kg feed mixture and 0.4 kg of garlic powder per 100 kg feed mixture and group E3 containing combination 1.8 kg of preparation Humac Natur per 100 kg feed mixture and 0.2 kg of oregano leaf powder per 100 kg feed mixture. The experiment was realized by methology **Haščík et al. (2018)**. Chickens in individual groups were stabled on deep budding, with a maximum occupation of the breeding areas 33 $kg.m^{-2}$. During the fattening period, the light regimen based on 24 h of dark was used. The temperature at the beginning of the experiment was 31-33 °C and decreased to 20 – 22 °C during the experiment. The temperature was maintained using electronic hen-like devices providing radiant heat.

The fattening lasted 42 days. The feeding program included three phases: starter (1^{st} – 21^{st} days of age), grower (22^{nd} – 35^{th} days of age), and finisher (36^{th} – 42^{nd} days of age). Feed and water were supplied *ad libitum*. The feed mixtures both starter and grower were produced without any antibiotics and coccidiostats. Composition of complete feed mixtures is presented in Table 1.

Humac Natur purchased from Humac s.r.o., Kosice is preparation of humic substances on base of oxihumolit contain min. 62% humic acids in dry matter, of this 48% free humic acids in dry matter, minerals and trace elements, carboxymethylcellulose complex with humic substances. Moisture was maximum 11%.

The garlic was added to the feed in the form of finely ground *Allium sativum* L. bulbs and the oregano was added as dried and finely ground of *Oreganum sativum* leaves (Vetservis a.s.).

Slaughter and measurements

At the end of the 42-d feeding period, broilers were weighed and slaughtered at the slaughterhouse of Slovak University

of Agriculture in Nitra. After evisceration, the carcasses were kept at approximately 18 °C for 1 h *post mortem* and thereafter carcasses were weighed and stored at 4 °C until 24 h *post mortem*. The breast and thigh muscles were separated from each half-carcass for the determination the AA composition. In breast and thigh muscles, measurements were made of the content of the EAAs (valine, leucine, isoleucine, phenylalanine, threonine, lysine, methionine) and NEAAs (histidine, arginine, cysteine) using an automatic amino acid analyzer AAA 400 (INGOS Prague, Czech Republic). This works on the principle of ionic exchange chromatography with post-column ninhydrin derivatization, based on procedure previously described by Moore and Stein (1963) approved by Bulletin of the Ministry of Agriculture and Rural Development of the Slovak Republic **(MARD SR, 2004)**. Post-column ninhydrin derivatization has become the official standard in recent years. There has been some methodological advancement, but the technique is still used very widespread. Total amino acid content of meat was determined by acid hydrolysis of proteins. Sulphur amino acids are first oxidized to a stable oxidized derivative, and then acid hydrolysed. Tryptophan was not determined because of its decomposition during acid hydrolysis of proteins. The resultant values of amino acids were re-calculated to 100% dry matter and expressed as g AA content per 100 g muscle.

Table 1 Composition of feed mixtures

Ingredients (%)	Starter (HYD-01) (1^{st} – 21^{st} day of age)	Grower (HYD-02) (22^{nd} – 35^{th} day of age)	Finisher (HYD-03) (36^{th} – 42^{nd} day of age)
Wheat	34.50	34.50	36.32
Maize	35.50	40.50	37.50
Soybean meal (48% N)	21.40	18.80	20.00
Fish meal (71% N)	3.70	1.90	-
Dried blood	1.25	1.25	-
Ground limestone	1.00	1.05	1.10
Monocalcium phosphate	1.00	0.70	1.00
Fodder salt	0.10	0.15	0.20
Sodium bicarbonate	0.15	0.20	0.25
Lysine	0.05	0.07	0.29
Methionine	0.15	0.22	0.29
Palm kernel oil Bergafat	0.70	0.16	2.50
Premix Euromix BR 0.5%*	0.50	0.50	0.50
Nutrient composition [g.kg^{-1}]			
Linoleic acid	13.52	14.20	14.92
Fibre	30.17	29.92	30.53
Crude protein	210.73	190.41	170.54
MEN (MJ.kg^{-1})	12.15	12.04	12.41
Ash	24.22	19.92	38.47
Ca	8.16	7.28	7.38
P	6.77	5.71	6.01
Na	1.68	1.75	1.74

Note: *active substances per kilogram of premix: vitamin A 2 500 000 IU; vitamin E 20 000 mg; vitamin D3 800 000 IU; niacin 12 000 mg; d-pantothenic acid 3 000 mg; riboflavin 1 800 mg; pyridoxine 1 200 mg; thiamine 600 mg; menadione 800 mg; ascorbic acid 20 000 mg; folic acid 400 mg; biotin 40 mg; cobalamin 8.0 mg; choline 100 000 mg; betaine 50 000 mg; Mn 20 000 mg; Zn 16 000 mg; Fe 14 000 mg; Cu 2 400 mg; Co 80 mg; I 200 mg; Se 50 mg.

Statistical analysis

A statistical analysis was computed using the ANOVA procedures of SAS software with using of Enterprise Guide 4.2 application (version 9.3, SAS Institute Inc., USA, 2008). Data were reported as mean ± standard deviation. Statistical significance was calculated using t-test. Differences between the groups were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of experiment with Ross 308 broiler chickens after addition of humic acid, garlic and oregano into their diet, which was aimed at amino acid composition of breast and thigh muscle are presented into Table 2 and Table 3. The method used allowed measurement of 7 dispensable amino acids (EAA) and 3 indispensable amino acids (NEAA) in the muscles. The most abundant AAs (in decreasing order) in both breast and thigh muscles were Lys, followed by Leu, Arg, His, Thr and Phe.

Four AAs (Leu, Phe, His, Arg) in breast muscle were significantly affected ($p \leq 0.05$) by dietary supplementation with humic acid (E1), combination of humic acid with garlic powder (E2) and combination of humic acid with oregano powder (E3) in comparison with control group (C). Their concentration in C group was Leu 1.94 g.100 g^{-1}, Phe 1.00 g.100 g^{-1}, His 1.12 g.100 g^{-1} and Arg 1.56 g.100 g^{-1}. Among EAAs, the concentration of Leu, respectively Phe has sligthly increased ($p \leq 0.05$) when chickens were fed with combination humic acid plus garlic powder (E2; 1.97 g.100 g^{-1} resp. 1.02 g.100 g^{-1}) and humic acid plus oregano leaf powder (E3; 2.02 g.100 g^{-1}, resp. 1.05 g.100 g^{-1}).

Table 2 Effect of natural feed supplements on amino acid composition of chicken breast muscle

Parameter\ Group	C	E1	E2	E3	*p*-value
EAA					
Thr	1.09 ±0.10	1.02 ±0.12	1.11 ±0.10	1.13 ±0.08	0.079
Val	1.04 ±0.09	0.98 ±0.08	1.04 ±0.08	1.08 ±0.06	0.052
Met	0.79 ±0.08	0.74 ±0.08	0.79 ±0.06	0.82 ±0.06	0.066
Ile	0.95 ±0.12	0.89 ±0.10	0.97 ±0.09	1.00 ±0.07	0.051
Leu	1.94 ±0.22^{ab}	1.81 ±0.20^{b}	1.97 ±0.16^{ab}	2.02 ±0.14^{a}	0.049
Phe	1.00 ±0.11^{ab}	0.94 ±0.10^{b}	1.02 ±0.08^{ab}	1.05 ±0.07^{a}	0.045
Lys	2.10 ±0.25	1.95 ±0.22	2.13 ±0.18	2.19 ±0.15	0.051
NEAA					
Cys	0.36 ±0.02	0.33 ±0.04	0.36 ±0.03	0.36 ±0.02	0.111
His	1.12 ±0.14^{ab}	1.01 ±0.14^{b}	1.13 ±0.12^{ab}	1.19 ±0.09^{a}	0.029
Arg	1.56 ±0.18^{ab}	1.45 ±0.16^{b}	1.58 ±0.13^{ab}	1.63 ±0.11^{a}	0.048

Note: Amino acids are expressed on a dry matter basis (g.100 g^{-1}). Values are given as mean ± *SD* (standard deviation); *n* = 50; C = control group; E1-E3 = experimental groups; $^{a-b}$ = means within the same row with different superscripts differ significantly ($p \leq 0.05$); EAA = essential amino acid; NEAA = non-essential amino acid; Thr = threonine; Val = valine; Met = methionine; Ile = isoleucine; Leu = leucine; Phe = phenylalanine; Lys = lysine; Cys = cysteine; His = histidine; Arg = arginine.

Table 3 Effect of natural feed supplements on amino acid composition of chicken thigh muscle

Parameter\ Group	C	E1	E2	E3	*p*-value
EAA					
Thr	1.09 ±0.01	1.08 ±0.04	1.12 ±0.09	1.05 ±0.08	0.167
Val	1.04 ±0.01	1.02 ±0.03	1.04 ±0.08	1.00 ±0.06	0.173
Met	0.79 ±0.01^{b}	0.81 ±0.04^{ab}	0.87 ±0.05^{a}	0.78 ±0.05^{b}	0.011
Ile	0.97 ±0.01	0.98 ±0.07	1.02 ±0.07	0.99 ±0.09	0.138
Leu	1.97 ±0.02	1.99 ±0.12	2.07 ±0.15	1.98 ±0.17	0.175
Phe	1.01 ±0.01	1.03 ±0.06	1.07 ±0.08	1.02 ±0.09	0.166
Lys	2.13 ±0.02	2.16 ±0.14	2.26 ±0.17	2.15 ±0.20	0.107
NEAA					
Cys	0.35 ±0.01^{b}	0.36 ±0.01^{b}	0.39 ±0.03^{a}	0.33±0.01^{c}	0.001
His	1.15 ±0.01^{a}	1.13 ±0.03^{a}	1.15 ±0.08^{a}	1.04±0.06^{b}	0.009
Arg	1.58 ±0.02	1.60 ±0.11	1.67 ±0.12	1.60±0.15	0.127

Notes: Amino acids are expressed on a dry matter basis (g.100 g^{-1}). Values are given as mean ± *SD* (standard deviation); *n* = 50; C = control group; E1-E3 = experimental groups; $^{a-c}$ = means within the same row with different superscripts differ significantly ($p \leq 0.05$); EAA = essential amino acid; NEAA = non-essential amino acid; Thr = threonine; Val = valine; Met = methionine; Ile = isoleucine; Leu = leucine; Phe = phenylalanine; Lys = lysine; Cys = cysteine; His = histidine; Arg = arginine.

The content of mentioned AAs has decreased ($p \leq 0.05$) after addition of humic acids (E1; 1.81 g.100 g^{-1}, resp. 0.94 g.100 g^{-1}) in comparison with control group. Among NEAAs, amount of His, resp. Arg was positively affected ($p \leq 0.05$) in E2 and E3 groups compared to C group. Their concentration was 1.12 g.100 g^{-1}, resp. 1.58 g.100 g^{-1} in E2 group and 1.19 g.100 g^{-1}, resp. 1.63 g.100 g^{-1}.

Supplementation with humic acids alone led to slight decerase in His, resp. Arg to 1.01 g.100 g^{-1}, resp. 1.45 g.100 g^{-1}. Other AAs in breast muscle were affected in the same way, but not significantly ($p \geq 0.05$).

In thigh muscle, three AAs (Met, Cys, His) were significantly affected ($p \leq 0.05$) by used dietary supplementations. Their concentration in C group was Met 0.79 g.100 g^{-1}, Cys 0.35 g.100 g^{-1} and His 1.15 g.100 g^{-1}.

Among EAAs, the concentration of Met, respectively, has sligthly increased ($p \leq 0.05$) when chickens were fed with combination humic acids (E1; 0.81 g.100 g^{-1}) and humic acid plus garlic powder (E2; 0.87 g.100 g^{-1}). The content of Met has slightly decreased ($p \leq 0.05$) after addition of humic

acids (E3; 0.78 g.100 g^{-1}) in comparison with C group. Among NEAAs, amount of Cys was very gently increased ($p \leq 0.05$) in E1 (0.36 g.100 g^{-1}) and E2 (0.39 g.100 g^{-1}) and decreased in E3 (0.33 g.100 g^{-1}) groups compared to group C. On the other hand, Arg was not positively affected by any of used supplementation and was even decreased by humic acids plus oregano powder supplementation (E3; 1.04 g.100 g^{-1}). As in the breast muscles, other AAs in thigh muscle were affected similarly, but not significantly ($p \geq 0.05$).

The most abundant AAs (in decreasing order) in both breast and thigh muscles were Lys, followed by Leu, Arg, His, Thr and Phe. The lowest obtained AA was Cys in both breast and thigh muscle. Comparing breast with thigh muscle, the breast was found to contain higher amounts of all the EAAs and NEAAs detected in chicken meat, either the essential or the non-essential ones, which contrast with results reported by **Chae et al. (2012)**, who observed higher AAs contents in chicken thigh compared with breast muscle. Similarly, higher values of AAs in chicken meat were found by **Sharipova et al. (2017)** except Lys and Tyr. According to **Strakova et al. (2006)** the general levels of individual amino acids in breast muscles of broiler chickens varied from 1.9 (Pro) to 11.0 (Glu) g.100 g^{-1}, while in thigh muscles ranged from 1.4 (Met) to 9.3 (Glu) g. 100 g^{-1}. The total sum of amino acids in broiler chickens was 78.7 g.100 g^{-1} in the breast muscles (essential AAs – 42.5 g.100 g^{-1} and non-essential AAs – 36.1 g.100 g^{-1}) and 59.1 g.100 g^{-1} in the case of thigh muscles (essential AAs – 30.1 g.100 g^{-1} and non-essential AAs – 29.2 g.100 g^{-1}).

When comparing between dietary groups, contents of most EAAs were the highest in experimental group E3 in breast muscle, with one expectation (Cys) and the lowest in E1 group. On the other hand, the breast muscle was affected different by our supplementation – E3 had the lowest AA content and the best affected group was E2.

Amino acids are generally seen as main precursors of flavour substances. In particular, Glu was shown to have considerable effect on taste of chicken meat. In addition to Glu, free aromatic AAs, such as Phe and Tyr, also play an important role in enhancing the savoury or umami taste at sub-threshold concentrations in the presence of salt and free acidic AAs **(Wattanachant et al., 2004; Huang et al., 2011)**. Other AAs, such as Cys, Gly, Asp, Arg, and Ala, are also considered the flavour-related AAs **(Liu et al., 2008)**. Independently from these results, it is important to emphasize the way of heat treatment to AA composition. From the results of **Shehab (2016)** it is clear that any heat treatment of fresh thighs or breasts causes some reduction in all amino acids. Samples from chicken breasts and thighs cooked under pressure retained the highest percentage of total, essential and non-essential amino acids. Methods such as ordinary cooking, oven and frying followed. In particular, their loss in juice separated during cooking as well as thermal decomposition may be responsible for the reduction of the amino acid content.

CONCLUSION

The addition of garlic and oregano powder in combination with humic acids can affect the AA profile of chicken meat. In breast muscle, supplementation with humic acids plus garlic povder and also oregano increased content of all AA. Unfortunately, humic acids alone decreased AA content in experimental groups compared to the control group. The AA content of the thigh muscle was increased only after the addition of the humic acids with garlic powder compared to the control. The effects of the tested supplements may positively influence AA content; however, we recommend further review to verify their effectiveness.

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MACRONUTRIENTS, MICRONUTRIENTS INTAKE AND INFLAMMATION IN HEMODIALYSIS PATIENTS

Siti Fatonah, Moh Sulchan, Muchlis Achsan Udji Sofro

ABSTRACT

Inflammation in hemodialysis patients occurs since before undergoing hemodialysis. Inflammation is associated with an increase in oxidative stress. Hemodialysis patients are at risk for macronutrients and micronutrients deficiencies which can influence the increase in oxidative stress and inflammation. The purpose of this study was to evaluate the intake of micronutrients and inflammatory status in hemodialysis patients. This study was a cross-sectional study with 76 hemodialysis patients (40 male and 36 female) who attended in two hemodialysis centers of Kendal, Indonesia. After obtaining the written consent, then patients were interviewed food intake consisting of macronutrient and micronutrient intake. Macronutrient and micronutrient intake are obtained by the semi quantitative food frequency method and classified as a deficit (<100% adequacy level) and normal/ more (≥100% adequacy level), according to specific recommendations for individuals undergoing dialysis. Serum albumin was examined using the Brom Cresol Purple (BCP) method with a low category (<3.5 mg.dL^{-1}) and normal (3.5 – 4.5 mg.dL^{-1}). The hs-CRP serum was examined using the ELISA method and categorized as low (<1 mg.L^{-1}), moderate (1 – 3 mg.L^{-1}) and high (> 3 mg.L^{-1}). A descriptive analysis was performed. The results of this study showed that 88.2% deficit energy intake, 84.2% deficit protein intake, 85.5% deficit of vitamin A intake, 85.5% deficit of vitamin C intake, 100% deficit of vitamin E intake, 98, 7% deficit zinc intake , 92.1% deficit copper intake. 63.2% subjects are low level of serum albumin and 61.8% subjects is high level of hs-CRP serum. Macronutrient and micronutrient intake in most hemodialysis patients shows deficit. The serum albumin of most hemodialysis patients shows low level. Serum hs-CRP most hemodialysis patients show high level.

Keywords: macronutrients; micronutrients; albumin; hs-CRP; inflammation

INTRODUCTION

End stage renal disease patients have high mortality rates which are not only caused by traditional risk factors of cardiovascular disease, but other factors such as inflammation and protein energy malnutrition (PEM) **(Kalantar and Kopple., 2001)**. End stage renal disease is characterized by a decrease in irreversible kidney function with a glomerular filtration rate below 15/mL/min/1.73 m^2 and must undergo renal replacement therapy **(Hill et al., 2016)**. A continuous decrease in glomerular filtration rate will worsen the condition of chronic kidney disease (CKD) patients and will increase their mortality **(Panichi et al., 2008)**. Hemodialysis patients will experience inflammation that occurs since before undergoing hemodialysis **(Kalantar et al., 2004)**. **Yao et al. (2009)** stated that oxidative stress will affect the occurrence of inflammation in hemodialysis patients. Oxidative stress in CKD patients occurs early in the disease and increases gradually with the development of kidney disorders **(Yao et al., 2009)**. Hemodialysis patients will experience an increase in proinflammatory cytokines such as elevated serum hs-CRP **(Verhave et al., 2005; Panichi et al., 2008)**. The hs-CRP serum is part of the process of atherosclerosis and has a direct effect on endothelial cells, monocyte-macrophages and smooth muscle cells and supports the occurrence of atherogenesis **(Stevinkel et al., 2005)**. Hemodialysis patients will also experience increased levels of urea which results in the occurrence of uremia. Urea levels in the blood will cause feelings of nausea and vomiting **(Kalantar et al., 2004)**. This will cause a decrease in appetite that can reduce nutrient intake. This in turn will cause nutrient intake to also decrease. Hemodialysis patients will also have poor eating behaviour such as high sugar, fat, low cereal, fruit and vegetables **(Lou et al., 2007)**. Hemodialysis patients had lower calorie, protein, and fiber intakes than recommendations **(Bossola et al., 2013)**. Decreased macronutrient intake will be in line with a decrease in micronutrient intake. Low micronutrient intake will also cause antioxidant intake to decrease. Antioxidants play a role in reducing oxidative stress **(Sahni et al., 2012)**. High oxidative stress in hemodialysis patients indicates inflammation and will increase cardiovascular

complications, mortality and increase the need for antioxidants **(Miura et al., 2002; Locatelli et al., 2002)**. Antioxidants in food ingredients are found in various active ingredients including vitamins C, E, pro-vitamin A, zinc, cooper, selenium, α-tocopherol, organosulfur, flavonoids, thymoquinone, statins, niacin, phycocyanin, etc. **(Sahni et al., 2012)**. Vegetables and fruits contain a lot of antioxidants, but hemodialysis patients sometimes limit vegetables and fruits because of high potassium. Whereas a diet high in fruits and vegetables is not only good for improving lipid profiles, but also reduces oxidative stress and inflammation **(Bossola et al., 2013)**. Hemodialysis patients will overhydrate and protein loss through urine and dialysate which can result in a decrease in albumin **(Gama et al., 2012)**. Low albumin is associated with increased lipid peroxidation and supports oxidative stress in hemodialysis patients **(Castro et al., 2014)**. **Kaysen et al. (2002)** states that low serum albumin in dialysis patients is strongly associated with inflammation. The aim of this study is to evaluate the intake of macronutrients, micronutrients and inflammatory status in hemodialysis patients.

Scientific hypothesis

We investigate several hypotheses in our study:

a. The intake of macronutrients (energy and protein of hemodialysis patients shows inadequacy

b. The intake of micronutrients (vit A, C, E, zinc, copper) of hemodialysis patients shows inadequacy

c. Albumin levels of hemodialysis patients show low levels

d. The levels of hs-CRP hemodialysis patients show high levels

MATERIAL AND METHODOLOGY

This research is a descriptive study with a cross sectional approach conducted from March to April 2019 in two hemodialysis centers in the city of Indonesia state. The study population was all patients undergoing outpatient care in two hemodialysis centers. Research subjects were all patients with chronic kidney disease undergoing hemodialysis at two hemodialysis centers were taken by consecutive sampling and obtained as many as 76 people. Inclusion criteria are hemodialysis patients who are aware and can communicate well and are willing to participate in research by signing an informed consent. Exclusion criteria were chronic kidney disease patients who have never undergone hemodialysis and patients who had sepsis.

The size of the sample with a confidence level (α) of 95%, a proportion of 13.4%, a degree of error of 10% so the minimum number of samples is 45 subjects. Samples who participated in this study were 76 people. This research has obtained ethics from the Ethics Research Commission of the Faculty of Medicine, Diponegoro University Semarang number 55/EC/FK UNDIP/II/2019.

The variables studied included macronutrient intake (which consists of energy, protein), micronutrient intake (which consists of vitamins A, C, E zinc, copper), serum albumin and hs-CRP serum. The research tool used was a semi quantitative food frequency questionnaire. Before dialysis, the patients' height and weight were measured with a health scale, respectively. All measurements are carried out according to standard instructions when the subject is not wearing heavy clothes and no shoes. The weight and heights were recorded with accuracy of 100 g and 1 cm, respectively. Ideal body weight is used to calculate nutritional requirements according to recommendations for patients undergoing hemodialysis. This study was conducted by researchers assisted by an enumerator who is a nutritionist. Blood sampling is performed by nurses on duty in the hemodialysis unit before the research subjects conduct the hemodialysis process. Serum albumin concentration was measured by using the Brom Cresol Purple (BCP) method. Hs-CRP concentrations were measured with enzyme-linked immunosorbent assay (ELISA) kits (DRG International Inc, USA). Serum albumin is divided into two criteria: low (<3.5 $mg.dL^{-1}$) and normal (3.5 – 4.5 $mg.dL^{-1}$). Inflammatory status of serum hs-CRP is divided into three namely low risk (<1 $mg.L^{-1}$), moderate (1 – 3 $mg.L^{-1}$), high (>3 $mg.L^{-1}$) **(Myers et al., 2004)**. The level of sufficient energy, protein, vitamins A, C, zinc and copper was obtained by interviewing food intake using a semiquantitative food frequency questionnaire. The data obtained is processed by performing a recapitulation of the frequency of the use of types of food and converted into grams and then processed using the nutrisurvey program. The adequacy levels of energy, protein, vitamins A, C, E, zinc and cooper are obtained by calculating the percentage of total intake divided by dietary requirement individual (DRI). Adequacy levels are categorized into deficits (<100% DRI) and normal / more (≥100% DRI).

Statistic analysis

Data were analysed using the version 16.0 of Stastitical Package for the Social Sciences (SPSS). Data distribution and normality were seen using the Kolmogorov Smirnov test ($p \geq 0.05$). A. descriptive analysis of each variable was carried out in which the categorical variables were expressed as frequencies and percentages and the continuous variables as mean and standard deviation or median (min – max). Result were expressed as mean ±*SD* (for normally distributed data) otherwise it expressed as median(min – max).

RESULTS AND DISCUSSION

This research was carried out on 76 subjects consisting of 52.6% men and 47.4% women. Hemodialysis patients are mostly male because the average glomerular filtration rate (GFR) and serum creatinine values in men are higher than those in female **(Hecking et al., 2014)**. The mean age of hemodialysis patient was 49.12 years, with the highest percentage (34.2%) at age 45 – 54 years followed by age 55 – 64 years (26.3%). Hemodialysis patients after the age of 40 the kidney will progressively decrease the glomerular filtration rate **(Kalantar and Kopple, 2001)**.

Age more fourthy years there will be a decrease of ±10% the number of functional nephrons every ten years after patients aged 40 years due to nephrosclerosis and glomerulosclerosis. As a result of nephrosclerosis and glomerulosclerosis will cause elderly patients experiencing chronic kidney failure and must be treated hemodialysis. Hemodialysis patients have a high prevalence of comorbidities **(Sarnak et al., 2003)**.

In this study the most common comorbid is hypertension (53.9%) and followed by cardiovascular disease (31.6%).

Table 1 Characteristics of hemodialysis patients.

Characteristics (n = 76)	n (%)	Mean ±*SD*
Gender		
Male	40 (52.6)	
Female	36 (47.4)	
Age (years)		
25-34	6 (7.9)	
35-44	20 (26.3)	49.12 ±10.03*
45-54	26 (34.2)	
55-64	20 (26.3)	
65-74	4(5.3)	
Comorbid		
Hypertension	41 (53.9)	
Diabetes	6 (7.9)	
Cardiovascular disease	24 (31.6)	
Serebrovascular disease	3 (3.9)	
Gastrointestinal disease	1 (1.3)	
Tuberculosis	1 (1.3)	
The number of comorbid		
1 comorbid	32 (42.1)	
>1 comorbid	34 (44.7)	
>2 comorbid	9 (11.8)	
>3 comorbid	1 (1.3)	
Frequency hemodialysis		
One per week	9 (11.8)	
Two per week	67 (88.2)	
Time of hemodialysis (months)		
< 6 months	26 (34.2)	10 (1 – 105)**
6 – 12 months	16 (21.1)	
>12 months	34 (44.7)	

Note: * Mean ±Standar deviation; ** Median (minimum–maximum).

Table 2. Kolmogorof smirnov test

Variable	*p*
Adequacy level of Energy	0.048
Adequacy level of Protein	0.175*
Adequacy level of Vitamin A	0.000
Adequacy level of Vitamin C	0.000
Adequacy level of Vitamin E	0.000
Adequacy level of Zink	0.200*
Adequacy level of Copper	0.001

Note : **p*-value ≥0.05 = normally distributed data.

The kidney damage especially the cortex will stimulate the production of the hormone renin which will encourage increased blood pressure resulting in hypertension **(Sarnak et al., 2003)**.

This study shows that 44.7% of study subjects had two comorbidities. **Steven et al. (2010)** states that the more comorbid hemodialysis patients have will affect physical function. This study shows that patients who have more than one comorbid have difficulty breathing problems, edema, lack of balance, difficulty walking, restlessness, using a wheelchair.

Hemodialysis is a kidney replacement therapy which is done 2 – 3 times a week with a length of 4 – 5 hours, which aims to remove the remnants of protein metabolism and correct disruption of fluid and electrolyte balance **(Daugirdas et al., 2015)**. In this study 88.2% did hemodialysis twice a week and 12.8% once a week. Despite hemodialysis, not all uremic toxins can be excreted. This can lead to various kinds of comorbidities.

Hemodialysis on schedule will reduce the accumulation of toxins. In this study the frequency of hemodialysis subjects was determined by the doctor and in accordance with kidney damage experienced. So, it will reduce nausea and increase appetite. At the time of the study there were one subject who did not keep the hemodialysis schedule due to being busy, so the subjects felt very nauseous, edema and have difficulty breathing problems.

Subjects of this study had undergone hemodialysis with different lengths of hemodialysis. The highest percentage is 12 months (44.7%) with a time span of 1 – 105 months. **Kalantar and Kopple (2001)** says that the provision of dialysis therapy in terminal kidney failure patients aims to prolong life and control uremia symptoms and maintain quality of life. Hemodialysis patients have experienced chronic inflammation since not undergoing hemodialysis **(Panichi et al., 2008)**. Hemodialysis patients will experience an increase in free radical production and lipid peroxidation which in turn will increase inflammation **(Bianchi, 2009)**. Dietary macronutrients and micronutrients may be especially important in protecting against human diseases associated with free radical damage to cellular DNA, lipids, and proteins **(Barakat et al., 2017)**.

Kolmogorov Smirnov test shows that the adequacy level of protein and zinc is normally distributed because of *p*-values ≥0.05. While the adequacy level of energy, vitamins A, C, E and zinc shows data not normally distributed as indicated by $p < 0.05$. Table 2 compares macronutrients and micronutrient intake with recommendations for hemodialysis patients. As It was seen that the intake of macronutrients and micronutrients in most hemodialysis patients is deficit. This research shows that 88.2% of the subjects experienced a deficit of energy and 84.2% deficit of protein. Macronutrient and micronutrient intake is very important to prevent the occurrence of protein energy malnutrition (PEM) that often occurs in hemodialysis patients **(Kalantar and Kopple, 2001)**. PEM is the state of decreased body pools of protein with or without fat depletion or a state of diminished functional capacity, caused at least partly by inadequate nutrient intake relative to nutrient demand and/or which is improved by nutritional repletion.

Indicators of PEM in maintenance hemodialysis patients include decreased dietary protein and energy intake. In this study there were 57.9% of subjects showed nausea so that food intake decreased, and nutritional adequacy was not fulfilled. The subject said that nausea made it difficult to accept food. The patient limits the protein to prevent increased in creatinine urea serum. PEM are associated with chronic inflammation **(Kalantar and Kopple, 2001)**. PEM in hemodialysis patients not only shows deficit of energy and protein. But it also deficit micronutrients that the body needs **(Kalantar and Kopple, 2001)**. This research shows there is a deficit of micronutrients needed by hemodialysis patients such as vitamins A, C, E, zinc and copper. **Slee (2012)** state about hemodialysis patients will experience a phase called anorexia cachexia syndrome (ACS).

Table 2 Intake macronutrients and micronutrients in hemodialysis patient.

Variables	N (%)	Average ±*SD*	Recomendation[2]
Energy			
Deficite	67 (88.2)	60.95 (28.5 – 128.3)**	30 – 35 kcal/kg bw/day (≥60 year)
Normal/ Higher	9 (11.8)		35 kcal/kgbw/day (<60 year)
Protein			
Deficite	64 (84.2)	69.36 ±30.3564*	1.2 gr/kgbw/day
Normal/ Higher	12 (15.8)		
Vitamin A			
Deficite	65 (85.5)	41.95 (1.2 – 143.7)**	≥900 µg/day (male)
Normal/ Higher	11 (14.5)		≥700 µg/day(female)
Vitamin C			
Deficite	65 (85.5)	42.4 (4.8 – 150.3)**	90 mg/day (male)
Normal/ Higher	11 (14.5)		75 mg/day (female)
Vitamin E			
Deficite	76 (100)	18.35 (2 – 64)**	15 mg/day
Normal/ Higher	-	-	
Zink			
Deficite	75 (98.7)	34.637 ±17.305*	≥10 mg/day (male)
Normal/ Higher	1 (1.3)		≥8 mg/day (female)
Cooper			
Deficite	70 (92.1)	53.3 (6.7 – 166.7)**	1.5 mg/day
Normal/ Higher	6 (7.9)		

Note:* Mean ±standar deviation; ** Median (min-max).

ACS is a collection of symptoms characterized by decreased appetite (anorexia) and increased resting energy expenditure (REE) accompanied by increased protein breakdown. Hemodialysis patients will also experience catabolism so that they need more calories than healthy people. Patients undergoing hemodialysis will usually experience an increase in uremic toxins that can reduce appetite and even loss of appetite due to nausea. Decreased appetite occurs because of an increase in proinflammatory cytokines and their effects peripherally on the skeletal muscle pathway that regulates turnover protein and centrally on the neurons in the hypothalamus that regulate appetite. ACS causes a negative energy balance in hemodialysis patients due to decreased food intake. Increased proinflammatory cytokines will increase the development of ACS, so that the activity of leptin and ghrellin may be disrupted. Leptin is anoxygenic peptide which can reduce appetite, while ghrellin is orexigenic peptide which increases appetite. Both are pathways regulating appetite in the brain/hypothalamus. In the normal pathway, neurons in the hypothalamus center produce melanocortins (proopiomelanocortin (POMC)/ cocaine and amphetamine-regulated transcript (CART)) activated by leptin, which have anorexic and catabolic effects. Whereas neuronpeptide Y (NPY) and angouti-related protein (AgRP) express neurons activated by ghrellin which have orexigenic effects. In hemodialysis patients, there is a disturbance in the circulation of leptin and ghrellin where the POMC/CART activity remains while the NPY/AgRP decreases. This has an impact on increasing resting energy expenditure and decreasing appetite. Besides that, the increase in creatinine urea also has the effect of reducing appetite. Decreased appetite is what will cause decreased food intake. **Barakat et al. (2017); Therrien, Byham and Beto (2015)** shows the same thing that hemodialysis patients are at risk of nutrient deficiency due to nutrient intake under the recommendation of KDOQI. Table 2 shows that the intake of vitamin A most (85.5%) of respondents showed a deficit. **Sahni et al., (2012)** shows that vitamin A intake in hemodialysis patients lower than healthy control groups . This study also showed that the median level of vitamin A adequacy was 41.95% with a minimum value of 1.2% and a maximum of 143.7 %. A range that is too far away indicates that there are study subjects who have very low and very high levels of sufficiency. According to KDOQI, the need for vitamin A in CKD patients with hemodialysis is 900 µg in males and 700 µg in females. Food sources that contain lots of vitamin A include all kinds of milk, butter, eggs, fish oil, vegetables with green and yellow leaves, fruits and liver **(Kalantar and Fouque, 2017)**. Subjects with deficit intake of vitamin A said they were afraid to eat vegetables and fruit because they thought it was high in potassium so they worried that blood potassium would increase. The study subjects only ate 1 – 2 tablespoons of vegetables each meal. Vitamin A is mostly contained in green and orange vegetables and fruits. Green vegetables and orange fruits are usually high in potassium which is automatically reduced by hemodialysis patients **(Bossola et al., 2014)**. The study subjects also did not consume liver containing high vitamin A. **Vas et al. (2014)** stated that the intake of vitamin A was only 373.98 (257.57 – 649.50) mcg from 700 to 900 mcg recommended. This shows a deficit intake vitamin A in hemodialysis patients. **Vas et al. (2014)** showed that 81.4% of subjects had inadequate vitamin A intake. This research also shows that most 85.5% had a sufficient level of vitamin C deficit. **Barakat et al. (2017)** which states that vitamin C intake in hemodialysis patients usually tends to be low because of the

limitation of potassium in diets recommended for hemodialysis patients. Hemodialysis patients restrict vegetables and fruit for fear of increasing potassium. **Kalantar and Kopple (2001)** states that hemodialysis patients have significantly lower intakes of vitamin C, fiber, potassium and carotenoids than healthy people. The Dietary Reference Intake Panel of the Institute of Medicine recommends a recommended vitamin C diet of 90 mg per day for men and 75 mg per day for women **(Kalantar and Fouque, 2017)**. The inadequacy of vitamin C in this study was due to the fact that most of the subjects thought that vegetables and fruits contained lots of potassium and should be avoided. Adequacy levels of vitamin E in study subjects showed that 100% had a deficit. **Kooshki, Samadipour and Akbarzadeh (2015)** points out the same thing that vitamin E intake is less than the recommendation. Vitamin E is found in many grains. **Lou et al. (2007)** said that hemodialysis patients also limit potassium intake by avoiding vegetables, fruit and seeds. The subject of this study also conducted restrictions on grains. This will cause the intake of vitamin E is also not enough to meet nutritional needs. Based on interviews with research subjects, they limited their intake of legumes such as tofu, tempeh, peas, green beans, cashews for fear of creatinine urea increasing. The need for Vitamin E in both male and female CKD patients is 15 mg/day **(Kalantar and Fouque, 2017)**. Sources of vitamin E are vegetable oils, unprocessed grains, nuts, fruits, vegetables and meat **(Kopple, 2001)**. Most of the research subjects (98.7%) had a zinc adequacy level classified as deficit. **Sahni et al., (2012)** states that the average zinc intake was found to be less than the recommendation for hemodialysis patients. **Sahni et al. (2012)** states that zinc is a mineral that has strong potential as an antioxidant, usually obtained from foods rich in protein. Low protein intake is significantly correlated with low zinc intake. The low zinc intake in the study subjects was due to the protein intake of the subjects also showing a deficit with the average protein intake of 69.361 ±30.356%. Zinc is found in many foods such as meat, fish, cheese, chicken, eggs and milk products, beans, almonds and parsley. This research shows that copper adequacy level was mostly (92.1%) indicating a deficit. **Szpanowska, Chowaniec and Kolarzyk (2008)** stated that copper intake was very low, less than 40% of the recommended. This research subjects carried out a restriction in their diet, especially those containing protein for fear that their creatinine urea had increased. Graph 1 shows that most (63.2%) had a relatively low albumin because it was less than 3.5 g.dL^{-1}. Low serum albumin is a sign of protein energy malnutrition. Protein energy malnutrition when energy and protein requirements are not met as needed. Deficit of food intake in hemodialysis patients in the long run will cause protein energy malnutrition. Low protein intake will cause hypoalbuminemia **(Kaysen et al., 2002)**. This study shows that the intake of macronutrients and micronutrients in most subjects is deficit. The origin of PEM appears to precede dialysis treatment, and it is engendered progressively as glomerular filtration rate (GFR) decreases to less than 55 mg.min^{-1} **(Kalantar and Kopple, 2001)**. Hypoalbuminemia have been shown to develop along with the progression of CKD stages. Serum albumin will decrease with decreasing glomerular filtration rate (GFR). Hemodialysis patients show a decrease in glomerular filtration rate up to <15 mL/min/1.72m^2. Serum albumin is a negative acute phase reactant as a marker of inflammation **(Kaysen et al., 2002)**. Graph 2 shows that most (61.8%) subjects had serum hs-CRP in the high-risk category because it was more than 3 mg.L^{-1}. Based on recommendations from the Centers for Disease Control and Prevention (CDC) cut offs point hs-CRP levels >3 mg.L^{-1} indicate a group at high risk of cardiovascular disease **(Myers et al., 2004)**.

This study also found that the median hs-CRP level was 4.85 mg.L^{-1} with a minimum level of 0.29 mg.L^{-1} and a maximum of 20.30 mg.L^{-1}. Increased levels of CRP as a consequence of the chronic inflammatory process that is found in conditions such as kidney disorders. The inflammatory process in hemodialysis is caused by the involvement of various factors such as accumulation of uremia toxin, malnutrition, oxidative stress, volume overload, carbonyl stress, decreased levels of antioxidant, low production of anti-inflammatory cytokines, comorbid, dialysis treatment **(Kalantar et al., 2003)**. Hemodialysis patients often experience a condition of uremia. Uremia in hemodialysis patients will increase proinflammatory cytokine levels associated with increased mortality. In addition, the dialysis process also contributes to the increase in cytokine secretion at the end of hemodialysis. In this case,

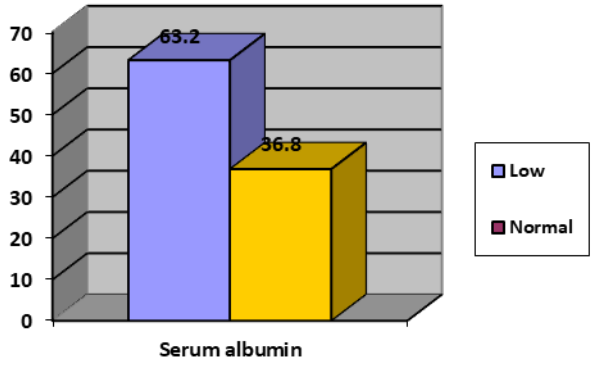

Figure 1 Frequency serum albumin level.

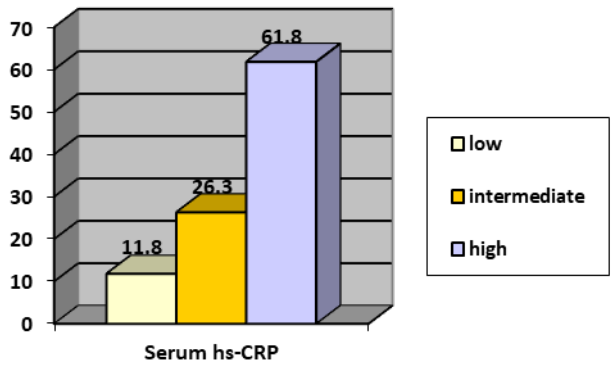

Figure 2 Frequency serum hs-CRP level.

the dialysis membrane can stimulate increased cytokine release. Increased proinflammatory cytokines can reduce appetite so that food intake decreases **(Kalantar et al., 2003)**. Decreased food intake such as energy and protein are likely to cause PEM which will increase inflammation by increasing hs-CRP levels. In this study the majority of hemodialysis patients showed micronutrient intake that was classified as deficit. vitamins A, C, E, zinc, copper are micronutrients that act as antioxidants. Malnourished dialysis patients may be deficient of antioxidant such as vitamin C or carotenoids which may lead to increased oxidative stress leading to inflammation **(Kalantar and Kopple, 2001)**. Antioxidants are very important to protect the body from free radical attack which can increase oxidative stress. Increased lipid peroxidation (LP) and reduced enzymatic antioxidant defence have been observed in predialysis patients. Loss or deficiency of antioxidant activity could also contribute to enhanced oxidative stress in uremia **(Kalantar et al., 2003; Tarko et al., 2013)**. Oxidative stress defines an imbalance between formation of reactive oxygen species (ROS) and anti-oxidative defence mechanisms. It occurs when there is excessive free radical production and/or low antioxidant defence and results in chemical alterations of bio-molecules, causing structural and functional modifications **(Sahni et al., 2012)**. Oxidative stress and inflammation status are well-known interrelated factors in hemodialysis patient with common underlying mechanisms including endothelial dysfunction and common complications, such as cardiovascular disease and death **(Bianchi, 2009)**. Serum hs-CRP is an inflammatory marker that is associated with an increased risk of death due to cardiovascular disorders **(Stevinkel et al., 2005)**.

CONCLUSION

This study shows that intake of macronutrients and micronutrients in hemodialysis patients is deficit. This research concludes that 88.2% deficit of energy and 84.2% deficit of protein. In addition, micronutrient intake also shows 85.5% deficit of vitamin A, 85.5% deficit of vitamin C, 100% deficit of vitamin E. Zinc intake at 98.7% showed a deficit and copper intake at 92.1% also showed a deficit. There were 63.2% of subjects had low albumin serum and 61.8% of subjects had high hs-CRP serum.

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THE INFLUENCE OF THE MOISTURE CONTENT OF RAW MATERIALS ON THE STRUCTURING OF THE EXTRUDATES

Davit Tsagareishvili, Otari Sesikashvili, Gia Dadunashvili, Nugzari Sakhanberidze, Shalva Tsagareishvili

ABSTRACT

The article presents the results of studies on the model systems of extrudates conducted with a view to determining the function of moisture during the process of forming the structure of starch pastes. There was studied the influence of the moisture content of raw materials on a starch gelatinization point. Studies showed that 15% moisture content in raw materials is sufficient for its constituent phase – starch gelatinization, as well as for the transition of the whole mass to a fluid-viscous state. Further increase in the moisture content is accompanied by a decrease in a gelatinization point. In order to study the influence of moisture on the formation of a porous structure of extrudates, we studied the relationship between the different-type starch pastes and the degree of its transparency and its embrittlement temperature. It has been found that during the process of thermal and mechanical impacts, there occurs the process of the formation of a structure of starch pastes, in particular, samples with the different moisture contents can have an amorphous or crystalline structure. There has been established the relationship between the moisture content of raw materials on the modulus of elasticity of starch pastes based on them. The modulus of elasticity of samples was determined one hour (cooling time to room temperature) and one week after obtaining the starch paste. The above studies showed that minimal physico-chemical and mechanical transformations occur in starch pastes, which are in an amorphous state, that is, in the conditions of a low moisture content. We have established that the moisture content of raw materials, on the one hand, ensures the transition of a high-dispersive phase to a fluid state, or implementing the ex process of extrusion, and on the other hand, influences on the formation of a porous structure in the extrudates.

Keywords: extrusion; moisture; gelatinization; structure; starch; paste

INTRODUCTION

The process of thermoplastic extrusion in the food industry is a new innovative technology, which is distinguished by high productivity and efficiency. It provides complex processing of raw materials, i.e. the processes of mixing, hydrothermal processing and structure formation occur in one machine **(Berk, 2012)**. It allows us for producing produce a wide selection of foods with high biological and nutritional values and balanced amino acid composition. The range of products obtained according to this progressive technology includes many commodities **(Berk, 2012; Saravacos and Kostaropoulos, 2016; Martirosyan, 2013; Kraus, 2004)**, which in turn are classified into three groups:

- Porous breakfast cereals, dry concentrates, dried biscuits and other similar products;
- Anisotropic analogues of meat and fish products;
- Isotropic instant noodles.

In order to determine the optimum conditions for hydro-thermo-mechanical processing of the mass undergoing treatment, as well as to produce high quality products, it is necessary to study the natures of the change in physico-chemical properties of the main components of raw materials during the extrusion process.

The base raw materials that are subject to extrusive processing for the production of porous products are the starch-containing raw materials, such as corn flour, wheat flour, rice flour, oat flour, and so on.

The main components of raw materials listed are starch and protein, which have a particular impact on their processing conditions and the quality of extrudates. **(Karpov, 2000; Alekseeva, 2007)**.

It is known that starch, whose content in raw material is 65 – 80%, is not a chemical individuum, but rather two main fractions, the biopolymers of amylose and amylopectin. The physicochemical properties of the starch are associated with primarily with its polysaccharide composition, the sizes of the molecules, as well as firmness and the compact arrangement of its grains.

As we have mentioned, protein is the second main component of raw materials. During the extrusive processing of starch-containing raw materials, proteins that make up about 15 – 20% of their total mass undergo denaturation because of a

hydro-thermo-mechanical impact resulting in physico-chemical changes in them, so starch gelatinization and denaturation of proteins occur in extruding raw materials during the process of thermoplastic extrusion **(Litviak, 2013; Karpov, 2000; Van Lengerich, Meuser and Pfaller, 1989)**.

The third main component of extruding raw materials is water - moisture. According to literature sources **(Litviak, 2013; Karpov, 2000)**, the moisture content of extruding raw materials is 15 – 40%. This amount of moisture affects the gelatinization point of the mass undergoing treatment, viscosity of the fluid mass, the functional properties of extrudates, and their storage conditions. In other words, moisture affects both the technological parameters of thermoplastic extrusion and the consumer properties of product, however, it is important to distinguish moisture as a functional additive for raw materials and the moisture content of product during storage of extrudates.

The analysis of literary sources has shown that there are very few scientific studies in this area. Based on the above, the goal of research is, using an example of the model and highly concentrated systems (starch-water)of extrudates, to study the impact of the moisture content of raw materials on the formation of their structure.

To achieve this goal, the following objectives should be resolved:

- Studying the impact of moisture on the starch gelatinization point and on the properties of starch paste (gel);
- Studying the hydro-thermo-mechanical impact on the structure formation of starch paste (gel).

SCIENTIFIC HYPOTHESIS

The process of obtaining extrudates by thermoplastic extrusion method can be seen as a thermotropic process of starch paste formation of biopolymers in the flow. It may be presumed that the study of a thermotropic process of starch paste formation in the highly concentrated systems containing 8 – 40% of moisture, and the study of the properties of obtained starch paste will provide information on the function of water in the process of thermoplastic extrusion.

MATERIAL AND METHODOLOGY

For research, to produce starch paste, we used corn starch **GOST 7697-82**, potato starch **GOST 7699-78** and drinking water **GOST 2874-82**. To conduct research on starch paste samples, we developed and created the test bench (Figure 1). The bench consists of a working chamber body 1, equipped with a spiral heating device 2, press 3, a temperature control unit 9 and the laboratory autotransformer 10.

The working chamber is a cylinder with an inner diameter of 26 mm. At the lower part of a cylinder, there is placed the lower fixed punch 7, on which the portion of test sample is sprinkled, and from above, the upper movable punch 6 is mounted. When current of a certain quantity passes through heating spiral, the working chamber is heated to the required temperature. We maintained the required temperature by means of temperature control unit, while the current strength required for the heating element, we generated by the laboratory autotransformer (LATR 1). The temperature inside the cell was monitored by a Ni-Cr thermistor with a multimeter DT9208A, and red the time with an electronic timer.

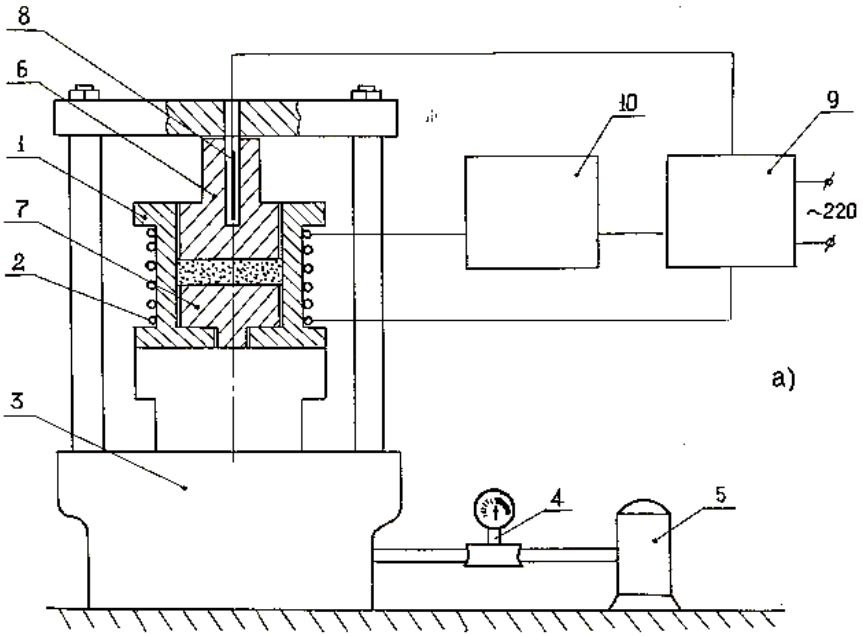

Figure 1 The test bench scheme: 1 – a working chamber body; 2 – a spiral heating device; 3 – press hydrocylinder; 4 – pressure-measuring instrument; 5 – pump; 6 – upper punch; 7 – lower punch; 8 – thermal converter 9 – temperature control unit; 10 – autotransformer.

We counted the assembled chamber with the portion placed inside it, in a press, which by acting on the upper punch, creates and retains some pressure in a working chamber. Samples are obtained by the following method: starch samples with different moisture content were placed in a working chamber under pressure of 30 $kg.cm^{-2}$, then we heated it to the temperature of 120 °C and retained for 5 minutes. After that, the system was cooled to room temperature and the obtained starch paste was taken from a working cylinder. The moisture content of starch paste was determined by drying method at the temperature of 105 °C until obtaining the constant mass. The mass was determined by means of analytical electronic scales.

We used the same test bench to determine the starch gelatinization point. We placed 10 g of starch sample with different moisture content in a working chamber between the punches under pressure of 30 $kg \cdot cm^{-2}$ and heated it in a chamber until the pressure in a chamber is dropped. We recorded the temperature corresponding to this moment, which corresponds to the point of transition of a friable mass into a fluid mass. The transparency of starch paste of various origins was determined by means of a spectrometer ,,Specord UV-VIS" (Karl-Zeiss, Jena, Germany). In a comparable cuvette filled with silicone oil, we placed 20 mm thick sample of test starch paste. The transparency percentage of test samples was determined according to wavelength of a beam spectrometer. In order to study the glass transition temperature or fragility of starch paste, we investigated the relationship between the relative elongation of samples and a temperature, under conditions of a scanning velocity of 5 $°C.min^{-1}$. As the glass transition temperature, or the embrittlement temperature, we have taken the intersection point of the linear values of the relative elongation and the temperature of starch paste. The tests were carried out on a thermomechanical analyser "TMA - 973" (USA).

We studied modulus of elasticity of different starch paste samples on a universal test machine "Instron TM-SM-1" with a ball indenter by method of penetration. Modulus of elasticity of starch pastes was calculated according to the following formula:

$$G = tg\alpha \cdot (K \cdot \sqrt{R})^{-1} \quad (1)$$

where α – angle of descent of a function $P = f(h^{1.5})$;

P – the axial load, N;

h – insertion depth in the indenter, mm;

K – non-dimensional coefficient, K = 10;

R – indenter radius, mm, R = 2.5 mm.

We placed the starch paste samples with various moisture contents on the machine table. We chose the mode of operation of machine by the following parameters: self-recorder chart's velocity $v_l = 1000\ mm.min^{-1}$ and the velocity of traverse $v_{tr} = 5\ mm.min^{-1}$, to which the indenter is fastened. The self-recorder describes the function P = f(h). The insertion depth of the indenter in a sample was calculated from a ratio $v_{tr}/v_l = h_{tr}/h = 1/200$.

We moved the P = f(h) function into the relationship $P = f(h^{1.5})$, and then we determined the tangent of an angle of descent of this function. By inserting the obtained value into the formula (1), we determined elasticity modulus of samples. The starch deterioration temperature was determined using a derivatograph Q-1500D (Hungary).

STATISTIC ANALYSIS

To analyse the test parameters (the moisture content of starch paste, gelatinization point, starch paste transparency, starch paste embrittlement temperature, starch paste modulus of elasticity) of extrusion products, there is conducted a statistical analysis of the obtained data, and the reliability of the obtained data was evaluated by method of mathematical statistics T-test, using the Windows IBM SPSS Statistics software program (version 20.0). To describe the ordered sample, we used statistical functions of the average arithmetic value and the average standard error. Graphical interpretation of the results was made by using Microsoft Excel. In Figure 2, Figure 3A, Figure 3 B, Figure 4, Figure 5A and Figure 5B there are presented the data of typical tests, and each value is an average of at least ten determinations. We selected the value of reliability $p = 0.05$.

RESULTS AND DISCUSSION

It is known that the maximum temperature required for the process of thermoplastic extrusion and the moisture content of processing raw materials have a particular impact on the quality of extrudates, as well as on the physicochemical and structural characteristics of starch paste.

The effect of hydration changes of snacks depended both on the hydration level and the extrusion process conditions. As a result of storage the shrinkage was more pronounced in case of extrudates obtained from raw material containing 16% of water regardless of the extrusion temperature. The hardness of extrudates depended both on the feed moisture and hydration. Samples obtained from the raw material containing 20% moisture were harder than those prepared at the feed moisture of 16%. Based on the results of molecular dynamics study by low field NMR technique, a model of hydration was developed and critical hydration value of extruded snacks was calculated. Higher values of the critical hydration were obtained for snacks extruded at temperature 135 °C than for those obtained at 175 °C **(Makowska et al, 2017)**.

In the extrusion of sorghum flour increasing feed moisture increased the peak gelatinization temperature (T_p), the degree of gelatinization (%) and starch crystallinity (%) while it decreased the gelatinization temperature ranges ($T_c - T_o$), starch gelatinization enthalpy (ΔH_G) and amylose-lipid complex (%) formation. With increasing die temperature, the degree of gelatinization and amylose-lipid complex formation increased and the starch T_p, T_c-T_o, ΔH_G and crystallinity decreased **(Morteza, Koocheki and Milani, 2017)**.

When studying the effect of leavening agents on lysine loss, determined by the furosine content in corn extrudates, it was found that furosine levels decreased by about 20% when the moisture content in the feed stuff increased from 22% to 26% **(Masatcioglu, Ng and Koksel, 2014)**.

Response surface methodology for evaluation of functional and physical properties of corn half products was developed. Response variables are: water absorption index (wai), water solubility index (wsi), degree of gelatinization (dg), water activity (a_w), specific volume and color properties of extrudates. increased screw speed decreased wai and a_w values and increased wsi values significantly, it has been established that specific volume of half products

was low for all extrusion combinations and negative correlated with screw speed, feed moisture and xg level. lightness and yellowness of half products were increased with increases in screw speed, feed moisture and xg level **(Gümüşay, Şeker and Sadıkoğlu, 2019)**.

There was studied the effect of flour moisture content during extrusion on in vitro fermentation properties of whole grain oats. Extrudates were processed at three moisture levels (15%, 18%, and 21%) at fixed screw speed (300 rpm) and temperature (130 °C). After 24 h of fermentation, samples processed at 15% moisture supported lower *Bifidobacterium* counts than those produced at other conditions, but had among the highest *Lactobacillus* counts. **(Brahma, Weier and Rose, 2017)**.

So given the fact that behaviour of extruding raw materials in the process of hydro-thermo-mechanical treatment is due to the continuous phase - starch properties, that is why we studied the relationship between the gelatinization point of starch of different origins and their moisture contents (Figure 2). The results obtained are consistent with the findings of studies conducted by above mentioned authors.

It is known **(Litviak, 2013; Blanshard, 1987)** that the process of starch gelatinization is accompanied by two events: the first one - diffusion between water and starch molecules; the second one - solubility of starch polysaccharides. In other words, the process of starch gelatinization is the process of the transition of a dispersive system to a viscous-liquid state.

As Figure 2 shows, if the moisture content in starch is up to 15%, the gelatinization point is lower than its decomposition point. Increasing the amount of moisture in the system causes a further drop of the gelatinization point. At 60% moisture content, there is a little link between the gelatinization point and the moisture content of starch. The results obtained are in compliance with the author's research **(Blanshard, 1987; Van Lengerich, Meuser and Pfaller, 1989)**.

During the process of heat treatment of starch of various origins, there occurs its structural modification, as our earlier studies show, during the process of heat treatment, the starch pastes may have an amorphous or crystalline structure.

There was studied the low moisture extrusion of rice starch fortified with pea protein and pea fibre. The addition of protein and fibre resulted in increased specific mechanical energy inputs, bulk densities as well as air cell densities. Microstructural analyses revealed that at the highest protein contents (42%), protein and starch were distributed in thin layers within the extrudate which is proposed to cause the decreased sectional expansion indices **(Beckab et al, 2018)**.

Figure 3A and Figure 3B illustrate the relationship between the transparency of the potato and corn starch pastes and their moisture contents. Figure shows that this relationship is of a "S"-like nature, however the change in the transparency of the aging process is observed only if the moisture content exceeds 15%.

It is noteworthy that the turbidity (the reciprocal of transparency) **(Litviak, 2013)** of both corn and potato starch pastes at a low moisture content, is one degree lower than the turbidity of samples with 25% or higher moisture content. Considering “S”- like nature of the relationship between the transparency and moisture content of starch paste, it can be assumed that the transition from a highly elastic state to a glass amorphous state occurs when the water content decreases. It is therefore natural that in a high-elastic state, there occurs the process of starch paste aging, since the movement of macromolecular chains leads to an increase in the turbidity of starch paste and in the number of crystals.

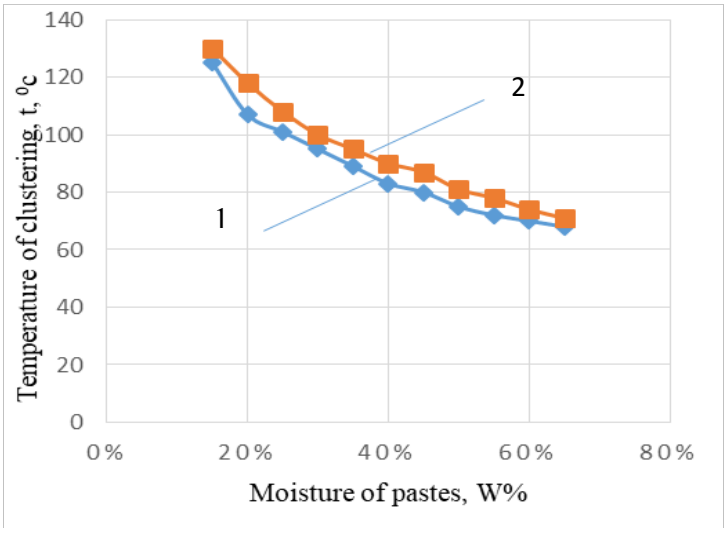

Figure 2 The relationship between the gelatinization point of the different-type starch pastes and the moisture contenmt. 1 – potato starch; 2 – corn starch

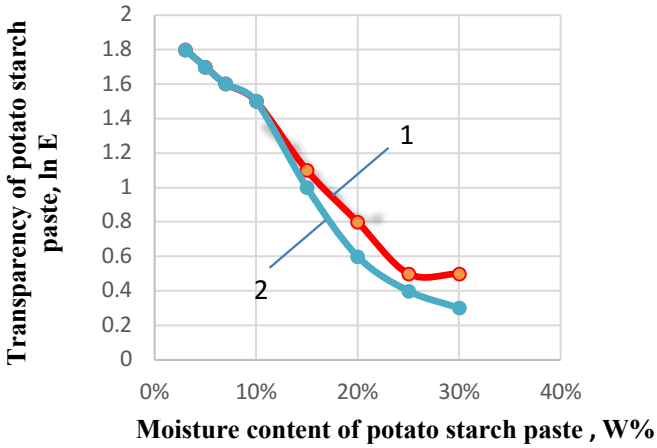

Figure 3A The relationship of the transparency of the potato starch paste on their moisture contents. Note: 1 – potato starch paste one hour later; 2 – potato starch paste 14 days later.

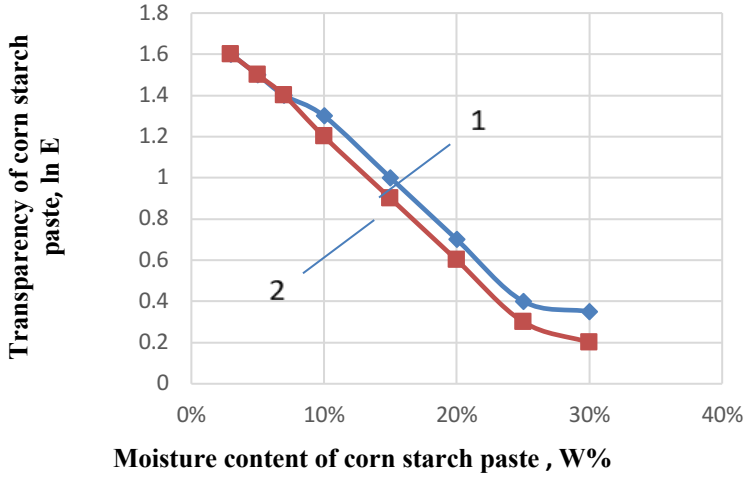

Figure 3B The relationship of the transparency of the corn starch paste on their moisture contents. Note: 1 – corn starch paste one hour later; 2 – corn starch paste 14 days later.

Physical state and mechanical properties of extruded grain-based products were studied as a function of the sucrose content and relative humidity (RH) to evaluate how the presence of sucrose affects the glass transition temperature. The Young's modulus showed that water acts as an anti-plasticizer at low a_w, and a plasticizing effect at high a_w. The stability map may explain the brittle-drictile transition that occurred when it was lower **(Masavang, Roudaut and Champion, 2019)**.

There was studied the effect of sucrose at concentrations ranging from 0 to 12.5 % wt. on the textural properties of extrudates of corn and wheat flours.

It has been established that (1) the internal structure of the extrudates evaluated by image analysis showed a reduction of the cell size with the sucrose whatever the flour; (2) the crispness of products evaluated by puncturing increased with the sucrose content until an optimum whatever the flour; (3) the addition of sucrose in corn and wheat mix reduced overall product expansion; and (4) increasing the amount of sucrose in the mix induced greater molecular degradation of corn starch than of wheat starch **(Mezreb, et al, 2006)**.

Figure 4 illustrates the relationship between the embrittlement temperature of the potato and corn starch pastes and their moisture contents. Figure shows that in samples with a moisture content below 10%, the embrittlement temperature is higher than 20 °C, the increase in a moisture content of starch pastes results in a lower embrittlement temperature, for example in the case of 25 – 30% moisture content, the embrittlement temperature is -7 to (-11 °C), This means that moisture plays the role of a plasticizer in such a system, while the increase in its amount leads to increasing fluidity micromolecular chains. The difference in the embrittlement temperatures for the potato and corn starch pastes are due not only to their different natures, but also to production conditions.

The results obtained are consistent with the findings of studies conducted by above mentioned authors.

It has been established that grain refinement using a reduced-size aperture grid caused a narrowing of the particle size distribution. The smaller size of particles of the crushed extrudate resulted in obtaining the tablets with higher tensile strength. This, along with a narrowing of the particle size distribution, led to obtaining the more homogeneous tablets, therefore, to less weak places for crack propagation and to obtaining a more durable tablet **(Hallam, Gabbott, 2019)**.

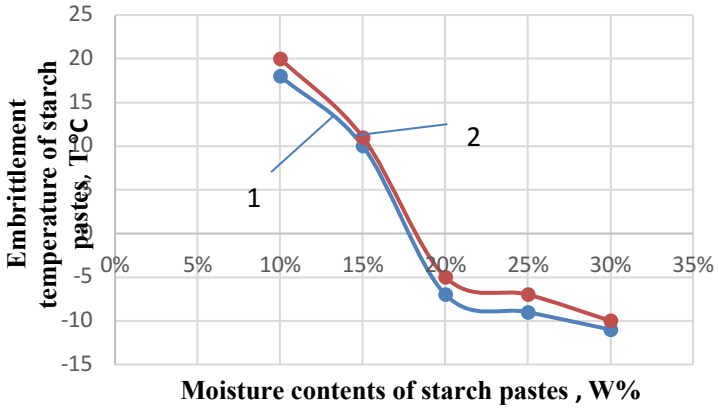

Figure 4 The relationship between the embrittlement temperature of the different-type starch pastes and their moisture contents. Note: 1 – potato starch paste; 2 – corn starch paste.

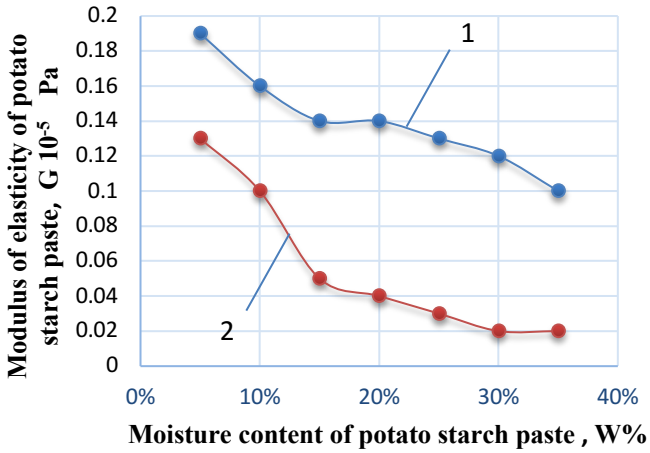

Figure 5A The relationship between the modulus of elasticity of the potato starch pastes on their moisture contents. Note: 1 – potato starch paste 1 hour later; 2 – potato starch paste 14 days later.

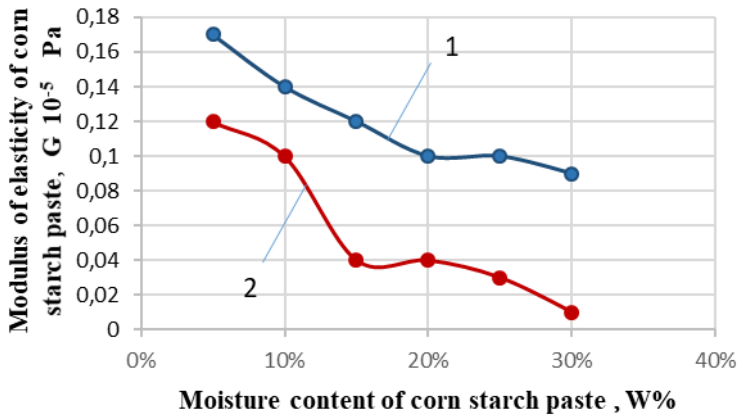

Figure 5B The relationship between the modulus of elasticity of the corn starch pastes on their moisture contents. Note: 1 – corn starch paste 1 hour later; 2 – corn starch paste 14 days later.

There was studied the effect of feed moisture (12 – 16% wet basis), barrel temperature (90 – 110 °C) and screw speed (100 – 200 rpm) on the extrudates using a blend of oat, green pea and fenugreek seed flour and leaf powder properties like lateral expansion (LE), bulk density (BD), water absorption index (WAI), water solubility index (WSI) and hardness was investigated. Desirable extruded products were obtained at moisture of 12%, 110 °C temperature and 200 rpm screw speed **(Wani and Kumar, 2016)**.

Figure 5A and Figure 5 B illustrate the relationship between the modulus of elasticity of the potato and corn starch pastes and their moisture contents. This relationship is also a "S"-like nature, and the reduction in a moisture content increases the modulus of elasticity by one degree, while in the process of aging of the test starch pastes, the modulus of elasticity increases, regardless of the change in their moisture contents.

An adequate nature of the relationships between the transparency degree and the modulus elasticity of the test starch pastes, as well as the dependence on embrittlement temperature, indicate that in the starch pastes with modulus of elasticity is within the limits of 10^4 Pa, are in an amorphous state, while the starch pastes having the one degree lower modulus of elasticity, are in a high-elastic state **(Litviak, 2013; Karpov, 2000; Abramov, 2011)**.

CONCLUSION

Studies carried out in the highly concentrated systems "starch-moisture", which focused on the process of starch gelatinization, as well as the study of the properties and structure of starch pastes based on them, allow us for drawing conclusion on the function of moisture in the process of thermoplastic extrusion.

In the highly concentrated systems and starch pastes based on them, which represent the model of extrudates, moisture plays the role of a plasticizer, the increased number of which in a system leads to an increase in the movement and fluidity of the macromolecular segments and structural elements of starch pastes. This process is accompanied by the transition of starch pastes from an amorphous to a highly elastic state. It is noteworthy that in the aging process of starch pastes in a highly elastic state, their turbidity and elasticity limit are increasing and the process of crystallization is intensified, also, the increase in the moisture contents of porous extrudates affects the functional properties of product, in particular, unlike the highly-elastic state, the intensification of the crystallization process results in a decline in the strength of extrudates and disruption of a porous structure.

Thus, studies on the model systems allow us for drawing the following conclusions:

- Moisture, during the extrusion process, on the one hand, determines the temperature of the process of raw materials gelatinization, and on the other hand, influences on the formation of a porous structure in the extrudates.
- In the production of porous extrudates, the moisture content in raw material in the case of corn flour should be 17 ±1%, while the process temperature in the extruder should be 140 ±5 °C. In the case of potato flour, the moisture content should be 15 ±1% and the process temperature in the extruder should be 130 ±5 °C.
- Minimal physico-chemical transformations are observed in starch pastes, which are in an amorphous state, that is, when the the moisture content in starch paste is minimal, we can conclude that porous extrudates should be stored in the conditions of a low moisture content (4 – 7%).

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ANALYSIS OF DEVELOPMENT OF RAW COW MILK PRICES IN THE CONDITIONS OF THE SLOVAK REPUBLIC

Ivana Váryová, Zuzana Poláková, Iveta Košovská, Alexandra Ferenczi Vaňová, Renáta Krajčírová

ABSTRACT

The paper is focused on the evaluation of the price development of raw cow milk in the Slovak Republic. The aim of the paper is to analyse the development of average prices of the raw Q class cow´s milk in 2006 – 2018 and to forecast the trend of these prices by June 2019. Monthly data from the Market Report of Milk and Dairy Products issued by the Agricultural Information Department – ATIS, as part of the Agricultural Paying Agency, were the base of our information resource. These data were analyzed by using the statistical software called SAS. Box-Jenkins methodology was used to model the future trend of average purchase prices of the raw Q class cow´s milk, designed for modeling stationary and non-stationary time series and time series with seasonal components. During the period of 2006 – 2018 the Slovak dairy market showed significant changes in the prices of raw Q class cow´s milk. Three crisis periods of the dairy sector have been identified, during which the milk price has fallen below 0.30 € per kilogram. Long-term low prices of raw cow milk led to the liquidation of primary milk producers. In the next forecast period, by February 2019 a moderate increase in the average purchase price of raw Q class cow´s milk is expected, followed by a decrease by June 2019.

Keywords: analyse; crisis; milk; price; trend

INTRODUCTION

Eating food is one of the most important needs of every person **(Nagyová et al., 2019)**. Milk and milk products are the essentials of human nutrition. The Central Europe is a region with long tradition of production and consumption of milk and milk products. There is quite strong competition between production capacities in the Central Europe **(Špička, 2015)**. Cow´s breeding can be considered as strategic, especially in relation to other categories of cattle and its connectivity to arable land and permanent grassland. Cattle´s breeding represents a crucial condition to maintain a balance between the plant production and breeding processes of agricultural business activities **(Siničáková, 2012)**. Raw cow´s milk represents one of the most important commodities in the agricultural market. Raw cow´s milk is an essential source of the nutrition of calves and a raw material for the production of liquid milk and dairy products, which have a unique place in the human nutrition and dietetics **(Šimo, Mura and Buleca, 2016)**. The dairy industry is one of the most important industrial sectors for healthy development of Europe. There is not a single country being part of the European Union that does not produce milk **(Pilvere, Nipers and Krievina, 2016)**. European vertical of milk production and processing is influenced by the Common Agricultural Policy. Milk producers (farmers) are supported through direct and indirect operational subsidies. Farmers are not able to generate profits without current subsidies **(Doubek et al., 2012; Foltínová and Špička, 2014)**. A relatively wide range of policy forms and tools have been used within Common Agricultural Policy, both with direct and indirect effects on the volume of production and production-cost relations of this production. Nonetheless, within the proclaimed single framework of the Common Agricultural Policy, size and level of production, and consequently also involvement in various stages of commodity chain, production and processing of milk vary considerably between individual European Union member states. Among the largest producers of milk and dairy products are Germany, France, Great Britain, and from the group of new member states Poland **(Zdráhal and Bečvářová, 2018)**. Sectors of milk production and milk processing play an important role in the development of the agrarian sector and global agribusiness in general. Particularly within the European Union, this sector is in addition to basic production features highly valued for its role in the practical implementation of the philosophy of multifunctional agriculture, i.e. in terms of its contribution to environmental and social field **(Bečvářová, 2011; Bečvářová and Zdráhal, 2013)**. The milk quota system, one of the most important instruments of the Common Agricultural Policy, was introduced in 1984 to the European Union dairy market,

in order to control the structural surpluses of milk. These surpluses arose because of imbalances between supply and demand for milk **(Costa-Font and Revoredo-Giha, 2018)**. The cow milk production volumes were not regulated in the European Union before the milk quota system has been introduced. Intervention purchases and guaranteed prices for milk producers were provided by previous system regardless of the milk production volume **(Vőneki, Mándi-Nagy and Stark, 2015)**. According to **Dreve, Călin and Bazgă (2016)**, the reason for milk quota system was to limit public spending on the sector, to control milk production, and to stabilize milk prices and the agricultural income of milk producers. The milk quota system has been removed since the 1st of April 2015. The development of more competitive and market-oriented dairy sector is expected by the European Commission after the milk quotas were removed **(Salou et al., 2017)**. After abolition of milk quota system, the European milk producing countries started to be exposed to the milk prices of the world market **(Buleca, Kováč and Šubová, 2019)**. The market conditions have been liberalized therefore the milk price volatility is expected. The milk producers will be more dependent on the milk prices of the world market **(Schulte and Musshof, 2018; Schulte, Musshof and Meuwissen, 2018; Parzonko, 2018)**. The dairy sector is one of the most important sectors of agriculture in the Slovak Republic. The natural conditions for keeping dairy cows are particularly suitable in Slovakia, therefore the dairy sector belongs to the prospective sectors of the Slovak agriculture **(Lajdová, Kapusta and Bielik, 2017)**. For several years, the dairy sector in Slovakia has faced a number of problems concerning reducing size of dairy herd, lowering amount of milk production, insufficient purchase price of milk and shrinking number of milk producers and processors that have raised as a consequence of declining milk consumption **(National Agricultural and Food Centre, 2015; Trend.sk, 2015)**. The dairy production has a longstanding tradition in Slovakia. Despite the fact that situation has changed significantly in recent years and the share of milk production in the total agriculture production tends to decline, milk and milk products still represent a significant part of the food components of Slovak households **(Jamrich and Vargová, 2018)**. The national milk quota of Slovakia was around 1.1 milliard kilograms. Slovakia never exceeded its national milk quotas from the beginning of the 2004/2005 quota year. From 1993 to 2010, the number of dairy cows in Slovakia decreased from 386,000 to 161,300, while the average milk yield per 1 dairy cow increased from 3,042 to 5,692 kg. Despite this substantial milk yield growth, the total volume production of raw milk declined from 1,250 to 918 million kilograms **(Weldesenbet, 2013)**. The number of cattle in the Slovak Republic reached 471,600 heads only at the end of 2012, out of which the number of dairy cows reached 150,800 animals **(Šimo et al., 2016)**. According to **Gurčík et al. (2016)** in the Czech Republic and the Slovak Republic remains long-term economic imbalances (loss) for breeding dairy cows, this affects the continuing reduction of livestock number. Farmers are replacing the reduction in number of dairy cows by a higher efficiency of dairy cows, which ensures adequate milk production for each country. Density of livestock at the level of the European Union average is achieved only in Poland, which has become a major exporter of animal products. Hungary and the Czech Republic come to about half of the level of the EU-28 and at the end comes the Slovak Republic with a continuous decline in recent years **(Szabo et al., 2018)**. Most popular kind of milk is cow milk in Slovakia. Consumption of cow's milk and dairy products made from cow's milk represents approximately 98% of total milk and dairy products consumption in the Slovak Republic **(Ministry of Agriculture and Rural Development of the Slovak Republic, 2019)**. The Milk Market System, issued by the Ministry of Agriculture and Rural Development of the Slovak Republic **(Decree of the Ministry of Agriculture of the Slovak Republic, 2002)**, sets the quality criteria for classification of the raw cow milk into individual quality classes. According to the content of somatic cells and the total number of micro-organisms, two quality classes are defined, namely quality class Q and quality class I. Other quality is considered non-standard milk. According to **Swinnen et al. (2006)**, there are indications that the quality of milk produced by Slovak farms has improved in recent years. For instance, the share of milk in the highest quality classes (class Q and class I) has increased from an already satisfactory level in the late 1990s up to 95% of all milk delivered belonging to class Q and I. Milk of that quality is of acceptable quality according to the European Union standards. Milk as a raw material to milk industry has been produced in adequate volume, even though the share of milk production at its consumption was markedly decreased. As to competitiveness of Slovak milk industry products, at domestic market their share was significantly decreased, at European Union markets their competitiveness was deteriorated namely by products with the higher value added **(Matošková and Gálik, 2014)**. The main aim of the milk production efficiency should be based on the definition of the objective value of costs per one production unit **(Michaličková et al., 2014)**. Contrary to the costs, the milk price could be less influenced by the farms. It is formed in the markets (international and national) through the interaction of supply and demand. It should be more influenced by the negotiating power of farmers. Dairy farmers should promote higher market prices of milk for example by marketing associations. In the future, a detailed analysis should be focused on the interaction of biological and economic parameters in the dairy cattle sector. From the results of the model calculations it can be concluded that with increasing capacity and rising capacity utilization rate considerable cost economies can be achieved. On the other hand, it appears that cost economies as a function of the number of production days are minimal because of the low share of the per diem fixed costs in total costs **(Schmidt and Krell, 1996)**. At present, agriculture in the Slovak Republic mainly uses traditional methods for the calculation of production costs. Traditional calculation formulas work with overheads (as opposed to modern methods of calculations that convert non-specific, anonymous overheads into direct costs). Traditional calculations do not reflect the needs of the market environment **(Hudáková Stašová, 2018)**. According to results of dairy inquiry organic milk products create higher costs at processing and marketing than conventional milk products within the added value chain. On collection level, apart from extra charges for organic milk, the collection costs of raw material are higher per kilogram organic milk. Further costs occur in the

dairies, particularly due to a low utilization degree of processing capacities as well as to higher costs for auxiliaries and additives, for distribution, for packaging and quality assurance **(Burchardi and Thiele, 2003)**. The milk prices in dairy industry within the food vertical in the territory of Slovakia is developed also by **Kadlečíková et al. (2012)**, **Brodová (2013)**, **Matošková and Gálik (2014)** and **Božík et al. (2016)**.

Scientific hypothesis

The aim of the paper is to analyse the development of average purchase prices of raw Q class cow milk in a time period 2006 – 2018. The analysis was based on monthly data from the Reports on the market of milk and dairy products. At the time of submitting the data, data were available until July 2018. The paper is aimed at analysing the trend of average prices of raw Q class cow milk ex post and based on this ex post analysis to carry out an ex ante prognosis by June 2019. The result of this are the following two hypothesis:

Hypothesis 1: Based on an ex-post analysis, we expect that the forecast for the further development of average prices of raw Q class cow milk will fluctuate but will not be affected by the seasonal component.

Hypothesis 2: As the retained sample was already being worked on at the time the paper was finalized (July 2018 to February 2019), we assume that the predicted prices for raw Q class cow milk will not significantly differ from the actual prices.

MATERIAL AND METHODOLOGY

In the process of writing this paper, information from the Dairy Market Reports issued by the Agricultural Information Unit – ATIS, which is part of the Agricultural Paying Agency, was used. Monthly data from January 2006 to June 2018 on average monthly purchase prices of raw Q class cow milk were used. These data were presented in Slovak Crown until May 2008, and therefore were converted at the conversion rate of 1 EUR = 30.1260 SKK. The statistical software SAS was used for the analysis.

Statistic analysis

Modeling the future development is a methodically demanding activity that can be realized by several methods. In general, more advanced methods and approaches, such as exponential smoothing or Box-Jenkins methodology (ARIMA and SARIMA models) are used to analyse time series and forecast their future development.

Box-Jenkins methodology

By analysing the average purchase price of raw Q class cow milk, the best model suitable for constructing future development forecasts is looked for. The Box-Jenkins methodology is designed for modeling of stationary and non-stationary time series and time series with seasonal component. It consists of three steps: identification, parameter estimation and model validation. Methodology has its advantages and disadvantages. Its advantage is flexibility and quick adaptation to changes in the character of the time series. The entire modeling process can be full automated using software applications, reducing the subjective human factor intervention. The intuition of the solver plays a decisive role in the forecasting process. The disadvantages include, for example, a need to have a sufficiently long time series (at least 50 observations) available for the modeling, the practical application is time consuming and the possibility of simple interpretation of the resulting models' parameters is lost.

ARIMA models (AutoRegressive Integrated Moving Average models) are constructed from time series values, which are a linear combination of intrinsic historical values and historical values of residual deviations (so-called random shocks). A presumption of stationarity is the condition of using Arima models, i.e. the time series statistical properties do not change over time. It is a random variable that has a constant mean value over time, the variance is constant over time, and the linear relationship between the two time shifted random variables is zero **(Obtulovič, Sojková, 1999)**.

The paper also uses combined models consisting of several models because, in some cases, they offer the best quality smoothing of the time series of the average purchase price of raw Q class cow milk.

Forecast accuracy rates

The result of the time series extrapolation is forecasts based on an estimate of the parameters of a mathematical model whose quality has been confirmed by various statistical tests. For this reason, it can be expected that the forecasts obtained will not differ significantly from reality. Over a longer period of forecasting, forecasting accuracy (ex-ante) is assessed using different average characteristics. Forecasting accuracy measures represent timing alignment accuracy and this accuracy (model quality) can be measured absolute or relative. The paper uses the evaluation of trend quality by mean absolute percentage error (MAPE).

$$MAPE = \frac{1}{n} \sum_{t=1}^{n} \frac{|y_t - y'_t|}{y_t}$$

In percentage terms, it represents the average magnitude of forecast errors compared to actual values over the entire forecast period t = 1, 2, ... , n. It is the most widely used indicator of the accuracy of forecasting. The accuracy of the ex-ante forecasts obtained by the Box-Jenkins methodology is sufficiently reliable for short horizons.

RESULTS AND DISCUSSION

The Slovak dairy market has been in an unfavourable situation in recent years. Several majors changes have been made to the dairy market through this situation. Figure 1 presents the evolution of the raw Q class cow milk average prices from early 2006 to July 2018. It is evident from the development that milk prices have changed significantly in the period under review, they were fluctuating. The prices of agricultural commodities have been volatile in recent years and the dairy sector has not been an exception **(Vargová and Rajčániová, 2017)**. During the evaluation period 2006 – 2018, three dairy crisis periods can be identified, during which the prices has fallen below 30 eurocents per kilogram of milk. Such low prices do not cover the costs of primary milk producers despite the aid granted. According to the Slovak Chamber of Agriculture and Food, production costs are between 40 and 42 eurocents per kilogram.

The 2008 – 2009 period is referred to as the „Great Dairy Crisis“, as this was the most significant drop in milk prices over the whole period under review. In the year before, in 2007, a rapid and significant price increase occurred due to a significant decrease in the quantity of milk delivered from Oceania. After the resumption of world milk supply, prices have returned to their normal levels, but the subsequent economic crisis has had a negative impact on European Union milk producers, which has contributed to price volatility. Despite the production of milk in the European Union remained stable, a decline in global demand and European demand for milk and dairy products have resulted in price collapse in European Union. As a result, the price of raw cow milk in Slovakia also dropped significantly. In the first months of 2008, the average price of raw Q class cow milk was 39 eurocents per kilogram of milk, the highest price for the whole period under review. In the following period, milk prices were falling until they fell below 30 eurocents per kilogram of milk in November 2008. The decline in milk prices continued in 2009 until April, when the price of milk fell below 18 eurocents per kilogram (EUR 17.9/100 kg). This price is at the same time the lowest price of raw Q class cow´s milk in the period from 2006 to 2018. In the following period the price gradually increased and in December 2010 exceeded the limit of 30 eurocent per kilogram of milk (30.63 EUR/100 kg).

As a result of the partial recession of the economic crisis, the price of raw Q class cow milk was stable in 2011, at around 32 eurocents per kilogram. Another crisis period, when the price of raw Q class cow milk fell below the critical level of 30 eurocent per kilogram, can be identified in 2012.

This decrease was only temporarily recorded during May to October and was the result of a previous significant increase demand. This fact also affected the price level of milk in Slovakia. The lowest price during this crisis period reached 27.24 EUR/100 kg in July 2012. In the following period, the price of milk continued to rise until February 2014, when the price of milk exceeded 36 eurocents per kilogram (36.04 EUR/100 kg). However, since that period, the price of milk has been falling steadily.

The unfavourable price development resulted in another milk crisis in 2015 – 2016. The price of milk fell below 30 eurocents per kilogram in February 2015 and remained below this level for more than two years until April 2017. The lowest price recorded during this crisis period was in June 2016, when 100 kg of raw Q class cow milk was purchased for 23.34 EUR. The unfavourable price development in the dairy sector during this dairy crisis was influenced by several factors. High prices of dairy products at the turn of 2013 and 2014 caused a global decline in demand for milk and dairy products.

Another reason for the decline in demand was the ban on agricultural products import from the European Union into the Russian Federation, which was introduced on 7th August 2014. The Russian Federation was the largest export market for dairy products from the European Union.

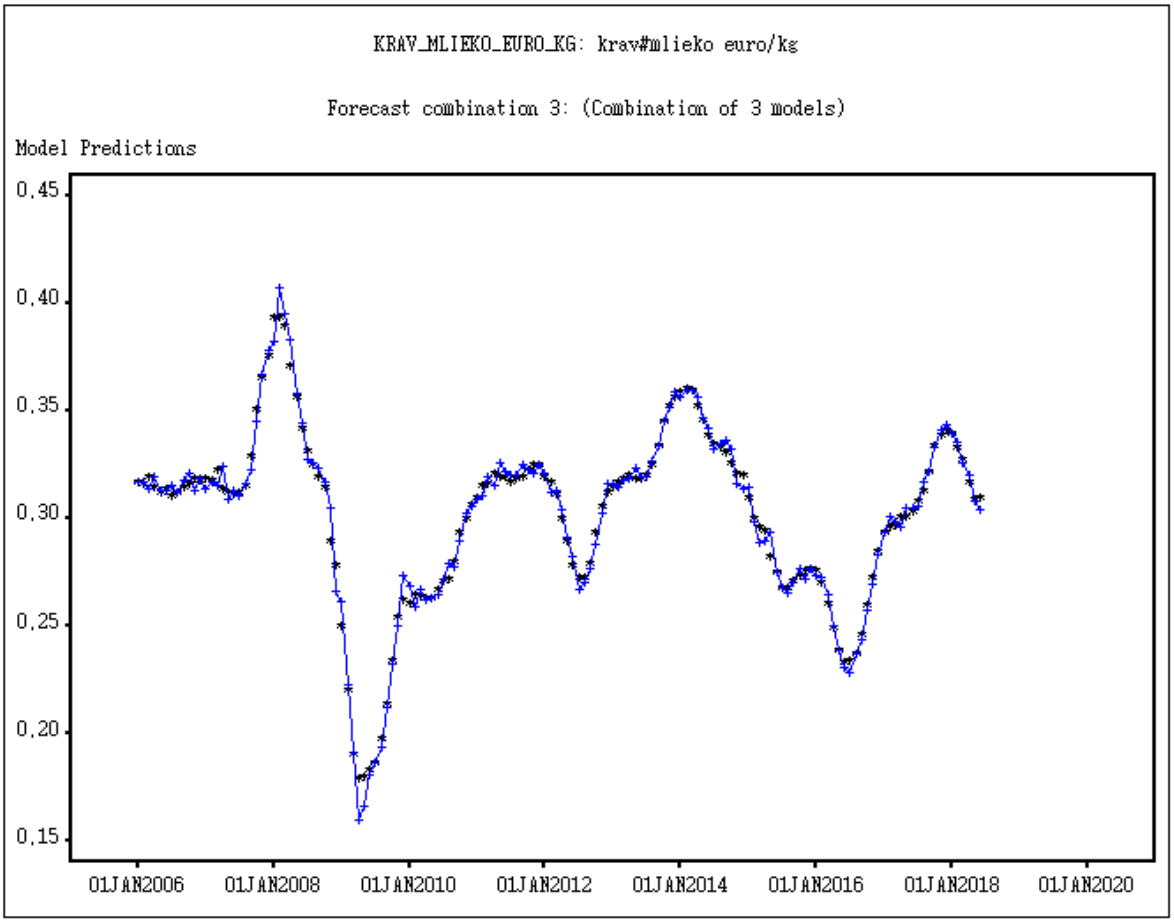

Figure 1 Development of the raw Q class cow milk´s average price. Note: Monthly reports on purchase of milk and cream and production of milk products (MARD SR), own calculations using SAS sofware.

Currently, the validity of the Russian embargo is extended until 31st December 2019. A declining demand from China for dried cow milk from European producers had also had a negative impact on the dairy sector crisis. The unfavourable situation during this period was also affected by the abolition of the milk quota system in the European Union on 1st April 2015. The abolition of milk quotas led to higher milk production, resulting in high milk surpluses on the European Union market and consequently a decline in milk prices. The new era of milk output without quota constraints will result in both opportunities and challenges for the European Union dairy industry. The opportunities will arise from the expanding global diary market. The challenges will involve the ability of the European Union dairy industry to achieve international competitiveness in servicing the increased global demand for dairy products **(Donnellan and Keane, 2015)**.

The long-term unfavourable situation in the dairy sector in Slovakia began to stabilize in the second half of 2017, since the purchase price of raw Q class cow milk has fluctuated between approx. 31 – 34 eurocents per kilogram.

The White Noise Test indicates a violation of zero mean conditions, constant scattering and residue independence. It is usually assumed that the residual component is the white noise. It consists of random movements (fluctuations) over the time series of the average purchase price of Q class cow milk, which are not of a systematic (recognizable) character. Figure 2 provides a preview of the white noise process from which it can be concluded that the conditions are met.

SAS offers 582 models. The MAPE value, also a graphical representation of the white noise process, are critical elements for model selection. It should be pointed out that there is no reliable and objective criterion that exhaustively and accurately determines the final shape of the model. The sensitive approach of the investigator plays a decisive role. Accordingly, all offered models are recalculated in the calculations and five top quality models are selected. Subsequently, the model that achieves the best quality results is chosen.

The Combined Model Variance, which is the same weight combination of Log Winters Method - Additive, Winters Method – Additive and Winters Method – Multiplicative, was used to analyse the ex post development and forecast the expected Q class cow milk purchase prices.

The degree of forecast accuracy in the model is determined, among other characteristics, by the MAPE value (1.22 %), which can be considered a very good result (R-Square = 0.986). The model was chosen not only based on the MAPE value, but also on the white noise and the presence of the unit root.

Based on the chosen model, a forecast of future development of the Q class cow milk´s average purchase price in the Slovak Republic was realized. The monthly trend by June 2019 is shown in Figure 3, which shows a graph of actual and estimated values.

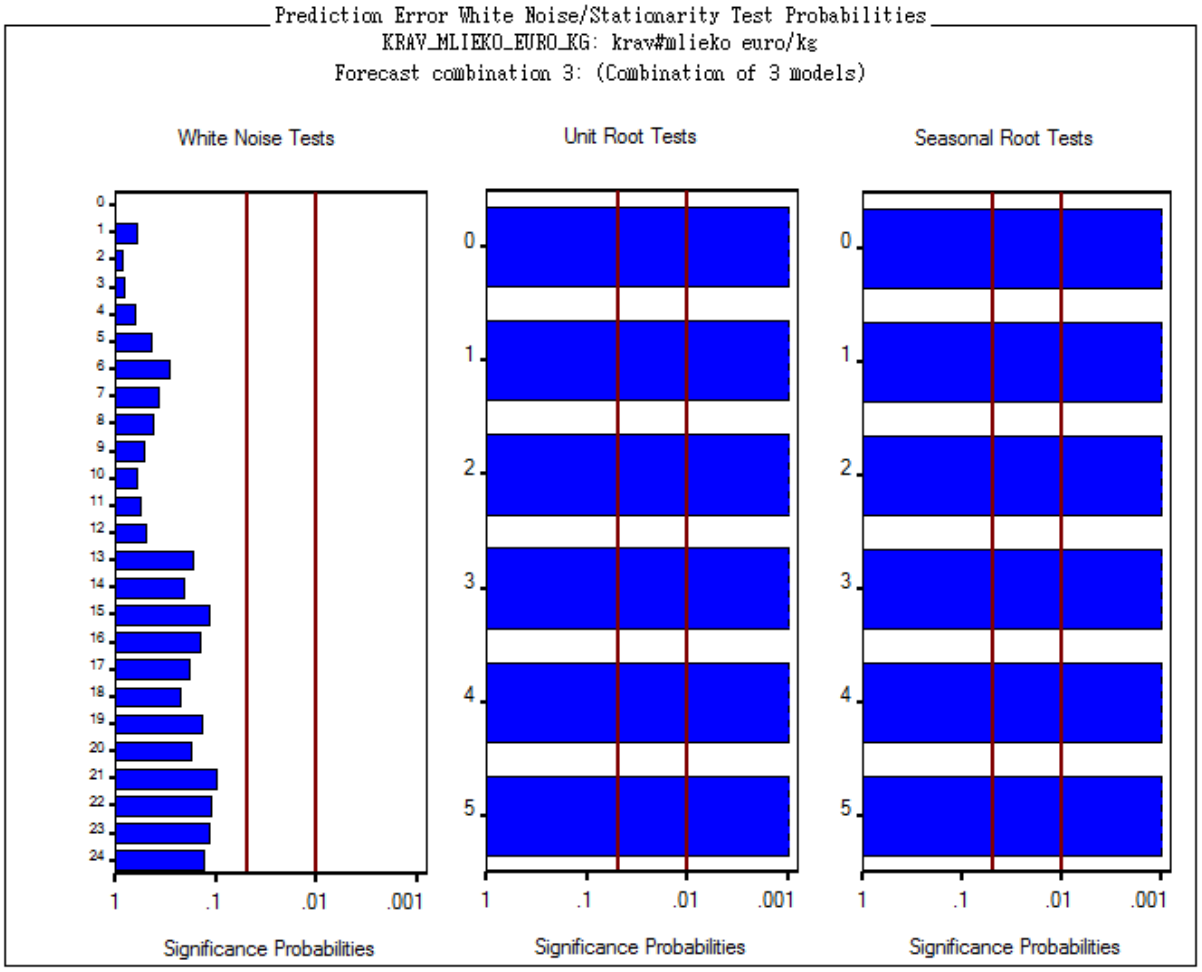

Figure 2 White noise and unit root test. Note: Monthly reports on purchase of milk and cream and production of milk products (MARD SR), own calculations using SAS software.

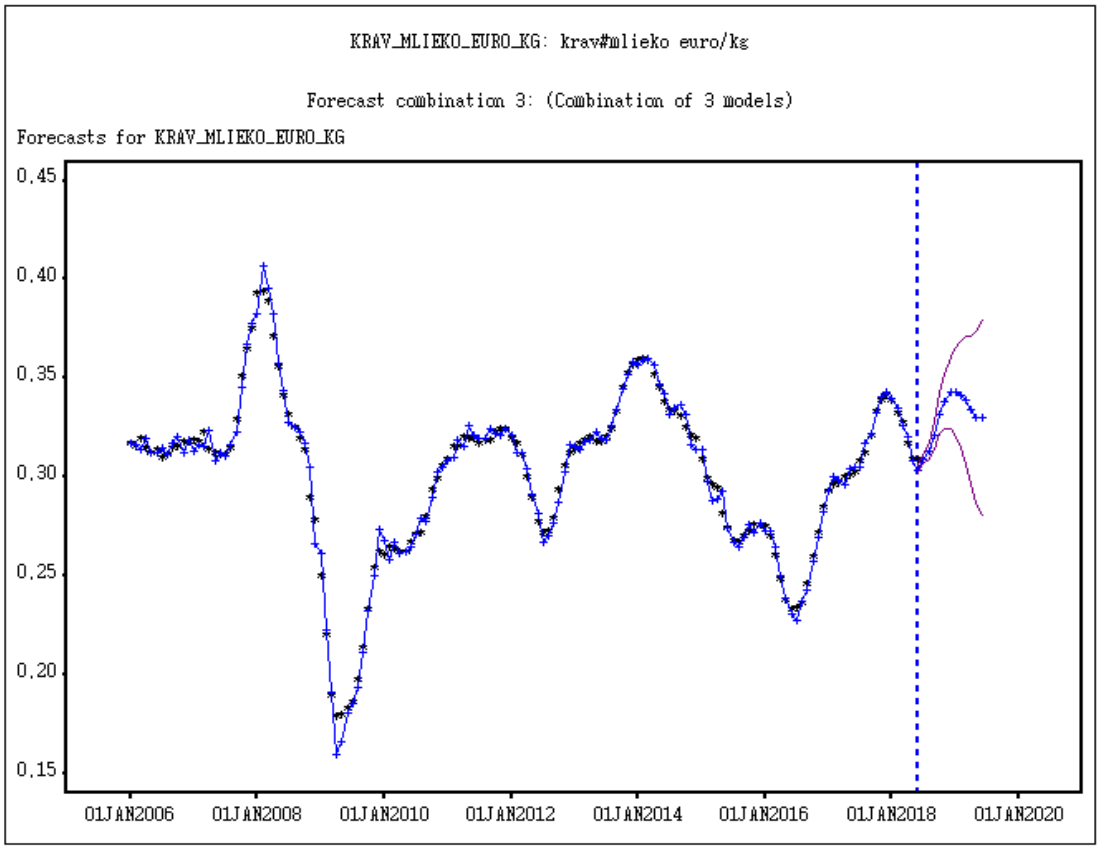

Figure 3 Development and forecast of the raw Q class cow milk´s average price. Note: Monthly reports on purchase of milk and cream and production of milk products (MARD SR), own calculations using SAS software.

Table 1 Actual and forecasted average prices for raw Q class milk. Note: Monthly reports on purchase of milk and cream and production of milk products (MARD SR), own calculations using SAS software.

Raw Q class cow milk prices	Period							
	7/2018	8/2018	9/2018	10/2018	11/2018	12/2018	1/2019	2/2019
Actual prices	0.312	0.315	0.322	0.328	0.333	0.334	0.336	0.334
Forecasted prices	0.309	0.313	0.321	0.332	0.338	0.343	0.343	0.342

The continuous line joins equalized values that represent a time series equalization using the selected combined model from January 2006 to June 2019. From the dashed vertical line, the forecast by June 2019 is shown in both a monthly point and a 90 % confidence interval estimate.

The results show that in the next forecasted period, that is for 2018 – 2019, the average purchase price of raw Q class cow milk is expected to increase slightly until February 2019, followed by its decline.

The retained sample prices of the raw Q class cow milk covered the period from July 2018 to February 2019. Table 1 shows the actual average prices of raw Q class cow milk together with the forecasted milk prices using the above-mentioned combined model. A comparison of the retained sample and the forecasted milk prices shows that the model used has provided a good forecast, as the differences are negligible.

As in the previous period and in the next forecast period, fluctuations in the average prices of raw Q class cow milk, as presented in Figure 3, can be expected. This confirmed the established research hypothesis.

CONCLUSION

The paper focused on the analysis of the trend of average purchase prices of raw Q class cow milk in Slovakia in the period from 2006 to July 2018. Based on the ex post analysis carried out, a forecast of milk price development by June 2019 has also been done. It can be stated that the average prices of raw Q class cow milk in both, the reference period and the forecast period, showed a fluctuation course, so hypothesis 1 was confirmed. At the time the paper was finalized, we worked with the retained sample (from July 2018 to February 2019), allowing forecast prices to be compared with actual milk prices. As the largest difference between the forecasted and the actual price is the difference of 0.9 eurocents, we confirm hypothesis 2, on the basis of which we assumed insignificant differences between the forecasted and the actual prices of raw Q class cow milk.

Based on the analysis of the development of average prices of raw Q class cow milk, three periods were identified as critical for the dairy sector during the reviewed period. During these periods, the price of milk has fallen below 30 eurocents per kilogram. Even at such low prices, despite the support provided to primary milk prouducers, there is a loss as production costs are high. According to the Slovak

Chamber of Agriculture and Food, the cost of raw cow milk production is between 40 and 42 eurocents per kilogram.

The lowest price of milk during the reviewed period was found in April 2009, when it fell below 18 eurocent per kilogram. This period 2008 – 2009 is also referred to as the period of the Great Dairy Crisis, when milk prices have been long below 30 eurocent per kilogram. Many businesses closed their milk production during the Great Dairy Crisis. Another milk crisis in 2015 – 2016 again led to the liquidation of this area of animal husbandry. At the beginning of the reviewed period before the Great Dairy Crisis, 777 primary producers of raw cow milk were registered in Slovakia, currently there are only 415 (as from 31 December 2018), which represents a decrease of 47 %.

The performed analysis of raw cow milk prices under the conditions of the Slovak Republic is a starting point for further scientific work in which the price disparities of raw cow milk according to quality classes, years and regions of the Slovak Republic will be identified.

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FORMATION OF MICROBIAL BIOFILMS ON STAINLESS STEEL WITH DIFFERENT SURFACE ROUGHNESS

Igor Stadnyk, Tetiana Hushtan, Ganna Sabadosh, Yana Yevchuk

ABSTRACT

The physical essence of the formation and influence of bacteria on the surface of technological equipment in the dairy industry is considered as an essential factor leading to contamination of dairy products and is a major hygienic problem. The ability of microorganisms on the surfaces of technological equipment to form biofilm forms and requirements for steel grade, relief, and its roughness were analysed. The effect of surface roughness on promoting or preventing adhesion and reproduction of biofilm forms of bacteria, which reduce the efficiency of sanitary processing of dairy equipment and thereby increase the microbial contamination of dairy products with shortened shelf life, is substantiated. Research about the process of bacterial adhesion to the surface of metals with different roughness depending on the size and shape is presented. It is found that on the surface of stainless steel with roughness 2.687 ±0.014 micron film formation process in *Escherichia coli* and *Staphylococcus aureus* are similar from 3 to 24 hours and does not depend on the size of the bacteria, and accordingly allows us to argue that rod-shaped and coccid bacteria attach freely in the hollows of the roughness are the beginning of the process of the first stage of biofilm formation. It is found that on the surface of stainless steel with roughness 0.95 ±0.092 micron film formation process in S. aureus is more intense than in *E. coli*. Thus, within 3 hours of incubation, the density of biofilms formed *S. aureus* was 1.2 times bigger than biofilms *E. coli*, by the next 15 hours of incubation formed biofilms *S. aureus* were, on average, 1.3 times denser. It is established that *S. aureus* due to its spherical shape is able to fit in the hollows of the roughness 0.95 ±0.092 μm and faster to adhere to the surface at the same time. *E. coli*, due to its rod-like shape, with such surface roughness, can adhere to the cavities only over its entire length. It is proved that by surface roughness 0.63 ±0.087 μm film intensity *S. aureus* was, on average, 1.4 times faster than *E. coli*, for roughness 0.16 ±0.018 micron film formation process took place equally for *S. aureus* and *E. coli*, but biofilms were lower in density than those formed on roughness 0.63 ±0.087 micron. Studies suggest that the use of equipment in the dairy industry with a roughness of less than 0.5 microns will reduce the attachment of microorganisms to the surface and reduce the contamination of dairy products.

Keywords: microbial adhesion; bacterial biofilm; adhesion; sanitary treatment; ejector; mathematical model

INTRODUCTION

With the widespread introduction into food production of modern automated, complex mechanized lines, when the processing speed of non-Newtonian food masses has increased significantly and new structural materials are widely introduced, there is always a need to study the adhesion strength and its effect on the passage of processes. Compliance with technological processes is directed to the separation zone taking into account both the type and condition of the surface of technological equipment and the structural and mechanical properties of the food masses. The main factor that reduces the shelf life and safety of food are microorganisms **(Kukhtyn et al., 2017; Shaheen et al., 2010; Verran et al., 2010)**. Is believed bacterial adhesion to the surface to be a complex physicochemical process that depends on surface properties such as topography, roughness, hydrophobicity, chemical composition, and surface energy; from the initial number of microorganisms, their size, temperature and pH of the environment, etc. **(Whitehead and Verran, 2007; Shaheen et al., 2010; Verran et al., 2010)**. However, among the many factors that influence the adhesion process are researchers **(Whitehead and Verran, 2007)** consider that surface properties play a major role. The presence of bacteria on the surfaces of technological equipment in the dairy industry is considered as an important factor that can lead to contamination of dairy products and is considered as an important hygienic

problem **(Kukhtyn et al., 2017)**. At the same time, microorganisms survive on the surfaces of process equipment due to their ability to form biofilm forms. In this regard, certain hygienic requirements are placed on the surface of the processing equipment used in the dairy industry, especially as regards steel grade, relief and roughness. Based on the interactions between the surface and the environment, the authors **(Stadnyk et al., 2019)** reveal the features of adhesion are given. The adhesion is increase or decrease in accordance with laws of Ammonon-Coulomb friction, the Euler relation, the terms as friction angle and friction cone, the friction coefficient. This is due to the fact that surface roughness can promote or hinder the adhesion and reproduction of biofilm forms of bacteria **(Vlková et al., 2008)**. Also, the development of biofilm helps to reduce the efficiency of sanitation of dairy equipment and thereby increases the microbial contamination of dairy products and shortens their shelf life. Consequently, studies of the formation of biofilms by bacteria of different shapes and sizes, depending on the roughness of the stainless-steel surface, are relevant in the dairy industry. Research in this direction will allow to scientifically substantiate the surface roughness parameters of dairy equipment, which would minimize the microbial adhesion process. Such studies may provide a basis for developing a method for evaluating stainless steel for the presence of adhesive properties.

Analysis of studies of adhesive bonds of biofilms

As mentioned above, microbial adhesion is a complex biological process that is influenced by the physiological features of the microorganisms, the environmental properties and the physicochemical properties of the surface **(Yi et al., 2004; Moons and Michiels, 2009; Whitehead and Verran, 2007; Götz, 2002)**. The results of the studies indicate **(Monds and O'Toole, 2009)**, that the nutrients for the microorganisms of the biofilm are contained in the solution, so the composition of the nutrient medium, its ionic energy, pH, temperature also affect the adhesion intensity of the microorganisms to the base. The formed biofilm consists of an inhomogeneous structure, resulting in a gas concentration gradient, in particular, a decrease in the amount of oxygen from the periphery to the depth, pH gradients and nutrients. These gradients of biofilm functioning provide physiological variability between individual biofilm cells - they grow more slowly at the depth of the cell than at the periphery of the film, leading to phenotypic stability and a dramatic change in environmental factors. The formation of the biofilm and the strength of its adhesion to the surface are influenced by the hydrophobic properties of the surface of the microorganisms, the presence of their villi and flagella. Most bacteria have a negatively charged surface that contains hydrophobic external components that cause hydrophobic interaction with the substrate. The presence on the surface of biofilms of polysaccharides or proteins helps bacteria to adhere to the surface, thereby providing a competitive advantage in the formation of biofilms for specific cells of the microbial association **(Yi et al., 2004)**. In addition, the structure of the biofilm is influenced by the presence in its composition of microorganisms, which in the course of their growth and development release the gaseous substances of metabolism. This leads to a decrease in the density of the biofilm and the uneven flow of nutrient substrates **(Götz, 2002)**. Among the many factors that influence the adhesion process, researchers highlight the role of surface properties, which is defined as the most significant **(Zogaj et al., 2003; Kania et al., 2007)**. As a result, three theories of microbial adhesion to the surface were proposed: thermodynamic, DLVO theory and XDLVO **(Kolari, 2003; Langsrud et al., 2016)**. The thermodynamic theory is based on the fact that when the microorganisms are attached to the surface there is a change in the total free energy of Gibbs, Van der Waals forces. Theory DLVO is based on the fact that the colloidal particles of the lyophobic dispersion system can move closer to each other without interruption until their liquid diffuse shells come into contact. Theory XDLVO is based on thermodynamic and DLVO theory **(Langsrud et al., 2016)**. However, researchers believe that all three theoretical models designed to reveal the essence of microbial adhesion to the surface have been designed for the perfect colloidal system. Under production conditions, microbial adhesion is a much more complicated process and attachment of microorganisms can occur in different ways.

Analysis of recent research.

In the dairy industry stainless steel corrosion-resistant steels of the following brands are most often used for the equipment AISI-304, AISI-316, AISI-321 **(Jullien et al., 2003; Dantas et al., 2016)**. These steel delivery conditions can have different surface roughness from 0.2 – 3.2 mm. Studies to investigate the effect of terrain and surface roughness on microbial adhesion are not straightforward. According to research **(Dantas et al., 2016),** there is a correlation between surface roughness and bacterial adhesion, the attachment of microorganisms to the surface increases with increasing roughness. From the analysis of works **(Kukhtyn et al., 2016)** found that the formation of biofilm was much slower on the surface with a roughness of up to 0.4 μm, compared with a roughness greater than 0.8 μm. Using electron microscopy, the researchers **(Dantas et al., 2016; Kukhtyn et al., 2016)** found that the primary adhesion of microbial cells occurs along the hollows of the surface roughness, since under these conditions the area of contact of the microbial cell with the surface increases. However, other studies indicate that there is virtually no correlation between stainless steel surface roughness and microbial adhesion. There are also various data on the effect of surface wettability on microbial adhesion. It was found that the number of adherent bacteria decreased with increasing surface hydrophobicity, and microorganisms that attached to hydrophobic surfaces were more easily removed by increasing the flow force during fluid circulation **(Dou et al., 2015)**. However, other researchers **(Kolari, 2003)**, indicate that there is no correlation between surface wettability and microbial adhesion. It is investigated that formed biofilms on surfaces with high roughness reduce the efficiency of heat transfer in condenser heat exchangers by about 15%. Thus, the hygienic quality and cleanliness of the processing equipment in the dairy industry after sanitation can be closely linked to relief and roughness. Therefore, research into the process of film formation on stainless steel with different roughness over

time and using different shapes and sizes of bacteria is promising. These studies will allow us to understand more deeply the process of the formation of microflora on the equipment and, accordingly, the contamination of food products. Also, finding out the effect of surface roughness on microbial adhesion will help to improve and modify the surfaces that interfere with adhesion.

The purpose of the work was to determine the effect of different surface roughness of stainless steel on the process of microbial adhesion and film formation, depending on the physiological and morphological features of the microorganisms that contaminate the equipment.

To achieve this goal, the following tasks were set:

- to experimentally investigate the process of film formation by rod-shaped and coccoidal bacteria in stainless steel with different surface roughness at a temperature of 25 °C;
- simulate the process of film formation on stainless steel with different surface roughness.

Scientific hypothesis

The successful formation of microbe biofilms is not possible without microorganisms' surface adhesion. The scientists have separated five main stages of biofilms formation and development on any surface. Bacteria surface adhesion depends on the initial number of microorganisms, their shape, temperature, environment pH, size and etc. The primary adhesion of microbial cells is taking place along the cavities of the surface roughness as under such conditions the contact area of the microbial cell and the surface is getting bigger. Nevertheless, any process of each biofilm formation has specific features that are regulated by both genetic, biochemical properties of the bacterial cell and environmental factors as well. We may claim that a biofilm is a microbial combination formed by the cells attached to the surface and to one another and are in the matrix of synthesized extracellular substances. The most serious biofilm hazard on the milk processing equipment is that the biofilm extracellular matrix protects the bacteria from the disinfection agents' actions. The survived microorganisms occupy the new surfaces and milk products. Thus, while studying the microbial adhesion and biofilms formation under laboratory conditions one can not always take into consideration the impact of production factors and physiological specific features of microbial cells. Conducting the research on studying the process of film formation on the stainless steel of different surface roughness during a certain period of time and using different bacteria forms and size will allow us to understand deeper the process of microbial flora formation on the equipment and also food contamination. To find out the impact of surface roughness on microbial adhesion means to facilitate the improvement and modification of the surfaces which resist the adhesion.

MATERIAL AND METHODOLOGY

In the dairy industry, stainless steel corrosion-resistant steels that can have a different surface roughness from 0.2 – 3.2 microns are most commonly used for equipment. Surface quality is judged by geometrical parameters and condition of the surface layer, which is determined by the physical, mechanical properties and structure. The characteristics of the geometric properties of the surface include macro- and microgeometric parameters. Stainless steel corrosion-resistant nickel-chromium austenitic steel plates were used for the study AISI 321 (standard of the American Institute of Steel and Alloys) size 30 × 30 mm and 4 mm thick, with surface roughness R_a = 2.687 ±0.014 microns, R_a = 0.95 ±0.092 microns, R_a = 0.63 ±0.087 microns, R_a = 0.30 ±0.065 microns, R_a = 0.25 ±0.035 microns, R_a= 0.24±0.026 microns and R_a = 0.16 ±0.018 microns. The process of forming the biofilm was carried out by strains *Escherichia coli ATCC 25299* and *Enterococcus faecalis ATCC19433* on brand stainless steel surface AISI 321 with different roughness over time.

Research methods

Experimental studies were carried out using modern standard and conventional methods: technical (determination of surface roughness of stainless steel), microscopic (light and electron microscopy of the process of film formation and degradation), spectrophotometric (optical density), biofilm, microbiology, microbiological parameters of dairy equipment and dairy products. Investigation of the process of film formation by microorganisms on the surfaces of metals with different roughness was carried out in a sterile Petri dish. The sterile stainless steel plates with appropriate surface roughness were placed in the cup and sterile meat-peptone broth was introduced into the cupand the appropriate test culture (*Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* or any other dedicated dairy equipment) in concentration to 1 cm^2 plate area accounted for from 1 to 50 thousand cells. The incubation was carried out at temperatures from +17 °C, +25 °C to +37 °C for 3, 6, 9, 12, 18 and 24 hours, depending on the purpose of the experiment. After incubation, the plates were removed from the Petri dishes, washed three times with planktonic (unattached) microorganisms with phosphate buffer. Recorded the formed microbial biofilms on the plates with 96% ethyl alcohol. After fixing, the biofilms were stained, for this purpose the plates were immersed in 0.1% crystalline violet aqueous solution. After staining, the plates were washed three times with phosphate buffer and dried. After drying, each plate was individually poured into 7.0 cm^3 of 96% ethyl alcohol and left for 20 min. After 20 min exposure, 5 cm^3 of the wash solution was removed from biofilms and the optical density t 570 nm was determined spectrophotometrically on the KFK-3 photometer (Ukraine) (Figure 1).

Electron microscopic studies of the process of forming biofilms on stainless steel were performed on an electron raster microscope (REM 106 I, Ukraine) with magnification from 2000 to 5000 times (Figure 2). In addition, the adhesion of microorganisms in the cavities or roughness projections was visually assessed using a microinterferometer MII-4Y4.2 with magnification in 1500 times (Figure 3).

Figure 1 Photometer (KFK-3).

Figure 2 Electron raster microscope (REM 106 I).

Figure 3 Microinterferometer MII-4Y4.2.

To determine the parameters of the surface layer, the following technique was used:

- scanning the surface with a 3D scanner David Laser Scanner SLS-1 using the software David Laser Scanner Professional Edition;
- processing the scanned surface using the program PowerShape;
- determining the required geometric characteristics of the scanned part of the surface.

A large number of dots were merged into the surface when scanned. To determine the roughness and waviness of the surface layer, we cut out a scanned area of 10 x 10 mm in software PowerShape, which is shown in Figure 4. We draw two two perpendicular planes (Figure 5) that will cut the plot. A fragment of a longitudinal profile with points (vertices and cavities) is shown in Figure 6. Similar operations were performed with a transverse profile, a fragment of which with vertices and cavities is shown in Figure 7. Determining the lengths of perpendiculars from cavities or vertices to the center line using the formula:

$$R_a = \frac{\sum_i^n |Y_i|}{n},$$

Where:

n – the number of points (in our case 107); $\sum_i^n |Y_i|$ – the sum of the lengths of all perpendiculars (in our case 2038 μm).

So,

$$R_{a_1} = \frac{203.8}{107} = 1.905 \text{ (microns).}$$

$$R_{a_2} = \frac{104.7}{64} = 1.6 \text{ (microns).}$$

Accordingly, the arithmetic mean, is:

$$R_a = \frac{R_{a_1} + R_{a_2}}{2} = \frac{1.905 + 1.635}{2} = 1.77 \text{ (microns).}$$

Using the standard table of accepted values of Ra, we can determine that the test area has a roughness within Ra 1.6 and Ra 2.0.

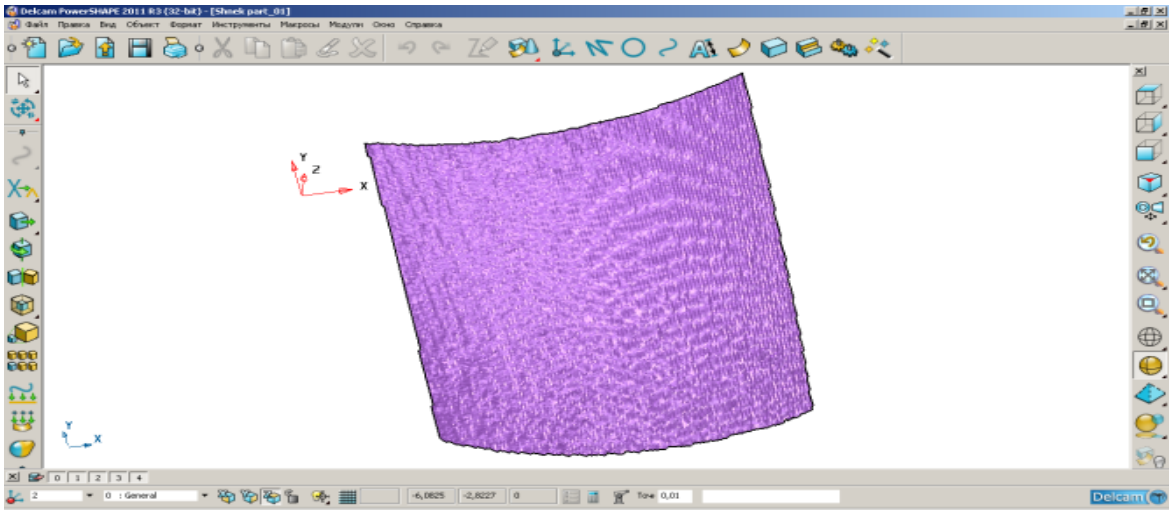

Figure 4 Fragment plot measuring 10 x 10 mm.

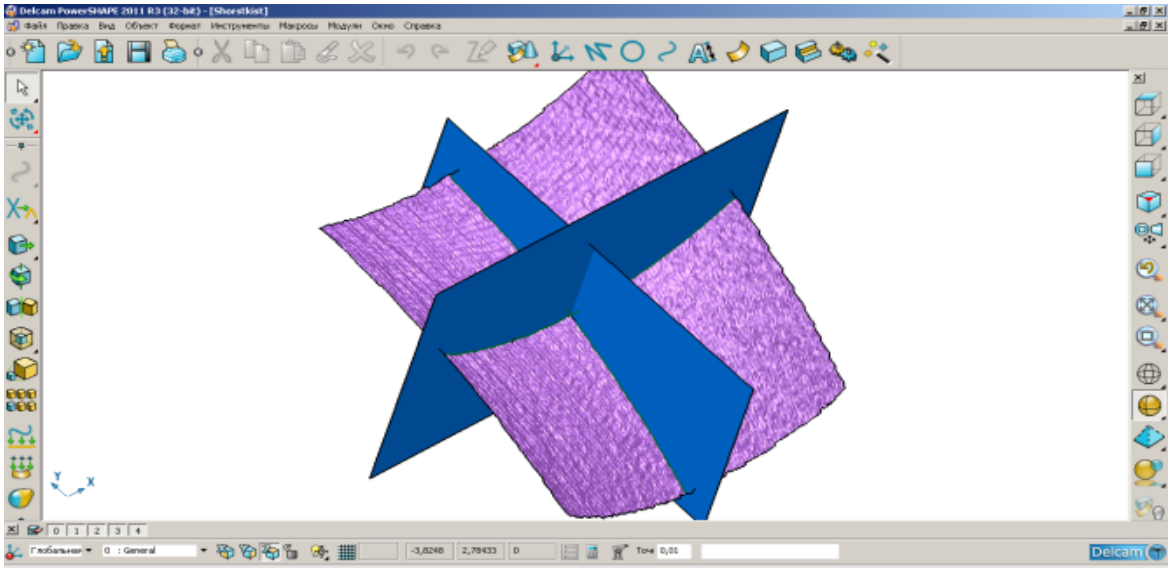

Figure 5 The section of the plot perpendicular to the planes.

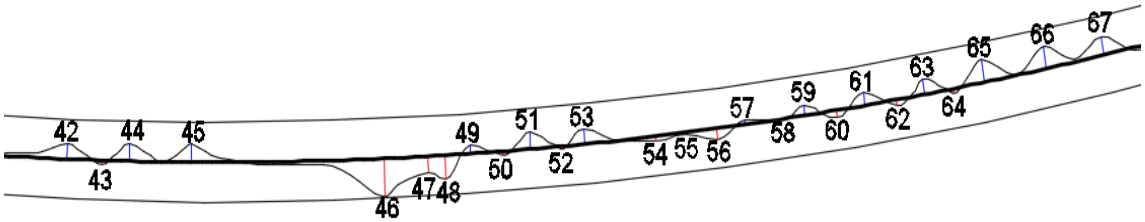

Figure 6 Fragment of a longitudinal profile with points of vertices and cavities.

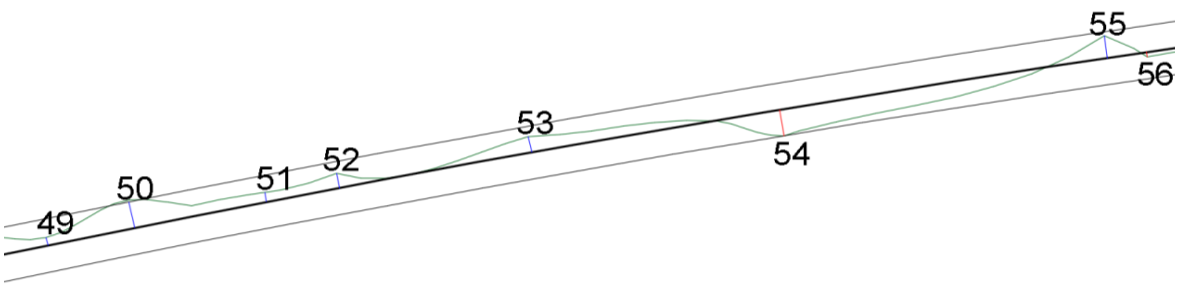

Figure 7 Fragment of transverse profile with vertices and cavities.

Statistic analysis

To obtain mathematical dependencies when comparing the results of the study of optical density by the factor of time and roughness for different temperatures and the initial bacterial count, regression equations of optical density and bacterial development were proposed.

RESULTS AND DISCUSSION

Obviously, in some cases the search for geometric connections to provide film formation by microorganisms on metal surfaces can be significantly simplified if the initial surface synthesis with the number of microorganisms are solved. However, the instability of the adhesion values and external conditions of operation of the surfaces, as well as variations in the film´s structured parameters, lead to the need to search for non-standard approaches to establish their bonds. The situation is complicated by significant differences in the initial surface areas, features of the microbial film, etc. According to research **(Dantas et al., 2016)**, there is a correlation between surface roughness and bacterial adhesion, with the attachment of microorganisms to the surface increases with increasing roughness. An analysis of the work **(Kukhtyn et al., 2016)** found that the formation of biofilm was much slower on the surface with a roughness of up to 0.4 μm, compared with a roughness greater than 0.8 μm. The theory of adhesion interactions of microorganisms with the the rough surface was based on research. The study of the phenomena of adhesion of microorganisms with a rough surface is conventionally divided into two stages: the application of the number of microorganisms on the surface, considering the time and temperature of the formation of the film, and when the relative formation of the film occure. Both cases occur in viscous materials transport systems whose properties are limited by support deformations, compression, and velocities. Studies have shown that the surface of stainless steel with roughness 2.687 ±0.014 microns, the process of film formation in *E. coli* and *S. aureus* passed over with the same from 3 to 24 hours of incubation and did not depend on the size and shape of the bacteria, but on the surface with roughness 0.95 ±0.092 microns, the process of film formation in *S. aureus* was more intense than in *E. coli*. Therefore, during the first 3 hours of incubation, the optical density of the formed biofilms *S. aureus* was 1.2 times higher compared to the density of biofilms formed *E. coli.* The next 15 hours of incubation formed biofilms *S. aureus* were, on average, 1.3 times denser, and no significant difference was found between 18 and 24 hours of incubation. This gives reason to believe that *S. aureus* due to its spherical shape, is able to be positioned in the roughness depressions of 0.95 ±0.092 microns and faster to adhere to the surface. Simultaneously *E. coli,* due to the rod-like shape, at such a surface roughness, adheres to the hollows only longitudinally and form biofilms. However, was found that the intensity of the film-forming process in bacteria on the surface with a roughness of 0.95 ±0.092 mm depends on the shape and size of the bacteria only up to 18 hours of incubation. The obtained data have corresponded **(Verran et al., 2010)** with the research which prove that *L. monocytogenes* adhesion on the stainless steel of the roughness less than 0.8 mkm was slower than on the surface of roughness 30 mkm. Although, we have found that apart from surface roughness the biofilm formation is also influenced by the form and size of microorganism cells. This can be explained by the fact that coccus forms of microorganisms at 0.95 and 0.63 mkm surface roughness was forming biofilms more intensely than the string formed bacteria. We agree with scientists idea **(Hočevar et al., 2014)**, that this phenomenon takes place due to the increased area of bacteria-surface contact. When used in experiments stainless steel with a roughness of less than 0.8 microns, as recommended in the food industry according to hygienic standards **(Council directive 93/43/EEC; Whitehead and Verran, 2007)** it is found that at a surface roughness of 0.63 ±0.087 microns, the intensity of film formation *S. aureus* was on average in 1.4 times faster than for *E. coli*, up to 18 hours of incubation. At the same time, at a roughness of 0.16 ±0.018 μm, the film-forming process proceeded equally for *S. aureus* and *E. coli,* but the formed biofilms were lower in density than those formed at a roughness of 0.63 ±0.087 μm. Figure 8 presents a schematic model of the formed biofilm in the depressions and on the projections of the roughness.

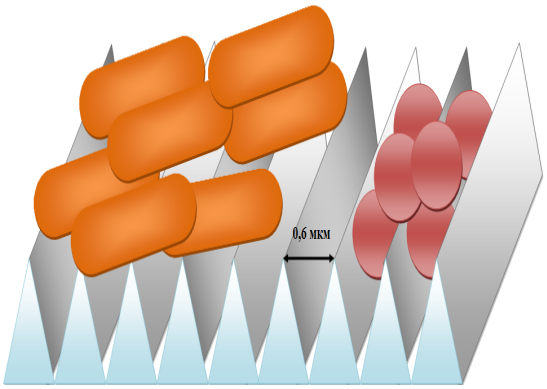

Figure 8 Schematic model of the formed biofilm in the depressions and at the projections of the roughness.

The technique of investigation of the optical density of the biofilm is developed, based on the use of the plan of experiment realization in the software complex Microsoft Excel, which allows to obtain a mathematical model of the film formation process in the form of regression equations. The function of the response of the obtained equation is the biofilm density from roughness and time. The optimization parameter used is the change in the number of bacteria during the process. What we don't know is the relationship between the input parameters and the number of bacteria, but we have a model that can be represented as:

$$Y = f(Ra, \tau),$$

Where:

Y – change in biofilm density, thousands; τ – time, hour; Ra – surface roughness of the equipment; Y_1 – *E. Coli*; Y_2 – *E. faecalis.*

Table 1a *E. coli,* T = 37 °C, Ra = 0.955 microns.

Initial bacterial count	0 Control	3	6	9	12	18	24
Up to 1 thousand	0.091	0.105	0.139	0.235	0.634	1.287	1.602
2 – 10 thousands		0.121	0.247	0.342	1.223	1.671	1.769
20 – 50 thousands		0.181	0.374	0.463	1.304	1.716	1.804

Table 1b *E. faecalis,* T = 37 °C, Ra = 0.955 microns.

Initial bacterial count	0 Control	3	6	9	12	18	24
Up to 1 thousand	0.091	0.112	0.156	0.307	0.672	1.295	1.670
2 – 10 thousands		0.136	0.263	0.395	1.246	1.683	1.774
20 – 50 thousands		0.196	0.392	0.467	1.415	1.743	1.851

Table 2 Optical density (number) by *E. coli* bacterial factors**.**

	37* <1	37* 2 – 10	37* >50	25* 2 – 10	17* 2 – 10
Y	-0.049	-0.181	-0.169	-0.027	0.08
y1	0.171	0.281	0.217	0.151	-0.028
y2	0.014	0.063	0.08	0.018	3.239*10^-3
y3	9.522*10^-3	4.579*10^-3	8.186*10^-3	0.01	0.01
y4	-0.055	-0.084	-0.058	-0.054	5.657*10^-3
y5	1.671*10^-3	3.785*10^-4	-2.485*10^-4	1.237*10^-3	7.219*10^-4

According to the results of the experiment (Table 1a and 1b), we obtain the regression quadratic equations of the law of change of density from surface roughness and time, which are shown graphically in Figure 9 for *E. Coli* (a) and *E. faecalis* (b) for 18 hours at Ra = 0.63 ±0.87 microns. Thus, from the obtained dependencies in Figure 9 it can be noted that the process of formation of biofilms *E. Coli* and *E. faecalis* on stainless steel in addition to surface roughness, the initial bacterial count is significantly affected. On steel plates with a roughness of 0.95 ±0.018 microns, the film formation process of *E. coli* is slower compared to *E. faecalis* (Figure 9). According to the data obtained and the dependence construction, it was found that the film density depends on the size of the bacteria and on the initial number of microbial cells on the steel surface That is why we consider that the surface maximum roughness for the most efficient sanitary treatment of milk processing equipment should be 0.5 mkm. Such a treatment is the best decision to prevent film formation both by coccus and string formed bacteria.

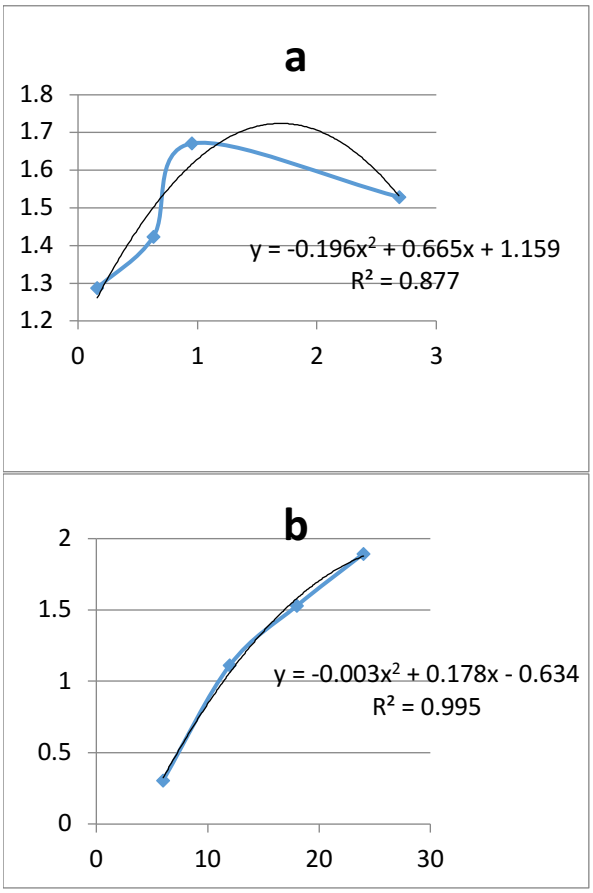

Figure 9 Change in optical density *E. coli* (a) and *E. faecalis* (b) for time 18 hours.

For bacteria E. coli

Optical density regression equation (quantity) **Y** by time factors and roughness **r** for different temperature ranges T °C and the initial bacterial count according to Table 2 will be:

$$Y_{k,i} := y_0 + y_1 \cdot r_k + y_2 \cdot t_i + y_3 \cdot r_k \cdot t_i + y_4 \cdot (r_k)^2 + y_5 \cdot (t_i)^2$$

The regression equation of the rate of change of quantity **Z** by time factors tand roughness **r** for different temperature ranges T °C and the initial bacterial count according to Table 3:

$$Z_{k,i} := z_0 + z_1 \cdot r_k + z_2 \cdot t_i + z_3 \cdot r_k \cdot t_i + z_4 \cdot (r_k)^2 + z_5 \cdot (t_i)^2$$

By comparing population intensities *E. coli* for T = 37*I(n<1), I(2<n<10), I(20<n<50) got that $Y(2<n<10)_{k,I} = s1_{k,i}* Y(n<1)_{k,I}$, $Y(20<n<50)_{k,I} = s2_{k,i}* Y(2<n<10)_{k,I}$,

Where:

$$s1 = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1.056 & 1.139 & 1.152 & 1.252 \\ 1.167 & 2.075 & 1.777 & 1.346 \\ 1.201 & 1.435 & 1.455 & 1.554 \\ 1.698 & 1.776 & 1.929 & 1.252 \\ 1.625 & 1.472 & 1.298 & 1.087 \\ 1.278 & 1.112 & 1.104 & 1.09 \end{pmatrix}$$

$$s2 = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1.053 & 1.165 & 1.496 & 1.396 \\ 1.889 & 1.198 & 1.514 & 1.56 \\ 1.6 & 1.178 & 1.354 & 1.226 \\ 1.138 & 1.085 & 1.066 & 1.363 \\ 1.051 & 1.125 & 1.027 & 1.199 \\ 1.05 & 1.089 & 1.02 & 1.133 \end{pmatrix}$$

k = 0 – 6; i = 0 – 3.
Thus, the initial number of bacteria is taken into account as a factor.

For bacteria E. faecalis

Optical density regression equation (quantity) **Y** by time factors**t**and roughness **r** for different temperature ranges **T** °C and the initial bacterial count according to the Table 4:

$$Y_{k,i} := y_0 + y_1 \cdot r_k + y_2 \cdot t_i + y_3 \cdot r_k \cdot t_i + y_4 \cdot (r_k)^2 + y_5 \cdot (t_i)^2$$

The regression equation of the rate of change of quantity **Z** by time factors**t**and roughness **r** for different temperature ranges T °C and the initial bacterial count according to the Table 5.

$$Z_{k,i} := z_0 + z_1 \cdot r_k + z_2 \cdot t_i + z_3 \cdot r_k \cdot t_i + z_4 \cdot (r_k)^2 + z_5 \cdot (t_i)^2$$

By comparing population intensities *E. faecalis* for T = 37*I(n<1), I(2<n<10), I(20<n<50) got that $Y(2<n<10)_{k,I} = s1_{k,i}* Y(n<1)_{k,I}$, $Y(20<n<50)_{k,I} = s2_{k,i}* Y(2<n<10)_{k,I}$,

Where:

$$s1 = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1.056 & 1.111 & 1.214 & 1.311 \\ 1.229 & 1.759 & 1.686 & 1.57 \\ 1.186 & 1.279 & 1.202 & 1.405 \\ 1.899 & 1.794 & 1.854 & 1.338 \\ 1.585 & 1.486 & 1.3 & 1.077 \\ 1.293 & 1.144 & 1.062 & 1.09 \end{pmatrix}$$

$$s2 = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1.042 & 1.133 & 1.441 & 1.729 \\ 2.007 & 1.459 & 1.49 & 1.404 \\ 2.039 & 1.259 & 1.266 & 1.361 \\ 1.118 & 1.084 & 1.136 & 1.307 \\ 1.104 & 1.129 & 1.036 & 1.203 \\ 1.051 & 1.061 & 1.043 & 1.128 \end{pmatrix}$$

k = 0,..,6; i = 0,..,3.

According to the regression equation, we construct response surfaces (Figure 10, 11 and 12).

Table 3 Changes in the number of *E. coli* bacterial factors.

	37* <1	**37* 2 – 10**	**37* >50**	**25* 2 – 10**	**17* 2 – 10**
Z0	-0.031	-0.056	-0.051	-0.033	-0.014
Z1	0.013	0.039	0.027	0.023	-3.509*10^-5
Z2	0.013	0.021	0.021	0.012	6.538*10^-3
Z3	-5.267*10^-4	-8.55*10^-4	-8.591*10^-6	6.023*10^-5	-1.62*10^-4
Z4	-3.2*10^-4	-7.675*10^-3	-3.668*10^-3	-5.458*10^-3	3.285*10^-3
Z5	-3.325*10^-4	-6.006*10^-4	-6.451*10^-4	-2.947*10^-4	-1.947*10^-4

Table 4 Optical density (number) by *E. faecalis* bacterial factors.

	37* <1	**37* 2 – 10**	**37* >50**	**25* 2 – 10**	**17* 2 – 10**
y0	-0.058	-0.177	-0.161	-0.065	0.074
y1	0.189	0.245	0.161	0.143	-0.028
y2	0.016	0.069	0.092	0.038	5.594*10^-3
y3	0.011	5.343*10^-3	8.453*10^-3	6.702*10^-3	0.012
y4	-0.061	-0.068	-0.034	-0.047	4.798*10^-3
y5	1.56*10^-3	1.715*10^-4	-6.781*10^-4	9.417*10^-4	7.161*10^-4

Table 5 Changes in the number of *E. faecalis* bacterial factors.

	37* <1	**37* 2 – 10**	**37* >50**	**25* 2 – 10**	**17* 2 – 10**
z0	-0.032	-0.027	-0.053	-0.032	-0.027
z1	0.028	0.021	0.05	0.033	0.021
z2	0.013	8.004*10^-3	0.02	0.013	8.004*10^-3
z3	-9.673*10^-4	-4.898*10^-4	-1.185*10^-3	-1.942*10^-4	-4.898*10^-4
z4	-3.618*10^-3	-2.157*10^-3	-6.644*10^-3	-8.725*10^-3	-2.157*10^-3
z5	-3.21*10^-4	-2.354*10^-4	-5.768*10^-4	-3.279*10^-4	-2.354*10^-4

Figure 10 (Y) shows that the number of bacteria *E. coli* highest value gets at roughness surface 2.68 μm after 20 hours of incubation. The speed of development (Z) *E. coli* increases most intensively with roughness values between 0.95 μm and 2.68 μm and time, starting after ten hours of incubation. Analysis of Figure 11 shows that similar trends in bacterial counts and growth rates, as well as in temperature +17 ±1 °C. However, the number of bacteria is increasing (Y) and fills roughness (Z) from 2.68 microns to 0.63 microns at 18 hour of incubation, and at 20 hour is practically up to 0.30 microns. From the temperature data +37 ±1 °C the number of bacteria is growing rapidly (Y) and the surface roughness is filled (Z) 2.68 – 0.63 microns within 12 hours of incubation. On the basis of mathematical modeling, it was found that the adhesion and intensive process of biofilm formation *E. coli* passes in the hollows of great roughness 2.68 – 0.95 micronsand it gradually fills the hollows with less roughness 0.63 – 0.16 microns. Figure 12 shows that there is a similar pattern in the process of film formation for *E. faecalis*, as in *E. coli* at this temperature. However, the rate of growth *E. faecalis* is more intense as the roughness depressions fill from 2.68 microns to 0.63 microns as opposed to *E. coli* to 0.95 microns. The process of film formation in *E. faecalis* for temperatures +37 ±1 °C, on the surface with different roughness, was more intense compared to *E. coli*, that is, the rate of filling all the roughness depressions took 12 hours. The data obtained indicate that the biofilm density depends on the initial number of microbial cells on the steel surface. It has been shown that the more contaminated the surface with microorganisms, the faster the process of film formation and formation of dense biofilms.

CONCLUSION

It is established that in addition to surface roughness, the shape and size of cells of microorganisms influence the process of biofilm formation. This is due to the fact that at roughness of 0.95 and 0.63 microns, cocoon forms of microorganisms more intensively formed biofilms than rod-shaped ones, since this phenomenon is associated with an increase in the area of contact of bacteria with the surface. The process of forming a biofilm *E. coli* on stainless steel, depended on the surface roughness and the initial number of microbial cells on the surface. The density of biofilms formed at the initial number of cells *E. coli* up to 1 thousand per cm^2 the area was on average 1.8 – 2.2 times ($p \leq 0.05$) lower, compared to the biofilm formed on the surfaces with the initial number of microbial cells 2 – 10 thousand and 20 – 50 thousand per cm ^{2} area of steel. This indicates that in order to prevent the formation of high density biofilms it is necessary to carry out careful sanitary treatment of dairy equipment. It was found that at a favorable temperature *E. faecalis* for 9 – 12 hours. capable of forming medium- and high-density biofilms on stainless steel surface with a roughness of 0.955 ±0.092 μm. However, the density of biofilms at the initial cell count *E. faecalis* up to 1 thousand per cm ^{2} the area was an average of 1.5 – 2.1 times ($p \leq 0.05$) ower, compared to the biofilm formed in the variants with the initial cell number of 2 – 10 thousand and 20 – 50 thousand per cm^2 area of steel.

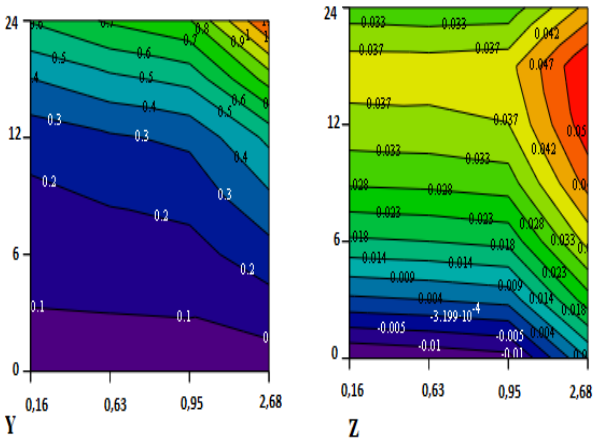

Figure 10 Charts of optical density changes (Y) and the intensity of development (Z) *E. coli* from surface roughness and incubation time in the process of biofilm formation at temperature +17 ±1 °C with the initial number of microbial cells 2 – 10 thous and sm^2 area.

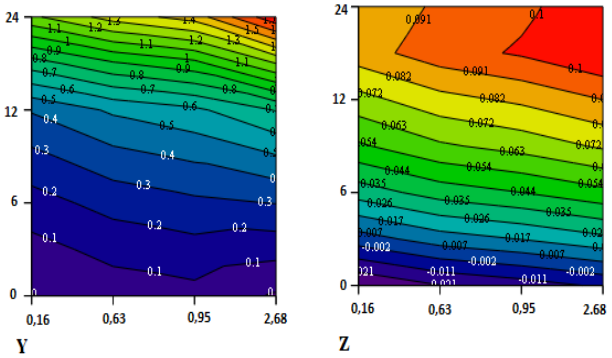

Figure 11 Charts of optical density changes (Y) and the intensity of development (Z) *E. coli* from surface roughness and incubation time in the process of biofilm formation at temperature +25 ± 1°C with the initial number of microbial cells 2 – 10 thous and sm^2 area.

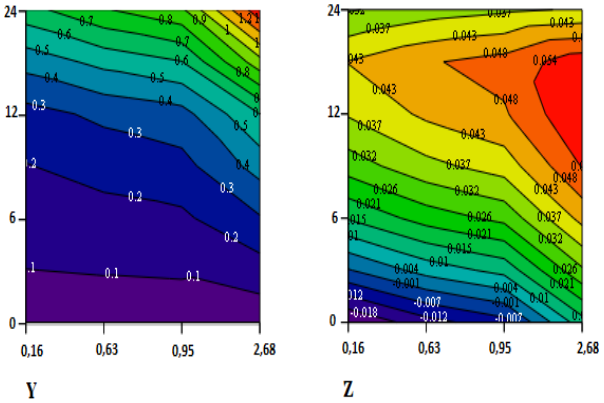

Figure 12 Diagram of change of quantity (Y) and the intensity of development (Z) *E. faecalis* from surface roughness and incubation time in the process of biofilm formation at temperature +17 ±1 °C with the initial number of microbial cells up to 2 – 10 thous and sm^2 area.

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INVESTIGATION OF CONSUMER BEHAVIOUR AT SELECTED MARKET COMMODITY

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ABSTRACT

Consumer behavior is an unexplored area of life for all buyers and sellers alike. Knowledge of consumer behavior brings better market orientation and more consistent establishment of individual products in consumers' shopping baskets. In examining this behavior, the authors of the paper focus on questions such as why, how, where, when, and how much consumers are willing and able to buy at a particular market price. The behavior in question is influenced by several factors (cultural, social, psychological and personality) that influence the final consumer decisions. The main objective of the present document is to evaluate consumer behavior, their purchasing preferences and also to make decisions when buying a particular food product – tea. The primary data were obtained through a questionnaire survey carried out on a sample of 640 respondents. The questionnaire was divided into two basic parts - the demographic part and the part dealing with consumer behavior at the selected market commodity. In the questionnaire processing the respondents were filtered based on their answers. The research has shown that more than 78% of respondents consume tea while women consume it more (50%). Flavor (56.3%) proved to be the most important factor in the selection of a particular tea. Almost half of the respondents reported consuming cut tea (46.5%). In the present document, assumptions were formulated which serve for a deeper analysis of the issue. The relevance of the formulated assumptions was verified by the XLSTAT statistical software. Data were evaluated by qualitative statistics – Chi-square test of good compliance, Fisher's exact test, Mann Whitney test and Friedman test.

Keywords: consumer; consumer behavior; tea; shopping behavior; purchase decision

INTRODUCTION

Consumer behavior is one of the constantly evolving and changing elements of today's world **(Carlucci et al., 2015)**. It can be defined as a certain behavioral process that reflects its internal and external properties **(Goel et al., 2010)**. Through the cooperation of the above-mentioned characteristics, consumers make their purchasing decisions in the market of goods and services **(Marriott et al., 2017; Pierański et al., 2017)**. Consumer behavior is generally defined as the behavior of people or groups of people whose primary role in this process is to meet needs at their best possible level **(King et al., 2010; Hoffman and Novak, 2015)**. There are several factors influencing good consumer choice **(Kacen et al., 2012)**. Among the basic influencing components are mainly price but also brand, quantity, and ultimately consumer preferences **(Huang et al., 2004)**. **Yoon et al. (2012)** claims that food trends and eating habits also influence consumer decision-making. Habit also plays an important role in purchasing behavior, i. what customers and consumers are taught to buy **(Major and Vincze, 2010)**. The habit is that consumers are willing to reach for a product they know, trust, and are satisfied with **(Behrens et al., 2010)**. An important factor in the purchase is also the impulse, i.e. consumers buy without a specific idea **(Amos et al., 2014)**. Last but not least, the consumer is also affected by advertising, which has a great weight in creating consumer opinion on a specific product **(Kopalle and Lehmann, 2006)**. Consumer opinion is created by direct and indirect marketing forms. According to **Chikweche and Fletcher (2010)**, direct form of marketing has more influence on decision-making than methods based on different kinds of other media (television, press, radio).

In general, consumers can be characterized as any individual who satisfies their needs by purchasing goods and services **(Hoyer et al., 2010; Golian et al., 2018)**. In terms of economic definitions, the consumer is an individual, an enterprise, a company or a family. So, consumers are actually all those who buy and consume goods and services **(Priest et al., 2013; Kozelová et al., 2011)**. Consequently, consumers are all people purchasing

in the market of goods and services. However, there are also customers in this market. The difference between customers and consumers is not perceived by ordinary people. However, for the professional public the difference is very significant. **Bordalo et al. (2013)** states that a consumer is a person who purchases and also consumes purchased goods. The customer is only the person who purchases the goods but does not consume them **(Vivek et al., 2014; Mokrý et al., 2016)**.

The consumer is able to orientate himself in the market of goods and services on the basis of several of the above menioned factors **(Bogomolova et al., 2018)**. More specifically, the document focuses on consumer behavior in the tea market. This commodity is considered as one of the most popular and cheapest drinks worldwide **(Raynolds and Ngcwangu, 2010; Trembecká et al., 2013)**. Given its increasing demand, this commodity is considered to be one of the main elements of world trade **(Lee and Chambers, 2010; Godoy et al., 2013)**.

Scientific hypothesis

Assumption no. 1: We assume that there is no dependence between whether sex has an impact on tea drinking.

Assumption no. 2: We assume that there are differences between the influence of individual factors on the purchase of teas.

Assumption no. 3: We assume that there is no dependence on whether the respondent's age affects the preferred type of tea.

Assumption no. 4: We assume that there is a dependence on whether the respondent's age affects the consumption of even loose teas.

MATERIAL AND METHODOLOGY

The methodological part of the paper was based on a questionnaire survey, which was carried out both in physical form and through the electronic platform Google Forms. The questionnaire survey started in January and lasted until May in 2019. The survey was carried out on the territory of the Slovak Republic. We obtained a sample of 640 respondents of different ages. The goal was to get a relevant number of responses to the questionnaire survey. The survey was divided into two parts. The first part dealt with the demographic categorization of respondents and the second part focused on consumer behavior in the market of a particular food product. The main task of the questionnaire survey was to find out the awareness and behavior of individual consumers. The survey was used to identify buying preferences, consumer behavior and decisions within a particular food market.

The structure of respondents by sex was as follows – 58% women and 42% men. The age structure was divided into 4 age categories and ranged from 21 to 51 years and over. The largest representation was in the age structure from 21 to 30 years (42.2%). The highest level of education was secondary school with GCSE (42%). More than 1/3 of respondents have finished university. Regarding to the economic status of respondents, most of them (49%) are employed. The second largest component was students – 200 respondents. In terms of marital status, most respondents marked free / single status. Their number reached more than half of the survey participants (54%). The second most marked response was the possibility of married. There were 39% of such respondents.

Statistic analysis

Prior to the questionnaire investigation, scientific assumptions were established and subsequently confirmed or rejected by selected statistical methods. The verification of the assumptions was carried out using the XLSTAT statistical software. Pivot tables that were processed in the program were applied to the obtained primary data, and the data were then evaluated using qualitative statistics – Chi-kvadrat goodness-to-fit test, Fisher's exact test, Mann Whitney test and Friedman test.

We will determine the probability level – alpha ($\alpha = 0.05$), which will be compared to the significance level (*p*-value). Based on alpha (α), we can evaluate the hypothesis with the *p*-value comparison. If *p*-value is lower than alpha (α), we will refuse H_0. If *p*-value is higher than alpha (α), we will not refuse H_0.

RESULTS AND DISCUSSION

640 respondents participated in the survey. Primary data were obtained to a greater extent from women than men. The number of women in the research reached 58%, with men accounting for 272 respondents (42%). Most respondents were between the ages from 21 to 30 (42%). This age limit will be the most important for us as we assume the most frequent consumption of tea. Other age groups were approximately equal. The second largest group was in age from 41 to 50 years old – 133 respondents. Furthermore, the age limit was from 31 to 40 years. Representation in this category was 20%. The last place with the number of 110 respondents placed the age limit of 51 years and more. Almost half of the respondents reported that their highest education was secondary school with a school leaving exam (42%). More than 1/3 of respondents have completed university. Regarding the economic status of respondents, most of them (49%) are employed. The second largest component were students - 200 respondents. The last and significant question of the demographic part was the respondents' net monthly income (Figure 1).

The figure clearly shows that the most respondents have a monthly income between 501€ and 800€. The second place with 18.5% ranked from 801€ to 1.000€. Least of respondents declare their monthly income was between 151€ and 300€. This result is probably due to the fact that some respondents still attend higher education and thus their income opportunities are limited.

The following group of questions dealt with questions about a particular type of food product we selected. We decided to focus our further implementation on a particular food type – tea. More specifically, we focused on consumption, purchase and related matters.

The first and basic question of this survey was the one that dealt with tea consumption. As shown in the Table 1 below, most of the respondents said they consumed tea.

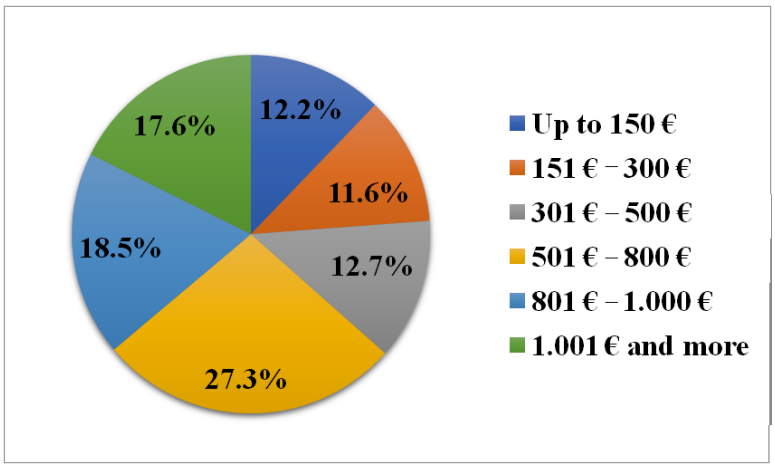

Figure 1 Monthly income of the respondent.

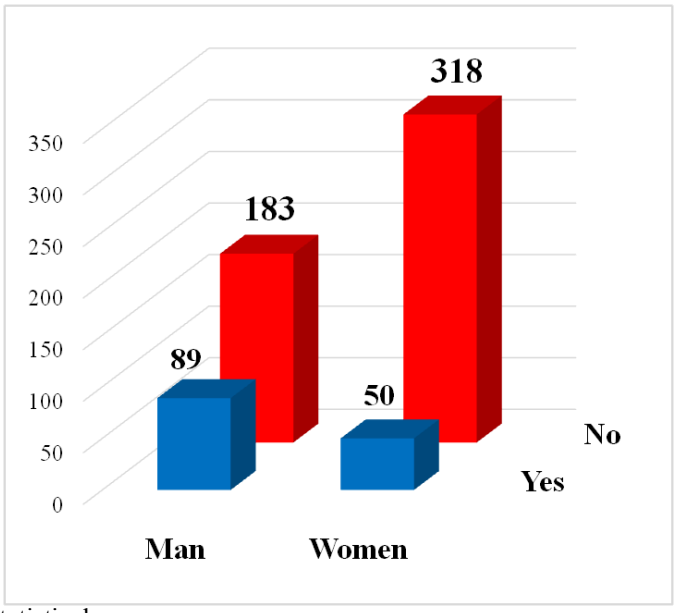

Figure 2 Output from the statistical program.

Table 1 Consumption of tea.

Consumption of tea	*Count*	*%*
Yes	501	78.3
No	139	21.7

From the information mentioned above we can state that most of the respondents consume tea. 78.3% of respondents expressed a positive answer when consuming this kind of not only hot but also cold drink. Positive figures for tea consumption were also achieved by a survey by the team of **Lee et al. (2010)**. 21.7% of respondents have a negative relation to tea consumption.

Figure 2 shows that women consume tea much more than men. If we look at the negative answers, we can see that 50 women do not consume tea. What is lower than men (89 respondents). A greater positive attitude of women to tea drinking has also been found in a survey by **Hegarty et al. (2000)**.

Based on the factors, statistical observations were made on the presumption - whether there was any dependency on whether sex had an impact on tea drinking.

H_0: There is no correlation between whether sex affects tea drinking.

H_1: There is a correlation between whether sex affects tea drinking.

We will use *p*-value from Fisher's exact test to verify the hypotheses. Testing will be carried out at the selected level of 5% significance, i.e. alpha will be 0.05. In this case, *p*-value = 0.0001, which means that we reject the null hypothesis. We accept the alternative hypothesis and assert that with 95% confidence there is a dependency on whether sex has an impact on tea drinking. Based on the test results, we consider our assumption to be incorrect. Subsequently, the Figure 2, which was implemented through the XLSTAT statistical program, is also presented.

That means that the survey is over for some respondents. They filtered out respondents that do not consume tea, so that testing could continue to evaluate the primary data collected. From the original number of 640 respondents, the evaluation continues with 501 respondents.

The following question concerned the frequency of tea consumption. We can say that a large number of respondents drink tea very often. Most respondents said they consume tea every day (27.9%).

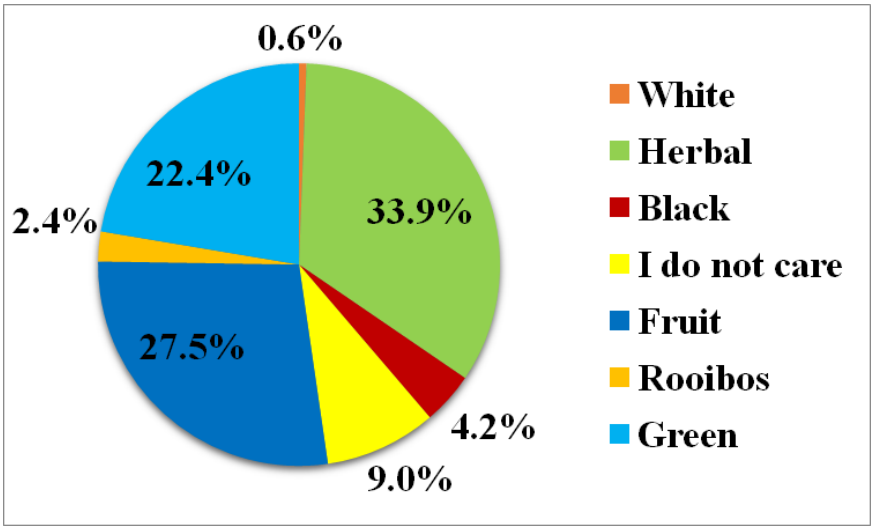

Figure 3 Tea flavor.

This was followed by a frequency of 1 – 2 times a week, which was indicated by up to 23.3%. The frequency was placed third place, that is 3 – 4 times a week with 24.2%. This frequency of tea consumption is also due to the fact that some people do not drink coffee and thus replace it by tea. Positive tea consumption also results from the drinking regime, as some types of tea do not contain any undesirable substances (especially caffeine) and so people who love tea can consume it (**Mineharu et al., 2011**). The last place was taken by the posibility of drinking tea occasionally. Only 13.7% identified this option, which is 78 respondents.

Furthermore, we were interested in the specific preferred type of tea among the respondents. As shown in the Figure 3, the most preferred type of tea is herbal. The figure shows that as many as 33.9% of respondents stated herbal tea preference. This type of tea, according to a team of authors **Bobková et al. (2015)**, is very popular with consumers because herbs are attributed to various medicinal effects (colds, diabetes, digestive problems, etc.). The importance of drinking herbal teas has also been confirmed in a survey by **Deetae et al. (2012)** and **Ulusoy et al. (2009)**. Fruit tea was on the second place (27.5%) and green tea was ranked as the third one with 22.4%. White tea is preferred by the smallest number of respondents (0.6%).

The following group of questions focused on respondents' buying preferences when buying teas. The questions were focused on the individual factors of the impact of the choice of a particular tea, the place of purchase of tea and also the price level that respondents are willing to spend for packing the tea. The first question was focused on selected factors that influence respondents when buying tea. The respondents were to give a rating of 1 to 5 with these factors (Figure 4). In this case, number 1 was the least important factor, and number 5 was the most important factor in choosing tea.

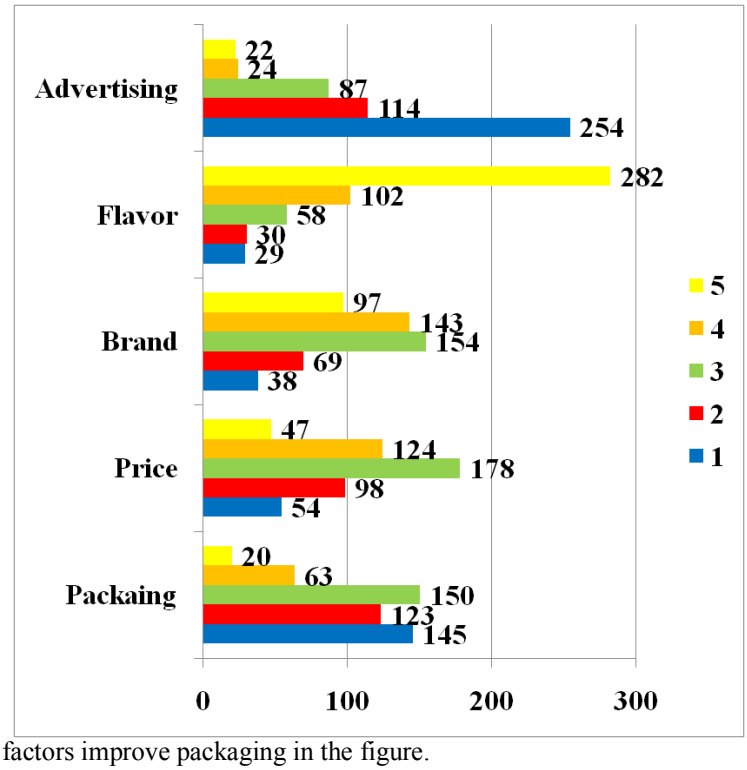

Figure 4 Tea selection factors improve packaging in the figure.

Figure 4 shows that the most important factor in tea selection is the particular flavor. As many as 56.3% of respondents stated that flavors were important. Taste was also found to be the most important factor in tea selection in a survey by **Cho et al. (2006)**. The brand ended up on the second place – 30.7%. The brand is important in the selection of teas for consumers, and this has been confirmed in a survey by **Lee and Lioa (2009)**. In addition, a very important factor has been placed in determining the choice of most products – price. This factor was on the third place with 35.5%. The last two places ended with packaging and advertising. For consumers, the color of the packaging does not play a big role and this survey also confirmed that neither advertising. The reason may be the time we live in, which is very saturated with online marketing tools.

Based on the factors, statistical observations were made on the assumption – whether there are differences between the influence of individual factors on the purchase of teas.

H_0: There are no differences between the influence of individual factors on the purchase of teas.

H_1: There are differences between the influence of individual factors on the purchase of teas.

The *p*-value from Friedman's test will be used to verify the hypotheses. Testing will be carried out at the selected level of 5% significance, i. alpha will be 0.05. In this case, *p*-value = 0.0001, which means that we reject the null hypothesis. We accept the alternative hypothesis and argue that with 95% reliability there are differences between the influence of individual factors on the purchase of teas. Based on the results of Friedman's test, we consider our assumption to be correct.

We used XLSTAT statistical software to verify the impact of factors on the consumer's decision-making process when buying teas. Table 2 again confirmed that the factor – flavor has the greatest impact.

In connection with the previous question, the Mann Whitney test was also used to investigate the dependence of gender and each of the factors (Figure 5). It follows that only one factor (shown in red) showed dependency.

Other dependencies were not demonstrated by the test as their *p*-value was higher than the alpha value (Table 3).

Another question, which falls under the category of consumer buying preferences, was the question of buying, i.e. respondents were asked where they most often buy tea. Most of the respondents buy tea in classic shops, it is up to 80.4%. The assumption of such a large number is clearly that respondents also buy tea during normal shop purchases. This saves effort but reduces the experience of taste, as in traditional shops (supermarket, hypermarket, retail stores) mostly loose teas are not sold **(Kalla and Arora, 2010)**. Pleasing news is that the second most frequently purchased place ended in specialized stores. The unflattering news is that only 10.8% of respondents buy tea in such shops. Tea shop, pharmacy and various e-shops and online stores have reached approximately the same percentage.

The last question about consumer buying preferences was price. Specifically, we asked for the price they are willing to spend on packaging tea. Most respondents said they were willing to spend between 1.1€and 3€ on packaging for tea. 62.3% of respondents indicated this answer. Commonly available teas in classic stores usually fall within this price range. The second most commonly referred response was the amount from 3.1€ to 6€. This price range allows respondents to purchase teas not only in traditional shops, but also in specialized stores. 26.1% of respondents voted for this price range. Other price ranges had approximately the same rating from respondents. Interestingly, 5 respondents saw a price range that they are willing to spend on packing tea, that is 14.1€ or more.

Table 2 Strength of influence of individual factors

Factor	Strength	Groups			
Advertising	1.881	A			
Packaing	2.302		B		
Price	3.139			C	
Brand	3.404			C	
Flavor	4.273				D

Table 3 Output from the statistical program.

Factor	*p*-value	Sing	Alpha
Packaing	0.163	>	0.05
Price	0.204	>	0.05
Brand	**0.022**	**<**	**0.05**
Flavor	0.099	>	0.05
Advertising	0.714	>	0.05

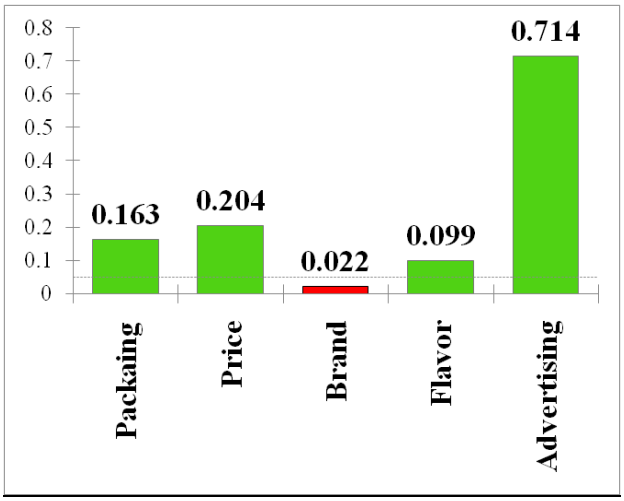

Figure 5 Output from the statistical program.

The following question dealt with the preferences of consuming selected types of teas. We found the most preferred type of tea. A larger number of respondents prefer teabags (46.5%). This form of tea consumption is the most convenient way of making tea for most respondents **(Keuskamp et al., 2013)**. Loose tea ended up on the sekond place with 37.7%. In the last place was the type of tea made from fresh ingredients - 15.8%.

Based on the factors, statistical observation was made on the presumption – whether there is any dependence on whether the respondent's age affects the preferred type of tea.

H_0: There is no dependency on whether the age of the respondent affects the preferred type of tea.

H_1: There is a relationship between whether the respondent's age affects the preferred type of tea.

To verify the hypotheses, we use the *p*-value of the Chi-square test. Testing will be carried out at the selected level of 5% significance, i. alpha will be 0.05. In this case, the *p*-value = 0.0250, which means that we reject the null hypothesis. We accept the alternative hypothesis and say that with 95% confidence there is a dependency on whether the respondent's age affects the preferred type of tea. Based on results. We consider the Chi-square test to be incorrect. Subsequently, we also present Figure 6, which was implemented through the statistical program XLSTAT.

It follows that more respondents also consume loose teas. It is 58.1% of respondents. Compared to men and women, we can say that loose tea is consumed more by women than by men. There were 98 men who also consumed loose teas and 193 women.

Based on the factors, statistical observations were made on the presumption - whether there is a dependency on whether the respondent's age also affects the consumption of loose teas.

H_0: There is no dependence on whether the respondent's age affects the consumption of even loose teas.

H_1: There is a correlation between whether the respondent's age affects the consumption of even loose teas.

To verify the hypotheses, we use the *p*-value of the Chi-square test. Testing will be carried out at the selected level of 5% significance, i. alpha will be 0.05. In this case, *p*-value = 0.0010, which means that we reject the null hypothesis. We accept the alternative hypothesis and assert that with 95% confidence there is a dependence on whether the respondent's age affects the consumption of even loose teas. Based on the results of the Chi-square test, we consider our assumption to be correct.

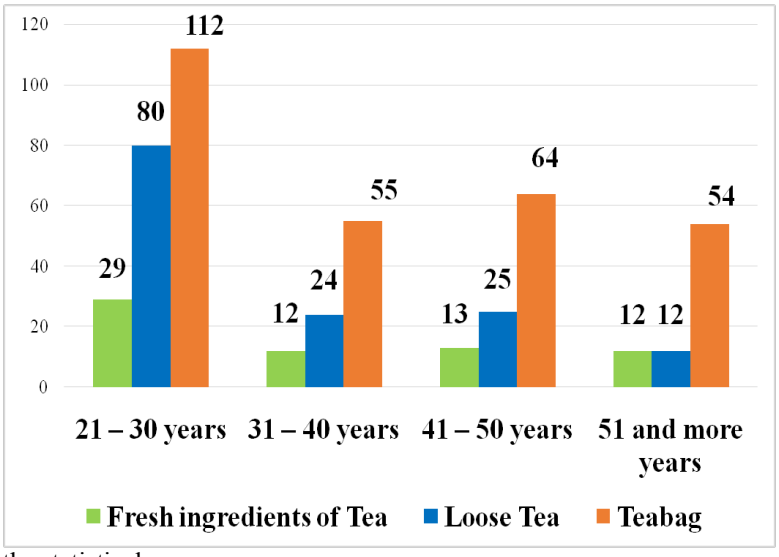

Figure 6 Output from the statistical program.

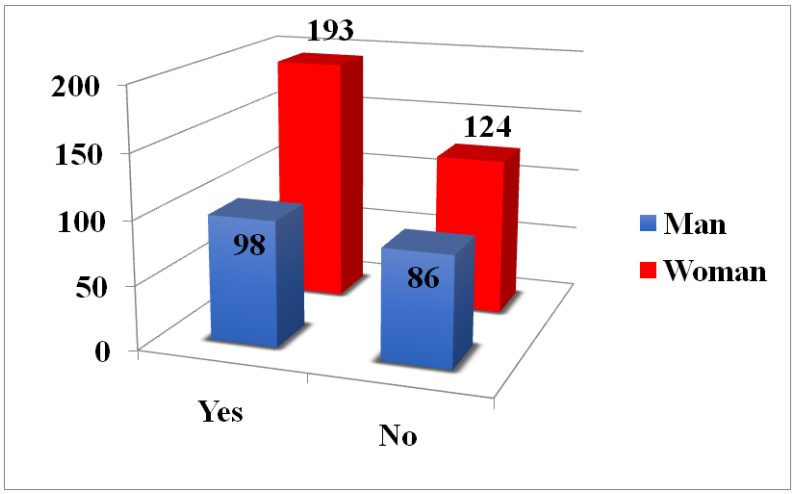

Figure 7 Eating loose teas.

CONCLUSION

Based on the survey, we can conclude that most of the respondents do not have a problem with consumption of tea (78.3%). Approximately 1/3 of the respondents consume tea every day. The most frequent consumption, more than 70%, takes place in the comfort of home. Most of the respondents stated that they consume tea mainly in the morning (26.3%). They do not need any special reason to consume it. 34.2% said they always drink tea when they taste it and most often drink herbal tea (33.9%). More than half of the respondents stated that they made a taste-based decision when buying tea. They buy tea mostly in traditional shops (80.4%), where they buy mainly teabags (46.5%) and are willing to spend from 1.1€ to 3€ for packaging tea. 58.1% of respondents also consume loose teas. They prefer it mainly due to quality (43.3%). The most common reason for buying loose teas is for most respondent's taste – 27.4% and are willing to spend from 2.1€ to 5€for loose teas.

In conclusion, however, we consider it necessary to state that the use of the concept of sensory marketing may encounter some limitations in practice, eg. preventing the use of olfactory, taste and tactile receptors for packaging. Also, in the aroma of marketing there are some unflattering facts such as allergic reaction or inappropriately chosen fragrance, which is likely to result in a negative assessment of individuals. Likewise, it remains only a matter of time before these concepts are actively used in the communication strategy. A big question mark hangs over the ethical aspect of recruiting respondents, as sensory stimuli are often subliminal without the customer being aware of and responding to them.

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COMPOSITION, QUALITY CHARACTERISTICS AND MICROSTRUCTURE OF THE GRAIN *TRITICUM DICOCCUM*

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ABSTRACT

The compositional and quality characteristics of two wheat varieties *Triticum dicoccum* (*Triticum dicoccum* var. *dicoccum*, *Triticum dicoccum* var. *rufum*) produced in the Republic of Azerbaijan have been tested and are relatively useful in assessing their applicability to bread production. The wheat species studied, *Triticum dicoccum*, were found to have a higher protein and cell content, as well as essential proteins of lysine, phenylalanine, leucine and isoleucine, methionine and valine, relative to Gorbustan wheat varieties. The chromatographic method was used to determine the carbohydrate composition of the *Triticum dicoccum* grain. The following redistribution of low molecular weight carbohydrate fractions is noted: the maltose content is higher, and galactose, glucose and fructose are much lower than those of the modern wheat variety Gorbustan. Such a distribution of carbohydrates can reduce the formation of toxic products when baking bread. In addition, the wheat grain *Triticum dicoccum* is characterized by a higher content of sterols, in particular β-sitosterol. The antioxidant activity expressed as percentage inhibition of DPPG free radicals in the *Triticum dicoccum* grain is twice as high as this indicator for wheat of the commercial variety Gorbustan. By scanning electron microscopy, it has been established that the microstructure of the grain surface and the cross section has varietal characteristics. Grain *Triticum dicoccum* var. *rufum* has a thicker shell, tighter and tighter, unlike the grain of *Triticum dicoccum* var. *dicoccum*. With all the benefits of the wheat grain *Triticum dicoccum*, its technological properties were even worse. But the use of technological methods to boost gluten will ensure the production of high-quality healthy bread from old wheat grain.

Keywords: wheat; grain; composition; microstructure; antioxidant activity; technological properties

INTRODUCTION

Recent large-scale epidemiological studies have shown that regular consumption of whole-grain cereal products can reduce the risk of cardiovascular disease and certain types of cancer by 30%, protect against obesity and second type of diabetes **(Chatenoud et al., 1998; Montonen et al., 2003; Larsson et al., 2005; Duchoňová and Šturdík, 2010)**. Nutritionists recommend cereal-based products to ensure dietary fiber, protein, vitamin and mineral diets, primarily found in cereal hulls **(Buddrick et al., 2014; Vitaglione et al., 2008)**. Grain products are a good source of biologically active compounds that inhibit oxidation processes in human plasma. In whole grain products, there are compounds with antioxidant properties: ferulic acid, caffeine, p-coumaric, synapic and other phenolic acids. The highest proportion of total antioxidant potential is found in the bran fraction **(Verma, Hucl and Chibbar, 2009; Ivanišová et al., 2011)**.

Recently, people have become more and more interested in natural and organic foods. In this regard, the older wheat varieties *Triticum monococcum*, *Triticum dicoccum* and *Triticum spelta* have been discovered for use in food technology. The main value of these varieties is their ability to produce good yields on poor soils and to resist fungal diseases. Some populations are tolerant to drought and heat stress **(Zaharieva et al., 2010; Konvalina et al., 2011)**. The nutritional value of the grain of *Triticum dicoccum* is mainly due to the high protein content (18 – 23%), the total proportion of essential amino acids in the protein **(Stehno, 2007)** and the high degree of digestibility of the protein compounds **(Hanchinal et al., 2005)**. An increased concentration of antioxidants has been found in the grain of *Triticum dicoccum* **(Piergiovanni et al., 1996)**. Grain starch is mainly represented by persistent fractions, resulting in slower absorption of carbohydrates **(Mohan and Malleshi, 2006)**. Non-digestible resistant starch is one of the factors that increase the functionality of food products **(Duchoňová and Šturdík, 2010)**. The low glycemic index makes the grain of *Triticum dicoccum* particularly valuable for diabetic nutrition **(Buvaneshwari et al., 2003)**. However, *Triticum dicoccum* grain has been found to have lower β-carotene values than traditional wheat varieties **(Hailu and Merker, 2008)**. A higher concentration of phytic acid has been found in the grain of

Triticum dicoccum compared to traditional raw materials in the bakery industry **(Cubadda and Marconi, 1996)**. Ancient wheat species are known to produce low yields but contain more protein and minerals **(Lachman et al. 2012)**.

The grain of *Triticum dicoccum* contains hard shells that attach closely to the weevil. *Triticum dicoccum* is used throughout the world for the production of traditional foods: roasted cereals, breakfast cereals, pancakes, cereals, baby foods, local pasta types. It has been established that wheat *Triticum dicoccum* can be used for bread making, but with a lower quality than traditional wheat varieties **(Hanchinal et al., 2005; Longin et al., 2016; Kissing et al., 2017)**.

Scientific hypothesis

The composition, quality characteristics and microstructure of the grain of *Triticum dicoccum* indicate the applicability of the varieties tested for the production of bread with yeast.

MATERIAL AND METHODOLOGY

We analysed the indicators of the technological qualities of the grain *Triticum dicoccum* var. *dicoccum*, *Triticum dicoccum* var. *rufum* and compared to Gobustan commercial wheat from *Triticum aestivum*. The tested crops grew in the Absheron peninsula in the Republic of Azerbaijan. The determination of crude protein, starch, cellulose and fat content was carried out according to the methods described by **Yermakov (1972)**. Free and protein bound amino acid content was determined after hydrolysis of the suspensions in sealed 6H HCl ampules for 24 hours using ion exchange chromatography using electrochemical detection on a BIOCHROM amino acid analyser (Biochrom Ltd., Great Britain). Antioxidant activity was determined by the spectrophotometric method in an alcohol extract described by **Silva et al. (2005)** based on percent inhibition of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. We have been determined by the optical density of the solutions in the interaction, Specord M40 (Carl Zeiss Industriel Messtechnik GmbH, Germany) at a wavelength of 515 nm. The concentration of sugars in the grain samples was determined by chromatography using electrochemical detection on an Agilent 1100 liquid chromatograph (Agilent Technologies, USA) with an ESA Coulochem III electrochemical detector. Separation of a mixture of sugars was performed on a grafted amino phase anion exchange column followed by electrochemical detection. The grain samples were milled in a laboratory mill and sieved through a sieve of 0.5 mm diameter. An acetate buffer (0.1 M, pH 5.0) was added to the weights of the resulting flour placed in conical flasks. The solids concentration in the suspension was 100 $g.L^{-1}$. The flasks were placed in a laboratory thermostatic shaker (40 °C, 250 rpm), where the water-soluble components were extracted for 2 hours. At the end of the process, the samples were centrifuged at 14,000 g for 20 minutes, the supernatant was collected and used to determine the sugar concentration with a 10-fold preliminary dilution. The lipid group composition was determined by thin layer chromatography on Silufol plates (Kavalier, Czech Republic) with a fixed layer of silica gel. The plate was previously moistened in a 2% solution of phosphomolybdic acid in acetone. About 10 μg of lipids in the ether solution were applied to the plate as a 10 mm long strip. The chromatogram was developed in the solvent system hexane (Merck, Germany):diethyl ether (Merck, Germany):acetic acid (Merck, Germany) – 80:30:1.5. The plate was air dried until the solvent odour disappeared and placed in a forced ventilation cabinet at a temperature of 80 °C until the appearance of blue spots on certain groups of lipids on the bottom yellow. The identification of individual groups of lipids was performed by comparing the Rf of standard substances with spots on the chromatogram. Quantitative determination of individual groups of lipids was performed by a densitometric method using a Chromoscan 200 instrument (Joyce Loebl & Co, USA). The calculation of the number of individual groups of lipids was performed by the internal standardization method using correction factors: polar lipids – 0.3; sterols – 0.1; free fatty acids – 0.5; triglycerides – 1.0; Sterol esters – 0.14. Microstructural studies were carried out using a JEOL JSM 6390 scanning electron microscope (JEOL, Japan). The pre-prepared samples were placed on a copper disk, a platinum layer was sprayed on a JEOL JEE 44E vacuum evaporator and scanned with a 15 kV accelerated voltage scanning microscope. Determination of the state of the carbohydrate-amylase complex was performed on the device "PChP-7" (LLC "Biophysical Apparatus", Russia) according to the method attached to the device. Gluten quality was determined on the IDK-1M ("Biofizpribor", Ukraine) device.

Statistic analysis

The results were evaluated statistically using the Analysis of Variance. Procedure compares the data in six varieties. The results assays were expressed as mean ±*SD* of eight repeated samples. To evaluate the reliability of the test differences, *t*-statistics (a two-sample t-test for independent samples) were used. The *p*-value used to test the null hypothesis in order to quantify the idea of statistical significance have to be provided. The tests were conducted at a level of significance $p < 0.05$ using the Statistica 7.0 software (StatSoft Inc., USA).

RESULTS AND DISCUSSION

In some countries, traditional foods are made from wheat *Triticum dicoccum*. It is thought to be rich in biologically active compounds and its starch has a slow digestibility. However, the content and composition of biologically active compounds would vary according to geographic location, seasonal variations, varieties used, and methods of analysis used **(Dhanavath and Prasada Rao, 2017)**. The population of *Triticum dicoccum* growing in the Republic of Azerbaijan is poorly studied. Basically, it is used as a material for wheat breeding. However, interest in the production of organic food is growing in Azerbaijan. Table 1 presents the results of the determination of the main nutrient content in the studied species of *Triticum dicoccum*.

Table 1 Essential nutrient content of *Triticum dicoccum* grain.

Component	Content, % (*t/p*) *		
	Triticum dicoccum* var. *dicoccum	***Triticum dicoccum* var. *rufum***	***Triticum aestivum* Gorbustan**
Raw protein	14.8 ±0.21 (9.69/0.001)	16.2 ±0.19 (16.11/0.001)	12.3 ±0.15
Starch	60.1 ±0.56 (4.71/0.01)	59.4 ±0.38 (7.06/0.001)	63.4 ±0.42
Cellulose	6.4 ±0.04 (56.0/0.001)	6.8 ±0.06 (47.7/0.001)	3.6 ±0.03
Fat	1.8 ±0.02 (4.47/0.01)	1.8 ±0.02 (4.47/0.01)	1.9 ±0.01

Note: * (t - student's criterion, P - level of significance).

Table 2 Amino acid composition of wheat protein.

Amino acid	Content, %		
	Triticum dicoccum* var. *dicoccum	***Triticum dicoccum* var. *rufum***	***Triticum aestivum* Gorbustan**
Arginine	0.42	0.31	0.40
Lysine	0.40	0.39	0.34
Tyrosine	0.29	0.21	0.23
Phenylalanine	1.08	0.86	0.76
Histidine	0.34	0.21	0.21
Leucine + isoleucine	1.25	0.87	0.94
Methionine	0.21	0.17	0.15
Valin	0.82	0.38	0.60
Proline	1.63	1.24	1.15
Prolamin	0.54	0.47	0.42
Serine	0.59	0.52	0.47
Alanine	0.71	0.55	0.50
Glycine	0.55	0.36	0.40
Cysteine	0.18	0.15	0.17
Glutamic acid	5.02	3.56	3.30
Aspartic acid	0.84	0.59	0.53
Tryptophan	0.12	0.14	0.11

Table 3 The carbohydrate composition of the grain *Triticum dicoccum*.

Carbohydrate	Content, $g.L^{-1}$ (*t/p*)*		
	Triticum dicoccum* var. *dicoccum	***Triticum dicoccum* var. *rufum***	***Triticum aestivum* Gorbustan**
Galactose	0.02 ±0.001	0.05 ±0.001	0.09 ±0.002
Glucose	0.21 ±0.014	0.24 ±0.012	0.32 ±0.018
Fructose	0.18 ±0.010	0.20 ±0.011	0.30 ±0.015
Maltose	1.85 ±0.028	1.78 ±0.018	1.16 ±0.020
Sucrose	0.14 ±0.003	0.12 ±0.001	0.11 ±0.002

Protein content is one of the most important characteristics of grain, which determines its nutritional value. Our data showed that both tested varieties of *Triticum dicoccum* had a higher protein content than commercial grade wheat. The highest value was obtained for *Triticum dicoccum* var. *rufum*. This is 3.9% higher than that of a modern commercial wheat variety. The high protein content of the grain of *Triticum dicoccum* is consistent with other data **(Dhanavath and Prasada Rao, 2017)**. Data from the literature on the fiber content of *Triticum dicoccum* in wheat grain is contradictory. Some researchers claim that *Triticum dicoccum* wheat is high in fiber **(Čurná and Lacko-Bartošová, 2017)**. According to **Shewry and Hey (2015)**, *Triticum dicoccum* may contain less dietary fiber than modern wheat varieties. According to our data, the fiber content of *Triticum dicoccum* wheat was 2.8 to 3.2% higher than in the grain of the modern variety. Our data on starch and fat content are consistent with those reported in the literature **(Giambanelli et al., 2013)**. Differences in the obtained values of the content of the main nutrients in the grain of *Triticum dicoccum* are significant, $p \leq 0.05$ in all studied parameters.

Table 4 Fractional composition of lipids in the grain *Triticum dicoccum*.

Fraction	Content groups of lipids in the grain, % (*t*/*p*)*		
	Triticum dicoccum* var. *dicoccum	***Triticum dicoccum* var. *rufum***	***Triticum aestivum* Gorbustan**
polar lipids	3.1 ±0.012 (8.9/0.001)	3.4 ±0.014 (4.5/0.01)	3.3 ±0.020
monoglycerides	0.3 ±0.002 (35.4/0.001)	0.5 ±0.001 (54.3/0.001)	0.4 ±0.002
diglycerides	0.27 ±0.01 (4.9/0.01)	0.31 ±0.03 (3.5/0.05)	0.2 ±0.01
sterols	1.3 ±0.010 (62.5/0.001)	1.5 ±0.010 (15.6/0.001)	0.5 ±0.008
β-sitosterol	2.8 ±0.018 (36.9/0.001)	2.9 ±0.014 (48.8/0.001)	2.0 ±0.012
free LCD	3.7 ±0.011 (66.9/0.001)	4.1 ±0.012 (85.0/0.001)	2.4 ±0.016
triglycerides	74.0 ±0.34 (2.86/0.05)	73.8 ±0.30 (2.6/0.05)	72.6 ±0.35
sterol esters	4.1 ±0.22 (11.1/0.001)	3.8 ±0.15 (14.4/0.001)	7.4 ±0.20

Note: * (*t* - student's criterion, *p* - level of significance).

It should be noted that the nutritional value of the grain is determined not only by the total amount of protein, but to a greater extent by its amino acid composition. Table 2 presents the results of the determination of the amino acid composition of the *Triticum dicoccum* grain protein and the winter soft wheat grain traditionally used in food production. Despite the fact that the protein content is higher in wheat varieties, *Triticum dicoccum* var. *rufum*, protein *Triticum dicoccum* var. *dicoccum* has a great nutritional value. He found a higher content of essential amino acids lysine, phenylalanine, leucine and isoleucine, methionine, valine. The grain of *Triticum dicoccum* is richer in lysine than modern commercial wheat, which is consistent with data from other scientists **(Abdel-Aal and Hucl, 2002)**. Chromatographic method was used to determine the carbon composition of the *Triticum dicoccum* grain of two varieties and the modern wheat variety. The results obtained are presented in Table 3. The results indicate that the amount of monosaccharides in the grain of *Triticum dicoccum* var. *dicoccum* and *Triticum dicoccum* var. *rufum* is lower than in the grain of the modern wheat variety, but the maltose content is higher. It is a positive fact that is important for the safety of cereal products, in the technology for which there is a heat treatment. Acrylamide is one of the products of the Mayer's reaction. This toxic product that forms in food products during their processing due to the reaction between reducing sugar and asparagine at a temperature above 120 °C. maltose > lactose **(Wang and Xu, 2014)**. **Žilić et al. (2017)** observed a similar composition of reducing sugars in the wheat grain *Triticum dicoccum*. Table 4 presents the results of the study of the fractional composition of the lipids of wheat grain. In recent years, plant sterols have received increasing attention because of their health benefits. We found that in the wheat grain *Triticum dicoccum*, the sterol content was 2.6 to 3.0 times higher, the β-sitosterol was 0.8 to 0.9% higher and the fatty acids were 1.3 to 1.7% higher than modern grain wheat. According to **Iafelice et al. (2009)**, the sterol profile present in tetraploid and hexaploid wheat species is the same, but there are differences in relative amounts and distribution. **Grela (1996)** found that *Triticum dicoccum* had a high proportion of monounsaturated fatty acids in its fatty acid composition, averaging 21.5% compared to 12.1% for wheat. The ranges of variability of sterols in the grain of *Triticum dicoccum* are consistent with data from other authors **(Giambanelli et al., 2013)**. Although data on the content and composition of bioactive components of ancient wheat varieties are limited, published studies show that they differ little from modern wheat varieties in terms of the content of most compounds **(Shewry and Hey, 2015)**. Wheat is known to contribute significantly to the antioxidant status, having a beneficial effect on human health **(Hejtmánková et al., 2010)**. The results of the study showed that the overall antioxidant properties were different among the three wheat varieties tested. The grain of *Triticum dicoccum* var. *dicoccum* had an antioxidant activity of 15.5% inhibition of DPPH, *Triticum dicoccum* var. *rufum* – 18.3% inhibition of DPPH, Gobustan variety of *Triticum aestivum* – 7.9% inhibition of DPPH. The antioxidant activity of the grain of *Triticum dicoccum* is probably associated with a higher content of flavonoids and vitamins **(Serpen et al., 2008; Calzuola et al., 2013)**.

Figure 1 shows microphotographs of shell surface and grain cross-section of three wheat varieties studied. The native surface has a characteristic relief consisting of parallel formations of cellulose fibrils. In different varieties of wheat studied, the epidermal derivatives of the components of the polysaccharide matrix have a different coating thickness; therefore, on the microphotographs, the cords of cellulose are not clearly visible everywhere. In wheat, *Triticum dicoccum* var. *dicoccum* and *Triticum dicoccum* var. *rufum* is probably the superficial layer formed by hemicelluloses is more pronounced. Approval by **Hanchinal et al. (2005)**, that the *Triticum dicoccum* grain contains rigid shells that attach closely to the weevil and can not treat all varieties of this species. The microphotographs show that the grain of *Triticum dicoccum* var. *rufum* has a shell of 70 – 100 microns, closely related to the grain.

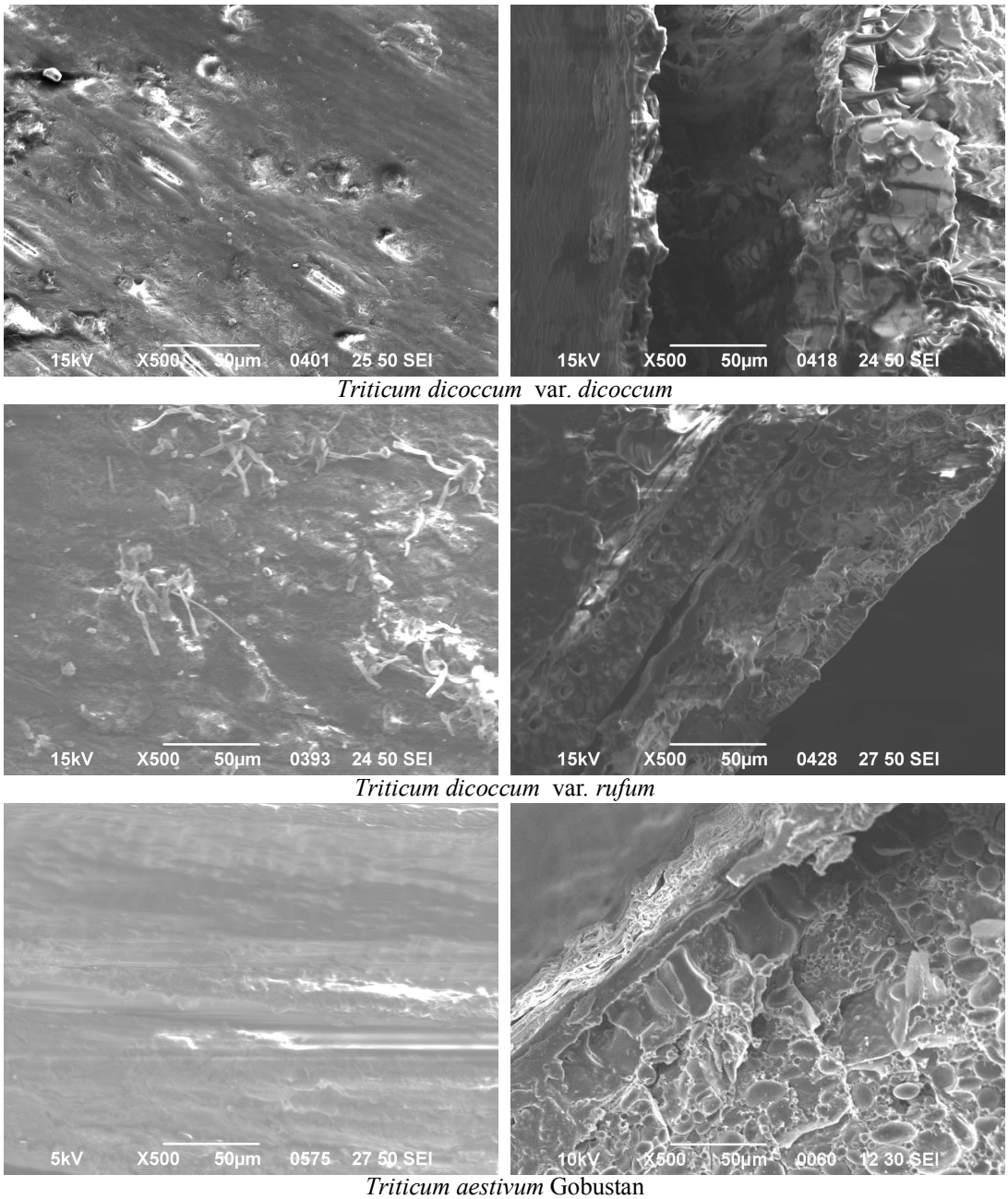

Figure 1 Microphotographs of superficial structures and cross-sections of the grain of three wheat varieties. Increase x500 zoom. Photo: Stolyarov (2019).

Table 5 Some technological properties of grain *Triticum dicoccum*.

Sample	Mass of raw gluten, g.100g^{-1} (*t*/*p*) *	Instrument reading IDK-1M, unit. (*t*/*p*) *	Value of the indicator PE, s (*t*/*p*) *
T*riticum dicoccum* var. *dicoccum*	14.8 ±0.21 (9.69/0.001)	16.2 ±0.19 (16.11/0.001)	12.3 ±0.15
Triticum dicoccum var. *rufum*	60.1 ±0.56 (4.71/0.01)	59.4 ±0.38 (7.06/0.001)	63.4 ±0.42
Triticum aestivum Gorbustan	6.4 ±0.04 (56.0/0.001)	6.8 ±0.06 (47.7/0.001)	3.6 ±0.03

Note: * (*t* - student's criterion, *p* - level of significance).

Grain *Triticum dicoccum* var. *dicoccum* has a pronounced detachment of the shell of the stone fruit. The thickness of the shell can be up to 35 microns, the cavity between the shell and the layer of aleuron has dimensions up to 90 microns. It can be assumed that the grain *Triticum dicoccum* var. *dicoccum* will be easier to peel during flour production. The composition of the seed coat of the grain *Triticum dicoccum* depending on the variety and area of

growth will be the subject of our further research. Some technological properties of the grain of *Triticum dicoccum* have been studied in relation to a modern commercial wheat variety. The experimental data are presented in Table 5.

The quality of the flour for bread baking depends both on the protein content and the type of protein, in particular the composition of the gluten. In general, it should be noted that the two wheat varieties studied, *Triticum dicoccum*, had the worst technological properties for bread production compared to the modern commercial variety. This confirms the results of research by other scientists who have shown that, in addition to their protein content, the technological properties of ancient wheat varieties are considered worse than those of ordinary wheat **(Geisslitz et al., 2018; Petrenko et al., 2018)**. The grain of *Triticum dicoccum* has a gluten content and a high gluten stress index. This suggests that you can get high quality bread. However, the index of the gluten strain is within the limits of using yeast bread from *Triticum dicoccum* flour by applying special methods to enhance gluten. The preliminary laboratory preparation of bread from *Triticum dicoccum* flour resulted in bread with a porosity of 60% and a specific volume of 2.1 $g.cm^{-3}$ confirming the reception of bread. These products can be used for the manufacture of flour confectionery.

CONCLUSION

Thus, it has been established experimentally on the grain of the varieties of ancient wheat *Triticum dicoccum* var. *dicoccum* and *Triticum dicoccum* var. *rufum* has a nutritional relationship to the modern commercial wheat variety *Triticum aestivum* Gobustan. *Triticum dicoccum* was 2.5 and 3.9%, grain fats 2.8 and 3.2% more. Results of the distress of the composition. That is, the grain protein of ancient wheat has a higher nutritional value. The study of the grain carbohydrate composition showed that the amount of monosaccharides in the grain of *Triticum dicoccum* var. *dicoccum* and *Triticum dicoccum* var. *rufum* is lower than the modern wheat grain, but the maltose content is on average higher by 0.65 $g.L^{-1}$. This suggests that the rate of acrylamide formation when baking bread from the studied grain will be lower, which will ensure product safety for consumption. We also found that in the wheat grain *Triticum dicoccum*, the sterol content was 2.6 to 3.0 times higher, β-sitosterol was 0.8 to 0.9% higher and that the acids free fat was 1.3 to 1.7% higher than that of modern wheat grain. Grain *Triticum dicoccum* var. *dicoccum* had an antioxidant activity of 15.5%, the grain *Triticum dicoccum* var. *rufum* – 8.3%, variety of *Triticum aestivum* Gobustan – 7.9% inhibition of the radical DPPH. Microphotographs of shell surface and grain cross section of three varieties of wheat tested showed that there were varietal differences in coating thickness of epidermal derivatives of the polysaccharide matrix and the thickness of the grain envelope. The rigidity of the seed coat structure and the density of its weevil adhesion may also vary according to the varieties of *Triticum dicoccum*. The two wheat species studied, *Triticum dicoccum*, had the worst technological properties for bread production compared to the modern commercial variety. However, our preliminary bread baking laboratory with *Triticum dicoccum* flour has enabled us to obtain a good quality yeast bread. Thus, the use of *Triticum dicoccum* in breadmaking can broaden the range of healthy breads containing biologically active components and having preventive properties.

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INFLUENCE OF SUSPENSION LIQUID TOTAL SOLIDS ON *E. COLI* O157:H7 SURVIVAL AND TRANSFER EFFICACY BETWEEN GREEN TOMATOES AND CARDBOARD

Oleksandr Tokarskyy, Mykhaylo Korda

ABSTRACT

The objectives of this study were: a) to determine *E. coli* O157:H7 survival on tomatoes and cardboard squares post-drying, stored at 25 ºC in humidified environment for four days, in buffered peptone water (BPW), and 0.1% diluted peptone (DP); b) to determine pathogen transfer rates (0, 1.5, or 24-hours drying post-inoculation), from inoculated tomato surfaces to uninoculated cardboard squares and conversely; and c) to evaluate SystemSure Plus ATP luminometer for recognizing contamination on visibly soiled (BPW) or visible clean (DP) cardboard. In tomato inoculation studies, *E. coli* O157:H7 survived better on the fruit when the inoculum was prepared using DP as compared to BPW. The 1.5-hours post drying counts of 5.34 and 5.76 log10 CFU.mL^{-1} in the rinsate substantially declined to 1.45 and 1.17 log10 CFU.mL^{-1} on day four, for DP and BPW, respectively. In cardboard inoculation studies, *E. coli* O157:H7 persisted for four days, with 1.5-hours post-drying counts and day four counts of 4.53 (DP) and 2.55 log10 CFU.mL^{-1} (BPW), contrary to 3.81 (DP) and 1.92 $\log_{10}$ CFU.mL^{-1} (BPW). Under the first impression, the slower die-off of *E. coli* O157:H7 on cardboard questions the possibility of reusing cardboard boxes due to the potential for cross-contamination. In wet transfer (0 hour drying) trials, both tomato-to-cardboard and cardboard-to-tomato yielded 100% positive transfers irrespective of diluent type. Dry transfer (1.5-hours drying interval post inoculation) from tomato-to-cardboard were 100% positive, but no positives were noted when inoculated, dried cardboard was contacted to tomatoes, irrespective of diluent. Results of transfers with BPW as the diluent showed 100% positive transfer from 24-hours dry tomatoes-to-cardboard, as inoculation spots on the tomatoes remained moist due to hygroscopic nature of solutes in BPW. Conversely, only a 40% positive transfer rate was observed under the same conditions with DP as diluent. No positive transfers were recorded from 24-hours dry cardboard-to-tomatoes, irrespective of diluent type. Though *E. coli* O157:H7 survived better on the surface of cardboard compared to the surface of tomatoes on day four, the dry transfers were more efficient from tomatoes-to-cardboard than conversely, possibly due to smooth and hydrophobic properties of the tomato, and rough and porous surface of the cardboard. ATP luciferase UltrasnapTM swab test showed 9/9 “pass” results for sterile liquid DP and BPW, while 9/9 “fail” results were observed with liquid peptone and BPW contaminated at ca. 9.0 log10 CFU.mL^{-1} *E. coli* O157:H7. Cardboard squares treated and dried, with sterile DP, showed 8/9 “pass” ATP luciferase results, and 1/9 “warning”, while cardboard squares with contaminated DP showed 9/9 “fail” result. Cardboard squares treated and dried, with sterile BPW, showed 7/9 “pass” ATP luciferase results, and 2/9 “warning”, while cardboard squares with contaminated BPW showed 9/9 “fail” result. Luminometer can simplify detection of microbial load, as well as organic residues, helping to check cardboard boxes for cleanness.

Keywords: tomatoes; cardboard; *E. coli* O157:H7; survival; transfer; cross-contamination; ATP luciferase test

INTRODUCTION

Tomatoes are important commodity, with the United States (US) an Ukraine among top-fifteen producers worldwide **(FAOSTAT, 2017)**. Morevover, the US is the fourth leading producer of tomatoes in the world, behind China, India, and Turkey **(FAOSTAT, 2017)**. Fresh tomatoes are produced in every state, with commercial scale production in 20 states. In addition, Florida has tomato production on ca. 30,000 – 40,000 acres, accounting for almost one-third of total US fresh tomato acreage **(FDACS, 2018)**. The food safety concerns associated with fresh tomatoes are related to absence of a terminal pathogen reduction step as tomatoes are often consumed fresh, not cooked **(Gurtler et al., 2018)**. Tomatoes are generally contaminated with various groups of microorganisms from the environment **(Tokarskyy and Korda, 2019)**. According to **Beuchat and Ryu (1997)**, enteric pathogens can contaminate tomatoes through

wildlife, irrigation water, handling by workers, wash water, or other contaminated surfaces. Fresh tomatoes prepared for a restaurant were implicated in a multistate outbreak of *Salmonella enterica* infection in 1999 **(Cummings et al., 2001)**. Other well-known outbreaks related to *Salmonella* contaminated Roma tomatoes occurred in the US and Canada in summer 2004 **(Croby et al., 2005)**. The common belief is that Gram-negative enteric pathogens will grow in the tomato pulp if introduced through a wound, cut surface, stem scar, or abrasions **(Wei et al., 1995; Zhuang, Beuchat and Angulo, 1995; Daş, Gürakan and Bayindirli, 2006; Shi et al., 2007; Beuchat and Mann, 2008; Bartz et al. 2015)**, however pathogens will die off if left on the undamaged or bruised skin of the fruit **(Lang, Harris and Beuchat, 2004; Allen et al., 2005; Tokarskyy et al., 2018)**. It is generally believed that *Salmonella* is more robust in surviving under harsh environmental conditions compared to *Escherichia coli* **(Hirai, 1991)**. **Lang, Harris and Beuchat (2004)** showed that *E. coli* O157:H7 spot-inoculated tomatoes showed counts decline by 3.17 log units, while *Salmonella* spp. declined only by 2.20 log units after 24 hours inoculum post-drying.

Several researchers showed that final resuspension diluent for the washed bacterial cells might influence their survival on the surface of tomatoes, with higher organic solids and protein favoring survival **(Wei et al., 1995; Guo et al., 2002)**. For example, **Wei et al. (1995)** showed rapid decline in *Salmonella* counts on the spot-inoculated tomato surface if deionized water was a diluent with counts declining from 5.5 $\log_{10}$ CFU.tomato^{-1} to below detection level in 3 days at room temperature, while pathogen suspended in tryptic soy broth showed minimal decline in numbers under the same conditions. Similarly, **Guo et al. (2002)** showed protective influence of soil favoring survival and growth of *Salmonella* on undamaged tomato surface compared to water alone causing rapid decline in counts. Conversely, the influence of humidity on *E. coli* O157:H7 and *Salmonella* survival in desiccated or humidified state might be more complicated **(Tokarskyy and Schneider, 2019)**. For example, **Møretrø et al. (2010)** showed that Shiga toxin-producing *E. coli* dried in brain heart infusion broth on plastic or steel had highest inactivation rate at 85% relative humidity (RH), while it survived best at 70% and even grew at 98%.

Raw tomatoes are transported to the distribution centers in various packaging, including cardboard, either waxed or unwaxed. The chemical nature of cardboard is a porous wood-derived material, which absorbs liquids, especially in unwaxed state. The question of possible cross-contamination by *E. coli* O157:H7 between tomatoes and unwaxed cardboard remains open.

The first objective of the current study was to determine survival rates of *E. coli* O157:H7, either in 0.1% diluted peptone (designated as low-solute liquid, DP) or buffered peptone water (designated as high-solute liquid, BPW), on the surface of unwashed and undamaged green mature tomatoes and cardboard squares stored at room temperature (25 ºC) in humidified environment within four days of storage. The second objective of the study was to estimate transfer rates of *E. coli* O157:H7 from inoculated surface of tomatoes to the surface of cardboard squares and conversely as influenced by the type of the diluent and timing of the transfer. The third objective of the study was to evaluate effectiveness of ATP luminescence SystemSure Plus luminometer to recognize contamination on heavily and visibly soiled (BPW) or loosely soiled and visible clean (DP) cardboard surfaces. All treatments were visually observed throughout experiments and appearance was subjectively noted, both in humidified 25 ºC incubator and non-humidified 25 ºC incubator.

Scientific hypothesis

We hypothesized that *E. coli* O157:H7 will survive better on the surface of porous cardboard than on the smooth surface of tomatoes, with protective properties of high-solute diluent used. We hypothesized that moisture and high solute would promote *E.coli* O157:H7 cross-contamination between cardboard and tomatoes. we hypothesized that ATP luciferase rapid test would be a helpful aid to identify dirty and contaminated cardboard.

MATERIALS AND METHODOLOGY

Rifampin preparation

Stock solution of rifampin (10,000 ppm) was prepared by dissolution of 0.4 g rifampin (Fisher Scientific, BP26795) in 40 mL HPLC grade methanol (Fisher Scientific) followed by filter sterilization (0.2 µm nylon filter, Fisher Scientific), and storage at 4 ºC in the dark. Antibiotic was added to cooled autoclaved media (Difco™ tryptic soy agar (TSA) or Bacto™ tryptic soy broth (TSB)) to yield 100 ppm final rifampin concentration.

Bacterial culture maintenance and preparation

Five rifampin (200 ppm) resistant *Escherichia coli* O157:H7 strains, MDD19 (alfalfa isolate), MDD20 (Odwalla juice isolate), MDD326 (cantaloupe isolate), MDD 327NA (spinach isolate), and ATCC 35150 (human feces) were used for this study. The first four strains were provided by Dr. M. D. Danyluk's lab (University of Florida, US), and the fifth strain was obtained from American Type Culture Collection (Manassas, WI). Rifampin-sensitive strains were adapted to 200 ppm rifampin as described previously **(Underthun et al., 2018)**. Cultures were maintained on TSA-rif80 ppm slants at 4 ºC with bi-weekly transfers to fresh TSA-rif80 slants.

E. coli O157:H7 strains were streaked on TSA-rif100 plates (37 ºC, 24 hours), and a single colony was transferred to 10 mL TSB-rif100 tube (37 ºC, 12 hours). Two more one loop transfers (ca. 10 µL) were done in 10 mL TSB-rif100 followed by 12 hours and 18 hours incubation at 37 ºC before cultures were ready for experiments. Two mL of each strain were mixed together (total 10 mL, 10^9 CFU.mL^{-1}) and centrifuged (4,300 g, 10 minutes, Sorvall RC-5B centrifuge, DuPont Instruments), followed by a single wash in 10 mL Dulbecco 'A' phosphate buffered saline (PBS, Oxoid, Hampshire, England), and final re-suspension in either 10 mL 0.1% Bacto™ peptone (DP, 0.1 g.L^{-1} of deionized water, Becton, Dickinson, and Co.) or 10 mL buffered peptone water (BPW, Becton, Dickinson, and Co.) using the same centrifugation procedure. Buffered peptone water contained 20 g.L^{-1} solutes, including enzymatic digest of protein (peptone) 10 g, sodium chloride 5 g, disodium phosphate 3.5 g, monopotassium phosphate 1.5 g, as

prepared by manufacturer's instructions. Inoculum concentrations were confirmed by pour plating using TSA-rif100 after serial dilutions in BPW.

Tomato and cardboard squares preparation, inoculation, and storage

Field mature green and breaker stage round tomatoes (*Lycopersicum esculentum,* variety Florida 47) were acquired from local packinghouses in Florida, USA, before processing, being unwashed and unwaxed for the experiments. Tomatoes were dry rubbed with sterile nitrile gloves to remove visible surface contamination. Cardboard squares (ca. 8 by 8 cm) were cut from the lid portions of cardboard boxes in which the tomatoes were packed and were considered as "used."

Tomatoes were inoculated with 0.1 mL of *E. coli* cocktail, either in BPW or in DP, as 10 spots of equal size around blossom end (10^8 CFU.tomato^{-1}). Similarly, cardboard squares were spot inoculated in the center with 0.03 mL of cocktail, either in BPW or in DP (3×10^7 CFU.square^{-1}). The fruit or squares were allowed to dry in a biosafety hood for 90 minutes (1.5 hours) ensuring complete dryness before moving into 25 ºC incubator. A shallow pan with deionized water was placed in the incubator to humidify environment, while humidity and temperature were recorded at 10 minutes intervals for four days (Hobo® U12 data logger, Onset Computer Corp, Pocasset, MA). Sets of three inoculated and dried tomatoes or squares with one negative control were tested immediately after drying (day 0), and sampled on days 1, 2, 3, and 4 for each diluent type from the storage incubator.

On three different occasions, sets of tomatoes and squares were spotted with 30 µL of inoculated DP or inoculated BPW. The specimens were visually observed after 90 minutes drying period and 24 hours later after storage at 25 ºC in either high (shallow pan of water for humidification) or low humidity atmospheres with temperature and humidity in both incubators being monitored as described previously.

Tomato and squares inoculation for the transfer studies

Two separate studies involved pathogen transfers from tomatoes to cardboard and from cardboard to tomatoes. Mature green and breaker stage tomatoes were spot inoculated on undamaged sharpie circle-marked spot on a side of the fruit with 30 µL drop of *E. coli* O157:H7 cocktail, either in BPW or in DP (3×10^7 CFU.tomato^{-1}). Two sets of three cardboard squares were firmly pressed against tomato surface for one second (one square per each tomato) either immediately (wet transfer), 90 minutes after the inoculum has dried up on the tomato surface (90 min dry), or 24 hours after tomato inoculation (24 h dry). The first set of wet transfer was analyzed immediately (W, day 0), while the second set of squares was placed under the biosafety hood to allow transferred liquid to dry on squares for 90 minutes. The second set was then moved to 25 ºC incubator and analyzed after 24 hours (W, day 1). Similarly, one set of 90 minutes dry transfer squares (90 min dry, day 0) was analyzed immediately and another set was placed in 25 °C incubator and tested for pathogen presence 24 hours later (90 min dry, day 1). The last set of inoculated tomatoes was placed for an additional 24 hours incubation at 25 °C including 90 minutes drying period inside biosafety hood before two sets of cardboard squares were pressed against inoculated spots and analyzed for pathogen transfer efficiency either immediately (24 h dry, day 0), or 24 hours later (24 h dry, day 1) after storage in the same incubator (25 °C). The shallow baking pan filled with deionized water was placed inside 25 °C incubator for the duration of the study to humidify atmosphere. Temperature and humidity were monitored as described previously. On each of three days, a negative control square was pressed against the marked surface of uninoculated tomato and analyzed as a negative control to ensure absence of rif-resistant microflora on tomatoes and squares. Transfers from cardboard to tomato surface were done as described previously, but in an opposite direction of inoculation and transfer.

Escherichia coli O157:H7 recovery from tomatoes and squares

A single tomato or square was transferred to a Stomacher® bag containing 20 mL BPW and subjected to vigorous manual shaking for 30 seconds, rubbing for 30 seconds, and final shaking for 30 seconds. The rinsate was either plated directly using spiral plater (WASP2 spiral plater, Don Whitley Scientific Limited, West Yorkshire England), or serially diluted in 9 mL BPW tubes before pour plating with TSA-rif100 medium. The plates were incubated for 48 hours at 37 °C before counting.

Cardboard squares cleanness evaluation by luminometer

The cleanness of uninoculated cardboard squares, as well as those spotted with either sterile DP and sterile BPW, or inoculated diluents, was accessed after 90 minutes drying period using Ultrasnap ATP test by swabbing 3.8 cm by 3.8 cm area including dried spot and measuring ATP activity in the swab following manufacturer's instructions (SystemSURE Plus luminometer, Hygiena, Camarillo, CA). A set of three cardboard squares were analyzed for each treatment. Liquid inocula and sterile diluents were analyzed as well by dipping three separate swabs sequentially in each liquid and proceeding as recommended by instructions.

Statistic analysis

Escherichia coli O157:H7 survival on tomatoes (three replications) and on the squares (four replications) results were analyzed separately using two-factorial experimental design with independent factors of diluent (BPW or DP) and storage timing (90 minutes dry, day 1, 2, 3, and 4). If significant influence of factors were observed (p <0.05), the means were separated using Fisher LSD procedure. Transfer studies were repeated three times and counts data were analyzed using two-factorial experimental design with independent factors of diluent (BPW or DP) and transfer timing with storage (wet transfer, day 0; wet transfer, day 1; 90 min dry transfer, day 0; 90 min dry transfer, day 1; 24 h dry transfer, day 0; 24 h dry transfer, day 1). Similarly, means were separated using Fisher LSD procedure. Percent positive samples for each data point

were calculated for transfer studies as well. Relative air humidity in storage 25 °C incubators was shown as average values with standard deviations. ATP luciferase Ultrasnap™ swab test results (three replications) were expressed as average values of Relative Luminescence Units (RLU) as defined by manufacturer, with standard deviations, as well as ratio of pass/total, warning/total and fail/total per treatments. Statistical analysis was performed using commercially available software Statistica ver. 10.0 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

A diluted peptone water (1 g peptone.L^{-1}, DP) represented a low solute inoculum, while buffered peptone water (Difco™, 10 g peptone, 5 g NaCl, 3.5 g disodium phosphate, and 1.5 g monopotassium phosphate per liter of deionized water, BPW) represented a high solute inoculum. Visual observation of 90 minutes dry inoculated squares and tomatoes followed by storage at 25 °C in either high humidity (RH = 72.5 ±3.0%) or low humidity (RH = 30.4 ±12.9%) confirmed differences between treatments. It was observed that 90 minutes dry inoculated spots appeared dry regardless of diluent. However, inoculated spots with BPW liquefied at high humidity but were dry at low humidity on tomatoes after 24 hours, while spots with DP remained dry in either environment. Spots remained dry on cardboard squares regardless of diluent or humidity; however, spots of DP were untraceable by naked eye, while BPW spots were visible. Therefore, the diluent in which the pathogens were resuspended and humidity where the tomato is stored, might cause moisture to be picked up by dried hygroscopic substances, as observed with BPW. Weather conditions in Florida, where approximately 45% of all tomatoes are grown, are known for high humidity **(FDACS, 2018)**. **Allen et al. (2005)** wrote that tomato packinghouse conditions in Florida late spring are 30 °C and 80% RH, while standard ripening room conditions are 20 °C and 90% RH. The recorded humidity conditions in 25 °C humidified incubator for all inoculation experiments are shown in Table 1.

For tomato surface survival studies, *E. coli* O157:H7 numbers declined from theoretical inoculation level of 6.75 and 6.73 $\log_{10}$ CFU.mL^{-1} rinsate to 5.34 and 5.76 $\log_{10}$ CFU.mL^{-1} upon 90 minutes drying time for DP and BPW, respectively. Both diluent types (BPW; DP) and storage factors, as well as their interaction, had a significant effect on *E. coli* O157:H7 recovery (p <0.05, Figure 1). Visual observation of inoculated spots of stored tomatoes (25 °C, humidified incubator) confirmed that spots with BPW liquified, while spots with peptone water were visibly dry. *E. coli* O157:H7 numbers significantly (p <0.05) declined by 2.4 and 4.9 $\log_{10}$ CFU.mL^{-1} from 90 minutes dry levels on day 2 for DP and BPW, respectively (Figure 1). Following next two days of storage, counts in BPW remained fairly stable (Figure 1). Overall, *E. coli* O157:H7 survived better in DP than in BPW in humidified 25 °C environment, but decline of 3.9 to 4.6 log on day 4 compared to day 0 dried tomato counts was observed in both cases. Similarly, **Lang, Harris and Beuchat (2004)** showed that *E. coli* O157:H7 counts in 5% horse serum on the dried spot-inoculated tomatoes decreased 1.07 $\log_{10}$ CFU.$tomato^{-1}$ after 1 hour drying and additional 2.10 $\log_{10}$ CFU.$tomato^{-1}$ 24 hours post-drying from initial 7.22 $\log_{10}$ CFU.$tomato^{-1}$. Studies by **Tokarskyy et al. (2018)** on survival of *E. coli* O157:H7 on the surface of undamaged raw tomatoes, inoculated at low levels, also showed substantial decline to 1.37 – 2.07 $\log_{10}$ CFU.$tomato^{-1}$ (day 1), 0.30 – 1.80 $\log_{10}$ CFU.$tomato^{-1}$ (day 3), and 0.04 – 0.33 $\log_{10}$ CFU.$tomato^{-1}$ (day 7) from inoculation level of 2.45 – 2.79 $\log_{10}$ CFU.$tomato^{-1}$ (day 0). To summarize, *E. coli* O157:H7 did not survive well on the intact surface of tomatoes.

Survival of *E. coli* O157:H7 on the surface of cardboard squares is shown in Figure 2. As cardboard material was porous and absorbent, inoculated spot liquefaction due to moisture absorbance from the air was not observed in case of BPW. However, dried spots remained visible in case of BPW, but not DP. Both diluent types (BPW; DP) and storage factors, but not their interactions, had significant (p <0.05) effect on *E. coli* O157:H7 numbers (Figure 2). *E. coli* O157:H7 numbers significantly declined from 90 minutes post-drying counts of 4.53 and 3.81 $\log_{10}$ CFU.mL^{-1} rinsate to 2.55 and 1.92 $\log_{10}$ CFU.mL^{-1} rinsate upon 4 days of cardboard storage at 25 °C in humidified atmosphere (p <0.05). These results are comparable to *Salmonella* data by **Kusumaningrum et al. (2003)**, who showed that *S. Enteritidis* was recovered from inoculated dried steel squares for at least 4 days at contamination level of 10^5 CFU.cm^{-2}. It appeared that *E. coli* O157:H7 survived better on cardboard compared to plastic (HDPE), stainless steel, and vinyl belt (PVC), where counts on average declined below 1.0 log unit on the fourth day under the same conditions (unpublished data). It can be speculated that porous organic surface of cardboard might have protective effect on *E. coli* compared to impervious plastic, steel, and vinyl surfaces. Similarly, **Allen et al. (2005)** showed that *Salmonella* survived better on unfinished oak wood compared to stainless steel, vinyl belt, and sponge rollers at 20 °C and 60% RH. However, **Siroli et al. (2017)** showed a rapid decrease in *E. coli* populations from ca. 6.0 $\log_{10}$ CFU.cm^{-2} to ca. 1.5 and 2.5 $\log_{10}$ CFU.cm^{-2} after 24 hours on the surface of cardboard and plastic, respectively. Our results showed better survival of *E. coli* O157:H7 on the cardboard surface, though with substantial decline over 4-day period, possible due to the use of nutrient-rich medium as suspension medium for inoculum. To support our hypothesis, **Wei et al. (1995)** showed *Salmonella* counts fast decline on spot-inoculated surface with deionized water as a diluent (>5.0 $\log_{10}$ CFU.$tomato^{-1}$ in 3 days), while pathogen suspended in tryptic soy broth showed minimal decline within same conditions. Additionally, **Guo et al. (2002)** showed protective influence of soil supporting survival and growth of *Salmonella* compared to water as a diluent, which caused rapid decline in counts. To summarize, *E. coli* O157:H7 can survive on the surface of cardboard for longer than 4 days at room temperature, creating concerns about possible cross-contamination if cartons are reused (Figure 2).

Cross-contamination by *E. coli* O157:H7 between raw produce and common packaging materials, kitchen surfaces, is possible **(Buchholz et al., 2012; Jensen et al., 2013; Jensen el al., 2017, Jung et al., 2017)**, and only

harsh food-processing technologies, such as cooking and ionizing irradiation, can kill pathogenic bacteria in various foodstuff **(Tokarskyy et al., 2009; Schilling et al., 2009)**. Transfer rates studies of *E. coli* O157:H7 between surfaces involved fresh-cut produce and common kitchen surfaces **(Jensen et al., 2013)**, gloved hands and raw fruits and vegetables **(Jensen et al., 2017; Jung et al., 2017)**, as well as commercial pilot plant equipment and raw produce **(Buchholz et al., 2012)**. **Buchholz et al. (2012)** studied transfer possibility of *E. coli* O157:H7 from contaminated produce (iceberg and romaine lettuce) to the commercial processing equipment, followed by processing of uninoculated produce in the same contaminated equipment. The researchers found the highest transfers from inoculated lettuce to the commercial shredder and conveyor belt, with the processed uninoculated produce getting contaminated as well **(Buchholz et al., 2012)**.

Results of the transfer studies were expressed either as percent positive (where at least one *E. coli* O157:H7 CFU.mL^{-1} of rinsate was detected) or as counts, total $\log_{10}$ CFU.item^{-1} (either a cardboard square or a tomato), and are shown in Figure 3, Figure 4, Figure 5, and Figure 6. Samples yielding no counts were assigned a limit of detection count (1.3 $\log_{10}$ CFU.item^{-1}). Wet transfers (W) yielded 100% positive transfers on both day 0 and 1 irrespective of diluent type (Figure 3 and Figure 5). Similar results were shown by **Jensen et al. (2013)**, who investigated transfer rates of E. coli O157:H7 from fresh-cut produce to common kitchen surfaces (ceramic, stainless steel, glass, and plastic). They found the highest transfer rates (over 90%) in case of moist, freshly inoculated produce, and 1-hour dry produce had lower transfer rates, at ca. 0.01 to 5% from inoculated celery, carrots, and lettuce, to ca. 5% from inoculated watermelon. The authors also stressed that surface moisture and direction of transfer had the highest influence on transfer efficiency **(Jensen et al., 2013)**.

Dry transfers from tomatoes to squares appeared to be more efficient comparing to the opposite direction (Figure 3 and Figure 5) possibly due to smooth and hydrophobic properties of the tomato and rough surface of the cardboard. Dry transfers (90 min dry) were 100% positive from tomato to cardboard, and 0% positive from cardboard to tomato. Cardboard squares were easily deformed by the transfer procedure, shaping their surface as tomato was pressed against it. **Jensen et al. (2017)** studied cross-contamination by *E.coli* O157:H7 from gloved hands to carrots, celery, and cantaloupe, and vice versa, and also noted influence of surface type and structure on the transfer efficiency. From gloves, 30% of *E. coli* population was transferred to carrots, 10% to celery, and 1% to cantaloupe **(Jensen et al., 2017)**. Regarding reverse transfers, 1% was transferred from carrots and celery to gloves, and only 0.3% from cantaloupe **(Jensen et al., 2017)**. Results of transfers where the diluent was BPW showed 100% positive transfer from 24 hours dry tomatoes to squares on day 0, as spots on the tomatoes were moist, with residual bacterial concentration found on the squares after 24 hours storage as well. Regarding bacterial counts, influence of both factors (diluent type and transfer timing with storage), as well as their interaction, was significant in case of 'tomato to cardboard transfer' (p <0.05). However, only individual factors, but not their interaction, had significant effect on *E. coli* counts in case of 'cardboard to tomato' transfer (p <0.05). *E. coli* O157:H7 counts on contaminated items after transfer, either immediately after transfer or 24 hours later, are shown in Figure 4 and Figure 6.

In case of successful dry transfers from tomato to cardboard, certain *E. coli* O157:H7 population remained viable on the next day after transfer (Figure 4). Dry transfers from cardboard to tomatoes were unsuccessful and bacterial counts were expressed at detection limit for statistical purposes (Figure 6).

The surface of used uninoculated cardboard squares passed Ultrasnap ATP swab test, as well as surface spotted with sterile DP or sterile BPW followed by 90 minutes drying period (Table 2). Squares inoculated with bacterial suspension in either DP or BPW followed by drying, failed ATP test (Table 2), however, DP inoculated spots appeared visibly clean compared to spots in BPW. Luminometer measures ATP activity, a universal energy molecule for all living cells, transferred to the swab from the surface. Food residues containing remnants of cells, as well as microbial contamination, may harbor ATP in significant quantities. Autoclaving does not destroy ATP **(Ceresa and Ball 2005)**. Though designed to measure organic residue/cleanness, and to a lesser extent, microbial contamination, the ATP test showed that uninoculated used cardboard squares passed cleanness test both if uninoculated or spotted with sterile diluents (with an exception of few "warnings"), while reported contamination when *E. coli* O157:H7 inoculum was used.,

Similarly, **Chen and Godwin (2006)** confirmed that microbial ATP bioluminescence assay can provide quick and convenient test to assess microbial contamination in refrigerators. Significant correlation coefficient between microbial ATP and psychrotrophic plate count PPC (r = 0.851) was slightly higher than that between microbial ATP and aerobic plate count APC (r = 0.823), which indicated a potential discrepancy in the populations of psychrotrophic and mesophilic bacteria on the refrigerator surface; nevertheless, microbial ATP assay appeared to have a potential as a reliable indication of the average of APC and PPC (r = 0.895) **(Chen and Godwin, 2006)**. However, a study performed by **Larson et al. (2003)** of comparing results between colony-forming units counts as natural microbiota on hands and kitchen table from one side, and ATP monitor readings from the other side, showed no significant correlation between the two. The authors noted a precaution of using ATP monitor test instead of aerobic plate counts for evaluation of microbial contamination **(Larson et al., 2003)**. A mini-review by **Shama and Malik (2013)** summarized observations: though significant correlations were shown between microbial numbers and ATP levels under certain conditions (but not within healthcare settings), intracellular ATP levels unfortunately vary between microbial taxa and also depend on environmental conditions. They warned that rapid ATP assays cannot be used instead of microbial pathogen culturing methods, but can be used to estimate effectiveness of cleaning and evaluate overall bacterial load **(Shama and Malik, 2013)**.

Table 1 Relative air humidity with standard deviations (%RH ±st.dev) in the incubators with stored tomatoes and cardboard during survival and transfer studies at 25 °C.

Experiment	Diluent	Trial 1	Trial 2	Trial 3	Trial 4
Survival on tomatoes	DP	58.8 ±3.6	59.4 ±3.8	59.5 ±4.1	NA
	BPW	65.2 ±6.2	64.8 ±6.4	65.3 ±6.0	NA
Survival on cardboard	DP	67.4 ±2.2	70.8 ±2.0	71.6 ±2.1	73.2 ±1.9
	BPW	72.5 ±2.1	72.7 ±2.0	73.0 ±1.8	72.5 ±1.9
T2C transfer & C2T transfer	DP	69.0 ±5.9	67.8 ±6.8	58.7 ±10.8	NA
	BPW	66.6 ±9.9	67.8 ±4.5	70.7 ±3.8	NA

Note: T2C – tomato to cardboard transfer; C2T – cardboard to tomato transfer.

Table 2 Cleanness of the media (sterile and *E. coli* O157:H7 inocula) and inoculated dried cardboard squares as assessed by ATP luciferase Ultrasnap™ swab test.

Liquid/ Squares	Diluent	Avg RLU ± st dev	Pass	Warning	Fail
Liquid	BPW, sterile	5.4 ±1.4	9/9	0/9	0/9
	BPW, 9.0 $\log_{10}$ CFU.mL^{-1}	5223.8 ±949.4	0/9	0/9	9/9
	DP, sterile	0.0 ±0.0	9/9	0/9	0/9
	DP, 9.0 $\log_{10}$ CFU.mL^{-1}	6848.1 ±434.5	0/9	0/9	9/9
Squares	Negative control square	2.9 ±2.9	9/9	0/9	0/9
	BPW, sterile	5.3 ±7.0	7/9	2/9	0/9
	BPW, 7.5 $\log_{10}$ CFU.square^{-1}	4033.7 ±2049.0	0/9	0/9	9/9
	DP, sterile	6.8 ±8.7	8/9	1/9	0/9
	DP, 7.5 $\log_{10}$ CFU.square^{-1}	1486.4 ±1451.7	0/9	0/9	9/9

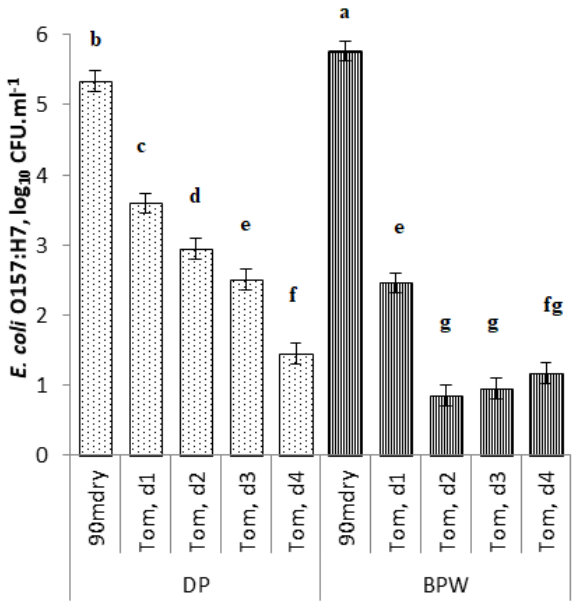

Figure 1 Recovery of *E. coli* O157:H7 (DP or BPW diluent) from inoculated tomatoes either immediately after drying (90 min dry), or after storage for four days (d1-d4) at 25 °C. Note: Counts expressed as $\log_{10}$ CFU.mL^{-1} recovered from 20 mL rinsate. Means with the same letters are not significantly different (p >0.05).

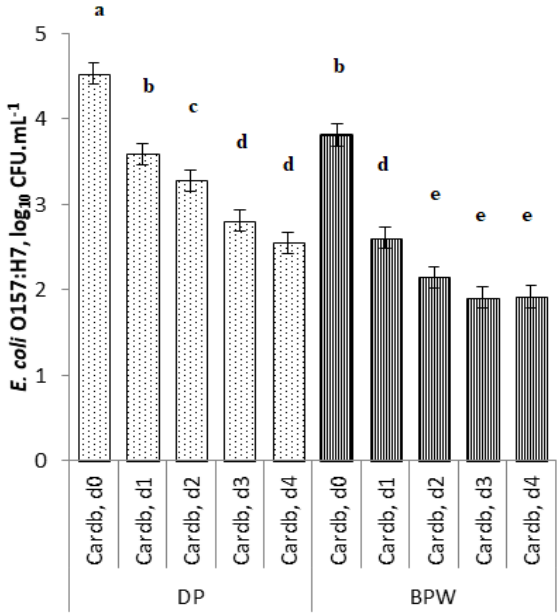

Figure 2 Recovery of *E. coli* O157:H7 (DP or BPW diluent) from inoculated cardboard squares either immediately after drying (90 min dry), or after storage for four days (d1-d4) at 25 °C. Note: Counts expressed as $\log_{10}$ CFU.mL^{-1} recovered from 20 mL rinsate. Means with the same letters are not significantly different (p >0.05).

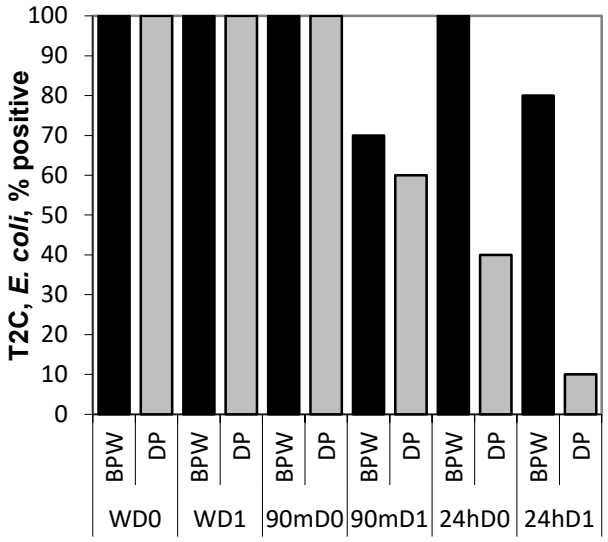

Figure 3 Percentage of squares yielding at least 1 cfu.mL^{-1} of *E. coli* O157:H7 in rinsate after inoculated tomatoes (w – wet; 90m – 90 minutes dry; 24h – 24 hours dry) touched cardboard squares. Note: Squares sampled for *E. coli* either immediately after the transfer (D0) or stored 24 hours after the transfer at 25 ºC (D1). T2C – Tomatoes to Cardboard transfer.

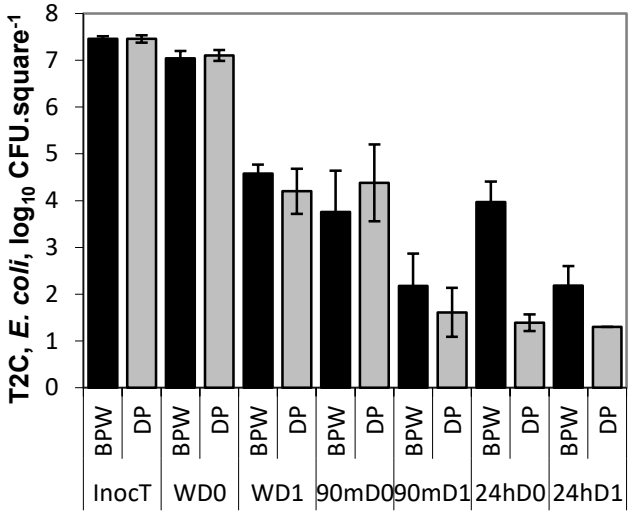

Figure 4 Total *E. coli* O157:H7 counts per square after pathogen transfer from tomato (w – wet; 90m – 90 minutes dry; 24h – 24 hours dry) compared to total inoculated log$_{10}$ CFU.tomato^{-1} (InocT). Note: Squares sampled for *E. coli* either immediately after the transfer (D0) or stored 24 hours after the transfer at 25 ºC (D1). Detection limit 1.3 log$_{10}$ CFU.square^{-1}. Tomato inoculation level (InocT) calculated theoretically based on stationary culture concentration and is shown for reference. Means with the same letters are not significantly different (p >0.05).

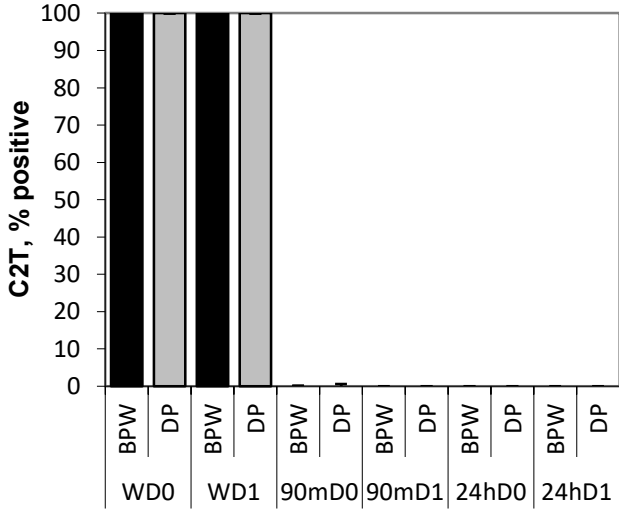

Figure 5 Percentage of tomatoes yielding at least 1cfu.mL^{-1} of *E. coli* O157:H7 in rinsate after inoculated squares (w – wet; 90m – 90 minutes dry; 24h – 24 hours dry) touched tomatoes. Note: Tomatoes were sampled for *E. coli* either immediately after the transfer (D0) or stored 24 hours after the transfer at 25 ºC (D1). C2T – Cardboard to Tomatoes transfer.

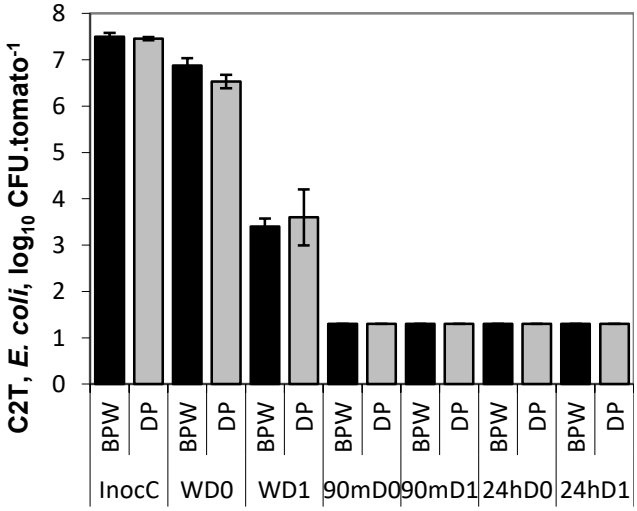

Figure 6 Total *E. coli* O157:H7 counts per tomato after pathogen transfer from square (w – wet; 90m – 90 minutes dry; 24h – 24 hours dry) compared to total inoculated log $_{10}$ CFU.square^{-1} (InocC). Note: Tomatoes were sampled for *E. coli* either immediately after the transfer (D0) or stored 24 hours after the transfer at 25 ºC (D1). Detection limit 1.3 log$_{10}$ CFU.tomato^{-1}. Cardboard square inoculation level (InocC) calculated theoretically based on stationary culture concentration and is shown for reference. Means with the same letters are not significantly different (p >0.05).

CONCLUSION

E. coli O157:H7 survived better on porous cardboard surfaces than on smooth tomato surfaces in humidified atmosphere. Bacterial cells survived for longer than 4 days on cardboard surfaces, questioning possibility of cardboard boxes reuse. Moreover, survival on smooth tomato peel was influenced by diluent type with BPW negatively impairing survival. The observed phenomenon was possible related to hygroscopic nature of solutes present in BPW, where dried inoculated spots liquefied during storage and possibly created environment of high osmotic pressure. Pathogen transfers are of great concern if the surface is wet, but less of a concern if the surface is dry. Though *E. coli* O157:H7 survived better on the surface of cardboard compared to the surface of tomatoes, the transfers were more efficient from tomatoes to cardboard than from cardboard to tomatoes. High humidity storage might cause decrease in bacterial counts of stationary phase cells inoculated in high solids/high salt diluent, therefore, choice of diluent of inoculation studies should be carefully decided. Rapid ATP measuring devices can simplify estimation of overall microbial load, and to some extent, present organic residues, questioning efficiency of surface sanitizing or checking cardboard boxes for cleanness and overall microbial contamination.

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HEALTH STATUS OF WOMEN FROM A SMALL AND A BIG TOWN IN POLAND: THE SUBJECTIVE AND OBJECTIVE ASSESSMENT

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ABSTRACT

The way of defining the concept of health varies depending on the age, sex, social position of a person conditioned by place of residence, economic and family situation. The aim of the study was to assess subjective and objective health status of women over 65 living in a small and big town. In the subjective assessment of the nutritional status were the MNA questionnaire used, in the objective assessment was BMI used. Body composition analysis was carried out using the bioimpedance method. Lab tests were made using standard methods. The quantitative assessment of the diet was made using the 24-hour intake method, the results calculated in software Dieta 5. Significantly more often women from Nysa than from Wroclaw assessed their health status in comparison with their peers as just as good or better (p <0.05). The BMI value among Nysa women shows the overweight, and among women from Wroclaw, obesity. The average content of adipose tissue among all examined women indicated significant fatness of the examined group (36 – 37%). The average energy supply in the food rations of women from Nysa and Wroclaw differed statistically significantly and did not meet the accepted norms. In conclusion, we can say that older women from a small city assessed their health as better or as good compared to older women in a large city. The implementation of dietary norms and recommendations in the food rations of the subjects was insufficient. However, women from a small town provided statistically significantly more nutrients in their diets.

Keywords: women; nutrition; BMI; food intake; health

INTRODUCTION

The assessment of health states five main perspectives for the analysis of the term "health": 1) as a lack of disease, 2) as vitality, physical fitness, 3) as beneficial social relations, 4) as a functional disability, and 5) as psychosocial well-being. The way of defining the concept of health varies depending on the age, sex, social position of a person conditioned by place of residence, economic and family situation. Being healthy can also mean broadly understood "good life", as an adaptation of an elderly person to the current life situation, level of isolation, but also social integration **(Hervik, 2016)**. The definition of health becomes particularly important in the case of people over 65, for whom the indicators determining them are significantly different. In a study of 505 people over the age of 65 led by Młynarska and colleagues **(Młynarska et al., 2015)**, it was shown that people over the age of 65 assign the highest-rank claims to "live late in old age", "not get sick, at most rarely get influenza, indigestion", "have functional all parts of the body "and" feel good "and are characterized by an instrumental approach to health. Medical health indicators make it possible to correct self-assessment of one's health with reference ranges, e.g. biochemical parameters compatible with age or human sex. In the last decade, important data on the health condition of older people in Poland has been presented in the multidisciplinary research project PolSenior **(Mossakowska, Więcek and Błędowski, 2012)** they are also systematically described in the Central Statistical Office (CSO) research and in the European Health Interview Survey (EHIS). In the PolPAN project from 2008 and 2013, additional health problems were assessed from three groups of ailments: physical, mental and social functioning. Social isolation is a major and prevalent health problem among community-dwelling older adults, leading to numerous detrimental health conditions. With a high prevalence, and an increasing number of older persons, social isolation will impact the health, well-being, and quality of life of numerous older adults now and in the foreseeable future The POLPAN 50+ report was devoted especially to the health situation of older Polish women.When comparing the percentage of negative health self-assessment in Poland in 2009 and 2014, it should be noted that it decreased in the group of people aged 50 – 59 from 55% to 50%, in the group 60 – 69 from 72% to 65%, and in 70 – 79 group from 85% to 79%. In the group of people aged 80 and more, it increased from 86% to 88% **(Piekarzewska et al., 2016)**. In the division of older people due to the level of education, it was found that poor or very bad self-esteem in the highest percentage was indicated by

people with lower education and this percentage in the group of 50 – 59 year olds was 18%, in the group of 60 – 69 years - 26%, in 70 – 79 years - 39%, and in the group of 80 years and more – 53%. People with secondary education three times more often claimed that their health is bad or very bad than people with higher education (13% vs. 31%). In the older age groups (80+), the percentage of people with health self-assessment as bad and very bad was definitely lower in the case of people with higher education (38%) than the average (47%) and vocational (53%). POLPAN research has shown that the process of aging in older age is different in men compared to women. The sense of health deteriorates in men after the age of 80, and between 60 and 80 years of age declared by respondents, the loss of health is small. At the same time, men feel an improvement in the subjective assessment of their own health and mood. Women, on the other hand, declare a decline in the assessment of their own state of health after the age of 60, and all medical indicators are deteriorating in the medical assessment **(Mikucka, 2015)**.

The aim of the study was to assess subjective and objective health status of women over 65 living in a small town (Nysa, Opole province, 40 thousand inhabitants) and Wroclaw (643 thousand inhabitants).

Scientific hypothesis

Subjective and objective health status of women over 65 living in a small and big town are different. Women from a small city assessed their health as better or as good compared to older women in a large city. Women from a small city provided more nutrients in their diets than women from big city. More often women from small city than big one assessed their health status in comparison with their peers as just as good or better.

MATERIAL AND METHODOLOGY

The criteria for including women in the study (n = 399) was age above 65 years of age, psychophysical condition enabling the examination and place of residence (Nysa, Wroclaw). All women participating in the study were informed about the purpose and methodology of the study. Each of them expressed their written consent to carry them out. The study obtained the consent of the Bioethical Commission at the Medical University of Wroclaw (KB309/2008) and at the University of Applied Sciences in Nysa (KB 4/2016). In the subjective assessment of the health status of the surveyed women, the answers to self-assessment questions about their state of health included in the MNA (mini nutritional assessment) questionnaire were used. In the objective assessment of the nutritional status of the subjects, the measurement of mass, body height was used and on this basis the BMI body mass index was calculated, expressed as: body mass (kg)/height (m^2). Body composition analysis was carried out using the electrical bio-impedance method using an analyzer TANITA (Tanita Europe B.V., Amsterdam, Netherland). Lab tests were made using standard methods in the analytical laboratory of selected clinics, and generally accepted ranges of reference values for women of a given age were considered norms **(Mahlknecht and Kaiser, 2010)**. The quantitative assessment of the diet was made using the 24-hour intake method, and the results calculated in the computer program Diet 5 were compared with the current standards developed by the Institue of Food and Nutrition (IFN) in Warsaw for older women **(Jarosz, 2017)**.

Statistical analysis

The normality of value distribution was checked by Shapiro-Wilk test. Due to the lack of normal distribution of quantitative data, the ANOVA-rank analysis of Kruskal-Wallis test was used. The Chi^2 test was used to examine the significance of differences between variables characterizing women from Nysa vs. women from Wroclaw. Statistically significant were the results for which $p < 0.05$ and $p < 0.001$ was calculated. Statistical analysis of the obtained results was made using the Statistica 12.0 computer program by StatSoft, using calculations of mean, min and max values, standard deviation and percentage calculations.

RESULTS

The characteristics of the examined elderly women are listed in Table 1. The survey included 187 women from Nysa (47%) and 212 women from Wroclaw (53%). The structure of the answer to the question regarding health self-assessment in comparison with peers was determined by the place of residence of the surveyed women. Statistically significantly more often women from Nysa than from Wroclaw assessed their health status in comparison with their peers as equally good (respectively 48.7% vs 32.6%) or better (29.9% vs. 14.6%, $p < 0.05$). On the other hand, resident of Wroclaw more often than residents of Nysa claimed that they cannot assess their health condition (27.8% vs. 14.4% respectively) or that it is not as good as in their peers (25.0% vs 7.0%; $p < 0.05$). Most of the women participating in the study had secondary education, however, significantly more often they were Nysa residents than group from Wroclaw (62.0% vs 44.8%; $p < 0.05$). Every fifth respondent had higher education. Statistically significantly more often inhabitants of Wroclaw than Nysa declared primary education (25.4% vs 4.8%; $p < 0.05$). Gross income per capita among respondents was not statistically different in relation to their place of residence. The majority obtained income above 1000 PLN.

Women living in Wroclaw significantly more often than those living in Nysa lived alone (50.9% vs 36.9%; $p < 0.05$), while Nysa resident statistically significantly more often lived with a spouse (49.2% vs 38.7 %; $p < 0.05$). The level of physical activity declared by the examined women depended on the place of residence. Significantly more often women from Nysa than Wroclaw women reported that they were physically active (58.8% vs 41.0%; $p < 0.05$). In turn, significantly more often the answers "I'm rather active" were given by Wroclaw residents (44.8% vs 24.1%; $p < 0.05$).

Table 2 presents the measured anthropometric parameters in the examined group of older women. A statistically significant difference was found between women from Nysa vs Wroclaw in relation to body weight and BMI. Senior women from Nysa, who in most (about 80%) in self-assessment of their health state declared good or better health from their peers, had statistically significantly lower body mass than Wroclaw women (72.4 vs 78.3 kg; $p < 0.001$).

Table 1 Charakteristics of studied group.

Variables		Women from Nysa		Women from Wroclaw		Chi^2 Test
		n = 187	%	n = 212	%	*p*-value
Self-health status in comparison with peers	not as good	13	7.0	53	25.0	0.00
	can not assess	27	14.4	59	27.8	0.00
	good as other	91	48.7	69	32.6	0.00
	better	56	29.9	31	14.6	0.00
Education	basic	9	4.8	54	25.4	0.00
	vocational	22	11.8	25	11.9	0.88
	secendary	116	62.0	95	44.8	0.00
	high	40	21.4	38	17.9	0.45
Income	≤ 1000 zł	79	42.2	101	47.6	0.32
	> 1000 zł	108	57.8	111	52.4	0.23
Type of family	along	69	36.9	108	50.9	0.00
	with a spouse	92	49.2	82	38.7	0.04
	with others/ with children	26	13.9	22	10.4	0.35
Activity physical declared	acitve	110	58.8	87	41.0	0.00
	rather active	45	24.1	95	44.8	0.00
	inactive	32	17.1	30	14.2	0.49

Table 2 Anthropometric parameters of the studied women.

Variables	Women from Nysa	Women from Wroclaw	*p*-value[4]
Body mass (kg)	72.4 ±14.4[1] 68.7[2]; 47.3 – 98.8[3]	78.3 ±20.5 75.0; 42.0 – 99.0	0.0002
Height (cm)	158.7 ±5.6 159.0; 144.0 – 173.0	158.7 ±6.4 159.0; 138.0 – 176.0	NS[5]
BMI index (kg.m^{-2})	28.7 ±5.1 27.9; 20.6 – 46.5	30.9 ±7,8 33.3; 17.4 – 45.1	0.0005
Body fat content (%)	36.4 ±5.7 36.6; 19.2 – 51.0	37.5 ±5.1 37.7; 19.2 – 51.2	0.0467

Note: [1]Data are expressed as mean ±*SD* (standard deviation); [2] *Mdn* (median), [3]min-max: minimal value-maximal value; [4]statistical significant differences were verifiet by the Kruskal-Wallis test, $p < 0.05$, $p < 0.001$; [5]NS not significant.

Table 3 Selected biochemical parameters of the studied women.

Variables	Women from Nysa	Women from Wroclaw	*p*-value[4]
Hematocrit (%)	39.8 ±2.4[1] 40.1[2]; 31.7 – 44.7[3]	40.3 ±3.0 40.4; 31.7 – 54.0	NS[5]
Hemoglobin (g.dL^{-1})	13.4 ±0.8 13.5; 11.3 – 15.4	13.7 ±1.0 13.0; 10.1 – 18.3	NS
Total cholesterol (mg.dL^{-1})	202.6 ±47.4 203.0; 2.5 – 310.0	213.5 ±38.4 212.5; 100.0 – 320.0	NS

Note: [1]Data are expressed as mean ±*SD* (standard deviation); [2]*Mdn* (median), [3]min-max: minimal value-maximal value; [4]statistical significant differences were verifiet by the Kruskal-Wallis test, $p < 0.05$, $p < 0.001$; [5]NS: not significant.

Similar conclusions concerned the BMI index, which was statistically significantly lower in the group of older women from Nysa than from Wroclaw (28.7 vs. 30.9, $p < 0.001$). The average BMI value among Nysa women was overweight, and among women from Wroclaw, obesity. The average content of adipose tissue among all examined women indicated significant fatness of the examined group (36 – 37%) and the risk of metabolic syndrome.

The biochemical parameters (hematocrit, hemoglobin and total cholesterol) determined in the blood of the studied women were not different in terms of the place of confusion. Increased total cholesterol was shown in the average sample of women from Wroclaw, wich amounted to 213.5 mg.dL^{-1} (Table 3). Table 4 presents data on the energy supply and selected nutrients in the food rations of the surveyed women.

The normative energy value of the diet of women over 65 with a body weight in the range of 55 - 65 kg (which corresponds to the average body height in the studi d group of 158 cm) is for low physical activity (PAL 1.4) from 6699 – 7327 kJ (1600 – 1750 kcal). Energy value of the food ration women with moderate physical activity (PAL 1.75) is 8583 – 9211 kJ (2050 – 2200 kcal) **(Jarosz, 2017)**.

Table 4 Energy and selected nutrients in the food rations of the studied women.

Variables	Women from Nysa	Women from Wroclaw	Norms IFN 2017	*p*-value[4]
Energy (kJ)	6382.8 ±2542.6[1] 5994.7[2]; 10256.6 – 3774.0[3]	5536.6 ±1977.4 5127.2; 1315.1 – 14318.9	7955	0.0009
Energy from protein (%)	16.5 ±5.0 15.9; 7.5 – 27.9	14.8 ±4.1 14.3; 6.0 – 21.2	15	0.0010
Energy from fat (%)	27.6 ±11.6 27.5; 7.6 – 3 .3	33.4 ±8.6 33.1; 13.4 – 38.6	25	0.0000
Energy from carbohydrates (%)	55.5 ±12.1 56.1;41.8 – 67.7	51.4 ±9.4 52.3; 44.6 – 67.9	60	0.0002
Saturated FA (g)	20.1 ±17.4 16.3; 1.6 – 32.8	20.6 ±10.5 18.5; 3.0 – 39.3	12 – 14	NS[5]
Monounsaturated FA (g)	18.5 ±14.1 15.2; 15.8 – 22.7	18.9 ±10.7 16.4;12.6 – 21.97	22 – 25	NS
Polyunsaturated FA (g)	6.8 ±5.4 5.7; 1.3 – 8.9	7.8 ±6.0 6.1; 1.2 – 9.0	16 – 17	NS
Calcium (mg)	543.5 ±313.6 488.8; 128.0 – 1091.8	391.8 ±259.8 322.5; 64.0 – 1003.7	1000	0.0000
Iron (mg)	11.4 ±5.5 10.0; 3.0 – 14.0	7.4 ±3.1 7.4; 2.6 – 12.6	6	0.0000
Vitamin D (μg)	2.1 ±2.5 1.5; 0.7 – 7.1	2.0 ±3.4 1.2; 0.5 – 5.8	15	NS
Folate (μg)	255.8 ±25.4 227.9; 17.5 – 3 9.3	126.2 ±27.3 118.8; 30.8 – 368.7	450	0.0000

Note: [1]Data are expressed as mean ±*SD* (standard deviation); [2]*Mdn* (median), [3]min-max: minimal value-maximal value; [4]statistical significant differences were verifiet by the Kruskal-Wallis test, $p < 0.05$, $p < 0.001$; [5]NS: not significant.

The average energy supply in the food rations of women from Nysa and Wroclaw differed statistically significantly (6382.8 vs 5536.6 kJ, $p < 0.001$) and did not meet the above standards. Higher energy supply in the Nysa seniors' food rations determined a better, close to the values recommended by the Institute of Food and Nutrition in Warsaw, the % energy from proteins (10 – 15%), fats (25 – 30%) and carbohydrates (55 – 65%).

The content of saturated TCs in the average food ration of all examined women was too high and amounted to an average of 20 g. The norms of Poland 2017 for the studied women for this nutrient are 12 – 14.4 g. The supply of calcium and folates in the food rations of Nysa and Wroclaw residents was deficient and differed in a statistically significant way. It should be emphasized, however, that women from Nysa followed accepted norms in a larger share (over 50% of the norm). In the food rations of the studied women, particularly low content of vitamin D was demonstrated, which in the average value was about 13 – 14% of the norm (15 μg) for this age group. In the face of such a low targeted implementation, it seems that dietary supplementation with this vitamin is used in the whole group of women surveyed.

DISCUSSION

The nutritional status of the subjects depends on many factors. In the face of the continuous extension of human life, psychological factors (feeling stress, coping with everyday life conditions, social relations) conditioning well-being and, subsequently, affecting the biological assessment of health condition are becoming more and more important. The majority of elderly women surveyed in this work living in a small town (about 80%) declared in the opinion of MNA that they assessed their health as compared to their peers as being good or better (Table 1). This was confirmed in anthropometric studies, where statistically significant differences between body weight and BMI were demonstrated. The body mass of seniors from Nysa was smaller and amounted to 72.4 kg on average and 78.3 kg from Wroclaw. BMI among Nysa women was overweight and women from Wroclaw were obese, 28.7 $kg.m^{-2}$, respectively 30.9 $kg.m^{-2}$ (Table 2). In the study of **Wasiluk et al. (2015)** from Biała Podlaska it was found that the average body mass of the examined women over 60 (n = 180) was 73.0 kg, and BMI 28.3 $kg.m^{-2}$. A high percentage of overweight and obesity in older people is also confirmed by international studies. Observations conducted in the USA showed that 78.4% of men over 60 and 68.6% women from the same age group are overweight or obese **(Flegal et al., 2010)**. **Sánchez-García et al. (2007)**, observing 60-year-old and older Mexicans, noted that BMI ≥25 $kg.m^{-2}$ concerned 65.4% of women. Similar results have been demonstrated on the European continent. **Lahti-Koski et al. (2000)** assessing Finnish residents noticed that 73.0% of women in this country aged 55-64 were overweight. On the other hand, in southern Italy, 86.0% of women aged 60 - 69 years were diagnosed with obesity or obesity **(Barbagallo et al., 2001)**. Representative Spanish

studies conducted among people over 60 also confirmed a very high percentage of overweight and obesity in women (82.1%) **(Gutiérrez-Fisac et al., 2004)**. Observations conducted in the Czech Republic indicate that 41.0% of women aged 55 - 64 suffer from overweight, whereas obesity is 30.0% **(Elmadfa et al., 2009)**. The changes in the BMI index are influenced by the increasing body mass and the progressive reduction in its height. According to research by **Zamboni et al. (2005)**, the regression of the somatic feature is 8 cm in women and 5 cm in men between the ages of 20 and 85, and every centimeter of body reduction translates into an increase in the value BMI by 0.3 $kg.m^{-2}$. In addition, changes in the body composition of the body are observed with age. They are mainly manifested in the re-production of lean body mass and the growth of adipose tissue. **Kyle et al. (2002)** found that the fat content between 20 and 85 years doubles. In addition, the BMI value alone in young people and seniors will reflect the body composition of the various components in terms of the contribution of individual components. Therefore, overweight and obesity should be determined on the basis of fat content for older people. This parameter in older Nysa women was 36.4% and was statistically significantly lower than the fat content of women from Wroclaw (37.5%), although both values indicated significant fatness of the examined seniors. The content of adipose tissue and fat free in the body is a particularly important parameter for predicting sarcopenia in older people. Sarcopenia is a syndrome characterized by loss of muscle mass, strength and performance **(Ożga and Małgorzewicz, 2013)**. This problem concerns 5 – 13% of 60 – 70 year old's and 11 – 50% of people >80 years of both obese and normal weight **(Cruz-Jentoft et al., 2010)**. In this study, an average of 202.6 $mg.dL^{-1}$ of total cholesterol was determined in the serum of older women from Nysa and 213.5 $mg.dL^{-1}$ in women from Wroclaw. These values did not differ in these groups in a statistically significant manner (Table 3). In the general classification, the level of this parameter should not exceed 200 $mg.dL^{-1}$. In observational studies in the elderly (> 65 years), a weaker relationship between total cholesterol and cardiovascular risk and deaths was demonstrated in comparison to younger people. Among the reasons for this low dependency are earlier deaths due to diseases of the cardiovascular system in people with higher cholesterol levels, more frequent occurrences of other chronic diseases, malnutrition and weight loss. In addition, nonlipid risk factors and their effect on instability of the atherosclerotic plaque, its rupture and/or thrombosis may play a greater role in increasing the risk of episode **(Catapano et al., 2016; Cybulska and Kłosiewicz-Latoszek, 2016; Bays et al., 2016)**. The energy value and content of individual nutrients in the food rations of the studied women from Nysa and Wroclaw differed statistically significantly (Table 4). Deficient energy value of food rations was demonstrated, which was conditioned by too low share of energy coming from carbohydrates. However, the percentage of implementation of energy standards among Nysa women was higher (80% vs 69%), there was an excessive intake of saturated fatty acids in the diets of the whole group of seniors tested and too low polyunsaturated fatty acids. The contents in the food rations of calcium and folates were deficient and statistically differentiated the examined groups of older women. However, the Nysa seniors realized a statistically higher percentage of the standard (Ca 54% vs. 39% of the norm, folate 56% vs. 28% of the norm). An important nutritional problem is the deficiency of vitamin D intake in the seniors' diets found in many works. Older women from Nysa and Wroclaw consumed about 13% of the norm on vitamin D. In the current literature, it is emphasized that a constant deficiency of this vitamin may have a negative effect on the immunomodulatory and neuroprotective functions in the body. If the concentration in the blood of this vitamin is too low, the incidence of cognitive impairment, Alzheimer's and Parkinson's disease is increased. There are many publications that link vitamin D deficiencies with an increased likelihood of mood disorders, including depression **(Jorde et al., 2008; Zdrojewicz et al., 2015)**.

CONCLUSION

Older women from a small city assessed their health as better or as good compared to older women in a large city. In both groups, however, excessive body mass and BMI were found, indicative of overweight and obesity. The implementation of nutritional standards and recommendations in the food rations of the subjects was insufficient. However, women from a small town provided statistically significantly more nutrients in their diets.

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AUTHENTICATION OF POULTRY PRODUCTS AT THE BREED LEVEL USING GENETIC MARKERS

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ABSTRACT

The Oravka tawny is a Slovak national breed of chicken. This breed has combined utility, which means it is valuable for both its meat and eggs. The Oravka tawny is linked to a specific region, Orava, and therefore these products could be protected by European geographical indication. The labeling and sale of chicken meat by the traditional breed of origin are widely used to promote quality and attract those products in the marketplace. For that use, we created the system and method of authentication that can reliably distinguish between the Oravka tawny, other chicken breeds, and other of Oravka's colorful characters. In our research, we analyzed 153 chicken feathers from the Oravka breed as well as from breeds used in the process of breeding the Oravka to their current state. They were divided into nine populations. To separate those populations, we used seven microsatellite markers recommended by FAO (Food and Agriculture Organization) and other authors. To create separate clusters of individual breeds, we used DAPC (discriminant analysis of principal components) analysis.

Keywords: Oravka; microsatellite; marker; DAPC; cluster

INTRODUCTION

By the term food authentication, we understand the process of verifying that the label description of the food is accurate and contains all the necessary information for customers. That information may include the origin (species, geographical or genetic), production method (conventional, organic, traditional procedures, free-range), or processing technologies (irradiation, freezing, microwave heating). The induction of select quality attributed to highly valuable products is an especially interesting topic. Those certain food products are often the target of falsification by deceptive labels. Proof of provenance is an essential topic for food safety, food quality, and consumer protection, as well as for compliance with national legislation, international standards, and guidelines **(Aung and Chang, 2014)**.

The information presented to consumers is also most important for them in the process of choosing specific foods over others. This choice could be driven by lifestyle. For example, vegetarianism, or religious practices such as those of Jews and Muslims, for whom pork meat should be absent. The occurrence of several food crises in recent years has emphasized food safety and protection of consumer's health as the primary goal for the food labeling legislation **(Cheftel, 2005)**. **Directive 2003/89/EC** of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs, Off J Eur Union L 308:15–18 says: "The increased awareness of consumers regarding the composition of foods has resulted in the need to verify the labeling statements. The incorrect labeling of foods represents commercial fraud, considering consumer acquisition. It is crucial to establish that species of high commercial value declared are not substituting, partial or entirely, by other lower-value species. The misleading labeling might also have negative implications concerning health, especially for sensitive consumers, to nondeclared potential allergens. Food allergies are considered a new public health problem, especially in developed countries."

The Oravka is a Slovak chicken named after the region of Orava, from where this breed originated. The Oravka was created in a crossbreeding process of the regional hens with Rhode Island, Wyandotte, and New Hampshire breeds. The breeding process started in the 1950s and continued with numerous individual stages. The Oravka was recognized as a breed in the year 1990. The main aim of the breeding process was to create dual-purpose poultry. That is, to create a breed with good egg production and growth ability. The ability to adapt to harsh outdoor rearing was requested as well. The agreement for the standard of the Oravka breed is yearly egg production of

180 to 200 eggs with a brownish eggshell; the minimum hatching egg weight is 58g **(Hrnčár et al., 2017)**. Authors **Kukučková et al., (2018)** published work of genetic diversity with aim to protect and preserve breed of Slovak Pinzgau cattle. We are aiming to protect national breed and preserve biodiversity of Oravka tawny chicken.

DNA-based analytical methods can provide much more information about processed food and foodstuff than methods based on proteins. This is due to the much higher thermostability of DNA in comparison to most proteins. Also, DNA is present in the majority of cells in organisms, potentially enabling identical information to be obtained from any appropriate sample from the same source, regardless of the tissue of origin. Furthermore, driven by the clinical arena, nucleic acid-based technologies are developing rapidly, and the informed adoption of suitable methods by the food industry has the potential to simplify methods of authentication **(Lockley and Bardsley, 2000)**.

Currently, microsatellite loci are the method of choice to study the genetic diversities within and between populations, because they are highly polymorphic, show co-dominant inheritance, and are found to be abundant and evenly distributed throughout the genome. So far, many studies have been conducted to assess chicken's genetic diversity using microsatellite markers, and the reported results are clear evidence of the usefulness of these panels for biodiversity studies. Understanding the genetic structure of native chickens is a vital step in setting up their conservation and genetic improvement programs **(Yacoub and Fathi, 2013; Khanyile, Dzomba and Muchadeyi, 2015)**.

In our study, 153 individual chickens were tested, and seven markers were used. We are aiming to create a useful method of authenticating products made from the Slovak national chicken breed – the Oravka tawny.

Scientific hypothesis

We are expecting that separation of the Oravka breed from other chicken breeds used in its breeding process will be possible with the usage of chosen microsatellite markers.

MATERIAL AND METHODOLOGY

In our study, we used 153 samples of hen feathers (Table 1). These samples were divided into nine populations. The most numerous population was the group of Oravka tawny (119 individuals). Other color variants of Oravka are genetically younger. All of them were bred later than the original autochthonous breed of Oravka tawny. We also included breeds used in the early stages of the breeding process of Oravka tawny and inbreeding processes, including the younger color variations. The Polymerase Chain Reaction (PCR) was performed as suggested **(Choi et al., 2015)** in total volume of 20 μL; 50 ng of genomic DNA, 10 pmol of labeled modified forward primer and standard reverse primer, 2.5 mM of each dNTPs, 10 X reaction buffer, 2.5 units of prime Taq DNA polymerase. The PCR was performed in an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 60 °C, 30 seconds of extension at 72 °C and final extension at 72 °C for 10 minutes. Seven microsatellite markers were used in our analysis: LEI0254, LEI0166, MCW0034, LEI0192, MCW0069, LEI0234 and LEI0228 (Table 2). Those markers were selected based on authors' **(Choi et al., 2015)** research and the recommendation of FAO. Genotyping was performed on ABI PRISM 310 Genetic Analyzer. The detection of genomic regions affected by natural selection was carried out based on the approach adopted in R package PCAdapt **(Duforet-Frebourg, Bazin a Blum, 2014; Moravíková et al., 2018)**.

Statistic analysis

To create clusters of selected chicken breeds, we used the DAPC analysis. For this analysis we used the software program Rstudio with additional packages installed.

RESULTS AND DISCUSSION

Seo et al. (2013) wrote: "In 1994, a program for the creation of lines of Korean national breeds began. The result was to document five breeds with nine chicken lines. A structured genetic analysis program based on microsatellite markers was used to investigate the genetic structure of the five Korean native chicken lines. Based on line specific clusters, the structure of the chicken line was estimated." Like the authors cited above, we have also sought to find microsatellite markers in order to improve the traceability of the national poultry breed.

Table 1 Populations of selected chicken breeds.

Breed	Number	Acronym
Oravka tawny	119	OTW
Oravka white	11	OWH
Oravka black	6	OBL
Oravka versicolor	6	OVC
Oravka tawny diminutive	1	OTD
Šumavka	4	SUM
New Hampshire	2	HAM
Rhode Island Red	2	RHI
Wyandotte	2	WYA

Table 2 Microsatellites used in analysis.

Microsatellite	Lenght (bp)	No.of alleles	Repetition
LEI0254	86 – 93	7	tetranucleotid
LEI0166	354 – 370	4	dinucleotid (CA)
MCW0034	220 – 240	9	dinucleotid (CA)
LEI0192	256 – 296	6	tetranucleotid
MCW0069	156 – 174	8	dinucleotid (CA)
LEI0234	219 – 315	8	tetranucleotid
LEI0228	165 - 255	14	tetranucleotid

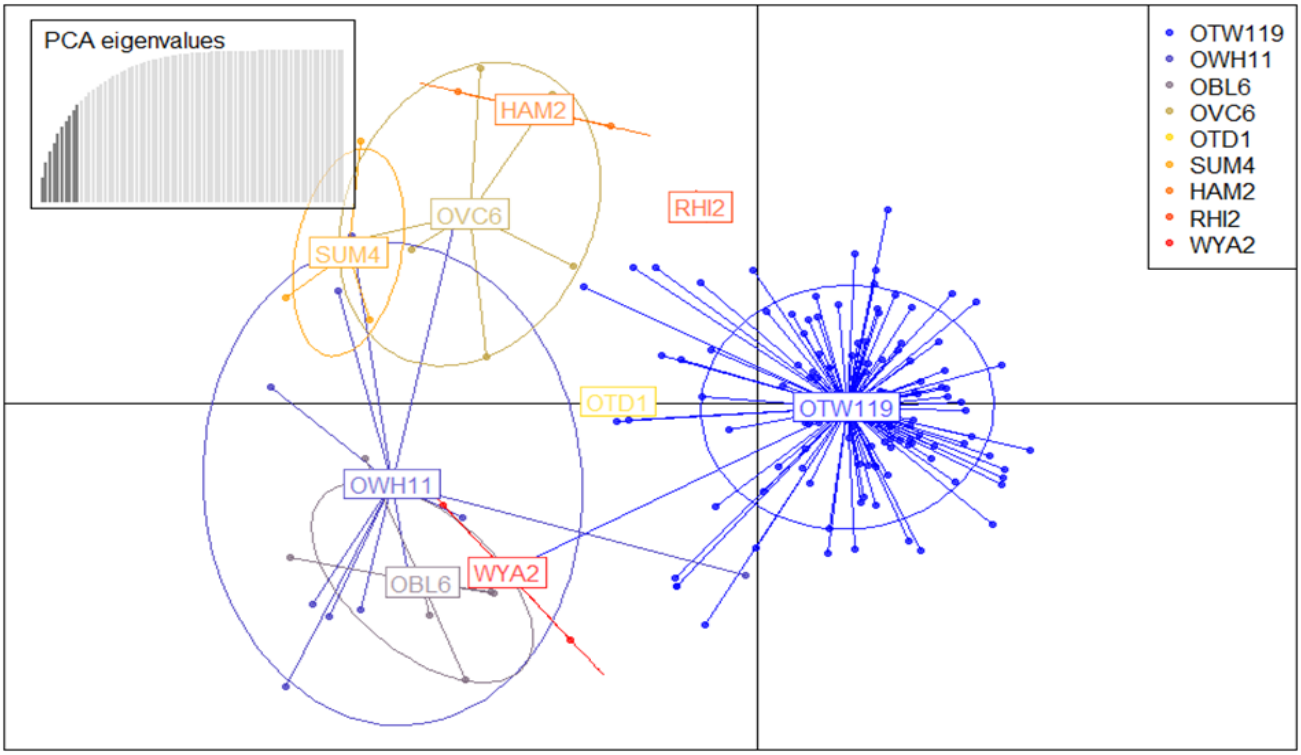

Figure 1 Clustes of selected chicken breeds after DAPC analysis.

In studies of human genetic variation, at the continental level, there is good agreement in the ordering by region of gene diversity measures among different kinds of markers, once ascertainment bias is removed **(Rogers a Jorde, 1996; Jorde et al., 2000)**. The same is true for the ordering of genetic distances among populations assessed for different markers **(Jorde et al., 2000)**. It is reasonable, therefore, to believe that the ordering among chicken breeds of diversity and distances seen here for microsatellites in DNA pools would not be very different than for other genetic marker systems. The microsatellite loci used here were selected to be polymorphic for use in gene mapping. However, **Rosenberg et al. (2002)** obtained diversity and genetic distance patterns for humans that largely agreed with those of **Bowcock et al. (1994)**. The recent study used microsatellites from a mapping set and the last used markers that were not selected. Ascertainment bias is, therefore, not expected to have had a significant effect on the ordering of statistics for our chicken data.

On the picture above (Figure 1) are visible clusters of populations used in our study. The most numerous cluster is that of our primary targeted population of Oravka tawny. We can see that this cluster is separated from others. Also, it is visible that the diminutive form of Oravka tawny is placed inside of the cluster. That means that with the usage of our chosen microsatellite markers, we cannot separate them from one another. Since the initial genotyping array has a large enough number of loci, even a low proportion of cross-amplifying SNPs may represent a useful set of markers for species which lack genomic resources. The distribution of SNPs over entire genomes of all chromosomes was not uniform and varied among the analysed groups **(Kasarda et al., 2015)**. The massive merging of breeding companies in recent years should call attention to the need for conservation of genetic variation among breeds and lines. Appropriate strategies for conservation of populations is out of the scope of the present report but is an important and controversial issue **(Fujihara, 1999)**.

Discriminant analysis of principal components (DAPC) uses Nei´s genetic distance to separate the observed populations of chickens. That means the longer the distance of the population on the created graph, the less they are related on the genetic level. With our analysis, we can differentiate the population of Oravka tawny from other color breeds as well as from breeds used in the early stages of its breeding process. Chosen microsatellite markers can be useful for the authentication of products made from this autochthonous chicken breed. With a functional system of authentication, products made from the breed of Oravka tawny could be eligible to be certified with European geographical indication (GI). Acquisition of this certification might help the preservation of this, the only Slovak national breed of chicken. Also, European certification would be able to support farmers and producers with increased credibility in the eyes of customers.

The European Union established measures to support breeds in danger of being lost to farming **(Commission Regulation (EC) No 1974/2006)**. The threshold under which a chicken breed is considered in danger is 25,000 breeding females; the number of Bionda and Bianca are below this value **(DeMarco et al., 2013)**. The AVIANDIV-FAO microsatellite tool meets the need to establish a standard approach to characterize animal genetic resources, and the number of loci ensures high differentiation power **(FAO, 2011; Gärke et al., 2012)**. The widespread use of these markers provides the most significant amount of data to perform comparisons among populations of different origins. Therefore, models for linking information are based mainly on this tool, and new data on indigenous poultry may be combined with available data sets of other breeds and commercial lines

(Granevitze et al., 2007; Zanetti et al., 2010). The markers of the present investigation are suitable to evaluate genetic relationships among populations and to assess whether a supported plan should be implemented. Although different markers were used, the number of alleles and the expected heterozygosity of the present analysis are in agreement with the investigation of **Guidobono Cavalchini et al. (2007)** on the same Piedmont breeds. Moreover, our results show that genetic variability has not changed in the short term.

Kumar et al. (2015) wrote: "In conclusion, this study clearly demonstrates the potential of locus-specific microsatellite markers in genetic diversity, phylogenetic relationships, and population structure analysis between wild and domestic chicken populations. The information generated by microsatellite marker analysis in the form of population-specific alleles may be used in the development of microsatellite assays for the identification of pure or admixed RJF in the wild chicken population. Apparent separate clustering of the four populations suggests pronounced population structure and minimal or no gene flow. High genetic diversity across the population and microsatellite panels with high levels of heterozygosity and PIC suggest the appropriateness of the methodological design."

As mentioned authors, we were able to prove the difference between the autochthonous breed of Oravka tawny and other color variants and other breeds.

CONCLUSION

There is a financial incentive for meat producers to substitute meat from chicken of cheaper breeds, thereby defrauding the consumer. There is a need for legal authorities to be able to authenticate the breed of origin of meat labeled by breed, in order to deter mislabeling fraud of breed of chicken. In our analysis, we proved that with the usage of seven selected microsatellite markers and discriminant analysis of principal components, we could separate clusters of Oravka tawny from other populations of chicken.

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CONSUMER PREFERENCES ON MILK MARKET: EVIDENCE FROM SLOVAK REPUBLIC

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ABSTRACT

Today the issue of healthy nutrition is very popular among consumers. The main task of nutrition is to ensure sufficient intake of substances that are necessary for the proper functioning of the human organism. These substances are divided into two types: sugars, fats and proteins, which are the source of energy and minerals, vitamins and water, which are substances necessary for metabolic processes. We distinguish five main food categories, from which people can obtain these necessary substances: cereals, fruits, vegetables, proteins and last but not least milk. Milk is a white liquid secreted by female mammals for feeding, and which is used (mainly from cows) as human food. Milk is sometimes even called a "super-food", as it contains all the necessary ingredients mentioned above. Its most important component is calcium, which is a key building block of bones and teeth. Milk sugar called lactose, in turn, is involved in the construction of brain cells. Among other things, the milk also contains 87% of water, making it suitable for maintaining a daily drinking regime, unfortunately its consumption is in Slovak Republic insufficient, so the main objective of this paper was to evaluate consumer preferences on the milk market to understand our consumers better. Based on the results od marketing and neuromarketing research we can state that 76.98% of respondents puchase milk, milk expenses range from 11 to 20 € (42.06%), it is purchased mainly in hypermarkets and supermarkets (36.71%), admissible price per liter is on average 0.89 € and decide according to milk quality taste and durability.

Keywords: milk; milk market; consumer; consumer behavior; Slovak Republic

INTRODUCTION

Animal source foods are important for people as they provide essential micro and macro nutrients for human development and functioning **(Iannotti et al., 2017)**. Since man domesticated ruminating animals 8,000 – 10,000 years ago, people started consuming milk and fermented milk products. Over the last 20 years milk consumption has plunged in developed countries. Adults in developed countries typically consume more milk than those in developing countries **(Petherick, 2016)** and adolescents and young adults tend to consume less milk than older adults because they replace milk with sweetened beverages or fruit juice **(Singh et al., 2015)**.

From a consumption point of view, dairy products have many benefits and are considered as key nutritious sources of proteins, fats and micronutrients with positive health impacts **(Garcia, Osburn and Cullor, 2019)**. On the positive side, milk contributes a significant proportion of daily requirements for protein and calcium at a population level **(Huth et al., 2013)**. When fortified, milk also contributes to vitamin D intake. As discussed further on, calcium, vitamin D, and dairy proteins are key nutrients for bone health **(Dawson-Hughes et al., 2010)**. Adequate vitamin D status has also been associated with a lower risk of some cancers and mortality, but conclusive evidence awaits the results from ongoing large trials of vitamin D supplementation. Milk consumption also contributes to dietary intake of magnesium, potassium, phosphorus, vitamin B12, riboflavin **(Lamarche et al., 2016)** and vitamin A, which is underconsumed nutrient **(US Department of Agriculture, 2015)**. Moreover, cow´s milk lower amount of vitamin C in human body **(Zeleňáková and Golian, 2008)**. Several recent reviews pinpoint a protective effect of dairy products on health outcomes **(Weaver, 2014)**, body weight **(Wang et al., 2014)** and obesity related comorbidities, including type 2 diabetes and cardiovascular disease **(O´Connor et al., 2014; Markey et al., 2014)**. Even though milk is a valuable nutritional resource, when collected, stored, distributed, and/or consumed under certain unhygienic conditions, it can serve as a favourable medium for pathogenic bacteria and thereby increase risk for foodborne illness **(Wu et al., 2018)**.

Because milk provides a direct and rich source of nutrients, it is a valuable dietary supplement **(Millward, 2017)**. Most countries have quantitative recommendations that usually range from 2 to 3 servings or cups of milk or another dairy product **(Weaver, 2014)**. Unfortunatelly, milk consumption has a downward trend (Figure 1) the average Slovak drank only half of what they should in last

year (**VUEP, 2019**). It is a consumer who ultimately influences the existence and prosperity of milk business in the future, hence studying consumer behavior and its influencing factors is interesting for both academicians and practitioners **(Kurajdová, Táborecká-Petrovičová and Kaščáková, 2015)**.

Scientific hypothesis

For the purpose of this paper, the following research assumptions were defined, respecting the main objectives:
-there is a relationship between the gender of the respondent and whether he/she purchases milk,
-more than 80% of consumers consume animal milk,
-consumers are willing to spend a maximum of 1 € per liter of milk,
-consumers are mostly affected by the quality and price of milk,
-factors affecting consumer behavior when buying milk affect women and men differently,
-the impact of factors in the purchasing process differs from the respondent's age.
-there is a relationship between the gender of the respondent and whether he/she consider milk as food beneficial to human health,
-there is a correlation between the age of the respondent and whether he/she believes milk consumption is beneficial to health.

MATERIAL AND METHODOLOGY

The main objective of this paper was to evaluate consumer preferences on the milk market.

To process the theoretical part of this paper, literature of domestic and foreign authors as well as professional articles were used. The questionnaire survey served as a source of primary information. The aim of this survey was to determine consumer habits when purchasing and consuming milk. Questions were trying to find out whether respondents buy and consume milk, what size of package, fat content and type of packaging they prefer, which factors influence them the most, how much money they spend per month to buy milk, how much are they willing to spend per liter of milk and whether they think that consumption of animal milk is part of a healthy diet. The Google Forms questionnaire was sent to potential respondents electronically in the form of a hyperlink. The questionnaire form included a greeting, an introduction of authors and the survey, description of the purpose for which obtained information will be used, and a request to complete an anonymous questionnaire. Firstly, people were asked to answer 9 classification questions and then 26 factual questions, which were primarily closed with pre-selected options for answering. Data were collected from 504 respondents in the time period from 22.4.2018 to 4.12.2018.

In total, 228 men and 276 women joined the questionnaire survey. Expressing these numbers in percentages, 45.24% of respondents were the male and 54.76% were female. Maintaining representativeness was also important when categorizing respondents by age. Respondents of all ages were approached in order to copy the structure of the population of Slovak Republic. Respondents aged from 36 to 45 (20.43%) represented the largest share. Approximately the same percentage of respondents (15%) have chosen the options "18 – 25 years old" and "46 – 55 years old". About 2% more (17.46%) were between 26 and 35 years of age. The lowest representation (11.31%) was in the age category over 66 years and the remaining 19.44% belonged to the age group from 56 to 65 years old. Since we itended to use statistical methods to evaluate questions, it was necessary to test the representativeness of the sample by Chi-square goodness of fit test. In terms of gender, the structure of sample was identical to the main population (p-value = 0.192 and alpha = 0.05). When it came to the age, the acceptance of the null hypothesis (p-value = 0.149 and alpha = 0.05) meant that the results also corresponded to the distribution of the main population. The questionnaire form also included a question about the highest educational attainment. 13 respondents completed primary education, 15.67% secondary education without a school-leaving examination and 50.60% secondary education completed with matricular exam. The rest of the respondents completed higher education of the 1st, 2nd or higher degree. When it came to the economic activity of respondents, the sample contained 71.72% economically active people (employees, employers, self-employed). Other 28.28% consisted of mothers at maternity leave, students, pensioners or unemployed. One of the classification questions categorized respondents per the number of people living in the household with the addressed individual. There were 30 people living alone (this alternative was mainly marked by pensioners), about 130 respondents inhabit a household with two, three or four members. Option "five family members" were chosen by 12.30% and a minority (3.7%) marked the response "more than 5". Based on the monthly income of household, respondents were divided into 6 income categories: up to 500 € (4.96%), 501 – 1000 € and 1001 – 1500 € (almost 30%), 1501 – 2000 € (22.22%), 2001 – 2500 € (7.54%) more than 2501 € (5.95%). In terms of place of residence, 69.64% came from the Western part of Slovakia, 13.89% identified with the option "central part of Slovakia" and the other 16.47% were inhabitants of Eastern Slovakia.

These data were supplemented by information obtained by neuromarketing research using eyetracker, which took place in the Czech Republic in February 2018 and was attended by 30 respondents. However, only 26 (19 women and 7 men) respondents were included in the results since 4 respondents did not meet the conditions for correct calibration values. Device called SMI RED 250 was used, which was produced by the German company SensoMotoricInstruments (SMI). During processing, we used SMI Experiment Center (for design research) and SMI BeGaze (for evaluation of data). The eyetracking device was controlled using sw: SMI iView X.

Statistic analysis

The results of each question were arranged in pivot tables and described in percentage and verbal terms. For a better understanding of the correlation relationships, pairs of hypothesis (null and alternative hypothesis) were formulated and were accepted or rejected using qualitative statistics methods by program XLStat. In the case of finding the dependence between two characters, we also

calculated the coefficients to determine the strength of the dependence. More specifically, the following methods have been used:
-Chi-square goodness of fit,
-Chi-square test for independence,
-Fisher´s test,
-Z-test
-Kruskal-Wallis test,
-Mann-Whitney U test,
-Wilcoxon test,
-Nemennyi test.

RESULTS AND DISCUSSION

For this survey, in the first place it was essential to find out whether consumers purchase milk (Figure 2). 76.98% of the respondents were milk buyers. 23.02% does not buy milk at all, or it is bought by another person living in the same household as a respondent. This option was chosen mainly by students or men. For this reason, we wondered whether there was indeed a statistically demonstrable difference in milk purchases between genders, for which Fisher's exact test was used:

H0: there is no dependency between the fact whether the respondent purchases milk and respondent´s gender.
H1: there is a dependency between the fact whether the respondent purchases milk and respondent´s gender.

p-value = 0.001
alpha = 0.05

The assumption of the dependence between purchasing milk and respondent's gender was confirmed because the value of alpha exceeds the *p*-value. It was also necessary to determine the strength of the dependency using the Cramer's V coefficient (0.156), which showed a weak dependence between chosen variables.

Participants of the questionnaire survey were differentiated based on their average monthly expenditures on milk as well. Mostly (42.06%) they were categorized between 11 and 20 €. Respondents who purchase milk worth less than 10 € per month were represented by almost 10%. 18.56% had costs between 21 and 30 € and the largest amounts of milk was purchased by 7.34%. The checked values increased in proportion to food expenditure.

The following question concerned the reason why people buy milk. In this question, respondents could mark more answers, so the results are expressed in relative values to the total number of respondents. Slovak consumers use milk mainly for direct consumption, when it is consumed by the individual or his/her family member (80.16%) or as a raw material for cooking or baking (75.40%). 18.45% are aware of the positive effects of milk on human health and therefore consume it as part of a healthy diet. The smallest group has chosen the variants “cosmetic purposes” and “other” and said that they do not consume milk directly, they use it only for their coffee, or buy it for visitors or other family members.

Moreover, respondents were differentiated based on the periodicity of buying milk. The majority (36.71%) buys milk into the stock. This option was mainly selected by residents of suburban areas and villages where local stores is usually owned by private individuals, where milk prices can be overestimated. Therefore, they usually make their large family purchases in a nearby town and take advantage of potential discounts in supermarkets. A similar amount (approximately 28%) visits a store to buy milk several times a month or several times a week. The smallest group (6.15%) goes to the store with intention of buying milk every day.

When it comes to the frequency of drinking milk, over half the group consumes milk with high frequency (29.17% daily and 34.72% several times a week). 13.30% identified with the answer “several times a month”. These high percentages may be caused due to the fact that dairy represents important industry in many European countries **(Buleca, Kováč and Šubová, 2018)** and milk drinking and dairy consumption in Slovakia have historical origin. 22.82% of respondents discarded milk from their diet. They could choose this option for a variety of reasons: they do not consider drinking milk healthy, it is difficult for them to digest milk, they have a milk allergy or simply they do not like it´s taste.

The main aim of the following question, was to find out how many liters of milk a person consumes on average per week (this question was answered only by milk drinkers).

Since this question had open character, respondents were able to freely write the volume of milk in liters and a variety of answers were obtained. The lowest recorded value was 2 dL and the highest was 7 liters. The most frequently respondents said that they consume from 1 to 2 liters of milk per week. The following question addressed the respondents' opinion on their milk consumption. 82.14% consideres their consumption to be sufficient and according to 17.86% is their consumption unsatisfactory. Unexpectedly, respondents whose consumption was low have declared their consumption to be sufficient and on the other hand, there were a lot of respondents declaring insufficient consumption even though, their consumption is well above the recommended annual intake reported by experts.

Consumers (97.02%) prefer to buy and consume cows' milk. This option was preferred perhaps, because customers find cow's milk on the store shelves the most. Goat's milk was selected only by 1.19%, but this is quite a detriment, as many studies point to the positive effects of this kind of milk, and even people who are allergic to lactose can drink it. The option "sheep milk" was not selected at all. 1.79% indicated the option “other” and wrote that they consume lactose-free milk (which can be classified as cow's milk) or plant based milk (coconut, almond and soybean), even though these drinks have “milk” only in their name, since only the liquid produced by the mammary glands of female mammals is considered to be milk and these beverages are produced by leaching and mixing the above mentioned ingredients.

In terms of durability 78.97% of consumers prefer to purchase durable pasteurized milk, because of its extended expiration date, which is often even half a year from filling the milk into packaging. 20.63% favored fresh milk that is only treated with basic pasteurization (not heated to 135 °C). It has a higher nutritional value than durable milk, but **Kunová et al. (2017)** claimed than the raw milk can sometimes contain undesirable microorganisms, so it can be dangerous for human consumption. Specific groups

in the society like the elderly people with aweakened immune system, young children and pregnant women (theunborn child) are recommended not to consume any raw milk or rawmilk products **(Baars et al., 2019)**. Two respondents (who were on maternity leave at the time) of the questionnaire survey marked the answer "dried milk", so we assume they meant baby formula.

More than half of the respondents (51.59%) prefer to purchase 1.5% semi-skimmed milk from a fat content point of view. Whole milk was also chosen by a relatively large proportion of respondents (41.47%). These two alternatives are the most advantageous purchase in terms of price and quantity of fat. The group represented by 2.78% chose the option low-fat and 0.40% defatted milk. These respondents were most likely careful about their daily fat intake and therefore choose alternatives with it´s lower proportion. Raw milk, which can be purchased mainly from dairy machines or directly from the farm, and which amount of fat is not artificially treated, specifically targets 3.80%. Although this is the original form of milk and it contains the most nutrients, its price is the highest and perhaps for that reason it has been chosen by such a small proportion of surveyed people.

One question was also dedicated to reveal which milk packaging is the most purchased from the consumer's point of view. Milk packaged in tetrapak (76.79%) clearly wins. Its greatest advantage is its very composition. It is made of cardboard that is recyclable (most commonly made from kitchen towels), polyethylene, which is impermeable to water and microorganisms and aluminum to protect milk from light, oxygen and bacteria. Another of its advantages is its relatively low weight and low production costs. In second place was a glass bottle (14.48%). It is also recyclable and thanks to the thread can be reused, but it is one of the more expensive materials, what is reflected in the price of milk packed this way. Only 11.37% of respondents prefer milk in a plastic bottle. This percentage is low because many people are skeptical of plastics because they can release harmful substances into the fluid.

The volume of milk packaging was also a point of interest of this survey. The current one-liter milk package suits the largest percentage of respondents (87.30%). 7.34% would like to buy a smaller package and only 5.36% would agree with enlargement of the packaging. From the consumer's point of view, the size of package which is currently offered in the market is the right one, so there is no significant reason for companies to change the volume milk is sold in.

For research purposes respondents, who were given multiple options, indentified places where they buy milk. Almost four fifths purchase milk mainly in hypermarkets and supermarkets, which are currently located on almost every corner in Slovakia, and moreover, they come up with a new flyer promoting discounts every week to lure as many customers into their stores as possible. In smaller villages where super and hypermarkets are not yet present, their role is represented by small local grocery stores, which are attended by 40.08% of respondents. Wholesale stores are visited with the aim of buy milk by 20.44%. While these stores are primarily designed for enterprenours, they are nowadays also visited by regular customers. Fewer respondents chose answer "milk from dairy machines" (10.32%), even though autors **Pereira et al. (2018)** highlight positives araising from short character of this supply chain because there are no intermediaries between the producer and the final consumer and all actors are geographically close to each other. Only 6.15% purchase milk directly from producers probably because of limited representation of farms in urbanized zones. Just 3 respondents from the sample order milk via the Internet. Yet such a form of food purchasing is not sufficiently developed in Slovakia and it is currently preferred to purchase electronics.

The fourteenth question of questionnaire form was conceived to find out how much euros is an individual willing to spend per liter of milk. Respondents who consider milk consumption to be unhealthy and do not buy it at all are willing to spend at least (0 €). The maximum value was 5 €. This respondent also stated that he prefers buying raw fresh farm milk, but its price is currently not so high, so he overstated the amount he was willing to pay. It can be concluded that this customer is ready and willing to continue buying milk even if the price increases. The average price was calculated at level of 0.90 €. This fact was proven even by neuromarketing research (Figure 3), when heat maps show that consumers payed more attention to free gift promotion when buying specific product, rather than a discounted cheap milk showed on A-board which was placed next to the entrance of the store "Môj obchod". We also tested the assumption that consumers are willing to spend a maximum of 1 € per liter of milk by Wilcoxon's one-sample one-sided test (more specifically, the one-sample t-test when normality is not met):

H0: consumers are willing to spend just € 1.
H1: consumers are willing to spend less than € 1.

p-value = <0.0001
alpha = 0.05

The results provided by the statistical program XLSTAT showed that the null hypothesis is rejected and the alternative hypothesis H_1 is accepted. Since the alpha is higher than the *p*-value, we conclude that with a 95% probability, consumers are willing to spend less than 1 € per liter of milk, and any price higher than one euro is overstated.

The country of origin is an important criterion at the milk purchasing process for 79.37% of participants, of which 98.00% want to support domestic milk producers and only 2.00% prefer to buy foreign milk. The information about the country of production on the packaging is not important for 20.64% as they make their decision on the basis of other factors.

One of the questions compared the quality of Slovak and foreign milk. The answer "Slovak milk is better than foreign" was chosen by more than 60% of the sample. The second most frequent response (32.34%) was "Slovak and foreign milk have of the same quality". The other 5.95% consider the quality of foreign milk better. The following question was devoted to the opinion of the respondent on the quality of milk on the Slovak market. Using the Likert scale we found out that almost half of the respondents (42.26%) were satisfied with the milk quality and 33.14% were rather satisfied. These results show that consumers

have no major reservations about the quality of milk on the domestic market. Even the survey of **Zajác et al. (2012)** showed that the quality and safety of cow's milk in Slovakia was satisfactory from quality point of view. On the other hand, only 1.39% were dissatisfied and 3.77% were rather dissatisfied. 19.44% of respondents were left without opinion. This answer was mainly marked by individuals who do not consume milk, so they can not objectively assess this fact.

Each respondent was asked to rate 10 predetermined factors influencing them when buyingmilk by using) a number from 1 (does not affect me when buying milk) to 7 (has the greatest impact on me). It can be said that taste and quality of milk are influencing consumer decisioncisions primarily, because if a person does not like the taste or lack the quality of milk, they will most likely not buy it for the second time. As far as the price is concerned, even if milk is not a big item in the consumer's shopping budget, individuals like to save and spend the saved amount to buy another product he/she might not otherwise be able to afford. Advertising and milk packaging have the least impact on decision-making process. In the last few years, everyone is exposed to innumerable advertising, which is perceived by customers rather negatively. Although milk is not widely promoted in the media, due to advertising supersaturation, buyers perceive these campaigns disapprovingly and therefore think their decisions are not affected. The packaging is also not a key factor in the selection in the shop, as its content (as described above) is more important.

We wondered whether there was a statistically proven difference of the impact of these factors. For this purpose, the Nemenyi method was used:

H0: there are no differences between the impact of individual factors on consumer purchasing process.
H1: there are differences between the impact of individual factors on consumer purchasing process.

p-value = < 0,0001
alpha = 0.05

P-value is significantly smaller than alpha, and therefore we confirm the alternative hypothesis. The impact of factors on consumers' purchasing and consumption decisions varies. Table 1 has again confirmed that durability, quality and taste have the greatest impact, and advertising and packaging are the least significant. Repeatedly, also these results were confirmed by gaze plot, which shows the location, rank and time spent looking at selected stimuls (Figure 3). As was mentioned before, customers care about objective features of milk and not marketing ones, so they purchase milk based on their previous experiences and do not pay any attention to its merchandising.

In connection with this issue, the Mann Whitney test was also used to investigate the dependence of gender on ranking of each factor. This test showed no dependency since the *p*-values of all factors were greater than alpha. Men and women therefore place the same importance to different factors in buying and drinking milk. The Kruskal-Wallis test was used to determine the relationship between age and selected factors. The graph shows the *p*-value of individual Kruskal-Wallis test applications for each factor is depending on age (Figure 4). The factors indicated by the red bars were evaluated significantly differently at the level of acceptance 0.05.

Using the Dunn method (Table 2 and Table 3), we can tell that people over the age of 66 and those between the ages of 18 and 25 rank factors brand and advertisement differently (there is a significant difference between these groups). For the youngest respondets they were more important than for the oldest ones. When testing the packaging and country of origin, no significant differences were confirmed.

The questionnaire survey also asked for consumer behavior when buying and consuming special types of milk. More specifically, the questions targeted flavored and lactose-free milk. Flavored milk favors 25.40%. The most sold flavor is chocolate (50.78%), 22.66% prefers vanilla, followed immediately by strawberry (17.97%), bananas (6.25%) and raspberries (2.34%), but authors **Park et al. (2019)** warn that sugar content within flavoured milk can cause chronic diseases such as obesity. Lactose milk purchases on a regular basis only 12.90% of the sample. The reasons for its purchase are: lactose intolerance of the respondent or family member (80%), 13.85% tastes more than traditional milk, and 29.23% is easier to digest.

The next open question was the expected recommended weekly milk consumption (in liters per person). One should drink up to 1 liter of milk according to 7.14%. One fifth of the respondents believe that everyone should consume from 1 liter (inclusive) to 2 liters of milk per week. The largest percentage of respondents (48.02%) thought that people should drink from 2 to 3 liters of milk per week. However, there have also been found individuals who consider optimal consumption of 3, 4, 5, 6, 7, 10, 12 or even 15 liters per week. As the recommended annual consumption is 91 liters per person, all of these people have exceeded the recommendations of specialists.

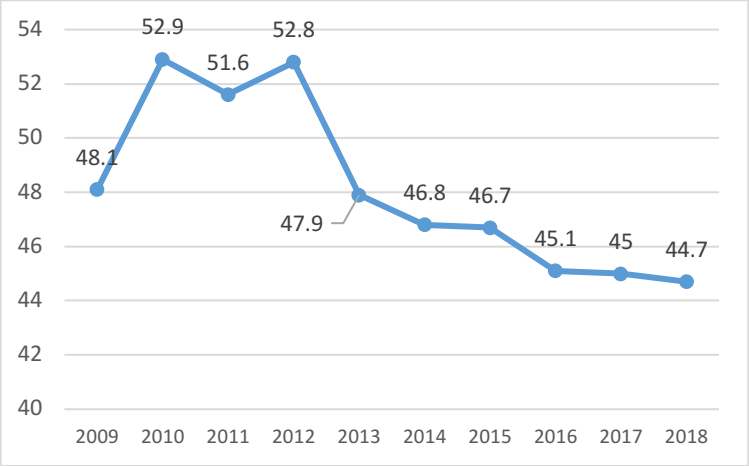

Figure 1 Annual milk consumption per person in liters.

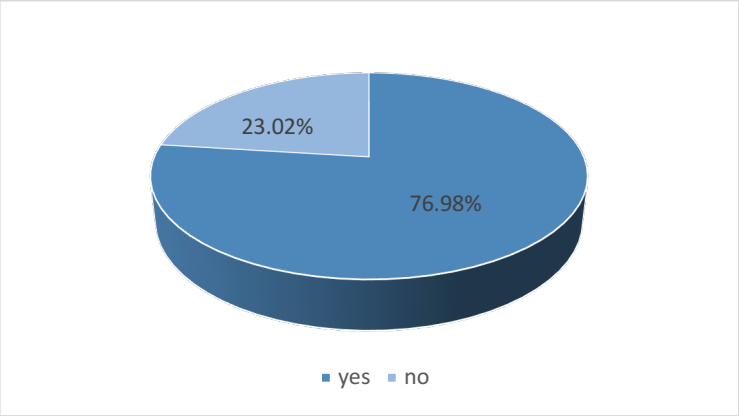

Figure 2 Fact, whether respondend purchases milk.

Figure 3 Stimul 3141 Heat maps; Stimul 3178 Gaze-plot.

Table 1 Importance of chosen factors when purchasing milk.

Factor	Mean	Groups				
Advertisement	3.432	A				
Packaging	3.912	A				
Size of packaging	4.668		B			
Brand	4.780		B			
Country of origin	5.886			C		
Fat content	5.912			C		
Price	6.243			C	D	
Durability	6.479			C	D	E
Quality	6.788				D	E
Taste	6.902					E

Table 2 Evaluation of brand by different age categories.

Sample	Mean of ranks	Groups	
over 66 years old	205.289	A	
from 26 to 35 years old	241.034	A	B
from 36 to 45 years old	246.218	A	B
from 56 to 65 years old	249.663	A	B
from 46 to 55 years old	267.419	A	B
from 18 to 25 years old	296.494		B

Table 3 Evaluation of advertisement by different age categories.

Sample	Mean of ranks	Groups	
from 36 to 45 years old	227.796	A	
from 56 to 65 years old	239.163	A	B
over 66 years old	248.263	A	B
from 46 to 55 years old	252.263	A	B
from 26 to 35 years old	261.636	A	B
from 18 to 25 years old	294.910		B

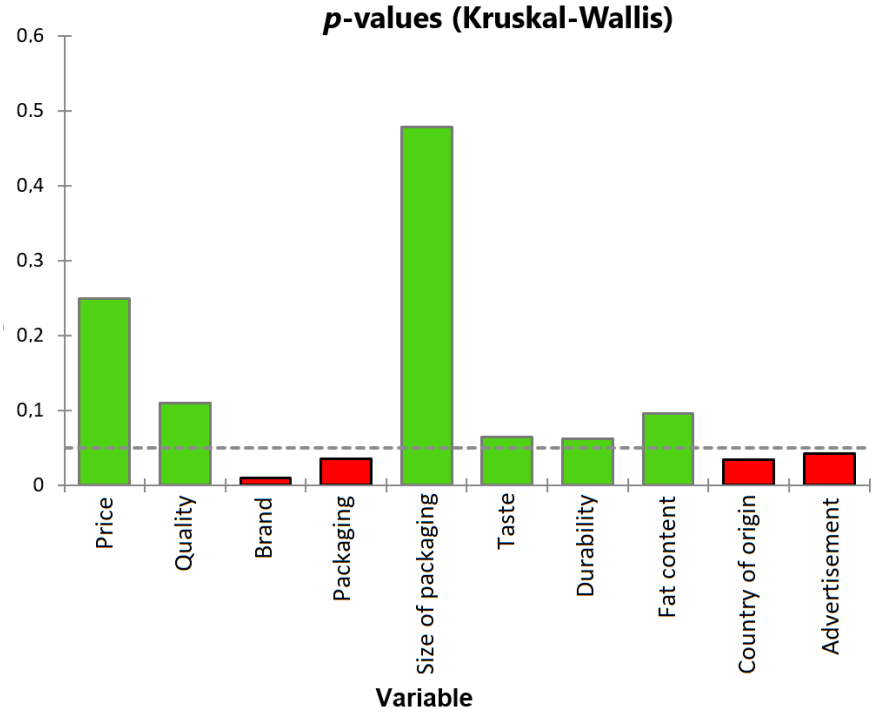

Figure 4 Results of Kruskal-Wallis test.

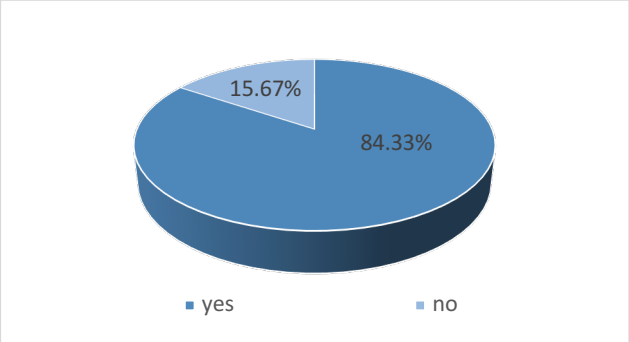

Figure 5 Perception of milk consumption as part of healthy lifestyle.

The last part of the questionnaire was focused on the issue of milk as a part of a healthy diet. 84.33% perceived consumption of milk positively, on the other hand 15.67% had opposite opinion (Figure 5). A consumer survey was also conducted by **Kubicová, Predanocyová and Kádeková (2019)**, in which they identified that 80% of consumers include consumption of milk and dairy products in a healthy lifestyle. When justifying positive impact of milk consumption, following responses were most commonly seen:
-strengthens bones and teeth,
-contains important nutrients for our organism,
-it is natural,
-easily digestible food,
-provides nutrients to the body,
-provides calcium and iron supply,
-supply the body with the necessary protein,
-vitamins and minerals,
-natural baby food,
-boosts immunity,
-no acid reflux,
-is simply tasty,
-fat content,
-source of fatty acids,
-in the case of goat's milk, it has medicinal properties.

Subjects who thought that milk should not be part of the human diet explained their belief as follows:
-the content of drugs and antibiotics consumed by animals,
-it is unnatural to consume breast milk of another species intended for the proper growth of the calf and not of human beings,
-milk is not as important as other foods,
-is not healthy because it contains dangerous bacteria,
-healthy nutrition is not about milk,
-diluted with water, so it´s quality is lower than in the past,
-personal beliefs,
-creates mucus in the body,
-for adults, it is useless, it should only be consumed by children,
-cow's milk decalcates, which is the exact opposite of what is presented to consumers,
-milk is healthy just when it is purchased directly from the breeder, because it is without additional treatment.

We tested the penultimate question using chi-square test for independence to find out if men and women and different age groups responded contrarily.

H0: there is no correlation between gender and whether the respondent considers milk as part of a healthy diet.
H1: there is a correlation between gender and whether the respondent considers milk as part of a healthy diet and its gender.

p-value = 0.99
alpha = 0.05

H0: there is no correlation between respondent´s age and whether he/she considers milk to be part of a healthy diet.
H1: there is correlation between respondent´s age and whether he/she considers milk to be part of a healthy diet.

p-value = 0.660
alpha = 0.05

Both tests showed that there is no significant dependence between variables, because *p*-value was greater than alpha and we accepted zero hypotheses. Thus, we can conclude that gender and age categories do not affect whether respondents consider milk consumption as part of a healthy diet.

CONCLUSION

The results of the questionnaire survey and conducted neuromarketing research showed the following: 76.98% of respondents purchase milk, but this does not mean that other households do not buy milk, it can be purchased by another person residing with the respondent. Most often (42.06%) milk expenses range from 11 to 20 € per month and the admissible price per liter is, on average, 0.89 €. Milk is mainly purchased in hypermarkets and supermarkets (36.71%) and 79.37% of the survey participants decide based on the country of origin (of which 98.00% wants to support domestic producers and only 2.00% buy foreign milk). Other important factors are taste and quality of milk. To characterize the most frequently purchased milk, the current liter packaging of milk suits the majority of respondents (87.30%) and they prefer cow (99.02%), long-life milk (78.97%), in a tetrapak packaging (76.79%) with 1.5% fat content

(51.59%). 84.33% believe that milk has a positive impact on their health.

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DEVELOPMENT OF GLUTEN-FREE NON-YEASTED DOUGH STRUCTURE AS FACTOR OF BREAD QUALITY FORMATION

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ABSTRACT

The article is devoted to the study of the influence of hydrocolloids and animal protein concentrates on the formation of the foam-like structure of gluten-free non-yeast dough as the main factor for bread quality formation. The use of CMC in a concentration of 0.5% is found to be appropriate. The bread volume increases to 236 $cm^3.100g^{-1}$ in comparison with the control sample in water – 202 $cm^3.100g^{-1}$. It is proved that the suggested additives in the amounts of 0.5 – 1.0% Helios-11 and 0.5% CMC solution cause 100% resistance of egg white foam. In this case, the foaming ability increases with the addition of Helios-11 only in amounts up to 1.0%, then decreases for higher amounts of Helios-11 or in the presence of CMC. This can be explained by the increase in density of the whipping mass and the ability of both additives to thicken solutions. In the presence of the additives, the foamy texture of the dough changes. The number of large pores (0.7 – 1.5 mm) decreases almost fourfold, and the number of small and very small pores (0.1 – 0.5 mm) increases significantly. The index of form resistance of the control sample is 32, and in the presence of 0.5% CMC with 0.5 – 1.0% APC is 20 – 21, which indicates a decrease in the surface tension of the aqueous solutions with additives, to a large extent, in the case of joint use.

Keywords: gluten-free; non-yeast; CMC; APC; dough structure

INTRODUCTION

Gluten-free products have a vital role in the prophylactic and curative diet of coeliac disease patients, and are crucial products for consumers with a variety of nutritional disorders such as gluten allergy or intolerance.

According to the World Organization of Gastroenterology, the global prevalence of coeliac disease is at least 1%, but varies greatly between countries. There are no accurate statistics on the incidence of the disease in Ukraine, which is connected to the complexity of diagnosis, but the number of people suffering from coeliac disease and gluten intolerance ranges from 400 to 500 thousand **(Kraievska and Stetsenko, 2018; WGO, 2016)** and even more.

The world medical community, food industry experts and public organizations are paying close attention to methods for identifying gluten in foods, and at the same time simultaneously providing consumers with all the necessary information about its content in certain foods **(Scherf and Poms, 2016)**.

Unfortunately, the problem of giving prior and objective information to consumers about the content of gluten in products in our country receives practically no attention. On the contrary, the legislative base of the European Union countries, the USA, Canada, etc. demands that food manufacturers and employees of the trade networks clearly label products with the sign "Gluten-free" **(Rizkalla Reilly and Green, 2012)**.

Technologies are being developed and the production of gluten-free bread, pasta, biscuits, muffins, pastry flour and others is being set up in many countries for patients with coeliac disease. These products are marked on their packaging with a "crossed grain" symbol. During their production, special attention is paid to the purity of the raw grain materials, from which the smallest impurities, which may be toxic for patients with coeliac disease, should be removed **(Rosell, Bajerska and El Sheikha, 2015)**.

Compared to the countries of North America, Europe, Japan, etc., in Ukraine the production of gluten-free products in sufficient assortment and volumes has, unfortunately, not been established. However, to provide this category of people with specialized dietary foods is a constant necessity.

Since May 2017, after the signing of a licensing agreement between the Association of European Coeliac Societies (AOECS) and the All-Ukrainian "Ukrainian Coeliac Union" and the registration of the "crossed grain" symbol in Ukraine, the licensing of Ukrainian producers

has become possible. Permission to license gluten-free products within Ukraine has greatly simplified the procedure for assigning a corresponding symbol, which should positively influence the development of the domestic market of gluten-free products **(Naumova, Doncova and Agramakova, 2017; Ukrainian Coeliac Society, 2019)**.

Bakery, confectionery and pastry flour products that do not contain gluten are one segment of this market. The range of gluten-free flour products in the Ukrainian market is formed mainly by imported products, which are fairly expensive. In addition, most gluten-free products in Ukraine are flour confectionery or baking mixtures, which can be used at home.

It is clear that for consumers the preparation of gluten-free food products is primarily a dietary matter. But in the production of gluten-free bakery products, the exclusion of gluten becomes a serious technological challenge and requires the solution of a number of technological problems. During the last decades, many studies have been conducted to improve the quality of gluten-free bread and its nutritional properties. However, there are still some issues with the development of gluten-free bread with a satisfactory structure, shelf life and cost.

Given the foregoing, not only the development of safe and effective therapeutic and dietary alternatives is justified, but also new approaches to the detoxification of gluten or gluten-free compositions. In addition, there is an obvious need for the development of formulations and technologies for the production of gluten-free flour products that have sufficient quality and an affordable price.

Investigations by specialists in the world food industry are aimed at finding gluten-free basic and additional raw ingredients (hydrocolloids, protein components, starches, pseudo-cereal raw materials, etc.), as well as the development of new technological approaches that involve the use of enzymes, high pressure, hydrothermal treatment, extrusion and sprouting of grain and flour raw materials, dough souring, etc. Each ingredient has a special role in gluten-free bread baking.

Rice flour and rice starch, corn flour and corn starch, potato, manioc and wheat starch are the most common and widely used raw ingredients **(Dotsenko et al., 2019; Morais et al., 2014; Mancebo et al., 2015; Gómez and Sciarini, 2015)**. As alternative raw materials, gluten-free flour from cereals (sorghum, millet, oats) **(Trappey et al., 2014; Marston, Khouryieh and Aramouni, 2014)** gluten-free flour from pseudo-cereals (buckwheat, amaranth, quinoa); flour from roots and tubers (cassava, sweet potatoes); legume flour (soybean, chickpeas, conifer, beans, lentils, peas); other flour (linen, chestnut, banana, teffi, etc.) **(Hager and Arendt, 2013; Mariotti, Pagani and Lucisano, 2013; Korus et al., 2015; Aguilar et al., 2016; Aguilar et al., 2015)** as well as flour mixtures are offered.

Starch and protein components are important because they cause the transformation of gluten-free dough as a foam-type system to the bread system **(Nitcheu Ngemakwe, Le Roes-Hill and Jideani, 2014)**. Since gluten-free bread contains a large amount of starch, the beginning of bread firming is faster than that of bakery products containing gluten. The origin of the starch (in particular, the size of the starch granules, the ratio of fractions, chemical and physical modifications) significantly influences its technological behaviour in terms of the ability to swell, water-binding, and the rate of gelatinization–retrogradation. This, in turn, is related to the rheological properties of the dough, the structure of the bread and the terms of its storage **(de la Hera, Martinez and Gómez, 2013)**.

Also, dietary fibres are considered as a special raw material for gluten-free bread production. As a rule, the higher the amount of dietary fibre in the dough, the greater the amount of water needed to obtain dough of a given consistency. In gluten-free bread production, the amount of water used to make the dough is often practically the same as (or more than) the total number of dry recipe ingredients.

Fibres can play a positive role in gluten-free bread quality. Psyllium is a generic name for several Plantago family members whose seeds are used for the commercial production of gum-substances **(Pal et al., 2019)**. Psyllium develops a "weak gel" network that captures carbon dioxide formed during fermentation, and therefore increases the gas content and bread volume. This bread is stable at different pH and temperature levels and is similar to gluten. Therefore, Zandonadi and co-authors suggested replacing gluten with Psyllium in gluten-free dough **(Puppin Zandonadi, Braz Assunção Botelho and Coelho Araújo, 2009)**.

It has been established that soluble Psyllium can play a crucial role in the development of gluten-free bread quality, as well as in its ability to form a film and to detect the anti-inflammatory effect, which it has due to its high water-binding ability. Its technological effect and functional properties can be enhanced in the presence of other additives **(Cappa, Lucisano and Mariotti, 2013)**.

The use of hydrocolloids together with thickeners or stabilizers, such as gum arabic, carboxymethylcellulose or guar gum, opens up significant prospects for the creation of alternative gluten-free products that are not worse in quality than those containing gluten.

Hydrocolloids are widely used as structuring agents to simulate the visco-elastic properties of gluten. These ingredients are generally used as a substitute for gluten due to their thickening ability, high water-binding and gel-forming characteristics. They are able to control the properties of the water phase, and stabilize the structure of emulsions, foams, suspensions and multiphase systems **(Morreale, Garzón and Rosell, 2018)**.

Hydrocolloids increase the volume of the dough, stabilizing its foam structure by increasing its viscosity, flocculation and coalescence. Hydrocolloids also prevent the influence of the water phase on the foam structure, improving the stability of the fluid in the films surrounding gas bubbles. Hydrocolloids can significantly affect the behaviour of the dough, even if they are present in very small amounts **(Moreira, Chenlo and Torres, 2012)**.

Proteins play a crucial role in determining the structure of many foods, including gluten-free bread **(Ziobro et al., 2013)**. Due to their specific functional properties, proteins of animal origin are widely researched and offered for use in food systems.

One review **(Deora, Deswal and Mishra, 2014)** aims to highlight the role of alternative protein components that

can be used to develop gluten-free products both functionally and nutritionally. There is enormous potential for the incorporation of proteins from various sources (dairy products, cereals and legumes) to improve the nutritional value of gluten-free products in addition to structural and texture-forming properties. In particular, the range of food proteins used in the development of gluten-free products include the following: soybean protein isolate, pea protein isolate, milk protein isolate, rice protein isolate, whey protein, egg white protein, zein protein, yeast protein, casein, albumin, kafirin, carubin etc.

An important functional component of the gluten-free bread formula is enzyme preparations. The paradigm of modern enzymatic modification of gluten-free bread is aimed at changing the structure of the dough by hydrolysis, oxidation or cross-linking, which leads to an improvement in the structure of the crumb, the quality of the fresh bread, and also predetermines the extension of shelf life **(Renzetti and Rosell, 2016)**.

The addition of guar gum and microbial transglutaminase leads to greater stability of gluten-free dough to the mixing process. The network of proteins formed becomes similar to the structure formed in the presence of gluten. The addition of transglutaminase has a positive effect on the yield of dough and the maintenance of moisture in the bread after baking. Negative effects of transglutaminase on the specific volume of bread may be levelled by the addition of guar gum **(Mohammadi et al., 2015)**.

It should be noted that the majority of the analysed scientific works concerning the improvement of gluten-free bread technology are devoted to the relevant issues of its production using yeast as a dough loosener. In other words, the rheological properties of such dough masses should ensure the maximum keeping of gas bubbles throughout the dough preparation, as well as in the first stage of baking.

In the technology of gluten-free products the main problem of creating high-quality goods is the absence from flour raw materials of a unique structure-forming agent – gluten. Because of this, the gas-forming ability of the dough significantly decreases, especially during prolonged fermentation. Despite the sufficient activity of yeast in gluten-free dough, the effectiveness of this method of dough loosening is very low. All technological efforts are aimed at keeping carbon dioxide during dough making (fermentation, proofing, etc.).

In other words, the active course of the sugar fermentation process and the formation of carbon dioxide in gluten-free dough is almost completely levelled off. Because its accumulation in dough is ineffective, since after the maximum stretching the dough is under the action of the generated gas, a continuous network ruptures, and as a result, the dough loses volume. All this leads to an increase in the loss of the dry matter of the dough – fermentation continues, and the gas is not retained.

In our opinion, another technological concept is needed for the production of gluten-free non-yeast dough. It is necessary to ensure, firstly, the maximum foaming capacity of the recipe mixture, and secondly, the maximum resistance of such foam during the short development of the dough (placing the dough in the baking moulds) and at the initial stage of baking. That is, to improve the porous structure of gluten-free non-yeast bread, another, non-microbiological method of loosening is needed.

Information on this issue is extremely limited in the sources investigated. In the absence of a microbiological leavening agent (yeast), enzyme preparations can be used. So, for gluten-free bread, **(Marti et al., 2015)** suggest using gluten inoculum in a mixture with gluten-free flour (1:1), which should be mixed with water. The fermentation stage lasts about 15 hours. The inoculum is obtained directly from the usual wheat yeast dough, which is kept in spring water for 24 hours. The dough is then dried and, together with water enriched with microorganisms, they are added to gluten-free flour raw materials. Then the dough is fermented for 24 hours. A refreshment step is recommended to be repeated every day at least five times. This study shows that from wheat fermented dough gluten-free dough can be obtained that is suitable for bread production without the addition of yeast or lactic acid bacteria, since the inoculum already contains live and viable microbial strains (lactic acid and yeast). However, in our opinion, it is possible to call this a non-yeast product only conditionally. This method of bread production is similar to that used in the production of sourdough or pre-dough. In addition, one of the obstacles to widespread adoption of this technology is its complexity and long-term sustainability.

To obtain a porous structure of bread without the use of yeast, a whipping operation can be used. In this area of focus, a number of papers have been published by **(Magomedov, Ponomareva and Aleynik, 2008)**. Thus, it is suggested to mix a dough using whole wheat flour, an enzyme preparation "GC-106" – (Döhler, Germany) – fungal protease produced by Aspergillus oryzae in the amount of 0.008 – 0.012% to the mass of flour, citric acid, salt and drinking water. Dough-making is carried out in two stages: first, all the recipe ingredients are mixed in the kneading chamber for 8 – 10 minutes, at 45 – 55 °C, then air is supplied to the chamber with a pressure of 0.35 – 0.45 MPa and the dough is kneaded for 6 – 10 minutes. The authors claim that this helps to increase the yield of the finished products, to obtain whipped non-yeast products with high nutritional and biological value, to slow down the process of bread firming, and to intensify the process of dough preparation. Analysing such a suggestion, one notes the complexity of the process flow diagram and the use of wheat as a grain raw material.

Another area of focus in non-yeast bakery is extrusion technology. The extrusion of supercritical fluid (SCFX) allows us to continuously produce non-yeast dough by the inclusion of supercritical carbon dioxide (SC-CO2). In this study **(Ruttarattanamongkol, Wagner and Rizvi, 2011)**, the optimal formation of dough, production and baking were developed. The combination of vacuum and common baking is recognized as an approach that can be useful for the continuous production of dough and the finished product. The total duration of dough-making is less than 1 hour and during proper baking the entire process can be continuous. The authors believe that since no yeast is present, no ethanol is emitted. And this means the absence of harmful volatile organic emissions and no need for an expensive catalytic converter.

In our opinion, the relevant area of the research focus in gluten-free non-yeast bread technology is the use of physical (whipping) and chemical (using chemical baking loosening agents) methods of dough loosening or their combinations, using improving additives such as sodium carboxymethyl cellulose (CMC) and animal protein concentrates (APC).

Scientific hypothesis

We assume that exclusion of dough fermentation stage will reduce the loss of carbon dioxide released during fermentation, prevent a decrease in volume of bread and formation of cracks on crust of baked product. In this case, it is necessary to prove the possibility of foam-like structure formation by mechanical whipping of gluten-free dough for the production of yeast-free bread. Therefore, the tasks were to study the surface properties of water-flour suspensions and the structural-mechanical properties of dough in the presence of improving additives, as well as the effect of CMC and APC on the quality of gluten-free non-yeast breads.

MATERIAL AND METHODOLOGY

During the experimental research and production testing, the following products were selected: flour mixture of "rice flour:corn flour – Frice:Fcorn" in the ratio of 70:30% (rice flour TM World's Rice; corn flour TM "Skvyryanka"); carboxymethylcellulose sodium salt (CMC – SMS 6500); animal protein concentrates (APC – Helios-11 and Scanpro T95); sodium bicarbonate (baking soda); model systems based on egg protein; gluten-free non-yeast breads; gluten-free non-yeast dough with various kneading options. The obligatory general quality indices of all these products correspond to the indicators of the current normative documentation.

The dough was mixed in various ways. Kefir (a source of high quality animal protein that is well absorbed and is able to enrich dough with lactic acid) was used as a liquid phase of the dough in variant 1. Rice-corn flour mixture was used as a flour raw material. Chicken egg and sodium bicarbonate were used as the dough loosener. In addition, sugar and salt were used. The dough loosening used a combined method (mechanical and chemical). Kneading by variant 2 used a 0.5% aqueous solution of CMC as a liquid phase. The dough loosening method was mechanical. APC was used as a technologically active recipe component in variant 3. This additive has good foam-forming and stabilizing properties. The dough loosening method was also mechanical. Variant 4 provides a combination of CMC and APC, which could improve the formation process of gluten-free non-yeast dough at the expense of a more developed and stable dough mass. The dough loosening method is mechanical.

The volume of finished products was measured by a volume meter. Baking loss was defined as the difference between the weight of the dough and the hot bread in percentage. Bread shrinkage was defined as the difference between hot and cooled bread.

The foam-forming ability of egg protein (FFA) and foam resistance (FR) were determined by the Lourie method **(Tikhomirov, 1975)**. Research of the dough foam structure was carried out as follows. A sample of dough was prepared according to the recipe, a portion of dough was poured into a cuvette, made of optical glass K-8 on the technology of UV bonding with an internal size of 20 mm. The sample was photographed in macro mode. The resulting photos were processed using the Photo M 1.21 program, calculating the amount and pore area of a certain size, the total area of pores, total area of dough, ratio of pore area to total dough area, and ratio of total area of pores to the total area of dough.

To determine the surface properties of the water-flour suspensions, we used the lying drop method **(Gorelov and Dranchuk, 2003)**. The diameter of the drop (in several repetitions) was 6 ±1 mm; this ensures that the edge angle will not depend on the diameter, since it is known that, in the case of very small droplets, the influence of the surface tension of the liquid itself (the tendency towards the formation of a spherical drop) is significant, and in the case of large droplets, the forces of gravity begin to dominate. By this method, the angle between the solid surface and liquid at the point of contact of the three phases was measured.

Additionally, a graphical method for determining the shape resistance (H/D) of the drop was used – due to the ratio of the height of the drop to its diameter.

Statistical analysis

Approximation of the obtained experimental data was carried out using the least squares method, as well as the MATHCAD mathematical package and EXCEL spreadsheet packet. The degree of credibility for all experiments is 0.95.

RESULTS AND DISCUSSION

Additives of polysaccharide (CMC, brand SMC 6500) and a protein nature (APC, namely Helios-11 and Scanpro T95) were chosen as enhancers of the gluten-free non-yeast dough structure. Hydrocolloids were used to increase the viscosity of the dough, and stabilize the distribution of the ingredients by preventing accumulation and foam destruction. These can significantly affect the behaviour of the dough, even if they are present in very small quantities. As was shown in the research **(Lazaridou et al., 2007; Sabanis, Lebesi and Tzia, 2009)** improvement of the specific volume of gluten-free bread from rice flour by the inclusion of gums, pectin, carboxymethylcellulose, agarose, xanthan or oat β-glucan was analysed previously. It was found that addition of insoluble fibres to gluten-free formulations significantly increased the bread volume.

At the first stage of the study, the concentration of CMC was chosen as the variation factor. It was also considered necessary to investigate the expediency of simultaneous use of another recipe component, namely bicarbonate sodium (baking soda), as a loosener. Experimental samples were prepared according to variant 2 of dough making without and with added soda. The water temperature was 30 °C. The composition of the flour mixture is "Frice:Fcorn" at 70:30%. The results of laboratory baking are presented in Table 1 and Figure 1.

It can be seen that the use of CMC in a concentration of 0.5% is appropriate. The bread volume increases to 236 $cm^3 \cdot 100g^{-1}$ compared with the control sample with water – 202 $cm^3.100g^{-1}$, a difference of 15%. According to the experimental data, the combined use of CMC and

baking soda is unsuitable, as it leads to excessive loosening of the crumb structure and weakening of its carcass.

An unbroken dough network develops, part of the gas gets lost, and the specific volume reduces. In addition, the colour of the crumb gets noticeably darker, and folds are formed on the lateral surface of the breads. The concentration of 0.7% of CMC is also considered to be excessive, as it leads to some deterioration in the structure of the breads.

Addition of non gluten proteins in the production of glutenfree bread is especially interesting, as those substances have both nutritional and technological role. Their addition reduces amino acid deficits, and impact structure and texture forming properties of the dough, as well as the color and sensory properties of the final product, in this way affecting its consumer acceptance. Their presence may influence storage of glutenfree products, and decrease bread staling. Protein could be added in various forms, as components of gluten-free flours (e.g., rice, soy, pea) or in the form of concentrates and isolates **(Deora, Deswal and Mishra, 2014)**. The formation of dough and bread structure by protein addition is often assisted by the introduction of other supplements such as polysaccharide hydrocolloids, enzymes or surfactants. Among cereal proteins zein and kaffirin have been applied for gluten-free bread supplementation **(Deora, Deswal and Mishra, 2014; Pontieri et al. 2013; Schober et al. 2011; Phongthai, 2016)**. **Andersson et al. (2011)** observed that the addition of corn protein in the presence of hydrocolloids positively influences dough rheology, improves bread structure and increase its volume.

The results of the study of the influence of animal protein concentrates on the specific volume and sample height (without CMC and with 0.5% CMC; water temperature – 30 °C) are shown in Figure 2. Analysis of the dependencies indicates the positive effect of APC on the structural and mechanical properties of the breads. In particular, the specific volume of the bread improves to a greater extent without the use of CMC. This can be explained by the high hydration power of CMC and its ability to increase the moisture retention capacity of the dough. Therefore, even with higher values of the products volume (confirming the fact that the height of the products is maximal when APC is added together with CMC), their mass is also slightly increased, but the specific volume decreases (the "APC + CMC" curves on the graphs are located lower than the APC curves).

It should be noted that the differences between these indicators are very small. However, the combined use of additives changes the structure of the bread – it becomes more elastic and less fragile when slicing and chewing. For the use of APC as an enhancer of gluten-free non-yeast dough, it is necessary to limit its concentration within the range of 0.5 – 1.0% to the mass of flour (higher concentrations lead to a slight deterioration of the structural and mechanical properties of the crumb, and are also inappropriate from an economic point of view).

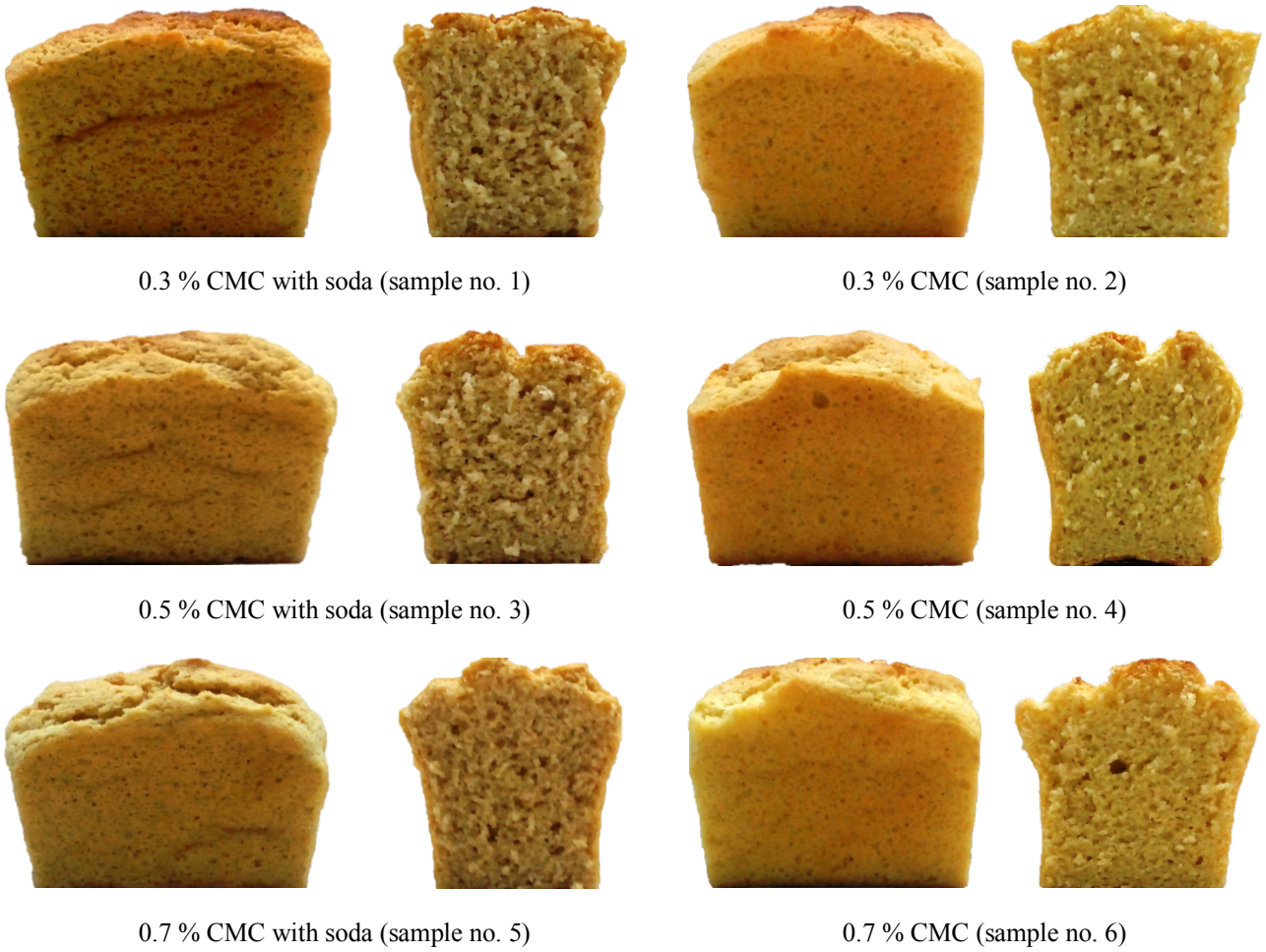
The influence of the CMC concentration on the specific volume and height of samples (without APC and applying 1.0% APC) was also investigated. We can interpret the data obtained as follows. When applying animal protein concentrates together with CMC, similar tendencies are detected, irrespective of the type of additive; namely, increase of the hydrocolloid concentration contributes to a rise of the specific volume and height of the breads at the cross-section if the concentration of CMC does not exceed 0.5%. Further, the structure of the products slightly deteriorates – the volume and height are reduced. Helios-11 has a slightly higher efficiency compared to Scanpro T95, but such advantage is not significant. In other words, any of the studied additives may be recommended for practical use. This approach will make it possible to take into account the availability of raw materials on the market and their market value for obtaining bakery products with a high competitive ability.

The proposed recipe components allow the exclusion both of yeast as a main ingredient of bread dough, and of long-term fermentation as the determining technological stage of dough making. The resulting technological effect requires a clear scientific justification. Considering this, in the next stage of the study, the foam-like structure of gluten-free non-yeast dough is studied.

In the production of traditional wheat bread, when the flour-water mixture turns into dough, gluten forms a visco-elastic network that can capture and retain gassed bubbles. The condition of dough aeration immediately after mixing has a huge impact on the texture of the bread. And the gassed architecture of the dough is regulated by various physical principles related to the formation of foam and stabilization. The specificity of the production of gluten-free non-yeast dough by whipping is that the resulting foam structure is exposed to unwanted external influences, which leads to a decrease in its stability. These factors include mixing the whipped mixture with flour and placing the dough in baking cups. In such conditions, it is important not only to get a foam system with given characteristics, but also to preserve it during the technological process. Accordingly, in our opinion, it is important to investigate the foaming ability and foam stability to fracture.

The suggested additives in the technology of gluten-free bread are intended to improve the properties of the flour protein substances in the absence of gluten proteins. To study its effect, the action of APC on the foaming ability and stability of egg foam without additives and with the addition of 0.5% CMC solution (in the amount of 10% of weight of egg protein) at fixed whipping modes and a temperature of 20 °C was investigated. At the same time, the foaming ability (FA) and foam resistance (FR) for an egg white + water model (10% by weight of egg protein) were studied. Usually, adding about 10% water to the weight of the egg white improves the foaming ability of the dough. Therefore, this precise amount was chosen for the control sample to minimize the effects of the water and to demonstrate the specific effects of the CMC additives.

In order to confirm the effective impact of the CMC, the improvement of FA and FR rates was expected in an egg white protein + 0.5% solution of CMC compared to an egg protein protein + 0.5% solution of CMC compared to the egg protein + water sample. The experimental data are presented in Figure 3.



0.3 % CMC with soda (sample no. 1) 0.3 % CMC (sample no. 2)

0.5 % CMC with soda (sample no. 3) 0.5 % CMC (sample no. 4)

0.7 % CMC with soda (sample no. 5) 0.7 % CMC (sample no. 6)

Figure 1 Appearance (section) of gluten-free non-yeast breads with the addition of baking soda and CMC.

Table 1 Physico-chemical indices of gluten-free non-yeast breads with addition of soda and CMC (n = 3, $p \leq 0.05$).

No. of sample	Type and concentration of additive	Specific volume, $cm^3.100\ g^{-1}$	Baking loss, %
1	0.3% CMC with soda	214	15.0
2	0.3% CMC	222	15.0
3	0.5% CMC with soda	220	12.5
4	0.5% CMC	236	12.5
5	0.7% CMC with soda	203	10.0
6	0.7% CMC	198	11.3

Table 2 Characterization of wetting angle (n = 3, $p \leq 0.05$).

Type and concentration of additive	Characterization of wetting angle	
	α	sin α
Without additives	83	0.993
0.5% CMC	80	0.985
0.5% CMC+ 0.5 Scanpro T95	74	0.961
0.5% CMC+ 1.0 Scanpro T95	71	0.946
0.5% CMC+ 1.5 Scanpro T95	80	0.985

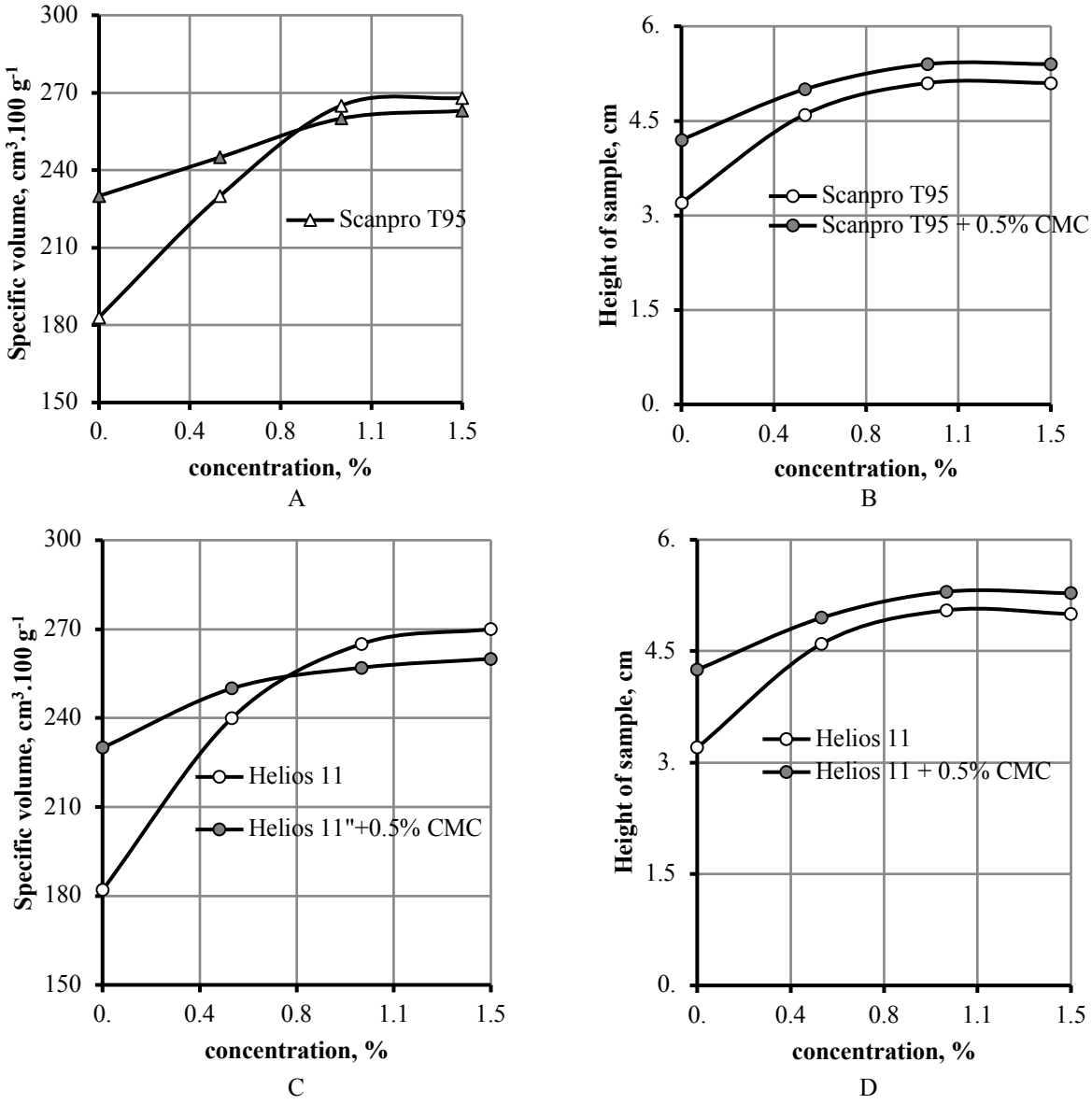


Figure 2 Specific volume and height of bread samples depending on concentration of animal protein concentrates (without CMC and with application of 0.5% CMC).

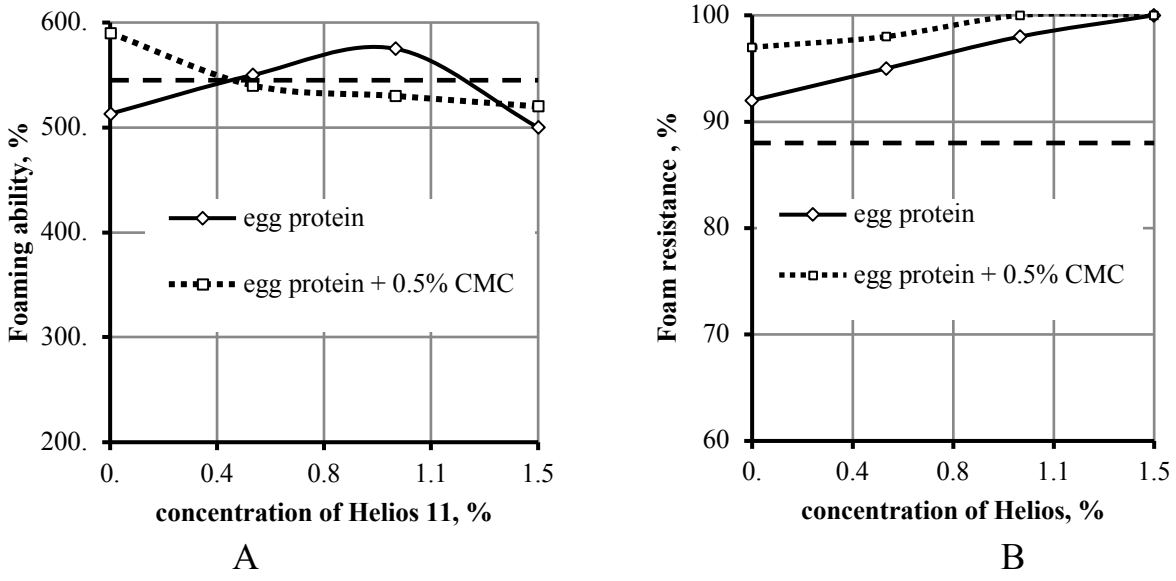


Figure 3 Influence of amount of APC (Helios-11) on foaming ability (a) and foam resistance (b) of egg protein without additives and with addition of 0.5% CMC solution (CMS 6500, 10% by weight of egg protein).

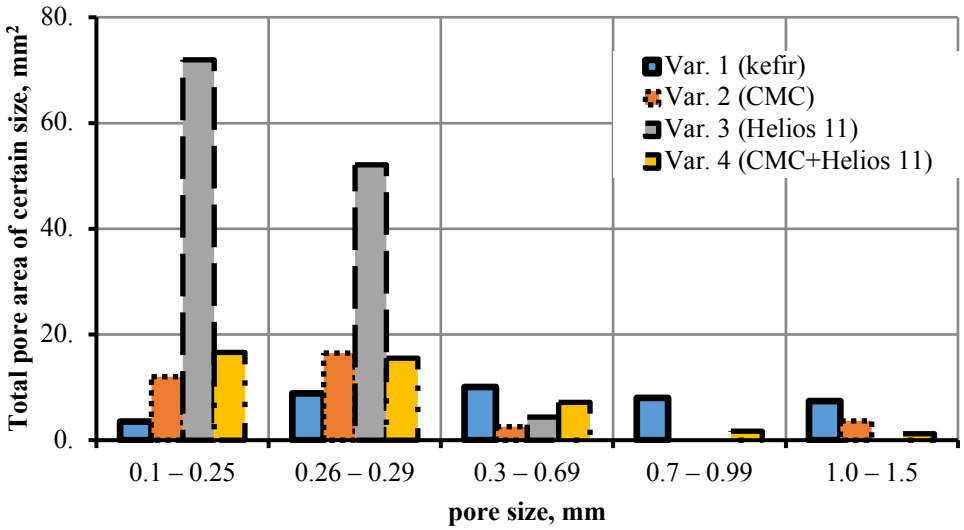


Figure 4 Total pore area of certain size of gluten-free non-yeast dough with different recipe compositions (variants 1, 2, 3 and 4).

Table 3 Results of mathematical processing of quantitative estimation of dough foam structure with different recipe compositions.

Indexes	Pore diameter, mm				
	0.1 – 0.25	0.26 – 0.29	0.3 – 0.5	0.7 – 1.0	1.1 – 1.5
Average area of one pore, mm²	0.03	0.07	0.13	0.57	1.23
Variant 1 (kefir)					
Number of pores in the field of view, pcs.	117	127	77	14	6
Total pore area, mm²	3.51	8.89	10.01	7.98	7.38
Total dough area, mm²	600	600	600	600	600
Ratio of pore area to total area of dough, %	0.59	1.48	1.67	1.33	1.23
Ratio of total pore area to total area of dough, %			6.3		
Variant 2 (CMC)					
Number of pores in the field of view, pcs.	400	235	20	0	3
Total pore area, mm²	12	16.45	2.6	0	3.69
Total dough area, mm²	600	600	600	600	600
Ratio of pore area to total area of dough, %	2	2.74	0.43	0	0.62
Ratio of total pore area to total area of dough, %			5.79		
Variant 3 (Helios-11)					
Number of pores in the field of view, pcs.	2400	744	34	0	0
Total pore area, mm²	72	52.08	4.42	0	0
Total dough area, mm²	600	600	600	600	600
Ratio of pore area to total area of dough, %	12	8.68	0.74	0	0
Ratio of total pore area to total area of dough, %			24.42		
Variant 4 (CMC + Helios-11)					
Number of pores in the field of view, pcs.	552	221	55	3	1
Total pore area, mm²	16.56	15.47	7.15	1.71	1.23
Total dough area, mm²	600	600	600	600	600
Ratio of pore area to total area of dough, %	2.76	2.58	1.19	0.29	0.21
Ratio of total pore area to total area of dough, %			7.02		

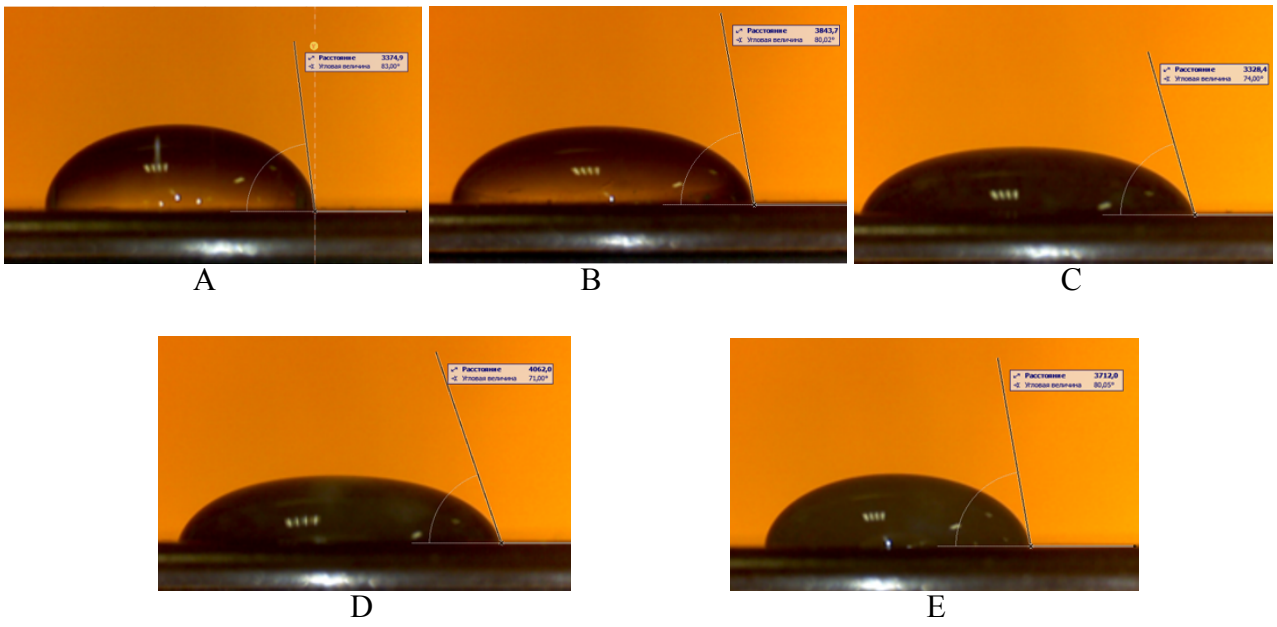


Figure 5 Appearance of experimental samples – water drops with supplements: a) without supplements; b) 0.5% CMC; c) 0.5 % CMC + 0.5 % Scanpro T95; d) 0.5 % CMC + 1.0 % Scanpro T95; e) 0.5 % CMC + 1.5 % Scanpro T95.

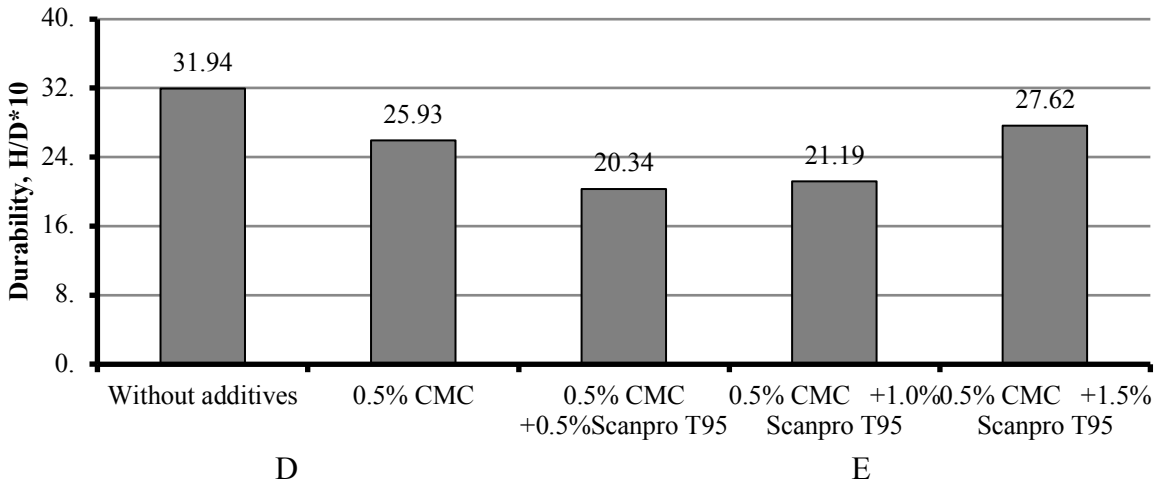


Figure 6 Durability of droplets of studied samples.

The graphs show that the addition of Helios in concentrations from 0.5 to 1.5% contributes to an increase in the foaming capacity of the egg protein, but the effect is extreme: with up to 1.0% of additive, the foaming capacity increases by 10 – 15%, while with an increase in the Helios up to 1.5%, the FA reduces to the control values.

In the presence of 0.5% CMC solution, the influence of Helios-11 on the FA changes to the opposite – the index gradually reduces with increase of the amount of Helios-11, reaching 520% (which is 88% of the control value (egg whites with 0.5% CMC). The foam stability improves with the addition of Helios-11 separately and in the presence of CMC, bringing the value closer to 100% at 1.0% Helios-11 and 0.5% CMC solution. The FA reducing tendency of egg protein can be explained by the increase of mass density while whipping due to the ability of both additives (CMC and APC) to thicken the solution, because the dilution of a colloidal solution of egg white protein (e.g., with the addition of 10% water) increases the FA of the system, reaching 545% compared with the native protein – 500%, although the foam resistance is reducing (to 88%).

At the next stage of the study, quantitative assessment of the foam structure quality of the dough was determined. Dough samples were prepared according to the suggested variants and placed in glass cuvettes. The macro-photography method fixed the visual structure. The number and size of pores were determined using the Photo M 1.21 program, and the results of their mathematical processing are in Figure 4 and Table 3. The analysis shows that in the presence of any additive, the structure of the foam/dough changes, namely: the number of large pores (about 1 mm or more) reduces almost fourfold. Thus, the total area of large pores of sizes 0.7 – 1.5 mm in the dough sample according to variant 1 (without additives) is 15.36 mm^2 in the field of view, and in the experimental

samples (variants 2 and 4) is 3.69 and 2.94 mm^2 respectively, while variant 3 is zero. At the same time, the number of small and very small pores (less than 0.5 mm), especially in the sample with added Helios-11 (variant 3), increases significantly. The data obtained correlates with the data of the foaming capacity, which is the highest with the addition of Helios-11 (Figure 3). The foregoing shows that the suggested supplements for improving gluten-free non-yeast dough contribute to the improvement of the porosity of the foam-like structure, forming a fine, uniform foam. This effect can be explained by the ability of the additives to improve the foaming ability and foam resistance to fracture. The formulation without gluten can only retain gas if another gel replaces gluten, being important that the ingredients form a continuous phase for stabilizing gas cells **(Gallagher, Gormley and Arendt, 2004; Sabanis, Lebesi and Tzia, 2009)**.

The substrate availability of gluten-free raw materials plays an important role for biological gas production through microorganisms, which can additionally improve the gas retention capacity by synthesizing hydrocolloids. Moreover, the deficient volume of gluten-free dough might be substantially improved by optimizing mechanical aeration via beating. High-speed mixing can provide a homogeneous distribution of small gas bubbles. Computed tomography is the method of choice to monitor gas bubbles if structure-conserving preparations and sufficient resolution are provided. To replace the traditional kneading stage, processing adaptions should provide maximum gas entrapment by mixing **(Elgeti, Jekle and Becker, 2015)**. Porosity of gluten-free bread crumb defined by the image analysis showed significant differences with the inclusion of buckwheat flour **(Wronkowska, Haros and Soral-Śmietana, 2013)**.

Foaming is a complex process due to the combined influence of numerous physico-chemical, physico-mechanical and other factors. Regularities that characterize the process of foam formation essentially depend on the conditions of a particular technological process.

For the study of surface phenomena at the interface between phases (gas-liquid-solids), it is expedient to apply a method based on measurement of the surface tension of this section boundary, which allows very reliable data to be obtained, provided that the temperature, volume of the system and the chemical potentials of all components in both phases remain constant. The experiment results are shown in Figure 5, and the data of its processing in Table 2 and Figure 6. During the experiment, the measurement time was 10 s (for all samples), since even after a short period of holding samples before measurements, the shape of the drop changed.

The wetting angle (edge angle) is the main characteristic of wetting. This is the angle of inclination of the surface of the liquid to the wetted surface of the solid. The liquid itself is always inside the edge angle. The top of the wetting corner is located on the line of wetting, which passes through the contact line of the three phases. It was found that during a certain period of time, dispersal of liquid on the surface occurs. Therefore, all samples were examined in identical conditions – after 10 seconds, placing a drop on the surface.

The suggested supplements-enhancers of structure generally reduce the foam resistance, to a greater extent in the case of joint application (Figure 6). Indeed, the H/D index of the control sample is 32, and in the presence of 0.5% CMC with addition of 0.5 – 1.0% of APC is 20 – 21. The decrease in the efficiency of 1.5% APC can be explained by a reverse increase in the surface tension of the liquid phase of the dough (this confirms an increase in the H/D index to 27.6), which occurs due to the possible processes of gelatinization with such an amount of additives.

CONCLUSION

The results obtained show that the use of CMC at a concentration of 0.5% is appropriate. The bread volume increases by 15% compared to the control sample. Consistent use of CMC and baking soda is inappropriate, as it leads to excessive loosening of the crumb structure and frame weakening.

The positive influence of animal protein concentrates on the structural and mechanical properties of bread has been proved. For the use of APC as an enhancer of gluten-free non-yeast dough, it is necessary to limit its concentration to within 0.5 – 1.0% of the mass of flour (higher concentrations lead to a slight deterioration of the structural and mechanical properties of the crumb, and are also inappropriate from an economic point of view). The suggested additives for improving gluten-free non-yeast dough contribute to improving the porosity of the foam-like structure, forming a fine and uniform foam. It was established that the total area of large pores in the range of 0.7 – 1.5 mm in the variant 1 dough sample (without additives) is 15.36 mm^2 in the field of view, and in the experimental samples, for variants 2 and 4 is 3.69 and 2.94 mm^2, respectively, while variant 3 is zero. At the same time, the number of small and very small pores (less than 0.5 mm), especially in the sample with added Helios-11 (variant 3), increases. The index of the foam resistance of the control sample is 32, and in the presence of 0.5% CMC with additives 0.5 – 1.0% APC is 20 – 21, which indicates a decrease in the surface tension of aqueous solutions with additives-enhancers of the structure, to a certain extent – in the case of consistent application.

Such data is in good agreement with the results of other research on the formation of an improved foamy texture of dough, and better organoleptic properties based on the results of laboratory baking, which determines the use of these additives in the indicated amounts. This also coincides with the interval recommended for effective concentrations.

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DETECTION OF MICROBIOTA IN THE VINEYARDS OF THE TOKAJ WINE REGION

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ABSTRACT

Tokaj is an important Central European wine-growing area with controlled planting and authorized varieties of white vines. This area has a specific microflora composition which changes based on its climate dependence, as well as during the fermentation process of wine production. Therefore, the aim of this study was, by culture examination of the samples, to detect the microbiota of soil, leaves, berries and fermentation must from two vineyards from the Slovak part of Tokaj. The highest total viable count (5.60 ±0.01 log cfu.g^{-1}) and the highest total yeast and mould count (4.32 ±0.01 log cfu.g^{-1}) in soil samples were recorded in vineyard Berecký. The highest total viable count in soil samples (6.71 ±0.01 log cfu.g^{-1}) was confirmed by examination of samples originating from the vineyard of Čierna Hora. When determining the total yeast and mould count, the highest numbers were recorded in the must samples (4.15 ±0.01 log cfu.mL^{-1}). Lactic acid bacteria were collected in samples from both vineyards, only in very low numbers. Overall, statistically significant differences (p <0.001) were detected by comparing the microbiota of the samples taken from the Berecký and Čierna Hora vineyards. The specific characterisation and identification of yeast was carried out using ITS-PCR-RFLP methods. The analysis confirmed the presence of yeasts of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Candida parapsilosis* and *Candida tenuis* and their subsequent transfer to the must at varying percentages.

Keywords: microbiota wines; terroir; ITS-PCR-RFLP; yeast

INTRODUCTION

In the countries with the highest wine production, the microflora of the oenological process is regularly monitored for variety, climatic conditions and geographical location **(Piknová and Jankura, 2019)**. Tokaj is an important Central European wine region located in the Bodrog river basin. The uniqueness of the Tokaj region is that it extends on the territory of two independent states - Hungary and Slovakia. The Slovak part consists of vineyards in the cadastral territory of 7 wine-growing villages (Bara, Čerhov, Černochov, Malá Tŕňa, Slovenské Nové Mesto, Veľká Tŕňa and Viničky). Planting is controlled and only white varieties of vine are allowed, namely Furmint, Lipovina and Yellow Muscat **(Furdíková, Kakaš and Malík, 2015)**.

The quality of Tokaj wines is influenced by several factors. Soil resulting from weathered ryoliths has an impact. The high potassium content of these rocks has a positive effect on the vine, as it is increasingly required during the growing season. Another important and specific factor is the location of Tokaj vineyards with positive climatic conditions **(Farkaš, 2002)**. These factors also have a significant impact on the biogeography of microorganisms (including yeast) in the ecosystems **(Nedomová et al., 2016).** Yeasts (especially *Saccharomyces* spp.), which are the most important group of microorganisms in wine production, also have a significant impact on quality. The population of non-Saccharomyces species decreases in fermentation processes and the wine yeast *Saccharomyces cerevisiae* completes the fermentation. The ability of *S. cerevisiae* to replace non-*Saccharomyces* species is associated with its higher fermentative power, alcohol tolerance and secretion of killer-like compounds **(Albergaria and Arneborg, 2016)**. During fermentation also occur to other genera and species of microorganisms, which ultimately positively affect the taste, smell the wine, but can also have a negative impact **(Fugelsang and Edwards 2007; Piknová et al., 2017)**. All these microorganisms can be found ecologically linked to the wine environment **(Spano and Torriani, 2016)**.

Molecular biological methods in yeast identification can reliably determine which species are found in a given wine-growing area as well as which species apply at a certain stage of fermentation and how they affect this process. From grapes and wine, many yeast genera have

been identified by molecular biological methods such: *Brettanomyces*, *Candida*, *Kloeckera*/*Hanseniaspora*, *Kluzveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces* **(Bartowsky, 2017)**.

Scientific hypothesis

The Tokaj wine region has a specific composition of microbiota, which, however, changes due to its climatic and geographical dependence, as well as during the fermentation and wine production process. Therefore, we expect differences in the composition of microbiota in two different vineyards in the Tokaj wine region, as well as changes in microbiota due to soil and vines on the must's microflora.

MATERIAL AND METHODOLOGY

Soil, grape leaves, white grape berries and must, from the Lipovina variety, from two vineyards in Slovak part of the Tokaj wine region were taken to determine the diversity of vine and must microbiota. Samples were taken from the Berecký vineyard, located in the wine village of Veľká Tŕňa, and from the vineyard Čierna Hora, located in the wine village of Čerhov. Sampling took place from May to October 2018 as follows: sampling of soil and vine leaves in May (1st sampling), subsequent sampling of vine leaves (2nd sampling) and grape berry in August and the first half of October, must samples 24 hours after pressing, were taken.

Culture microbiological examination of samples

Total viable count (TVC)

A 10 g base suspension and a further ten-fold dilution were prepared from the 10 g samples. Inoculum of 1 mL was inoculated in parallel to sterile Petri dishes from three consecutive dilutions. The inoculum was flooded with agar medium Plate Count Agar (PCA) for at least 15 minutes (18 ±2 mL). After agar solidification in labeled Petri dishes, the inoculated broths were incubated in a thermostate at 30 ±1 °C for 72 hours. After the incubation period, colonies were counted in inoculated Petri dishes. The results were evaluated according to **STN EN ISO 4833-1:2014**.

Lactic acid bacteria count (LABC)

A basic suspension and ten-fold dilutions were prepared from the samples according to **STN EN ISO 6887-1:2017**. From three consecutive dilutions, 0.1 mL was spread onto the surface of De Man, Rogosa and Sharpe agar (MRS; Oxoid, UK) selective diagnostic medium. Samples were prepared and evaluated in parallel. Subsequent incubation was performed under anaerobic conditions to propagate mesophilic lactic acid bacteria. The inoculated plates were placed in an anaerostat and incubated at 37 °C for 48 hours. The Anaerobic environment was provided by the AnaeroGen (Oxoid, UK).

Total yeast and mould count (TYMC)

The determination of the number of microscopic fungi and yeast was performed according to a standard procedure **(STN ISO 21527-1:2010)**. Three ten-fold consecutive dilutions were spread on the surface of Dichloran Rose Bengal Chloramphenicol (DRBC; HiMedia, India) agar medium at 0.1 mL and incubated for 5 – 8 days at 25 °C. Solitary yeast colonies taken sterile from the surface of DRBC agar were used for further studies.

Identification of yeast by ITS-PCR-RFLP

To obtain pure and concentrated DNA from yeast, a column isolation kit for the isolation of yeast DNA, NucleoBond® AXG Columns 20 (Macherey-Nagel GmbH & Co. KG, Germany) was used. DNA purity and concentration was detected using a BioSpec nanometer spectrometer (Shimadzu, Austria). The obtained supernatant was used as a DNA source in PCR reactions. The rRNA gene region of interest was amplified in a Thermal Cycler (Techne, Cambridge, UK). Primers used to amplify a given ITS region, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), were synthesized and used according to **(White et al., 1990)**. The PCR reaction was carried out in the following steps: initial denaturation at 95 °C/5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 53 °C/2 minutes and extension at 72 °C/2 minutes, and final extension was performed at 72 °C for 10 minutes. The PCR products were sequenced by the Sanger method (GATC Biotech AG, Germany) and the subsequently obtained strains of those that were being studied were submitted to the GenBank-EMBL database for homology to the sequences available in the GenBank-EMBL database using the BLAST program (NCBI software package).

After evaluation, for accurate species identification, PCR products were digested with restriction endonucleases *Hha*I, *HaeIII* and *HinfI* (New England BioLabs®inc., USA), according to the supplier's instructions. The size of PCR products and restriction fragments was determined based on their mobility in agarose gels, in comparison to the 50 bp standard (Sigma-Aldrich, USA). PCR products and restriction fragments were visualized by UV transillumination using Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Israel).

Statistic analysis

Data analysis was carried out with R – statistics software **(R Core Team. R: 2019)**. A two-way analysis of variance (ANOVA) and Tukey test for multiple comparison of means with a confidence interval set at 95% was conducted according to **Semjon et al. (2018)**. The differences between the vineyard location and the wine processing phase were set as the main factors.

RESULTS AND DISCUSSION

Previous studies of grapes and grape musts microflora revealed valuable indigenous yeast strains, which could serve as the contributors to the regional character of wines specific to different winemaking regions **(Varela and Borneman, 2016; Raymond Eder et al., 2017)**.

Table 1 Culture microbiological examination of samples.

	Vineyard		Two-way ANOVA table (p-value)		
	Berecký (log cfu.g^{-1})	Čierna hora (log cfu.g^{-1})			
Variable	TYMC		**Vineyard (V)**	**Processing (P)**	**Interaction (V x P)**
Soil	4.32 ±0.01^{Ab}	3.82 ±0.01^{Bb}	<0.001	<0.001	<0.001
Leaves first sampling	2.81±0.01^{Bd}	3.3 ±0.01^{Ad}			
Leaves second sampling	4.78 ±0.01^{Aa}	3.64 ±0.01^{Bc}			
Grape berries	3.17 ±0.02^{Ac}	2.61 ±0.01^{Be}			
The must	3.17 ±0.02*^{Bc}	4.15 ±0.01*^{Aa}			
Variable	**TVC**				
Soil	5.6 ±0.01^{Ba}	6.71 ±0.01^{Aa}	<0.001	<0.001	<0.001
Leaves first sampling	4.18 ±0.01^{Ad}	4.15 ±0.01^{Be}			
Leaves second sampling	5.15 ±0.01^{Ab}	4.23 ±0.01^{Bc}			
Grape berries	4.2 ±0.01^{Ac}	4.18 ±0.01^{Bd}			
The must	4.2 ±0.01*^{Bc}	5.18 ±0.01*^{Ab}			
Variable	**LABC**				
Soil	-	-	ns.	ns.	ns.
Leaves first sampling	-	-			
Leaves second sampling	<3.00 ±0.00^{Aa}	<3.00 ±0.00^{Aa}			
Grape berries	-	-			
The must	<4.00 ±0.00*^{Aa}	<4.00 ±0.00*^{Aa}			

Note: V - the main effect of different Vineyard location; P - the main effect of processing phase of the vine production; V x P - interaction effect between the vineyard location and processing phase; ns. - not significant (p >0.05); $^{A–B}$ - in a column means (Vineyard) without a common superscript letter differ (p <0.05); $^{a–e}$ - in a row means (Processing) without a common superscript letter differ (p <0.05); * log cfu.mL^{-1}.

According to **Baroň (2017)**, the soil in the vineyard serves as a reservoir of microorganisms - yeast and bacteria. These are influenced by different selection pressures during the year, reflecting the composition of the soil microclimate, or the given terroir.

These selection pressures change the species representation of microorganisms that come from roots, trunk, leaves to the grapes and affect the microflora of the grapes during the season, and this therefore affects the wine.

To determine the differences in the microflora of soil, vines and musts from different vineyards of the Tokaj wine region, we used soil, vine leaves, grape berries and musts samples. Culture microbiological examination of the samples determined TVC, LABC and TYMC.

As shown in Table 1, the highest TVC was recorded in soil samples (5.60 ±0.01 log cfu.g^{-1}) and vine leaves at the second sampling (5.15 ±0.01 log cfu.g^{-1}). These results also correlate with yeast and fungi counts, where the TYMC also recorded in soil samples of 4.32 ±0.01 log cfu.g^{-1} and in vine leaf samples taken at the second harvest 4.78 ±0.01 log cfu.g^{-1}. LABC were detected only in very low numbers, namely in leaf samples (first sampling) and must collected in October.

Examination of samples originating from the vineyard of Čierna Hora confirmed the highest TVC in soil samples of 6.71 ±0.01 log cfu.g^{-1} and must 5.18 ±0.01 log cfu.mL^{-1}. When determining TYMC, the highest numbers were recorded only in the must samples (4.15 ±0.01 log cfu.mL^{-1}). In contrast, the lowest TYMC were recorded in grape berries (2.61 ±0.01 log cfu.mL^{-1}). The presence of LABC was confirmed in must and leaf samples (first sampling), but in very low numbers (Table 1). Similar to **Kačániová et al. (2019)**, which confirmed LABC counts in the must ranged from 0.48 to 2.06 log cfu.mL^{-1}.

Overall, statistically significant differences (p <0.001; Table 1) were detected by comparing the microbiota of samples taken from the vineyards of Berecký and Čierna Hora originating in the same wine-growing region.

After the cultivation examination of samples from the Tokaj wine-growing region, the team was, in turn, able to proceed with species identification of yeasts isolated on the surface of the DRBC agar medium.

Yeast DNA isolation was performed according to the commercially available NucleoBond® AXG Columns 20 kit. Isolation was followed by amplification of the ITS region of the rDNA using non-specific primers ITS1 and ITS4. Visualization of the resulting PCR products contained 450 bp - 880 bp fragments in the agarose gel. Individual PCR products were subjected to sequencing and subsequent comparison of the nucleotide sequence with the GenBank-EMBL database. Accordingly, PCR products

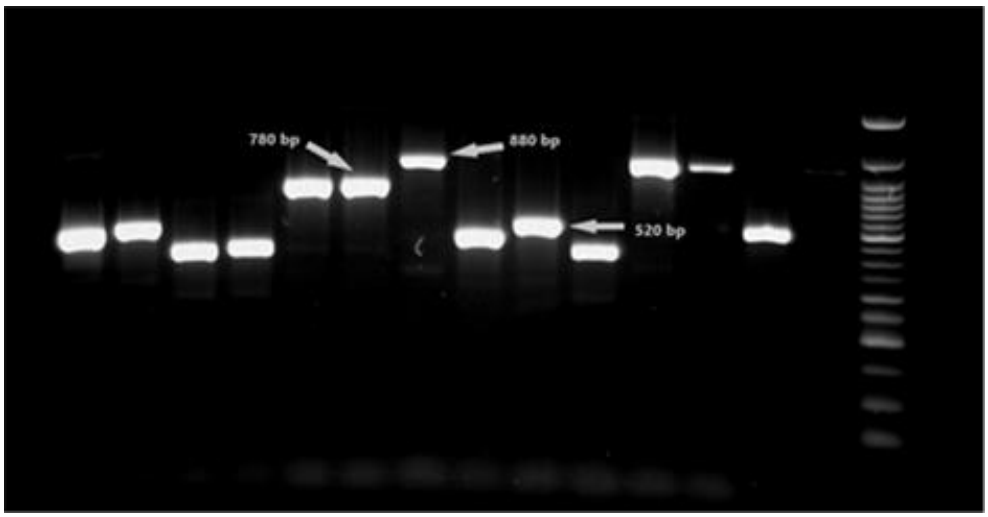
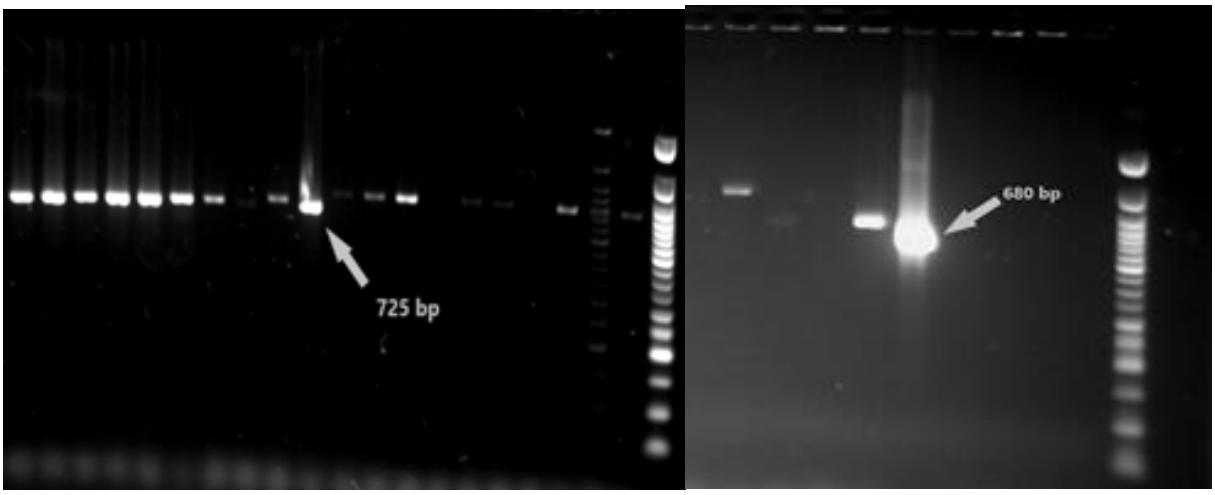


Figure 1 Visualization of PCR products in species *Saccharomyces* spp. 880 bp, *Zygosaccharomyces* spp. 780 bp, *Candida* spp. 520 bp.



A B

Figure 2 Visualization of PCR products in genus: *Zygosaccharomyces* 725 bp (A), *Candida* 680 bp (B).

of 880 bp size corresponded to yeasts of the genus *Saccharomyces*. PCR products of 780 bp and 725 bp corresponded to yeasts of the genus *Zygosaccharomyces* and fragments of 680 bp and 520 bp corresponded to the yeasts of *Candida* (Figure 1 and 2).

PCR products of smaller size (450 bp) were considered to be non-specific products that did not show relevant homology to any sequence in the GenBank-EMBL database, therefore such isolates were excluded from the study. Similarly, **Kačániová et al. (2018)** confirmed the presence of yeasts of the genus *Sachcaromyces* and *Candida* in grapes berries using molecular methods.

After the initial identification at the genus level, the individual PCR products were digested with restriction endonucleases *HhaI*, *HaeIII* and *HinfI* into fragments of different sizes, which generated species-specific restriction profiles (Figure 3 and 4). The restriction profiles of the yeast reference strains (CCM8224, CCM8239, CCM8260, CCM8191) were used to verify the correct yeast species, whereby identification took place via ITS-PCR-RFLP method. As shown in Figure 3 and 4, the restriction profiles of the individual yeast species differ from each other. This is confirmed by the study of **Sadel (2016)**, which also identified various types of yeast from grape must by ITS-PCR-RFLP.

After yeast species identification using the ITS-PCR-RFLP method, the percentages of individual yeast species in the soil, vine and must samples be originating from the Berecký and Čierna Hora vineyards were calculated.

As shown in Table 2, the species distribution of yeast varied in individual samples taken from the Berecký vineyards. In soil samples, the largest proportion of yeasts of the species *Zygosaccharomyces bailii* (90%) was confirmed. This type of yeast was detected at a lower percentage in other samples and this type of yeast was not detected in must. In contrast, *Zygosaccharomyces rouxii* was detected in leaf samples (May and August) and grape berries. The highest percentage was found in samples of vine leaves taken in May (57%).

The genus *Zygosaccharomyces* comprises some of the most feared species in the industries of high sugar and high acidic food products. *Zygosaccharomyces rouxii* is the microbe has the ability to live in environments of extreme salinity or saccharinity, and spoil your food, yet they can also facilitate the production of the subtle and nuanced characteristic flavors we appreciate so much in soy sauce

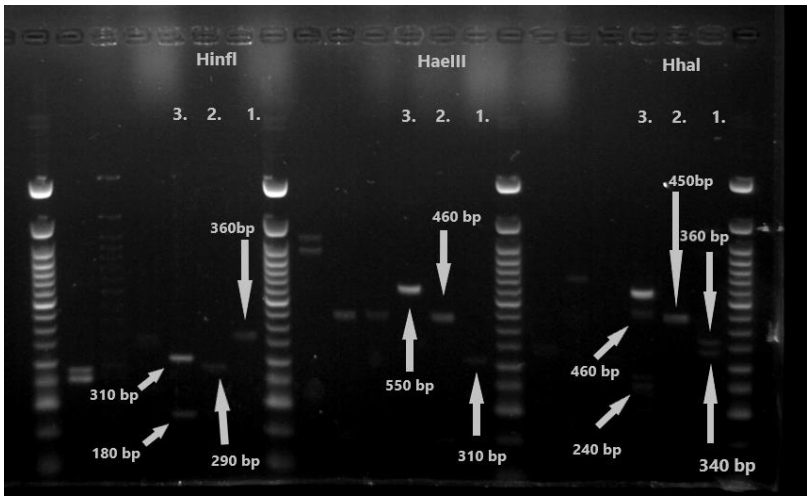
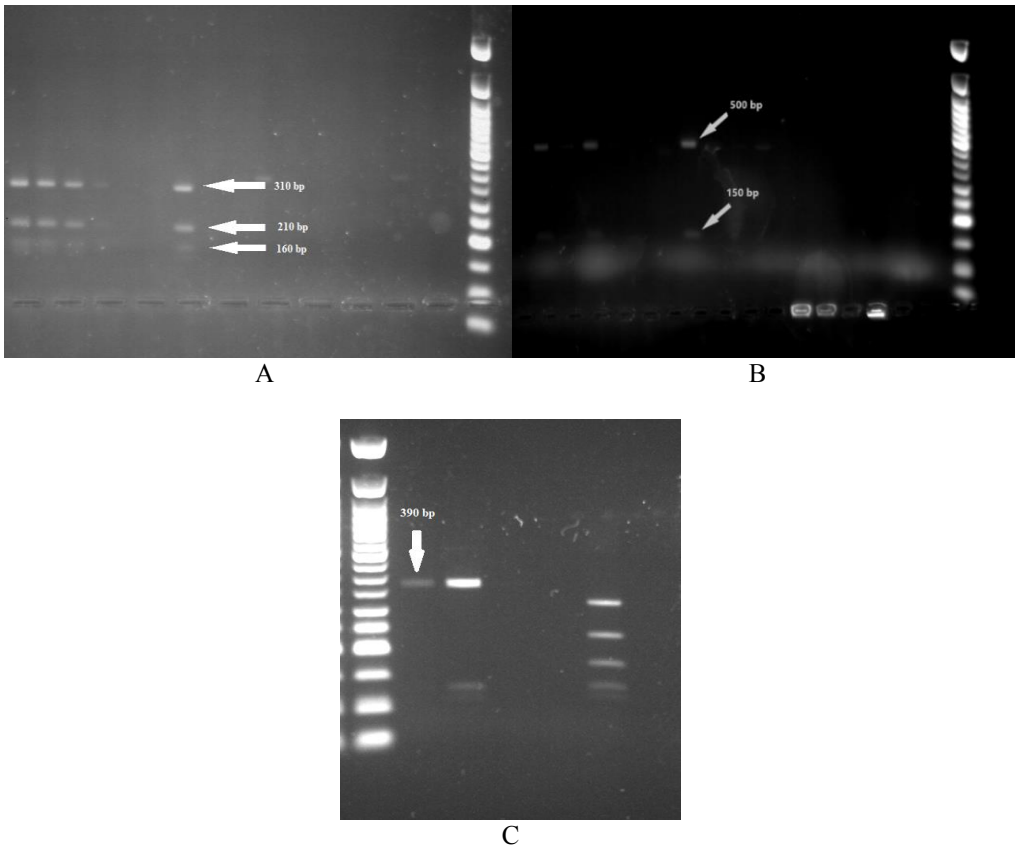


Figure 3 Restriction profiles of species: 1. *Saccharomyces cerevisiae*, 2. *Zygosaccharomyces rouxii*, 3. *Zygosaccharomyces bailii*



A B

C

Figure 4 Digestion of the PCR product with the restriction endonuclease *HinfI* (A), *HaeIII* (B) and *HhaI* (C) of *Candida tenuis*.

and other fermented foods **(Maffezzoli, 2017)**. On the other hand, *Zygosaccharomyces bailii* is notorious for its resistance to low pH, high concentration of organic acids, including preservatives, and possesses a rather high osmotolerance.

It causes off-flavours and vigorous alcoholic fermentation with abundant gas formation **(Rossi et al., 2010)**. It is the most problematic species for wines, with respect to its activities, which lead to visible sediment formation, cloudiness, or haziness in dry wines, and refermentation in sweet wines. Given the visual nature of the spoiling effect, it is of greater concern in white wines **(Fugelsang and Edwards, 2007; Barata, Malfeito-Ferreira and Loureiro, 2012)**.

Another species detected in the samples taken from the Berecký vineyard was *Saccharomyces cerevisiae*. Like *Zygosaccharomyces bailii*, it was detected in all samples of the Berecký vineyard. *Candida tenuis* and *Candida parapsilosis* species were also detected in vine leaves, berries and must. The percentages for both species varied significantly between samples (Table 2).

The same yeast species were detected in the samples of soil, vine leaves, berries and must taken from the vineyards of Čierna Hora as in the samples from the

Table 2 The percentage of yeast from the vineyard Berecký and Čierna Hora.

Samples	Vineyard Berecký Yeast species	Vineyard Čierna Hora Yeast species
Soil		
	90% *Zygosaccharomyces bailii*	90% *Zygosaccharomyces bailii*
	10% *Saccharomyces cerevisiae*	10% *Saccharomyces cerevisiae*
Leaves		
first sampling	57% *Zygosaccharomyces rouxii*	42% *Candida tenuis*
	29% *Candida tenuis*	33% *Zygosaccharomyces bailii*
	14% *Saccharomyces cerevisiae*	17% *Zygosaccharomyces rouxii*
		8% *Saccharomyces cerevisiae*
Leaves		
second sampling	55% *Candida tenuis*	80% *Candida tenuis*
	18% *Zygosaccharomyces bailii*	20% *Saccharomyces cerevisiae*
	9% *Zygosaccharomyces rouxii*	
	9% *Saccharomyces cerevisiae*	
	9% *Candida parapsilosis*	
Grape berries		
	32% *Candida parapsilosis*	54% *Candida tenuis*
	27% *Zygosaccharomyces bailii*	33% *Zygosaccharomyces rouxii*
	17% *Zygosaccharomyces rouxii*	13% *Saccharomyces cerevisiae*
	17% *Candida tenuis*	
	7% *Saccharomyces cerevisiae*	
The must		
	53% *Saccharomyces cerevisiae* 37% *Candida tenuis*	80% *Saccharomyces cerevisiae*
	10% *Candida parapsilosis*	20% *Candida tenuis*

Berecký vineyards, but at different percentages (Table 2). One of the yeast species detected was *Zygosaccharomyces bailii*. Its presence was confirmed in soil samples (90%) and vine leaf samples taken in May (33%). In contrast, the species *Zygosaccharomyces rouxii* was detected only in samples of vine leaves (harvest in May) and grape berries with the same percentage (33%).

Another yeast species detected was *Saccharomyces cerevisiae*, which was present in all percentages of samples from the vineyards of Čierna Hora. Only *Candida tenuis* was detected from yeasts of the genus *Candida*, namely samples of vine leaves, grapes and must (Table 2).

According to **Knight et al. (2015)** wine shows the strongest geographical signatures of all agricultural products and is therefore an excellent model for evaluation with respect to what extent it is affected by microbial terroir. Microorganisms, predominantly yeast, can significantly affect the "phenotype" of wine primarily by affecting the health and development of the vine and hence the quality of the wine. Also, their action during fermentation creates a number of secondary metabolites, including volatile compounds that have an impact on the flavor and aroma of wine. As **Fleet (2008)** states, microorganisms can very easily switch from soil to berries, changing the overall composition of the microflora. An important factor is the health of the berries because the microflora of healthy and rotten, damaged or bitten berries is significantly different. Also, insects greatly affect the diversity of yeast species, as they contribute to the transfer of different species of microorganisms from one environment to another. As reported by **Kántor et al. (2017)**, the species representation of yeasts on grape berries also depends on factors such as temperature, soil, rain, pesticide treatment and grape varieties. In the study, he identified a total of 65 bacteria and yeast species from 19 samples of Slovak grapes. A total of 123 yeast isolates were identified, mainly from the *Saccharomycetaceae* family (45%). **Kántor et al. (2017)** dealt with the microflora of grape berries, and the following three species were detected by *Candida* yeasts: *Candida saitoana*, *Candida magnolia* and *Candida parapsilosis*.

According to **Jackson (2008)**, the most common yeast species on ripe grapes are *Kloeckera apiculata*. Other yeasts that were also isolated from grapes include the genera *Brettanomyces*, *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Nadsonia*, *Pichia*, *Saccharomyces* and *Torulopsis*. In our study, however, only a total of 5 yeast species have been identified, which may be due to adverse climatic events between May and October 2018, whereby this period was characterized by below-average rainfall as well as above-average air temperatures **(SHMU, 2018)**. Such climatic conditions do not support the proper development of

microbiota on berries and vine leaves, as our findings suggest.

Saccharomyces cerevisiae (53%), to a lesser extent *Candida tenuis* and *Candida parapsilosis*, were detected in the must from Berecký vineyards. Only species of *Saccharomyces cerevisiae* (80%) and to a lesser extent *Candida tenuis* were detected in the vine originating in Čierna Hora (Table 2).

Despite the fact that *Candida* belongs to skin-forming undesirable microorganisms in wine ripening, *Candida tenuis* is the main producer of gluconic acid, which is found mainly in the must in the amount of 100 – 300 $mg.L^{-1}$. In musts of botrytic grapes the concentration may be increased to 6.0 $g.L^{-1}$. This causes the characteristic sensory properties of the mature wine. This species is found only sporadically in about 0.25% of total wine production **(Pandey et al., 2016)**.

Similarly, *Candida parapsilosis* appeared at a low frequency, in comparison to the total number of yeasts isolated from both musts, but **González et al. (2007)** found a large percentage (18.5%) of these yeasts at Agustín Díaz winery, thus, they may be responsible for distinctive and interesting wine properties. **Sipiczki et al. (2001)** studied the diversity of yeast microflora in spontaneously fermented wines in Tokaj. They found that fermentation began with a mixed population of yeast species, but on day 4, 59% of YPGA colonies belonged to *Saccharomyces* spp. and from day 8 already 98% of *Saccharomyces* spp. In their study, **Ženišová et al. (2014)** was also involved in mapping the diversity of wine yeasts and moulds in the Small Carpathian Wine Region. Genotypic identification was performed using the real-time PCR method. They have identified the presence of various yeast species, including *Saccharomyces cerevisiae*.

CONCLUSION

The work focused on finding differences in microbiota of soil, vine leaves, grape berries and must from two vineyards of the Tokaj wine region. Simultaneously, by means of ITS-PCR-RFLP method, yeast species representation in individual samples was found. The following species were identified: *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Candida tenuis*, *Saccharomyces cerevisiae*, *Candida parapsilosis*. The total microbiota as well as the species representation of yeast in the samples from the vineyards examined varied in percentage. However, since these vineyards are located in nearby locations, the second representation of yeast was similar. In addition, differentiation in the microbiote during the wine production process was confirmed. Yeast, especially non-*Saccharomyces*, has been confirmed in soil, leaves, and vine berries, but the presence of the genus *Saccharomyces* has already predominated in the musts.

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PLUM (*PRUNUS ROSSICA* EREM.) FRUIT FIELD AND LABORATORY RESEARCES DEPENDING ON THE SCION-STOCK COMBINATIONS

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ABSTRACT

The results of the field observations and biochemical researches of plum (*Prunus rossica* Erem.) fruits grown in the garden of intensive type with agrogenic soil in row spacing are presented in the article. The fruits of plum breeds Kubanskaya kometa and Naidena were gathered from the trees grafted on different stocks. The influence of the stock on the trees productivity, the fruit weight and biochemical structure of the plum fruits were studied. Basic chemical analyses, ascorbic acid concentration, antioxidant activity and fruit ash content were determined. The variation limits of the parameters under study depending on the used stock were shown. The best degustation evaluation parameters were found at the combinations of Kubanskaya kometa on stocks OPA-15-2 and Novinka, Naidena on stocks OPA-15-2 (more than 4,5 points). The high positive correlation (r =0.92) was marked between soluble solids and sugars content. The researches about the stock influence on the plum fruits titratable acidity change we have not found. The correlation between AA and ascorbic acid content in the fruits was determined (r =0.7). The decreasing row of the elements content in the plum fruits ash is the following: K>P>Mg>Ca>Mo>Cu>S>Ni>Fe≈Si>Mn. The proportion of K is from 18.67 (Naidena/OPA-15-2) to 27.48 (Kubanskaya kometa/Novinka); P from 3.35 (Kubanskaya kometa/seedlings *P. cerasifera* Ehrh. to 6.39 (Kubanskaya kometa/Novinka), Mg from 1.11 (Naidena/OPA-15-2) to 2.43 (Naidena/seedlings *P. cerasifera* Ehrh.) and Ca from 0.39 (Naidena/13-113) to 1.85 (Naidena/OPA-15-2) mass % respectively. The proportion of the rest elements is not more than 1 mass %. Under the OPA-15-2 stock influence the trees productivity and the single fruit weight increased, soluble solids, sugars and ascorbic acid concentration in the fruit in comparison with standard stock by the seedling rootstock of alycha (*Prunus cerasifera* Ehrh. seedlings). While ingrafting by Novinka stocks the parameters under study decreased.

Keywords: plum; clonal stock; scion-stock combinations; biochemical structure of fruit

INTRODUCTION

One of the most perspective plum breeds cultivated on the European territory of Russia is plum Russkaya (*Prunus rossica* Erem.). The first breeds were received by the member of the Academy of Siences G. V. Petrov on Krymskaya EBS ARIR (Krandodarskiy Krai) as the result of cross-species hybridization of breed Skoroplodnaya (*Prunus ussuriensis* L.) and alycha breed Pionerka (*Prunus cerasifera* Ehrh.) **(Eremin, 2003)**. At the present time thanks to winter-hardy breeds *Prunus rossica* Erem. they are cultivated not only in the southern areas, but in the Central region of Russia **(Upadysheva, 2017)**. It is appreciated for the high eating qualities of the fruit, their curative and dietic properties **(Motyleva et al., 2017)**. Plum fruit are rich in organic mineral substances: mono- and disaccharides, pectin, vitamins, flavonoids and other biologically active substances **(Kukurová et al., 2015; Kulichová et al., 2016)**.

The researches of the totally different fruit antioxidant ability measurements showed that plums have one of the highest antioxidant activities **(Imeh and Khokhar, 2002)**. Among the molecules that show antioxidant activity in plums it was discovered that the phenolic compounds contribution is essentially higher than vitamin C and carotenoids contribution **(Gil et al., 2002)**. For the plum reproduction and the intensive gardens creation the clonal stocks are used **(Dekena et al., 2017)**. As our experiments with sweet cherry show the stock influences the grafted plants development and metabolism determining the harvest quality **(Upadysheva et al., 2018)**. For the plums the question of the stock influence on the fruit quality is not studied enough. The plum hybrid rootstock forms either are self-sterile or have small non-edible fruit with

bitterness. This may cause the deterioration of grafted scion fruit taste and properties. The purpose of our work is to study the productivity and plum (*Prunus rossica* Erem.) fruit quality depending on the stock-scion combination.

Scientific hypothesis

The productivity, the plum (*Prunus rossica* Erem.) fruit quality and their biochemical composition depending on the stock-scion combination are not studied enough. We have checked the influence of the stock on the formation of productivity, quality and nutritional value of *Prunus rossica* Erem fruit Kubanskaya kometa and Naidena grown in Moscow region conditions. We supposed that on the base of the field and laboratory experiments the optimal stock for each breed that will provide the high productivity and valuable biological active substances accumulation in the plum fruit in comparison with the traditional seedling rootstock will be found.

MATERIAL AND METHODOLOGY

The field researches were held in 2017-2018 on the experimental plum *Prunus rossica* Erem. plantations of Federal State Budgetary Scientific Institution "All-Russion Horticultural Institute for Breeding. Agrotechnology and Nursery", Moscowregion (Figure 1). The plantation overall area is 0.5 ha. The garden of intensive type is set out using the pressed scheme. The soil in the row spacing is agrogenic. The experimental researches object was the fruit of plum Kubamskaya kometa and Naidena breeds grafted on four stocks: 13-113, Novinka, OPA-15-2, *Prunus cerasifera* Ehrh alycha seedling (standard). Under study there were 8 scion-stock combinations totally, not less than 6 trees in each combination were studied. The biochemical researches were held in the Laboratory of Physiology and Biochemistry of Federal State Budgetary Scientific Institution "All-Russian Horticultural Institute for Breeding, Agrotechnology and Nursery".

The determination of the productivity and fruit weight and the sensor evaluation

In the period of fruit ripening the trees productivity was determined by the fruitage weighing from each tree in five-time repetition. The average fruit weight and the stone-fruit weight relation were determined by the weighing of 100 fruits in three-time repetition. The sensor evaluation was fulfilled by the group of high qualified specialists for the evaluating products. They estimated three main quality parameters: taste (sweet, sour, with bitterness), after taste and fruit external appearance – the form, colour, the surface condition.

Chemicals

All chemical substances chosen for the analysis were of analytical sort and were bought from Sigma Aldrich (USA).

Sample preparation

From average 1000 g probe 100 g fruit without stone were prepared and extracted by double-distilled water (to determine antioxidant activity) and metaphosphoric acid (for ascorbic acid determination) with the help of highspeed homogenizer (10 000 rpm, 1 min, UltraTurrax T25 Basic, IKA). After centrifugation at 4000 g (Sigma, Germany) within 10 min the supernatant was used for measuring. The extraction as well as the measurements were held in three-time repetition.

Basic chemical analyses

Studied biochemical parameters: dry matter - by drying samples to constant weight at t = 100 °C. Titratable acidity. Total titratable acidity (TTA) – titration with 10 N NaOH – was estimated by alkalimeter and expressed in $g.kg^{-1}$ malic acid equivalent of fresh matter. The soluble solids content (SSC) was expressed by the index of refraction (°Bx). Content of sugars (glucose, sucrose, fructose) by methods of Bertrand **(Ermakov, Arasimovich and Jarosh, 1987)**.

Figure 1 Plum plantations: Left – blossoming garden, row spacing – agrogenic soil. Right – fruitage, Kubanskaya kometa breed.

Ascorbic acid (AsA) determination

Ascorbic acid determination was held using HELC method **(Stan et al., 2014)**, the chromatograph KNAUER (Germany) was used. Chromatoghraphic conditions: HELC column Silasorb C18 (5 mkm), 150 x 4.0 mm (Biohimmac, Russia), the column temperature is 25 °C, flow speed 1.0 mL.min^{-1}, the detector UV, the wave length l = 251 nm, the mobile phase MeOH: water – 5:95 (r./r.), aliquote for injections 20 mkL, the retention time Rt = 4.4mm.

Antioxidant activity (AA) determination

Antioxidant activity was measured by the **Brand-Williams et al. (1995)** method using a compound DPPH· (2.2-diphenyl-1-pikrylhydrazyl). The spectrophotometer Thermo Helios Ɣ (Thermo Fisher Scientific, made in England) was used. The homogenized by the distilled water samples were put on the shaker Lab-PU-01 (Russia) for 8 hours, and then they were filtered, and the antioxidant activity was measured in 10 minutes after interaction between the extract and reagent at wavelength 515 nm. The calculation of antioxidant activity values was fulfilled using the formula:

$$\text{Inhibiting DPPH} = (AC - AAt) = AC / 100\ (\%),$$

Where:
AC – DPPH solution absorption; AAt – absorption at the antioxidant presence.
Three-time repetition.

EDS - analysis

The chemical composition of the basic ash components (Na, P, S, K, Mn, Fe, Mg, Ca, Al, Si, Cl, Zn, Se, Mo) was determined by the method of energy dispersive spectrometry (ESD) on the analytical raster electron microscope JEOL JSM 6090 LA. The microscope solution is 4 nm at accelerating voltage 20 kV (secondary electrons image), zooming is from x 10 till x 10 000. While performing the elemental analysis the working distance (WD) is 10mm. Energy-dispersive spectrometer allows to carry out the quantitative X-ray microanalysis with the desired analysing area: in a point or areally, and to receive the maps of elements allocation. X-ray microanalysis data are presented in the form of standard protocols which contain the microstructure picture of the sample under study, the table of the data in weighting and atomic correlation, spectra and histograms. The spectrum example is shown in Figure 2.

Taking into consideration the spectrum lines intensity the concentration of the desired element can be determined. The fractional accuracy of the chemical analysis is spread in the following way: at the element concentration from 1 till 5% the accuracy is less than 10%; from 5 till 10% the accuracy is less than 5%; at the element concentration more than 10% the accuracy is less than 2%. 100 ash areas of each sample were studied. The local analysis is 3 mm, the scanned area is not less than 12 um.

Statistic analysis

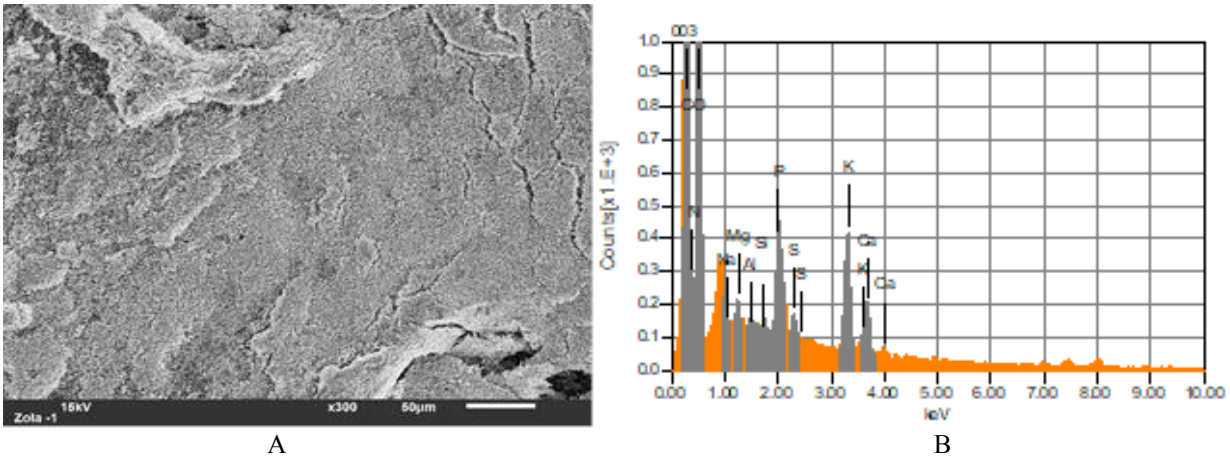
All analyses were three-time repetition. Results were expressed as mean values (n = 3) ±standard deviation (*SD*).

To determine the differences – significance between the data one-way and two-factor experience ANOVA test was used (p <0.05) via the program Statgraphics Centurion XV (USA).

RESULTS AND DISCUSSION

As a result, it was determined that the plum trees productivity at average for the period of 5 years was 14.2 kg/tr. and depending on the stock varied from 9.2 kg/tr. (Naidena on seedlings) to 21.8 kg/tr. (Kubanskaya kometa on OPA-15-2). Higher than the average value this parameter was at the trees of scion Kubanskaya kometa grafted on the stocks 13-113 and Novinka. It should be mentioned that scion Novinka productivity depending on the stock varied in less degree (Table 1). The influence of stock on the productivity of plum trees was noted in **(Grzyb, Sitarek, and Kozinski, 1998; Magyar and Hrotkó, 2006)**.

Scions under study belong to large-fruited group with the fruit weight more than 30 g. The single fruit weight of scion Kubanskaya kometa with average value of 32 g depending on the stock was changing from 29.5 g (seedlings) till 34.8 g (OPA-15-2). The fruits of the scions under study were significantly bigger because of stocks OPA-15-2 and Novinka influence. Scion Kubanskaya kometa stone separated from the flesh badly, its portion in the fruit weight was 7% at average with variation depending on



A B

Figure 2 The microstructure picture of the sample under study (A) and the general view of the X-ray spectrum lines (B).

Table 1 The productivity, fetal mass and organoleptic estimation of *Prunus rossica* Erem. fruits depending on the stock at average in 2017 – 2018.

Stock-scion combination	Parameters under study		
	Productivity (kg/tree)	Average fruit Weight (g ±*SD*)	Degustation evaluation, point
Kubanskaya kometa/ 13-113	16.8± 2.41	31.22 ± 0.41	4.1
Kubanskya kometa/ Novinka	19.4± 1.19	32.33 ± 0.44	4.6
Kubanskaya kometa/ OPA-15-2	21.8 ± 2.54	34.82 ± 0.51	4.8
Kubanskaya kometa/ Seedlings	11.7 ± 1.87	29.54 ± 0.31	4.0
***P.cerasifera* Ehrh**	10.9 ± 2.07	28.64 ± 0.61	4.1
Naidena/ 13-113	11.8 ± 2.17	32.01 ± 0.41	4.3
Naidena/Novinka	12.3 ±1.25	32.53 ± 0.61	4.5
Naidena/OPA-15-2	9.2 ± 0.87	28.07 ± 0.73	4.0

from 6.8% (Novinka) to 7.5% (seedlings).

At scion Naidena the stone was slightly smaller, separated from the flesh better and was nearly 6% for all stock-scion combination. According to the degustation results the fruits of the scions under study were characterized by the harmonized sore-sweet taste and smell (4.0 – 4.8 points), no taste deterioration and bitterness were observed on either of the stocks. The best degustation evaluation parameters were found at the combinations of Kubanskaya kometa on stocks OPA-15-2 and Novinka, Naidena on OPA-15-2 (more than 4.5 points). The plum (*P. rossica* Erem.) fruits were characterized by the high content of soluble solids and sugars which depended on the stock-scion combination. The solutable solids content variated from 11. 2% (Kubanskaya kometa on seedlings) to 15.2% (Naidena on OPA-15-2) at average value of 12.9 % (Figure 3.).

However, there is conflicting evidence that confirm that the solids soluble content/titratable acidity ratio weren't affected by soil type or rootstock **(Ratoa et al.,2008)**. The sugars content in the fruits was within 7.2 – 10.0%. The sugars higher accumulation was found at scion-stock combinations Naidena on OPA-15-2 (10%) and on Novinka (9.3%) (Figure 4).

The high positive correlation (r = 0.92) was marked between soluble solids and sugars content. While evaluating the stock influence it should be noted that the soluble solids content on stock OPA-15-2 is on 13 – 20% higher in comparison with alycha standard seedlings. The received data correlate with **Hatton et al. (2015)** researching results about the stock influence on the plum trees productivity and the apricot fruits productivity and quality **(Milosevic et al., 2011)** and plum fruits bio-chemical parameters change (**Świerczyński and Stachowiak, 2009**).

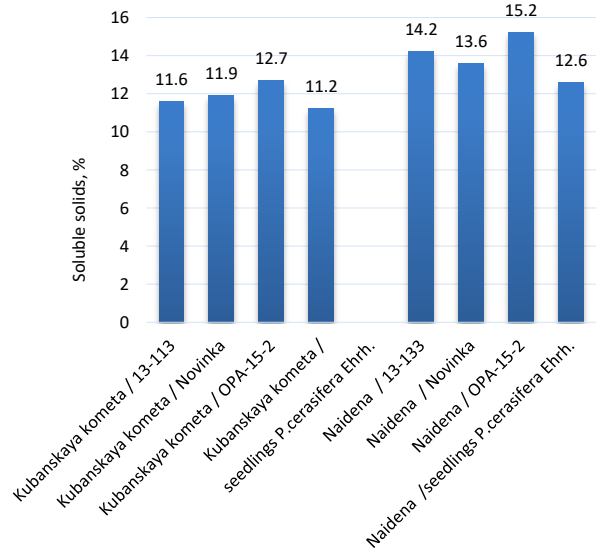


Figure 3 Soluble solids content in plum fruits.

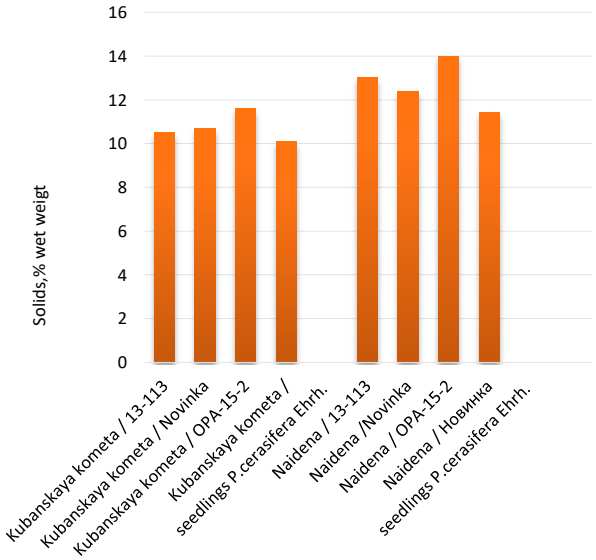


Figure 4 Solids content in plum fruits of *Prunus rossica* Erem., depending on the stock at average in 2017 – 2018.

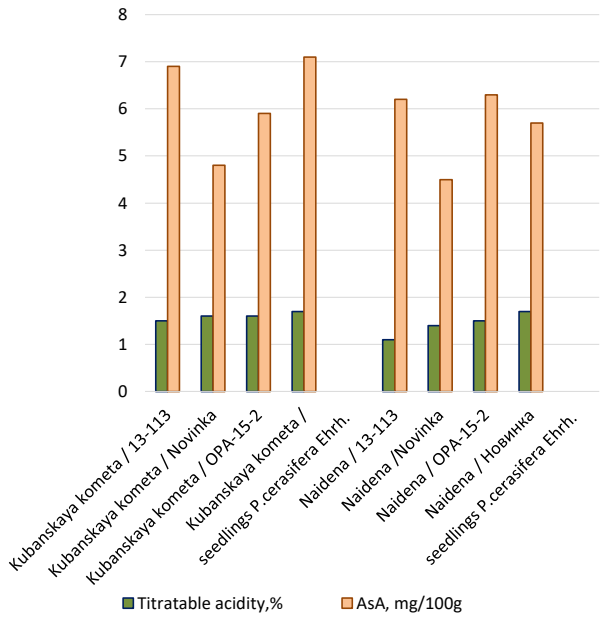


Figure 5 Titratable acidity and ascorbic acid content in plum fruits of *Prunus rossica* Erem. depending on the stock at average in 2017 – 2018.

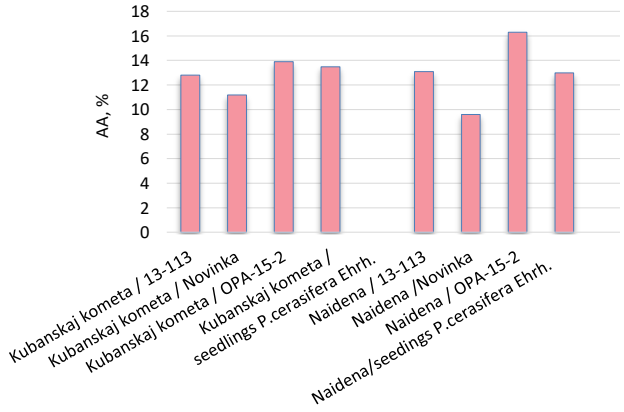


Figure 6 Antioxidant activity in plum fruits of *Prunus rossica* Erem. depending on the stock at average in 2017 – 2018.

Organic acids determined the plum fruits titratable acidity. The scion-stock combination under study showed the titratable acidity variation from 1.1 to 1.7%. The maximum values of the plum fruits titratable acidity (1.6 – 1.7%) were determined for the scion Kubanskaya kometa on the stocks Novinka, OPA-15-2 and on the seedlings *P. cerasifera* Ehrh. Studies performed by **Milošević and Milošević (2012)** showed that the influence of rootstocks on most of the analysed quality attributes plum was variable because strong and complex interactions, rootstock × cultivar, rootstock × year and cultivar × year, were observed. Several traits, such as yield, soluble solids/acid content ratio and total sugars/acid content ratio, seem to be harder conditioned in rootstocks. For the scion Naidena on the same stocks the titratable acidity values were maximum (1.4 – 1.7%). On the seedlings the influence on the fruits acidity was expressed by some increase of titratable acidity value and was marked at the level of 1.7% for both scions, the titratable acidity minimum value for the both scions was marked on the stock 13-113 and was marked at the level of 1.5% (Kubanskaya kometa) and 1.1% (Naidena) (Figure 5). The received titratable acid values are lower than in the fruits *Prunus spinosa* L., as it is described in the paper **Erturka, Ercislib and Tosun (2009)**. The researches about the stock influence on the plum fruits titratable acidity change we have not found. AsA content in plum fruits is not relatively high – not more than 7,1 mg/100 g. At average AsA content in scion Kubanskaya kometa fruits is 6.2 mg.100g^{-1}, in scion Naidena fruits – 5.7 mg.100g^{-1}.

It should be mentioned that on the stocks 13-113 and Novinka both scions accumulate the same AsA content. We could not find any papers about the stock influence on AsA content as well, however, the received quantites correspond to the literary data **(Gil et al., 2002)**. The fruits total AA that determines their value for the functional nutrition was nearly 13% depending on the used stock. It varied greatly at scion Naidena: from 9.6% (Novinka) to 16.3% (OPA-15-2), Figure 6. Plums are characterized by a relatively high content of antioxidants. Our results are consistent with those of **Najafabad and Jamei (2014).** We did not find in the scientific literature information on the effect of stock on the antioxidant activity of plum fruits. But when analyzing the antioxidant activity of cherry plum leaves, we found a significant effect of the stock **(Upadysheva and Motyleva, 2019)**.The correlation between AA and ascorbic acid content in the fruits was determined (r = 0.70). **Kalt et al., (1999)** reported that the ascorbate content and the antioxidant activity, being negatively correlated (r = −0.80) for strawberries, raspberries and high- and low-bush blueberries. High correlation between antioxidant activity and vitamin C was likely to be found only in fruits that contain high vitamin C such as citrus fruits **(Gardner et al., 2000)**. The high nutritional value of plum fruits is determined not only by the organic bioactive substances content, such mineral components as K, Na, Ca, Mg play an important role **(Agbede and Ibitoye, 2007; Motyleva, Simonov and Kulikov, 2017)**. We analysed the content of 11 elements in the plum fruits ash – K, P, Mg, Ca, Mo, Si, Mn, Fe, Ni, Cu and S (shown in Table 2). K was a prevailing element. The decreasing row of the elements content in the plum fruits ash is the following: K>P>Mg>Ca>Mo>Cu>S>Ni>Fe≈Si>Mn. The proportion of K is from 18.67 (Naidena/OPA-15-2) to 27.48 (Kubanskaya kometa/Novinka); P from 3.35 (Kubanskaya kometa/seedlings *P. cerasifera* Ehrh. to 6.39 (Kubanskaya kometa/Novinka), Mg from 1.11 (Naidena/OPA-15-2) to 2.43 (Naidena/seedlings *P. cerasifera* Ehrh.) and Ca from 0.39 (Naidena/13-11) to 1.85 (Naidena/OPA-15-2) mass % respectively.

Table 2 The elemental composition of fruit joint of *Prunus rossica* Erem., depending on the stock at average in 2017 – 2018, mass. % in the ash.

Elements	Kubanskaya kometa/stock						Naidena/stock					
	13-113	Novinka	OPA-15-2	seedlings *P. cerasifera* Ehrh.	Mean ±*SD*	Variation coefficient (%)	13-113	Novinka	OPA-15-2	seedlings *P. cerasifera* Ehrh.	Mean ±*SD*	Variation coefficient (%)
K	25.21	27.48	21.19	19.12	23.25±3.79	16.29	23.45	22.09	18.67	24.98	22.30±2.7	12.07
P	4.72	6.39	3.36	3.35	4.46±1.44	32.41	3.38	4.70	3.78	5.15	4.25±0.8	19.10
Ca	1.16	1.45	1.13	1.24	1.25±0.14	11.46	0.39	1.20	1.85	1.26	1.18±0.6	20.88
Mo	1.36	1.11	0.96	0.45	0.97±0.38	39.40	0.52	1.42	1.96	1.28	1.29±0.5	45.78
Mg	1.71	1.78	1.26	1.41	1.53±0.24	15.47	2.34	1.81	1.11	2.43	1.92±0.6	31.52
S	0.52	0.78	0.52	0.52	0.59±0.13	21.88	0.23	0.64	0.59	0.41	0.12±0.2	40.77
Si	0.11	0.18	0.21	0.23	0.18±0.05	29.86	0.09	0.06	0.12	0.22	0.61±0.1	36.80
Fe	0.25	0.26	0.18	0.09	0.15±0.46	32.43	0.11	0.15	0.07	0.26	0.07±0.1	36.77
Mn	0.08	0.11	-	0.08	0.06±0.08	39.54	0.01	0.08	0.04	0.13	0.47±0.1	41.39
Cu	0.54	0.42	0.97	0.68	0.67±0.31	40.03	0.19	0.59	0.79	0.28	0.23±0.3	40.55
Ni	0.21	0.39	0.31	0.19	0.24±0.15	36.17	0.23	0.39	0.25	0.07	0.0.14±0.1	35.68
∑	48.0	51.1	52.7	59.4			59.05	55.9	59.0	58.2		

Note: Results represent mean values (n = 3) ±*SD*.

The proportion of the rest elements is not more than 1 mass %. The coefficient of variation of elements in the ash plum Kubanskaya kometa / stock ranges from 15.47% (Mg) to 40.03% (Cu); Naidena/stock ranges from 12.07% (K) to 40.77% (S), which indicates the relative homogeneity of the data. The average values of the coefficients of variation in the ash plum Kubanskaya kometa / stock were found in the elements S (21.88%) end Si (29.86%); in the ash plum Naidena/stock – In the elements Ca (20.88%) and Mg (31.52%). The maximum value of the elements sum was determined in the fruits of scions Kubanskaya kometa
and Naidena on the stock seedlings P.cerasifera Ehrh. (59.4 and 58.2) and in the scion-stock combination Naidena/OPA-15-2 (59.0) mass % respectively. At average the maximum value of K, P, Ca, Mo, S, Cu and Fe is found in the plum fruits of scion Kubanskaya kometa in comparison with the value of Mg which is on 37% higher in the scion Naidena. The regularities of the elements content in the ash of both scions on the stocks is identical to the necessary microelements for the human health (Figure 7, 8).

The content of macronutrients in plum fruits is considered in the article **(Ertekina et al., 2006)**.

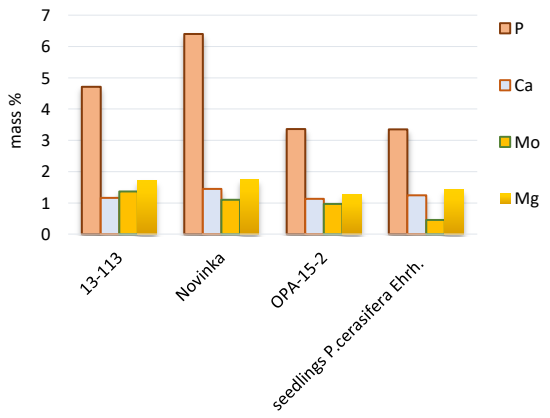


Figure 7 The comparative content of macrolements in the plum fruits ash of scion Kubanskaya kometa on different stocks.

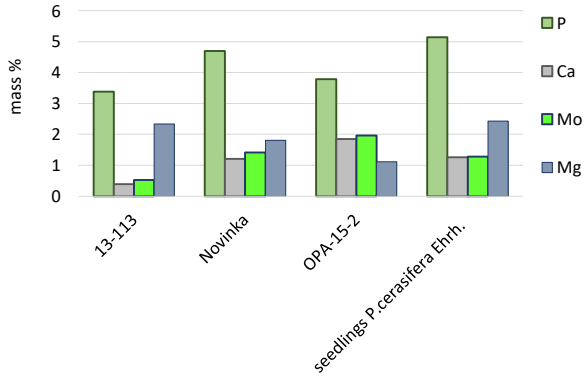


Figure 8 The comparative content of macrolements in the plum fruits ash of scion Naidena on different stocks

CONCLUSION

In the present paper the main focus was given to the study of the essential biochemical parameters that characterize the nutritional and dietic value of plum fruits (*Prunus rossica* Erem.) of different scion-stock combinations. As the result of the researches the stock influence on the trees productivity, the plum fruits (*Prunus rossica* Erem.) weight and chemical composition was identified and the limits of soluble solids, sugars and ascorbic acid content variation depending on the used scion/stock combinations were determined. Under the stock OPA-15-2 influence higher content of soluble solids, sugars and ascorbic acid accumulated in the fruits. While grafting on alycha seedlings the reduction of the parameters under study was observed. Using the data of the field and laboratory researches the conclusion can be made that the optimal stock for scions Kubanskaya kometa and Naidena is OPA-15-2 may be a better solution to achieve higher yields and better fruit. The results received while working at this paper give new information about the stock influence on the biochemical characteristics of the scions.

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KEY FACTORS AFFECTING CONSUMPTION OF MEAT AND MEAT PRODUCTS FROM PERSPECTIVE OF SLOVAK CONSUMERS

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ABSTRACT

Nowadays, meat and meat products are considered as a part of the daily diet of most people. Therefore, it is necessary to deal with meat and meat products and their consumption according to individual types of meat. Based on the above, the paper is focused on the issue of consumption of individual types of meat in the Slovak Republic and the identification of key factors affecting the consumption of meat and meat products from Slovak consumers' point of view. Secondary and primary data is used to fulfil the aim of the paper. Secondary data is obtained from the Statistical Office of the Slovak Republic, on the basis of which the development of consumption of individual types of meat is predicted by 2020. It can be stated that poultry and pork meat and meat products consumption is constantly increasing and there is a slight change in beef and fish meat and meat products consumption, which is currently at a very low level. In the context of the above, a questionnaire survey is realised and based on its results it could be concluded that the price is a main reason for the inadequate consumption of different types of meat. However, consumers consume meat and meat products mainly because of taste, which can be considered as an aspect of irrationality in the diet of Slovak consumers. Furthermore, a number of factors affecting the purchase and consumption of meat and meat products have been identified. The results show rational and irrational aspects in the decision making of Slovak consumers. The most important factor is the quality of meat and meat products, which consumers perceive differently, mainly on the basis of their own personality and other aspects of meat quality (price, origin, freshness, and sensory characteristics of meat). Other important factors are the perception of composition, freshness, price and country of origin.

Keywords: consumer; consumption; factor; meat; meat product

INTRODUCTION

The demand for food, including meat and meat products, is relatively inelastic and is influenced by a number of factors. The main factors are the gross domestic product and its redistribution among the population, the standard of living, the market structure, the intensity of international trade and individual consumer behaviour **(Skořepa, 2009)**. In relation to meat consumption, the factors determining the consumption of meat and meat products can be divided into three basic categories. The first category is represented by commercial and political factors, which include trade liberalization, globalization and political issues linked to the common agricultural policy **(Thow, 2009)**. The next factors are related to the production of meat and meat products. This group of factors includes the industrialization of production, problems with livestock farming and subsequent production, as well as the possibility of falsifying meat and meat products in the production process **(Rivera-Ferre, 2009)**. The last category of factors affecting the meat and meat products consumption includes determinants related to socio-economic aspects. These factors include, in particular, the standard of living and the degree of urbanization resulting mainly from changes in eating habits, as well as individual consumer behaviour **(York and Gossard, 2004)**.

Individual consumer behaviour has a significant role, because it influences consumers most in the process of purchase and consumption of meat and meat products. Consumer behaviour is influenced by several factors, including personal, cultural, social and psychological factors.

The first group of factors influencing consumer behaviour are personal characteristics. **Kotler and Armstrong (2004)** define gender, age, level of education, employment, income and lifestyle as the main personal factors determining individual consumer behaviour. **Keyzer et al. (2005)** states that consumer behaviour on the meat and meat products market is determined by an increase in population incomes. Increased incomes or lower prices lead to an increase in

food consumption, especially of animal origin. Consumers with a higher income maintain a healthy lifestyle and prefer the consumption of beef, which is more nutritionally. The effects of increased consumer income can be considered beneficial and lead to a higher quality of eating, better health care, a lower risk of obesity and other health diseases **(Marmot, 2001)**. Consumer behaviour is thus determined by the purchasing power of the consumers, which is a reflection of their income and savings **(Benda-Prokeinová and Hanová, 2016; Sans and Combris, 2015)**. Consumers' gender also has an impact on meat consumption, women tend to avoid eating red meat more than men and prefer white meat especially chicken **(Kubberød et al., 2002)**. Another demographic factor is age. According to **Jongen and Meulenberg (2005)** it is expected that the population older than 65 years will represent 21% in the European Union. This part of the population perceives consumption of meat and meat products differently compared to other age categories **(Bleiel, 2010)**. The size of households is another demographic factor affecting meat consumption **(Jongen and Meulenberg, 2005)**. The following demographic aspect is the level of education. Nowadays consumers have better education, which enables faster and better understanding of information presented in advertisements or information contained in product labelling **(Frewer and Van Trijp, 2006)**. Lifestyle has a significant factor in relation to personal influences and is influenced by general factors affecting the consumption of meat and meat products. **Buitrago-Vera et al. (2016)** diversify consumers according to their consumers' lifestyles and their relationship to meat and meat products into four groups: indifferent consumers, cooks, comfortable consumers and rational consumers.

Cultural factors are another group of factors influencing consumer behaviour in relation to meat and meat products. Culture has a significant impact on consumers, which is reflected in the shaping of their behaviour and in providing standards and rules on the facts that affect the consumption of the selected type of food **(Schiffman, Kanuk and Hansen 2012)**. In the context of meat and meat products, it is necessary to emphasize the symbolic importance of meat **(Beardsworth and Keil, 1997)** or the tradition of eating meat and meat products based on cultural and religious elements such as rituals, myths, taboo **(Mathijs, 2015)**. In some cultures, there have been changes in the practices of killing animals, presenting meat on the table, and in what is considered edible and not in recent years **(Dvořáková-Janů, 1999)**. Currently, there are cultures where meat is considered as a tool of hierarchical consolidator or social connection of the population **(Mathijs, 2015)**. Within the culture, the subcultures are also created and divided into four groups: ethnic groups, religious groups, racial groups, geographical areas **(Richterová et al., 2007)**.

Consumer behaviour on the meat and meat products market can be also influenced by social factors. Social factors – consumer groups, family, the role of the individual in society, etc., have a significant impact on the resulting consumer behaviour **(Kotler, 2007)**. In the context of consumer behaviour on the meat market, opinion leaders have an important role in terms of marketing. There are people influencing the consumer behaviour of other members of the group, and they may be relatives, friends, doctors, nutritional advisers **(Font-i-Furnols and Guerrero, 2014)**. The family also plays an important role in the consumer's behaviour in the process of purchase and consumption of meat. With regard to social factors, it is also important to point out that consumers also demonstrate their position in society by the consumption of individual types of meat and meat products **(Ingr, 2011)**.

Another group of factors are psychological factors. The incentive aspect plays an important role and lead consumers to buy and consume meat and meat products. In particular, physiological needs and the fact that the consumption of meat and meat products is essential for the nutrition of consumers could be considered as motivation **(Dostálová and Kadlec, 2014)**. Motivation encourages consumers to ask questions with the purpose to buy meat products. On the other hand, it is important to point out the demotivation of consumers to meat and meat products and the orientation of consumers towards healthy eating with the absence of a given type of food. Another factor is perception. Perception of sensory aspects of meat, such as the taste and visual appearance of meat, plays an important role in consumer behaviour in the meat and meat products market. The appearance of fresh meat and meat products, especially their colour, is influenced by genetics, nutrition, livestock farming, climatic conditions or the product packaging **(Kerry, 2009)**. The juiciness and taste of the meat are very difficult to identify in the process of selection of meat and meat products. These factors are mainly influenced by the breed of animals, age, and the process of ripening of meat **(Ingr, 2011)**. According to **Pirvutoiu and Popescu (2013)** consumers prefer domestic meat and meat products mainly because of their lower risk. There are mainly risks related to meat and meat products that do not meet the quality requirements for a given type of food **(Kameník, 2014)**. All these factors are considered as aspects affecting the quality of meat and meat products. If the consumers do not have necessary information, they often assess the quality of the products on the basis of their perception. The choice of specific meat products could be influenced by the personality of the consumer. The last factor is the emotions that affect consumers and can change depending on the experience of buying products. Emotions are recorded in memory depending on the degree or intensity of the sensory experience, and it leads to the recall of past experiences **(Horská and Berčík, 2017; Rybanská, Nagyová and Košičiarová, 2014)**.

Scientific hypothesis

The aim of the paper is to point out the consumption of meat and meat products in the Slovak Republic and to identify the main factors affecting the consumption from the point of the Slovak consumer. For a deeper analysis of the research objectives, the following hypotheses were formulated:

Hypothesis no. 1: We assume that Slovak consumers evaluate the factors influencing the purchase and subsequent consumption of meat and meat products differently.

Hypothesis no. 2: We assume that there is a difference in noticing percentage of meat in meat products depending on different types of meat.

Hypothesis no. 3: We assume that there is a difference in consumer perception of freshness depending on different types of meat and meat products.

Hypothesis no. 4: We assume that there is a difference in consumer perception of prices depending on different types of meat and meat products.

Hypothesis no. 5: We assume that there is a difference in the preference of Slovak origin of meat and meat products depending on different types of meat and meat products.

MATERIAL AND METHODOLOGY

The aim of the paper was achieved by using and processing secondary and primary data.

The secondary data was obtained from the Statistical Office of the Slovak Republic. The obtained secondary data became the basis for the calculation of the average growth coefficient (k') and for the prediction of the development of meat and meat products consumption by 2020 using the determinant coefficient R^2.

The primary data was obtained from the consumer survey aimed at the identification of key factors affecting the purchase and subsequent consumption of meat and meat products in the Slovak Republic. The questionnaire survey was conducted on a sample of 498 respondents in the Slovak Republic and was realised in the electronic version from April to December 2018.

The respondents involved in the questionnaire survey were diversified into 8 categories by gender (women 62.0%; men 38.0%), age (up to 25 years 44.4%; 26 – 35 years 22.3%; 36 – 50 years 19.3%; more than 51 years 14.1 %), education (elementary 2.0%; secondary 46.0 %; university 52.0%), residence (village 47.8%; city 52.2%), economical status (student 37.8%; employed 47.8%; the self-employed person 4.8%; unemployed 1.0%; maternity leave 2.2%; retired 6.4%), monthly income of respondent (up to 400 Euro 40.4%; 401 – 800 Euro 38.8%; more than 801 Euro 20.9%), monthly household income (up to 1,000 Euro 20.1%; 1,001 – 2,000 Euro 56.0%; more than 2,001 Euro 23.9%) and by number of members in the household (1 – 2 members 25.7%; 3 members 21.1%; 4 members 35.7%; more than 5 members 17.5%).

Statistic analysis

Collected data was processed by using Microsoft Excel and then evaluated in the statistical program XL Stat.

The formulated hypotheses were tested by applying the following statistical methods:

- Cochran's Q test,
- Friedman test,
- Nemeny's method.

In hypothesis testing, if the *p*-value is lower than significant level, in case of XL Stat software, it is 0.05, the null hypothesis is rejected and the alternative hypothesis is confirmed.

RESULTS AND DISCUSSION

In the Slovak Republic, the development of meat and meat consumption was at a relatively stable level with an average annual growth rate of 1.12% (k' = 0.0112) in the period 2009 – 2018 (Figure 1) **(Statistical Office of the Slovak Republic, 2019)**. Meat and meat products consumption was sufficient and exceeded the recommended amount in terms of food rationalization (meat 57.3 kg, fish 6.0 kg) during the analysed period except for 2014 and 2015. The lower consumption of meat and meat products may have been due to food scandals in the last years, mainly related to poultry and beef, as well as rising prices for selected types of food. Since 2015, there has been a relatively fast growth rate in the consumption of meat and meat products because the consumption has increased by 16.7 kg per person and year over the last four years. The trend of the development of meat and meat products consumption in the monitored period can be expressed by the following quadratic function:

$$q_t = 69.592 - 5.4243 * t + 0.5572 * t^2$$

$$R^2 = 0.8125$$

On the basis of the chosen quadratic function, it is possible to expect the trend of the development of meat and meat products consumption in the future, which will have an increasing tendency and the consumption of analysed types of food should increase to 85 kilograms per person and year in 2020. In this context, it is necessary to point out the possible reasons for the overall high consumption of meat and meat products. These reasons may include lower prices for pork and poultry meat compared to fish and beef, the level of prices of meat and meat products in relation to consumers' incomes, standard of living in the Slovak Republic, insufficient information about the positive and negative effects of meat consumption on human health, or inadequate promotion of individual types of meat **(Skořepa, 2009)**.

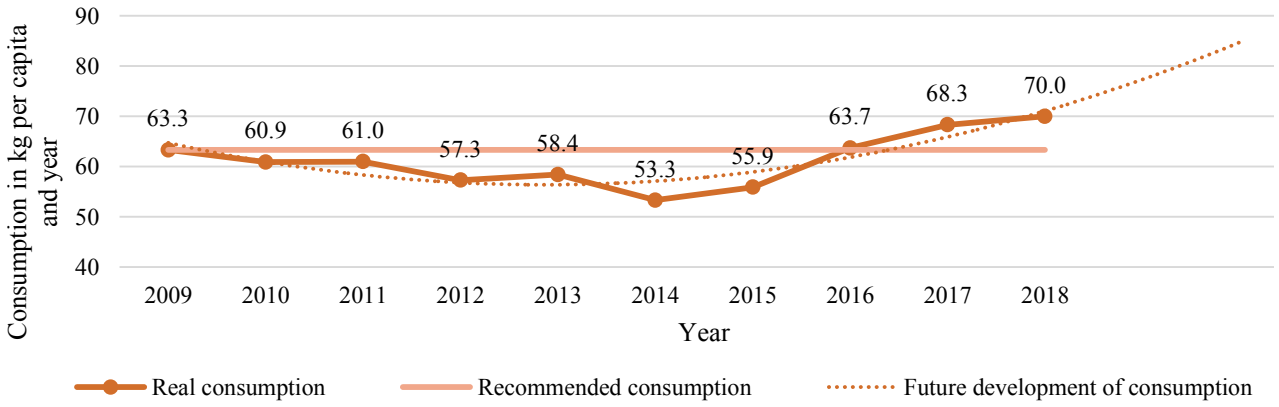

Figure 1 The development of meat and fish consumption in kilograms per capita and year in the Slovak Republic. Note: Statistical Office of the Slovak Republic, 2019.

In connection with the consumption of meat and meat products, it is important to highlight the proportion of individual types of meat in the diet of Slovak consumers.

The consumption of pork meat and meat products had a slightly increasing tendency and ranged from 32.0 kg to 35.8 kg per inhabitant of the Slovak Republic and year during the monitored period 2009 – 2018, which implies an average annual growth of consumption of the given type of meat by 1.25% (k' = 1.0125) (Figure 2). In 2014, a greater decrease in pork meat and meat products consumption was recorded, mainly due to lower prices of poultry meat. From 2015 to 2018 the sharp increase in consumption was recorded by almost 8 kilograms, which represents an increase of 27.9%. We have chosen quadratic function with the following parameters as a suitable function to describe the trend in the development of pork meat and meat products consumption:

$$q_t = 33.748 - 1.7749 * t + 0.209 * t^2$$

$$R^2 = 0.7174$$

Based on the quadratic function, it is possible to assume the consumption of pork meat and meat products with a perspective for the next two years. The consumption should increase and exceed the level of 40 kilograms per inhabitant of the Slovak Republic in 2020. The increasing trend in consumption may be caused by the availability of pork meat in commercial establishments, various marketing activities for sales support in retail, the prices of substitute products and by the existence of food scandals related to other types of meats, in particular, beef and poultry meat **(Matošková and Gálik, 2016)**.

Poultry meat and meat products consumption was similar in the first and last year of monitored period 2009 – 2018 (Figure 3). The development of consumption of poultry meat and meat products was accompanied by slight fluctuations in both growth and decline. Average growth coefficient k' reached the level of 1.0073, so it means stagnating and relatively stable development of poultry meat and meat products consumption. In 2014 and 2015 the consumption did not reach the recommended dose level and covered 95% of recommended intake. However, the tendency in poultry consumption has increased since 2016 and nowadays poultry meat and meat products are consumed at the level of 22.1 kg. We chose a quadratic function with the following parameters to express the trend of poultry meat and meat products consumption in the monitored period:

$$q_t = 24.89 - 3.2686 * t + 0.2932 * t^2$$

$$R^2 = 0.7215$$

Based on the quadratic function, we expect that the consumption of poultry meat and meat products will increase in the following two years. In 2020 the consumption will reach the annual level of 28 kg per inhabitant of the Slovak Republic. Increasing tendency in poultry meat consumption can be influenced mainly by favourable price relations, purchasing power of the population or new nutritional trends and lifestyles **(Kubicová, 2008)**.

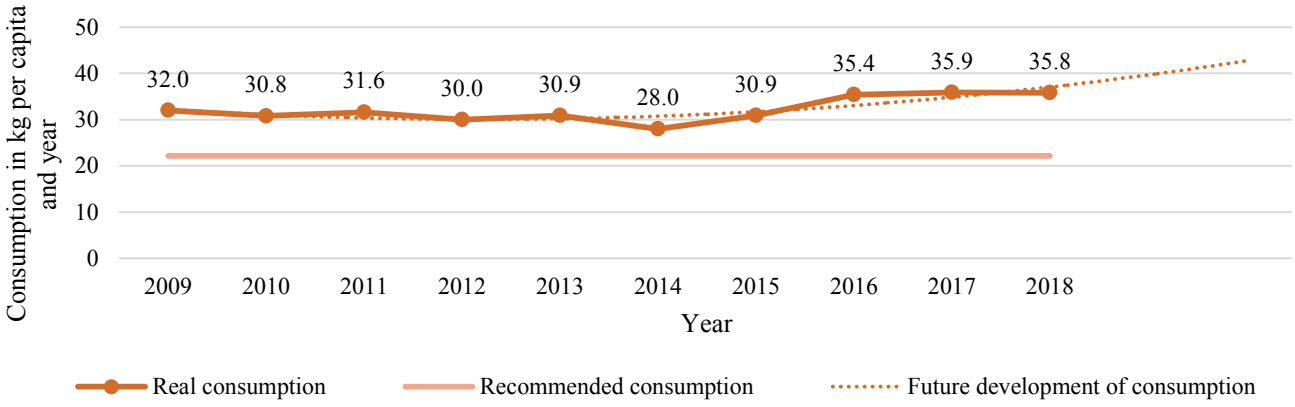

Figure 2 The development of pork meat consumption in kilograms per capita and year in the Slovak Republic. Note: Statistical Office of the Slovak Rebulic, 2019.

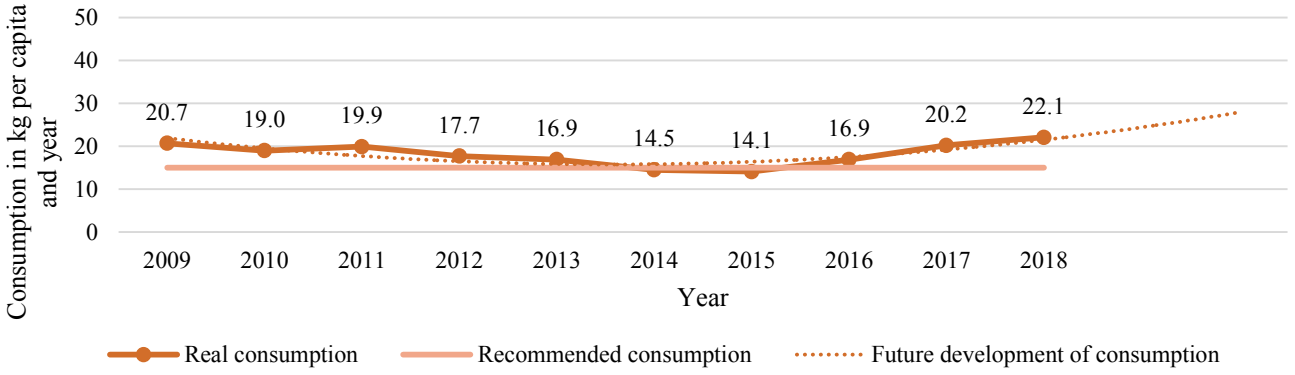

Figure 3 The development of poultry meat consumption in kilograms per capita and year in the Slovak Republic. Note: Statistical Office of the Slovak Republic, 2019.

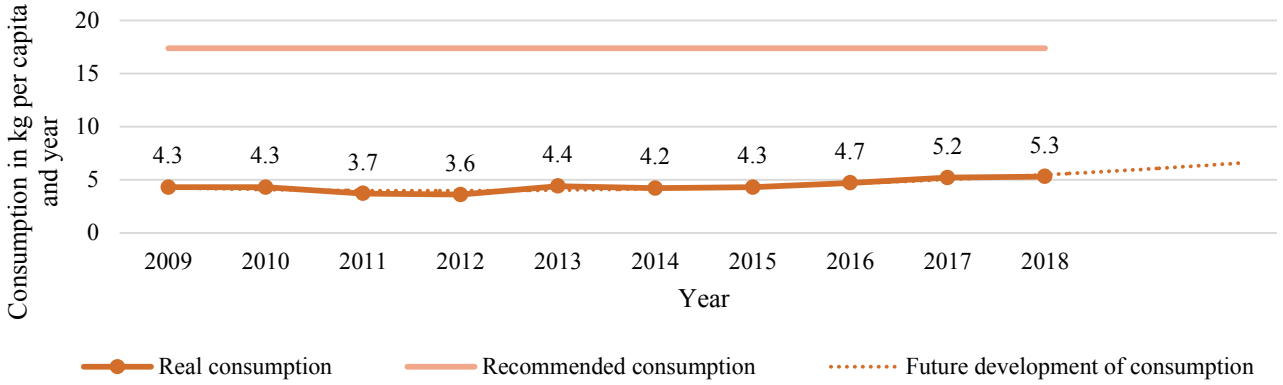

Figure 4 The development of beef meat consumption in kilograms per capita and year in the Slovak Republic. Note: Statistical Office of the Slovak Republic, 2019.

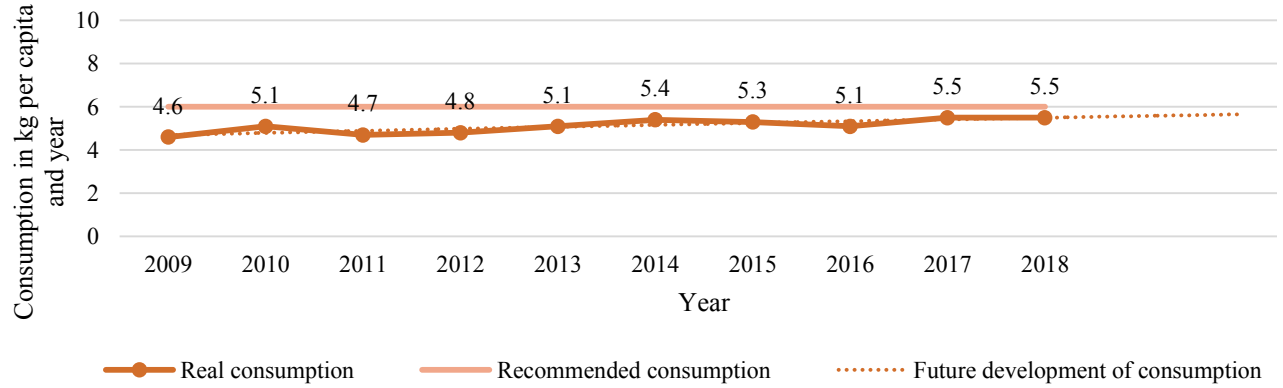

Figure 5 The development of fish meat consumption in kilograms per capita and year in the Slovak Republic. Note: Statistical Office of the Slovak Republic, 2019.

Consumption of beef meat and meat products had a decreasing tendency in the period 2009 – 2018 and an average growth coefficient k' was at the level of 1.0235 (Figure 4). Beef meat and meat products did not reach the level of recommended consumption resulting from the rational diet. In the last year of the monitored period the annual consumption of beef meat and meat products was only 5.3 kg per Slovak citizen. Low beef consumption was due to its unfavourable prices compared to other types of meat, and a decline in consumer confidence caused by the occurrence of food scandals related to beef quality and technology. We chose the quadratic function with following parameters to express the trend of development of beef meat and meat products consumption in the period 2009 – 2018:

$$q_t = 4.5017 - 0.2863 * t + 0.0383 * t^2$$

$$R^2 = 0.8271$$

Based on the quadratic function, it is possible to assume the future development of beef meat and meat products consumption to the next two years. We estimate that the consumption should increase moderately and reach almost 7 kilograms per inhabitant of the Slovak Republic in 2020. The slightly increasing tendency in beef meat and meat products consumption can be influenced by the growth of real wages and living standards of the population, changing eating habits, and expansion of health education and awareness of positive effects on consumer health **(Matošková and Gálik, 2016)**.

The trend in the development of fish consumption had an increasing tendency with an average growth coefficient k' at the level of 1.02 in the period 2009 – 2018 (Figure 5). Fish consumption ranged from 4.6 kg per person and year to 5.5 kg per person and year, so it means an increase on the level of 19.6% compared to the first year of the monitored period. The annual consumption of fish per capita of the Slovak Republic is lower by approximately 8% compared to the recommended doses of consumption in terms of rational nutrition. The trend in the development of fish consumption in the years 2009 – 2018 was expressed by the quadratic function with the following parameters:

$$q_t = 4.595 + 0.1016 * t - 0.0011 * t^2$$

$$R^2 = 0.6907$$

Based on the quadratic function, it is also possible to assume fish consumption in the next two years. Consumption of fish meat and meat products should rise slightly and should reach approximately 5.7 kilograms per inhabitant of the Slovak Republic in 2020. The slightly increasing trend in fish consumption may be influenced by increasing incomes of the population, rising living standards of the population, awareness of consumers about the benefits of fish consumption, changing eating habits, and by existence of food scandals related to other types of meat.

Analysis of the development of meat and meat products consumption in the Slovak Republic shows sufficient total consumption of meat and meat products, but inadequate distribution among individual types of meat. In the context of the above, we were interested in the opinion of Slovak consumers why there is a relatively high consumption of pork and poultry meat and low consumption of beef and fish in the Slovak Republic. Consumers ranked 4 possible reasons for the high consumption of pork and poultry meat, 1 being the most important reason and 4 the least important and the reasons were ranked according to the average order.

Based on the research results (Figure 6), it can be concluded that consumers consider the lower price of monitored products compared to the disposable income (1.58) as the main reason for the relatively high consumption of pork and poultry meat and meat products followed by a higher quality (1.55), offer of substitution products (2.70) and insufficient education related to excessive consumption of poultry and pork (3.17).

Furthermore, consumers also ranked 4 reasons for low consumption of beef and fish, 1 being the most important reason and 4 the least important. Based on the results of the questionnaire survey (Figure 7), it can be concluded that consumers consider the higher price of monitored products compared to disposable income (1.62) as the main reason for the low consumption of beef and meat products and fish, followed by lower product quality (2.48), offer of substitution products (2.78) and inadequate education in the consumption of these type of meat (3.11).

In connection with the consumption of different types of meat, the main reasons for consumption were evaluated. The results of the questionnaire survey (Figure 8) showed that Slovak consumers consume poultry meat and meat products mainly because of taste (30.3%), rational diet (29.9%) and high level of nutritional values (21.1%). Pork is consumed mainly due to its taste (32.3%), high levels of nutritional values (19.5%) and addiction since childhood (19.5%). Beef is preferred due to its taste (32.3%), high nutritional values (25.7%) and part of a rational diet (12.9%).

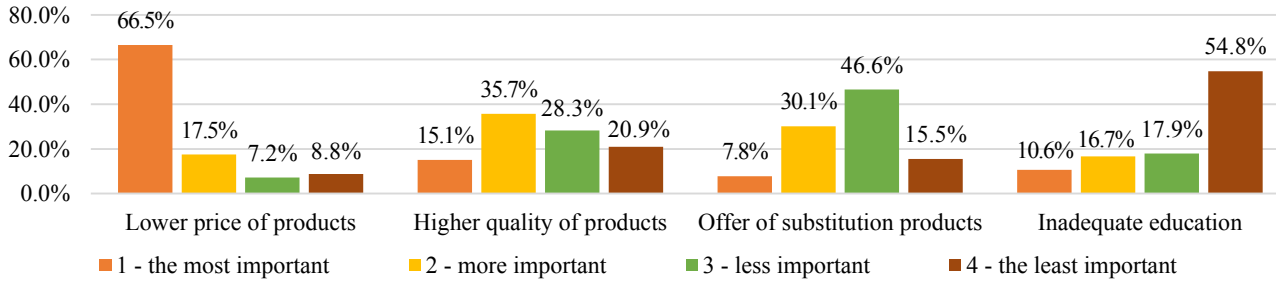

Figure 6 Reasons for high consumption of pork and poultry meat and meat products. Note: questionnaire survey.

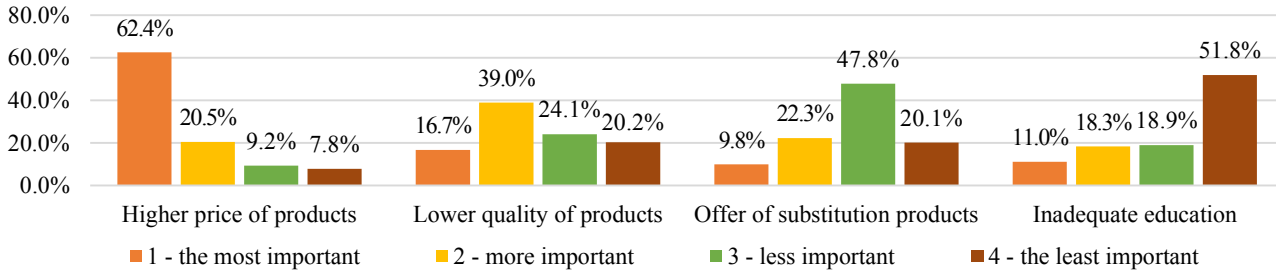

Figure 7 Reasons for low consumption of beef and fish meat and meat products. Note: questionnaire survey.

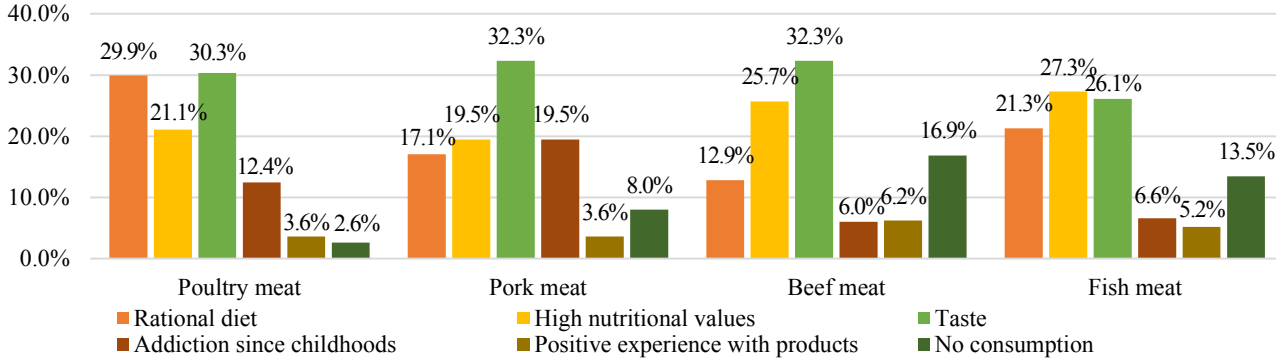

Figure 8 Reasons for meat consumption. Note: questionnaire survey.

Fish meat and meat products are consumed because of high nutritional values (27.3%), taste (26.1%) and rational diet (21.3%).

The questionnaire survey was also focused on factors that influence Slovak consumers in the process of purchase and subsequent consumption of meat and meat products. On the basis of the results (Figure 9) it can be stated that quality (99.0%), composition (94.8%), price (93.1%), freshness (91.7%) and country of origin (81.5%) are the main factors which are the most important for Slovak consumers in selection of meat and meat products. On the other hand, Slovak consumers do not consider product promotion (75.3%) and the appearance of the product packaging (63.1%) as the important criteria in deciding to buy meat and meat products. The survey of **GfK (2017)** has shown that for the Slovak consumers is still a very important price when deciding about purchasing chosen food, including meat and meat products, but the emphasis on quality is clearly rising. 59% of consumers say they closely monitor the prices of food in different stores and shop where the best deal is offered. As a result, consumers are focusing on quality, but at the same time they are looking for quality for the best price. In relation to the assessment of the various factors affecting the choice of meat and meat products by consumers, we found differences in the assessment of these criteria among the respondents. It is possible to identify differences in factor evaluation that is confirmed by applying Friedman test and the statistical calculation of the *p*-value (<0.0001). By using Nemeny's method and based on the data in the Table 1, we conclude that quality and composition are the most important criteria when choosing meat and meat products (Group A), another group of significant factors is created by composition, price and freshness (Group B), followed by a set of criteria created by the country of origin, nutrition data and producer (Group C), the other group of factors consisted from the country of origin, the producer and the package size (group D), and the last group of factors is the appearance of packaging and the product promotion (Group E). By dividing the factors determining consumer behaviour when choosing meat and meat products into these groups, it is possible to point to the differences in the assessment of individual criteria (groups) by consumers. The composition criterion is placed in two groups (Group A and Group B), which can be explained by the fact that there is no statistically significant difference in their ratings among Group A and Group B factors.

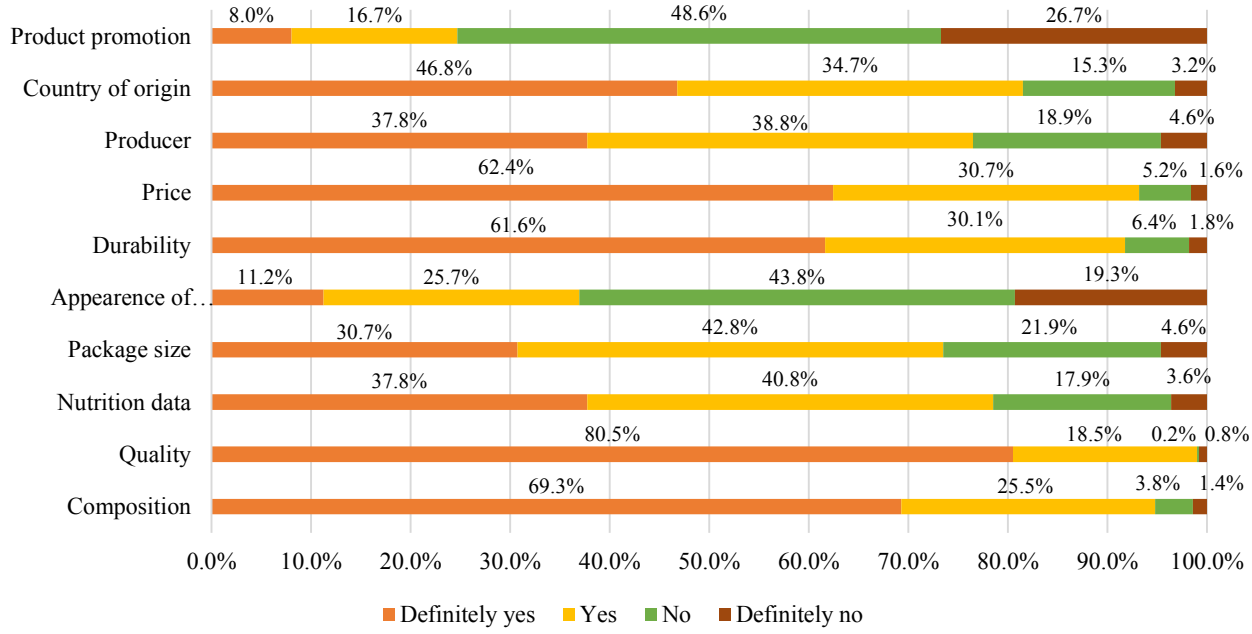

Figure 9 Factors affecting the purchase and consumption of meat and meat products. Note: questionnaire survey.

Table 1 Differences in factor evaluation when choosing meat and meat products by applying the Friedman Test and Nemeny's Method.

Sample	Frequency	Sum of ranks	Mean of ranks	Groups				
Quality	498	1710.000	3.434	A				
Composition	498	1989.500	3.995	A	B			
Price	498	2134.000	4.285		B			
Freshness	498	2151.000	4.319		B			
Country of origin	498	2591.500	5.204			C		
Nutrition data	498	2812.000	5.647			C	D	
Producer	498	2849.000	5.721			C	D	
Size of package	498	3007.500	6.039				D	
Appearance of package	498	3940.000	7.912					E
Product promotion	498	4205.500	8.445					E

Note: questionnaire survey.

However, between groups A and B there is a difference in the assessment of factors by consumers. The similar explanation has also two factors, nutrition data and producer, which are placed in two groups (Group C and Group D).

The quality of meat and meat products as the most important factor in purchasing and subsequent consumption can be perceived differently by each consumer. The perception of the quality of meat and meat products therefore depends mainly on the personality of the consumer. This is also confirmed by **Stávková, Stejskal and Toufarová (2008)**. Consumers evaluate the quality of selected foods based on several aspects, for example, price, composition, sensory, origin, experience with the products, product promotion and so on. These aspects may have a rational and irrational explanation by consumers. However, in the questionnaire survey, we focused on factors that consumers consider as important and perceive them in the process of purchase and subsequently consumption of meat and meat products.

The first factor is the composition. The main component of meat products is meat, so its percentage should be as large as possible. For this reason, we were interested in whether consumers notice the percentage of meat in meat products and how they perceive it in the process of buying products. In general, consumers have started to notice the percentage of meat in their purchase and subsequent consumption **(Tovar&Predaj, 2017)**. On the other hand, we assume the possible differences in the noticing the percentage of meat depending on the individual types of meat. We identified these differences on the basis of the applied Cochran's Q test, by calculating of which we confirmed our assumption (p-value = <0.0001). The results (Figure 10) showed that most consumers perceive the percentage of meat when buying poultry meat products (83.5%) and pork meat products (80.3%). On the other hand, the percentage of meat in the process of buying beef meat products is not perceived by 25.3% and in the case of fish meat products the percentage of meat is ignored by 35.3% consumers. These findings could be caused by two factors. Firstly, consumers do not buy these products because of their high prices and the second factor is the fact that consumers buying these products choose from the cheapest ones. It is probable that these products contain a low percentage of meat, so consumers do not look for this information on the product packaging.

The results of the questionnaire survey showed that another important factor in the process of purchase and subsequent consumption of meat and meat products is their freshness that is connected mainly with the taste of selected foods. **Nagyová et al. (2012)** confirm that freshness is one of the most important aspects for consumers buying food, especially meat and meat products. Our results reached by applying Cochran's Q test showed the existence of a difference in the perception of meat freshness depending on the individual types of meat (p-value = <0.0001). The results of the questionnaire survey also showed (Figure 11) that most consumers prefer fresh products in the case of poultry meat and meat products (81.3%), pork meat and meat products (75.1%) and beef meat and meat products (66.9%). In terms of fish meat and meat products, 46.0% of consumers perceive their freshness. On the other hand, more than 50 % do not perceive the freshness in buying fish, which could be caused by preference of frozen fish meat and meat products, semi-finished products or by the fact that consumers do not consume this type of meat.

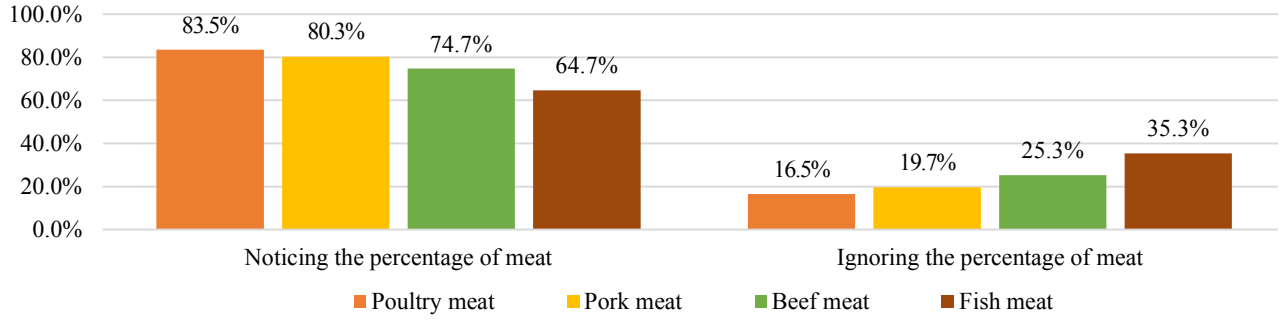

Figure 10 Noticing the percentage of meat in meat products according to different types of meat. Note: questionnaire survey.

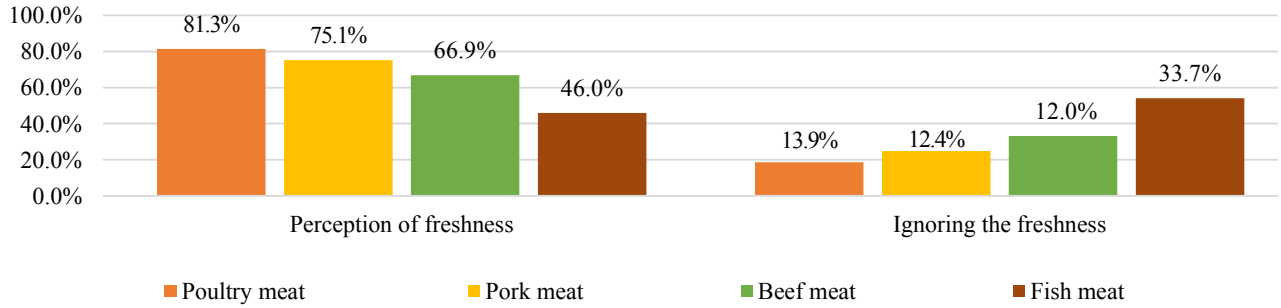

Figure 11 Perception of freshness of meat and meat products according to different types of meat. Note: questionnaire survey.

The following factor determining Slovak consumers in the process of purchase and subsequent consumption of meat and meat products is their price. Price has shaped consumer habits and eating habits since the past, and this can also be seen in the purchase and consumption of analysed foods **(Kubíčková and Šerhantová, 2005)**. Within the questionnaire survey, we were interested in the perception of prices of individual types of meat from the perspective of Slovak consumers. In the context of the question, we assumed the differences in the price perception depending on individual types of meat and meat products. We confirmed our assumption based on the calculation of the Friedman test (p-value = <0.0001). Based on the survey results (Figure 12), it can be also concluded that most consumers perceive prices of poultry meat and meat products (69.9%) and pork meat and meat products (70.7%) as reasonable. On the other hand, it is probably that Slovak consumers will start to perceive prices of pork meat as above average, because of the increased prices. Risen prices may be caused by the spread of African disease of pigs, which will have a negative impact on pork production. Furthermore, an increase in demand for European pork by China may also have a negative impact on the development of pork prices **(Ministry of Agriculture and Rural Development of the Slovak Republic, 2019)**. Prices of beef meat and meat products are perceived by most consumers as high or very high (63.9%). In the case of fish meat, prices are considered reasonable (47.2%) and high or very high (46.4%) by Slovak consumers. These results are also confirmed by **Schmid et al. (2017)**, which note the high prices of beef and fish. In this context, consumers are relatively sensitive to changes in the prices of different types of meat **(Kubicová and Kádeková, 2012)**. This fact is also confirmed by **Souček and Turčínková (2015)**, who emphasize that the impact of the price is particularly important for people with lower purchasing power and is reflected in the focus on cheaper types of meat and meat products.

Another important factor for Slovak consumers in the purchase and subsequent consumption of meat and meat products is their origin. Orientation to the domestic origin of meat and meat products is becoming a global trend, so we were interested in the questionnaire survey whether consumers prefer Slovak meat and meat products. In the context of the above, we assume that differences in preference of Slovak origin of meat and meat products depending on the individual types of meat. These differences were confirmed by calculating the applied Cochran's Q test (p-value = <0.0001) and are demonstrated in Figure 13. Based on the results of the realized questionnaire survey it can be concluded that consumers prefer Slovak products in the case of poultry meat and meat products (85.6%), pork meat and meat products (82.3%) and beef meat and meat products (76.6%). Consumers may prefer the Slovak origin of these products mainly due to the absence of any food scandals related to Slovak meat, shorter distance from the supplier, elimination of carbon footprint, perception of safety and risk of meat, or other perceived characteristics of Slovak meat. These findings were confirmed by **Nagyová et al. (2019)** and **Golian et al. (2018)**. The Slovak origin of fish meat is not preferred by 51.1% of consumers, which may be due to several factors, such as the limited offer of Slovak fish products, the import of fish products from abroad or the price of foreign fish products is lower compared to Slovak substitutes. However, it is assumed the future widespread of fish farming in the Slovak Republic, so the possibility of a greater preference of Slovak fish products by consumers is probable **(Pol'noinfo, 2017)**.

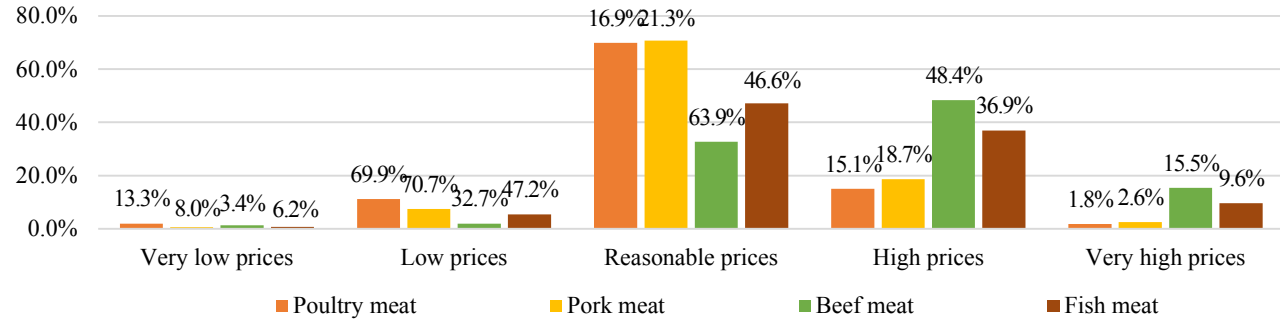

Figure 12 Perception of price of meat and meat products according to different types of meat. Note: questionnaire survey.

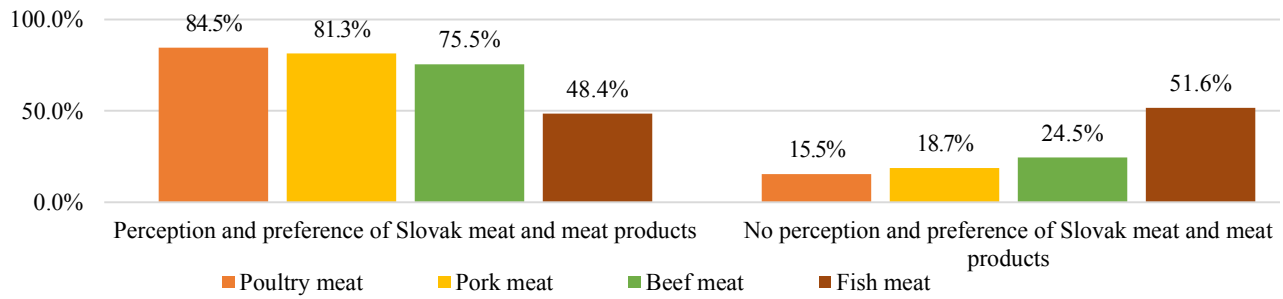

Figure 13 Perception and preference of Slovak origin of meat and meat products according to different types of meat. Note: questionnaire survey.

Based on the reached results, it can be stated that significant changes in the consumption of individual types of meat should not occur and will still be the relatively high pork and poultry meat and insufficient beef and fish meat consumption in 2020 in the Slovak Republic. Consumption is mainly influenced by individual consumer behaviour, which is related mainly to personal and psychological factors. The perception is the main factor from the psychological ones and was identified on the basis of the results of the questionnaire survey. The most important factor in the purchase and subsequent consumption of meat and meat products is the perception of quality, percentage of meat, freshness, price and origin.

CONCLUSION

The paper was focused on the meat and meat products consumption in the Slovak Republic, as well as on the identification of key factors affecting the purchase and consumption. Based on the obtained secondary data, we predicted the development of consumption of poultry, pork, beef and fish meat by 2020 and expect a stronger growth of poultry and pork consumption and slight growth in beef and fish consumption. Although this progression has demonstrated that the total consumption of meat and meat products will be sufficient, the distribution between the different types of meat will still be inadequate. High consumption of pork and poultry meat, as well as low consumption of beef and fish, are mainly caused by price relations of meat in relation to disposable incomes of Slovak consumers. This was confirmed by the primary data from the questionnaire survey, which showed that the price together with the quality of products and the existence of substitution products have a significant influence in the inadequate consumption of individual types of meat. On the other hand, it is important to note that approximately one-third of Slovak consumers consume different types of meat, mainly because of taste. We have also identified the perception of quality, composition, freshness, price and origin as the main determinants in the purchase and consumption of meat and meat products from the Slovak consumer's point of view. In relation to the identified factors, we have analysed in more detail the influence of these factors in the purchase of individual types of meat. The percentage of meat in meat products is most noticed by consumers in the process of purchase and consumption of poultry and pork meat products. Consumers look for the freshness of meat and meat products in the case of poultry, pork and beef. Consumers perceive the prices of poultry and pork as reasonable, but prices of beef and fish as high, which can be considered as the main reason for the low consumption of these two types of meat. The origin of meat is perceived by consumers mainly in the process of purchase and consumption of poultry and pork. Results show that Slovak consumers do not perceive the percentage of meat in products and the origin of meat, especially for beef and fish. It is also important to note that factors such as the perception of the meat and meat products quality, the perception of the percentage of meat in meat products, the perception of freshness, perception of price and perception of meat origin are crucial for consumers. However, consumers approach them individually and explain them rationally and irrationally, depending mainly on their personality.

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RURAL PRODUCTION OF TROPICALLY ADAPTED BREEDS OF CHICKENS IN RURAL AREAS OF KWARA STATE, NIGERIA

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ABSTRACT

Chicken keeping is a common thing with most household in rural areas of Nigeria. The birds are raised under extensive system of production with little or no feed provided by the farmers for the birds, hence there is the need to compare the rate of egg production of six different breeds of chickens reared under the same conditions in the rural areas. Twelve villages were randomly selected from the long list of villages in Kwara, 20 households per village and 4 villages per senatorial district, with a total coverage of 240 households for the study. The birds that were used for the study were indigenous chicken (Fulani), improved indigenous chickens (Shika Brown, Funaab Alpha and Noiler) and imported tropically adapted birds (Sasso and Kuroiler). Thirty six weeks old pre-vaccinated and brooded chickens of different breeds given to the farmers were managed under the traditional poultry scavenging system in all the three senatorial districts. The non-parametric Kruskal–Wallis test was used for the comparison between districts and breeds. There were no significant differences in egg production per senatorial district, egg production from different breeds and production from different senatorial districts remained averagely low. Low egg production by all the breeds showed that scavenging way of rural chicken production should be improved on for better productivity. This can be achieved through supplemental feed formulated and produced from locally available feed ingredients for the chickens.

Keywords: breeds; chickens; egg; production

INTRODUCTION

The demand for meat and egg is on the increase as a result of increase in the rate of population growth worldwide. Notably, poultry production development in Nigeria has taken a quantum leap in the last two decades; however, the development has been mainly restricted to commercial poultry production and not rural poultry production. The rural poultry production accounts for about 70 percent of the total poultry population **(Ogunlade and Adebayo, 2009; Akinola and Essien, 2011)**, indigenous chicken eggs and meat has always fetched a much higher price than that from the commercial poultry and also, the products from both rural chickens and commercial birds have been running parallel with their own market segment and specific clientele **(Omprakash and Pandian, 2011)**, yet, indigenous chicken has been totally neglected by both government and the commercial poultry investors.

A systematic and planned development of indigenous chicken production into small commercial units thus holds a tremendous potential for growth in rural areas, especially owing to consumer preference for its egg and meat **(Bett et al., 2012)**. The consumers' preference for indigenous chickens is based on the perception that locally produced poultry products are from natural and safe feed crops and hence good for the family's consumption. Indigenous chickens are considered as valuable asset for rural household and has contributed significantly to food availability for the household. This because, poultry mainly provides meat and eggs which increase households' consumption of animal sourced food. Eggs, however small in quantity provide micronutrients and high-quality protein in bioavailable forms to balance nutritionally the consumption of common staple food that is basically energy-based diet **(De Bruyn et al., 2015)**.

Moreover, the contribution of poultry to food security can be related with income **(Magothe et al., 2012; Abebe and Tesfaye, 2017)** from sales of poultry and poultry products, which are often, used for purchase of addition food items necessary for the household from the market **(Assa, 2012)**. More so, indigenous chickens are genetically envied for genetic exploration and hybrid vigour exploitation **(Adeleke et al., 2011)**. Indigenous chicken farming is, however, faced with several challenges **(Billah et al., 2013)**, including inherent slow growth rates, high rearing mortalities and susceptibility to diseases, poor housing, insufficient health care **(Alders, Bagnol and Young, 2010)**, high feed cost, poor nutrition and poor layers laying small sized eggs, all these challenges has adverse effect on production **(Sharaunga, Darroch and Mudhara, 2014; Ruel, Quisumbing and Balagamwala, 2018)**. Many improved breeds of broilers have been

imported into Nigeria and presently, there is an improvement in the potential of broiler strains to provide high quality meat at lower cost. The improved breeds grow faster and produce more meat and eggs than the local breeds. The objective of this study was to compare the rate of egg production of six different breeds of chickens reared under the same conditions in the rural areas.

Scientific hypothesis

Management practices has direct effect on rate of egg production than breeds of chicken

MATERIAL AND METHODOLOGY

The study was conducted between December 2017 and April, 2018 within the three Senatorial Districts of Kwara State of Nigeria. Kwara State is located in the North central geopolitical zone, commonly referred to as Middle Belt. The state comprises of rainforest in the Southern parts with wooded savannah covering the larger part of the state. Average maximum temperatures vary between 30 °C and
35 °C.

A total of four villages per Senatorial District were selected for the study. In each of the selected villages, twenty households were randomly selected for study, making a total of 240 households. The birds that were used for the study were indigenous chicken (Fulani), improved indigenous chickens (Shika Brown, Funaab Alpha and Noiler) and imported tropically adapted birds (Sasso and Kuroiler), as shown in Figure 1. The distribution of birds in each of the villages were as follows:

- four households with Sasso birds,
- four households with Kuroiler birds,
- three households with Fulani birds,
- three households with Shika Brown birds,
- three households with Funaab Alpha birds,
- three households with Noiler birds.

Thirty-six-week old pre-vaccinated and brooded chickens of different breeds given to the farmers were managed under the traditional poultry scavenging management system. The feeding of birds was supplemented with readily available commercial feeds, agricultural products (maize, rice, sorghum, wheat, millet, etc.) , agricultural by-products (corn bran, wheat bran, rice bran, groundnut cake, etc.) and kitchen wastes (leftover food, leafy vegetables, etc.). Based on the capacity of the farmers, health management practice was also carried out. In handling health challenges, traditional medicine was practiced by the rural resource-poor poultry farmers and this was done by practical application of indigenous medicinal herbs/plant extracts were used in controlling health challenges.

Statistic analysis

Statistical analysis was carried out using the SPSS Staistical Software. The non-parametric Kruskal–Wallis test was used for the comparison between districts and breeds. Statistical significance was determined at *p*-value of 0.005 (SPSS 2015).

RESULTS AND DISCUSSION

Indigenous chicken breeds are important in rural economies with respect to income generation and provision of nutritious chicken egg and meat for consumption **(Vali 2008; Mahendra, 2016)**. The system of production adopted in the rural communities affect the availability of these products. The scavenging system of chicken production in the rural area is important **(Alders and Pym, 2009)** and cheap for the farmer but results in low productivity in terms of body weight gain and egg production **(Natukunda et al., 2011)**. The interview conducted revealed that the farmers gave their chickens a handful of grain early in the morning and evening as supplementary feed but not compounded balanced diet. A Kruskal Wallis One Way ANOVA tests revealed that there was no significant difference in the total number of eggs produced in the senatorial districts (Table 1). However, egg production was observed to be significantly higher in the local breeds than the imported birds (Table 2).

Kruskal Wallis One Way ANOVA tests gives a probability of 0.000 which implies a significant difference in total number of eggs produced in the breed categories. From this result, the Noiler breed has superior and better performance in egg production, followed by Shika Brown, Kuroiler and Sasso. The breeds with least egg production were the Fulani and Fuuaab Alpha (Table 3).

A comparison of egg production of the breeds across the senatorial districts showed that the rate of egg production by the improved indigenous chickens (Noiler and Shika Brown) were higher than the imported chickens (Sasso and Kuroiler) as shown in Figure 2. From the study, egg productivity from different breeds and senatorial districts remained averagely low for each of the districts and this is largely due to poor management practices of birds scavenging on their own for feed with little or no supplementary feed **(Turk, 2013; Tadelle and Ogle, 2001)**, consequently, low and poor feed intake will result in low output productivity even with improved breeds of chickens. Hence performance of birds reared in the rural areas can be improved by change in husbandry, feeding, and better health cover **(Fiorella et al., 2016)**. Another factor responsible for low production is that most of the rural chicken producers do not have the mindset of rearing of chickens for profit making **(Dumas et al., 2016)** most of the rural chicken producers raise chickens for consumption, entertainment of visitors during festive periods, dowry payment or to give as gift but not as a viable business **(Melesse, 2014; Dhaka et al., 2017)**.

The result on egg production per breed shows that noiler breed has superior and better performance in egg production when raised under scavenging system of poultry management. The production of eggs by Sasso and kuroiler chickens were low compared to Noiler, an improved tropically adapted indigenous chicken. This could be due to inappropriate feeding regime and poor management practices **(Varguez-Montero et al., 2012)** which led to the low laying performance of the imported chickens that are known to have good laying ability **(Javed et al., 2003)**. The scavenging system without adequate supplementary feed is a major contributory factor to low egg production even with breeds that has better genetic potential for growth and production.

Table 1 Egg Production in the senatorial districts (n = 2132).

Senatorial District	No. of Collection Point	Mean	Total Eggs	95% Confidence Interval for Mean	
				Lower	Upper
Kwara Central	478	12.87	6154	10.19	15.56
Kwara North	481	7.57	3642	6.65	8.49
Kwara South	1173	11.66	13676	10.53	12.79
Total	2132	11.01	23472	10.12	11.90

Note: Kruskal Wallis Value =0.329, Degree of Freedom = 2, *p*-value = 0.848.

Table 2 Egg production of imported and local breeds (n = 2132).

Breed	No. of Collection Point	Mean	Total Eggs	95% Confidence Interval for Mean	
				Lower	Upper
Imported	796	9.76	7771	8.65	10.87
Local Breed	1336	11.75	15701	10.49	13.01
Total	2132	11.01	23472	10.12	11.90

Note: Kruskal Wallis Value = 218.049, Degree of Freedom = 5, *p*-value = 0.000.

Table 3 Egg production per breed (n = 2132).

Breed	No. of Collection Point	Mean	Total Eggs	95% Confidence Interval for Mean	
				Lower	Upper
Fulani	342	5.70^{c}	1950	4.59	6.82
Sasso	356	10.31^{b}	3671	8.63	11.99
Noiler	212	31.03^{a}	6579	25.58	36.48
Kuroiler	440	9.32^{b}	4100	7.84	10.80
Shika Brown	446	12.36^{b}	5512	10.41	14.31
Funaab Alpha	336	4.94^{c}	1660	3.59	6.29
Total	2132	11.01	23472	10.12	11.90

Note: n = 2132, Kruskal Wallis Value = 218.049, Degree of Freedom = 5, *p*-value = 0.000.

Figure 1 The chicken breeds used for the study **(Adebambo et al., 2018)**.

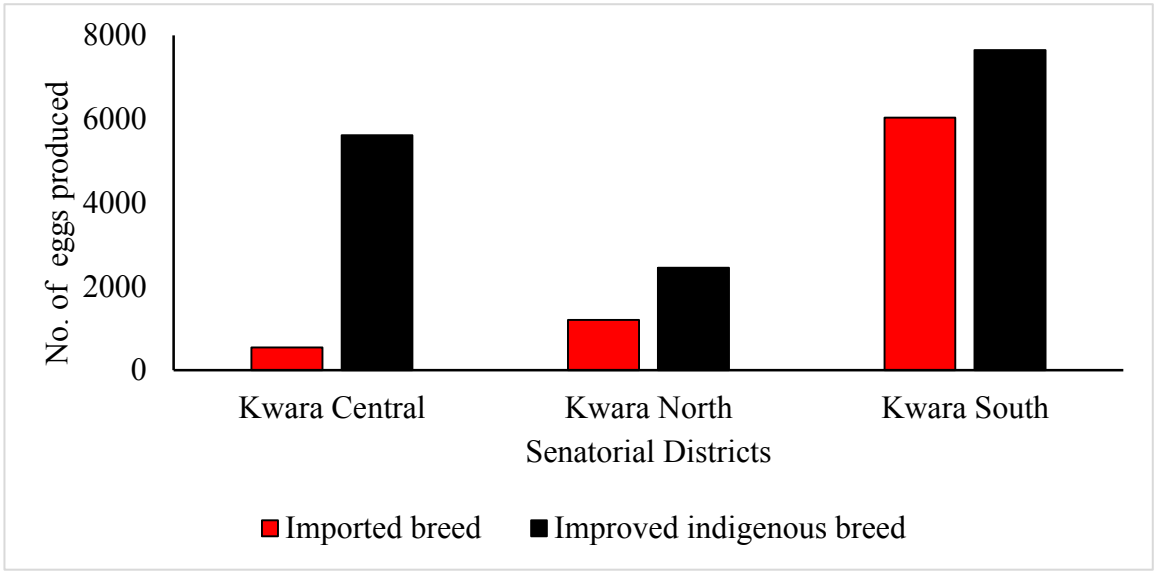

Figure 2 Egg production of the imported and improved indigenous breeds.

Mutayoba et al., (2012) reported that supplementation can lead to improved performance of local chickens in terms of growth, egg production and quality.

The cost and availability of commercial feed influenced its use by people of low resources in the rural areas however, supplementary feed can be compounded from locally available feed ingredients in the rural areas. It is of importance that scavenging system must be supplemented either with compounded feed or left over meal from individual household.Left over meals can only be possible where the household has more than enough to eat but in situations where there are no leftovers as a result of poverty or food scarcity, it will have negative effect on the chickens resulting in low body weight and egg production **(Gondwe et al., 2005)**.

Another factor that may be responsible for this low egg production by the imported chickens may be adaptability problem. The variation in temperatures, high humidity, excessive heat and rainfall exact significant effects on poultry birds in terms of egg production, body weight, health, diseases, income of farmers, diet of the people, quality and quantity of poultry products and the economy of the developing countries **(Tumova and Gous, 2012; Diarra and Tabuaciri, 2014)**.

Though the six breeds are tropically adapted birds but the rate of adaptability may vary among breeds having effect on the productivity of the chickens. Probably the rate of adaptability may also be reason why noiler birds did better than sasso and kuroiler birds. The performance of any chicken is also affected by genotype and the environment **(Dessie et al., 2012)**, important attributes of indigenous chickens are their hardiness in the ability to tolerate harsh environmental condition and poor husbandry practices in terms of climate, handling, watering, and feeding without much loss in production are all for survivability and not for production as observed in this study.

CONCLUSION

Low egg production by all the breeds shown in Figure 2 showed that scavenging method of rural chicken production should be supplemented with home formulated feed produced from locally available feed ingredients . Most of the chicken producers in the rural areas do not keep chickens for profit making hence, the need to educate them on improved management system of poultry production. Improved practices will not only increase income level generation but will also bring about positive change in the socio-economic level and food security of the rural communities.

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VEGETABLE INGREDIENTS IN CHEESE PRODUCT

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ABSTRACT
Sesame seeds are a functional food ingredient with vasoprotective, antioxidant, prebiotic, chondro- and osteoprotective characteristics. In this study, sesame seeds were used to enrich a cheese product. The dose, method and technological production stage of the cheese product in which to add sesame seeds were determined, in addition to the effect of sesame seeds on the product's quantitative indicators. The nutritional value of sesame seeds, their total amino acid and fatty acid compositions and microbiological parameters were evaluated, depending on the method of the filler temperature treatment. The appropriate heat treatment method was holding the functional component in milk at 73 ±2 °C for 25 min, followed by cooling to 30 ±2 °C. Adding the filler into the cheese mass before moulding the cheese head was determined as the appropriate technological step to introduce the previously prepared sesame seeds. The cheese product was found to have the best sensory characteristics at the 3% sesame seed dose compared with the doses of 1% and 5%. The cheese product enriched with sesame seeds can be recommended as a functional product for systematic consumption without restrictions for all groups in a healthy population.

Keywords: cheese product; sesame seed; nutritional value; functional product

INTRODUCTION
One of the promising directions in the development of the dairy industry is the creation of functional products that provide food rations with nutrients and energy for improving physiological functions in various population groups **(Gorlov et al., 2014)**. In the production of functional dairy products, plant raw materials (seeds, berries, fruits, vegetables and spices) are attractive ingredients that are nutritionally-valuable, primarily due to the specific combination of biologically active components **(Klimenko et al., 2017; Kryuchkova et al., 2015)**.

At present, a healthy lifestyle and proper nutrition are gaining popularity. Followers of such a diet often add various seeds, for example, chia, flax, sunflower and sesame seeds (SS) to habitual dishes **(Suprunova et al., 2010)**. These additives and toppings positively affect the nutritional value of the dish and its benefits in general. Of great interest are SS because of their sweet taste, nut aroma and wide range of valuable macro- and microelements and vitamins **(Sanzharovskaya, Sokol and Khrapko, 2018)**.

Sesame (*Sesamum indicum* L.) is an oilseed plant that is grown everywhere in the East. In the countries of the Commonwealth of Independent States, sesame is grown mainly in India. SS are rich in manganese, copper and calcium, but also contain B vitamins and vitamin E (tocopherol) **(Katserikova and Lipatova, 2009)**. Sesame exerts diverse health benefits attributed to the biologically active components, including phytosterols, tocopherols, polyunsaturated fatty acids and lignans. The main SS lignans are sesamin, sesamolin and sesaminol – a group of phenolic compounds of plant origin **(Martinchik, 2011)**. Phenolic compounds exhibit pronounced antioxidant activity, bind heavy metal ions and serve as free radical acceptors **(Kumar and Singh, 2015)**. When exposed to moderately high temperatures, sesamin and sesaminol are converted to a more powerful antioxidant – sesamol **(Gerstenmeyer et al., 2013)**.

Research on the antibacterial activity of sesame lignans has revealed that the antimicrobial spectrum of sesamol, sesamolin and sesamin includes most pathogenic Gram-positive and Gram-negative microorganisms **(Kumar and Singh, 2015)**. Moreover, an *in vitro* fermentation study of sesaminol triglucoside by human intestinal microbiota showed a marked increase in the number of lactobacilli, enterococci and bifidobacteria, without stimulating the growth of *Eubacterium rectale*, *Clostridium coccoides* and *Clostridium histolyticum* **(Zhu et al., 2013)**.

As a dietary component, sesame helps the blood vessel endothelium to restore its functional state. This action is due to the antioxidant activity of its lignans and vitamin E, as well as recovery of the blood lipid spectrum **(Asgary et al., 2013)**. In addition, sesamin induces endothelium-dependent vasorelaxation, which is one of the important mechanisms of the anti-hypertensive effect of sesame lignans **(Nakano et al., 2006)**.

Substantial evidence has been collated to suggest that sesamol has chemopreventative potential *in vitro* and *in vivo* **(Majdalawieh and Mansour, 2019)**. When administered for 9 weeks in the diets (500 $mg.kg^{-1}$) of mice with induced polyposis of the small intestine, sesamol reduced the number of polyps in the middle part of the small intestine to 66.1%, compared with the control group **(Shimizu et al., 2014)**. Sesamol inhibited the synthesis of melanin in melanoma in mice by 63%, besides inducing apoptosis and limiting the proliferation of tumour cells **(Kumar et al., 2011)**. This same bioactive compound demonstrated chondroprotective effects in IL-1β-induced inflammation in an osteoarthritis model *in vitro* **(Kong et al., 2016)**. Sesamin, another major bioactive component in sesame, inhibited ROS-induced apoptosis of primary osteoblasts in the femoral head of rats **(Deng et al., 2018)**. The stimulated differentiation of osteoblasts and inhibition of osteoclastogenesis, by sesamin has also been investigated **(Wanachewin et al., 2017)**.

As exemplified by these cited experimental, clinical and epidemiological studies of the biological properties of bioactive SS components, sesame is a functional food ingredient that maintains the cardiovascular system and bone tissue and normalises the intestinal microflora and antioxidant activity.

In this context, it is valuable to investigate the effect of functional plant ingredients in the production of dairy products. Therefore, this study aimed to use SS to develop an enriched cheese product.

Scientific hypothesis

We asumed that the dose, method and technological production stage of the cheese product in which the SS were added would have a significant effect on the quality of the cheese product.

MATERIAL AND METHODOLOGY

Cheese product samples incorporated with different doses of SS were prepared, using various strategies to improve the nutritional and sensory qualities of the product. The fat mass-fraction was evaluated empirically by the Gerber method, as detailed in the **Government Standard (GOST) R ISO 2446-2011:2012**, which provides the mass fraction of fat. Briefly, milk was placed in the test bottle and centrifuged to separate the fat after dissolution of the protein with sulfuric acid, and the addition of a small amount of isoamyl alcohol. The value of the indicator was measured by the calibration of the test bottle.

The mass fraction of protein was determined by the Kjeldahl method, in accordance with **GOST 34454-2018:2019**. In brief, the organic matter in the analysed product sample was mineralised by the addition of concentrated sulphuric acid in the presence of a catalyst. Ammonium sulphate was formed and converted to ammonia that was distilled into a boric acid solution and quantified by the titrimetric method for calculation of the protein mass fraction.

The mass fractions of moisture and solids were ascertained based on **GOST R 8.894-2015:2016**, using an Evlas-2M moisture analyser. An infrared thermogravimetric method to determine the mass fractions of moisture and dry matter was applied, which involved measuring the weight of the sample before and after it was dried under the infrared radiation. Sensory properties were assessed by a group of experts by adhering to the requirements of **GOST R ISO 22935-2-2011:2011**. Microbiological properties were analysed based on **GOST 32901-2014:2016**. The amino acid content was determined by capillary electrophoresis using a Kapel-105M system. A deuterium lamp was applied as a light source in the system, and a diffraction monochromator with a spectral range of 190 – 380 nm and a spectral interval width of 20 nm was used as a dispersing element. Fatty acids were determined on a Tsvet-164 gas chromatograph, using the polar liquid phase, polydiethylene glycol adipam.

Statistic analysis

Statistical data were processed using the Statistical program (Statistica, version 6.0 (Dell, USA). The data are presented as averages. The differences between the samples were assessed using unpaired t-test. Correlation analysis with calculation of pair correlation coefficient, for establish the dependence of parameters was used. The significance of differences was determined by the Student's criterion (t). The level was considered significant at $p \leq 0.05$. The study was repeated three times.

RESULTS AND DISCUSSION

One of the most important steps in determining the quality of SS as a phyto-enriching component of a cheese product is the analysis of its chemical components. The quantitative analysis of the protein, fat and fatty acids were important because of their association with the potential contribution of SS to satisfying the physiological needs of these substances in the human body. After a series of tests using conventional methods, the nutritional SS values were obtained (Table 1).

From the data, it was evident that SS had a high content of vegetable protein and fat per 100 g of the product. The integration of plant-based bioactive ingredients in dairy products to provide a source of not only healthy fats but also dietary protein would allow creating complete protein-rich cheese products that are considerably cheaper compared with products consisting entirely of expensive animal protein. Due to its high nutritional value, sesame is used as a filler in various food products: sweets, bakery products, dairy products and others **(Poddubny and Zhurbenko, 2019; Koneva et al., 2017; Ovchinnikov et al., 2016; Pashchenko et al., 2008)**.

For a deeper understanding of the value of SS in the formulation, the total content of amino acids and fatty acids was analysed. Amino acids are the “building” material for a healthy organism **(Singelot et al., 2018)** and structural, chemical units that form proteins, and, in turn, the structure of the tissues of the human body. The amino acids are important for the body because proteins play an essential role in all life processes. There are 20 essential amino acids. To construct the vast majority of human body proteins, all 20 amino acids are required in certain proportions **(Lysikov, 2012)**.

Table 1 Nutritional value of sesame seeds, per 100 g.

Indicator	Value
Weight fraction of fat, %	49.7
Weight fraction of protein, %	19.7
Weight fraction of carbohydrates, %	16.0
incl. weight fraction of dietary fibre, g	5.6
Weight fraction of minerals, g	3.8
Weight fraction of moisture, g	5.2
Calories, kcal	590.1

Table 2 Total content of amino and fatty acids in sesame seeds.

Acids	Content, g.100g^{-1}	Percentage of daily amount, %
Proteins and amino acids		
Total protein content	19.70 ±0.3	25.6
Essential amino acids	8.6 ±0.06	26.8
Non-essential amino acids	11.03 ±0.1	16.2
Fats and fatty acids		
Total fat content	49.7 ±0.4	56.4
Unsaturated fatty acids:	41.1 ±0.2	66.7
Monounsaturated fatty acids:	20.8 ±0.01	139.4
Polyunsaturated fatty acids	20.2 ±0.4	101.8
Saturated fatty acids	8.6 ±0.3	32.6

Table 3 Microbiological properties of sesame seeds depending on the temperature-time modes of treatment.

Indicator	Weight of product (g), where not allowed	Characteristic/Temperature (T)–time (τ) mode		
		T = 65 ±2 °C τ = 25 min	T = 73 ±2 °C τ = 25 min	T = 85 ±2 °C τ = 25 min
QMAFAnM, CFU/cm^3 (g), not less than	1×10^7	2×10^6	–	–
Listeria monocytogenes	–	–	–	–
Enterobacter sakazaki	–	–	–	–
Yersinia bacteria	–	–	–	–
Mould, cfu per g	50	15	–	–
Yeast, cfu per g	50	12	–	–

Note: QMAFAnM: quantity of mesophilic aerobic and facultative anaerobic microorganisms.

The human body's capacity to conserve individual amino acids varies considerably, so the proportions of the amino acids needed in the diet to match their catabolic rates are not directly proportional to the composition of body protein. An imbalance in the amino acid composition of dietary protein leads to a disruption in the synthesis of body proteins and shifts the dynamic equilibrium of protein anabolism and catabolism towards the predominance of the breakdown of the body's proteins, including enzyme proteins **(Stepuro and Khaprova, 2010)**. The lack of an essential amino acid limits other amino acids being used in the protein biosynthesis.

Fatty acids form the basis of cell membranes and participate in the synthesis of the most important hormone-like substances, so they are vital for the body. Correct results in analysing amino and fatty acid compositions depend on the reliability of the qualitative identification of the components analysed. The total content of amino acids and fatty acids is presented in Table 2. The percentage of the normal daily intake of protein and fatty nutrients was calculated for an average person aged 30 – 39 years, group II of physical activity, and provides for 2650 kcal of a daily diet **(Federal Center for Hygiene and Epidemiology of Rospotrebnadzor, 2009)**.

A detailed analysis of the chemical composition of SS found that they contain a considerable amount of essential amino acids and unsaturated fatty acids. Essential amino acids include valine, phenylalanine, lysine, leucine, tryptophan, threonine, arginine, histidine, isoleucine and methionine. These amino acids are not synthesised in the human body or formed in insufficient quantities for sustaining a healthy life. Both types are essential for health. According to the **FAO WHO (1974)** recommendations, the daily requirement for essential and non-essential amino acids is 32 and 68 $g.100^{-1}g$ of protein, respectively. We found that 100 g of sesame protein accounts for 43.6% of the essential amino acids, which corresponds to 2.9% of the daily requirement. Unsaturated fats are divided into monounsaturated and polyunsaturated fats. In percentage terms, the unsaturated fatty acids accounted for 82.6% of the lipid portion of the seeds, composed by 41.8% monounsaturated and 40.8%, polyunsaturated fatty acids, respectively (Figure 1).

In the manufacture of dairy products, any ingredient added must be heat-treated, while preserving its nutritional value to the fullest extent, besides preventing the destruction of biologically active substances and all the necessary vitamins **(Mazhaeva, Kozubskaya and Sinitsyna, 2017)**. The heat treatment is one of the most important steps in the technological food preparation process. Effective processing preserves the taste and nutritional characteristics of the product, destroys the pathogenic microflora and thus, ensures the sanitary and epidemiological safety of the product.

Raw peeled sesame seeds have a slightly bitter taste and a mild nutty flavor. Heat treatment positively affects the color change of sesame seed, enhances aroma, improves taste, improves digestibility. Seeds acquire the taste inherent in nuts. The known parameters of heat treatment of sesame seeds in the production of cottage cheese desserts 65 – 70 °C for 6 – 7 min **(Katserikova, Solopova and Lipatova, 2011)**. We proposed various temperature treatments of SS in pre-warmed milk: 65 ±2 °C for 25 min, 73 ±2 °C for 25 min and 85 ±2 °C for 25 min. After the treatment, SS were cooled to 30 °C. Then the microbiological properties and sensory qualities of the treated SS were determined (Table 3). The qualitative and quantitative compositions of the microflora of the product are of great importance for establishing its purity and sanitary condition. The microbiological control of the raw materials and finished products allowed us to timely identify the source of contamination of products with microorganisms that cause their spoilage, as well as judge the possible presence of foodborne infections and poisoning pathogens.

The temperature treatment of SS was followed by analysis of the microbiological properties that indicated their high rates at 73 ±2 °C for 25 min and no foreign microflora. Moreover, SS acquired a pleasant, pronounced taste of pasteurised milk, complemented by the sour-milk taste of the cheese base. When SS were processed in milk below this temperature, the cheese product was likely to be contaminated with microorganisms, such as mesophilic aerobic and facultative anaerobic microorganisms (MAFAnM), coliform bacteria, yeast and mould (fungi), which is not permissible in the cheese production. It was not economically feasible to apply the higher temperature of 85 ±2 °C for 25 min due to the destruction of the B vitamins and 30 – 50% decrease in their properties during heat treatment. Therefore, it was necessary to use the minimum temperature modes that ensured the destruction of foreign microflora of SS.

In the production of fortified dairy products, the method, process step and dose of adding plant materials into the base product are important. The experimental samples were developed according to the same technology and differed in the stage of manufacture when the SS treated at 73 ±2 °C for 25 min were added into the product. To determine the method and technological stage of the SS application, two additional options were tested, followed by an examination of the sensory qualities of the cheese product. Sample I was added into the milk base before coagulation, that is, the coagulation process of the milk mixture was evaluated in the presence of SS. Sample II was added to the cheese mass before forming the head, that is, SS did not affect the coagulation of milk and, therefore, the quality of cheese grain. The results of the experimental study are presented in Table 4.

It was found that the SS added to the milk base before coagulation (Sample I) caused a part of the filler being lost with whey, which reduced the quality of the product, its nutritional value and functional efficiency. The emergence of seeds on the surface of the milk base led to economic loss in production. Adding the SS to the cheese mass before forming the head (sample II) was the appropriate approach. This method caused the SS to swell, soften, and become evenly distributed throughout the cheese mass. It also led to high sensory characteristics and eliminated the technological loss of SS. Studies are also known that confirm the effectiveness of making fillers in the cheese mass after the formation of cheese grain **(Aravina and Arsenyeva, 2016; Ryabkova et al., 2011; Kharenko et al., 2010)**.

Table 4 Sensory characteristics of the cheese product of sesame seeds (SS) depending on the method of adding SS.

Method of SS addition	Appearance and consistency	Taste and smell	Colour
Sample I SS were added to milk at 73 ±2 °C, held for 25 min, cooled to 30 ±2 °C and added to the milk base until coagulated	SS swelled, softened and floated to the surface of the milk base	Moderately-expressed cheese taste, complemented with pleasant SS taste and aroma	Milky-white, heterogeneous, interspersed with white SS
Sample II SS were added to milk at 73 ±2 °C, held for 25 min, cooled to 30 ±2 °C and added to the cheese mass before moulding the cheese head	SS swelled, softened and distributed evenly throughout the cheese mass	Moderately-expressed cheese taste, complemented with pleasant SS aftertaste and aroma	Milky-white, homogeneous, interspersed with white SS

Table 5 Sensory characteristics of the cheese product depending on the dose of sesame seeds (SS).

SS weight fraction	Appearance and consistency	Taste and smell	Colour
Sample I (1% SS)	Soft, spreading and slightly fluffy consistency, with single, white SS	Clean cheese taste with a slightly pronounced flavour of SS	Milky-white, with single interspersed white SS evenly distributed throughout the mass
Sample II (3% SS)	Soft, spreading and slightly fluffy consistency, with single SS	Clean cheese taste with a pronounced SS flavour complemented by the cheese base	Milky-white, with single interspersed white SS evenly distributed throughout the mass
Sample III (5% SS)	Soft, spreading and slightly fluffy consistency, with SS	Cheese taste with a predominant pronounced flavour of SS	Milky-white, interspersed with white SS distributed throughout the mass

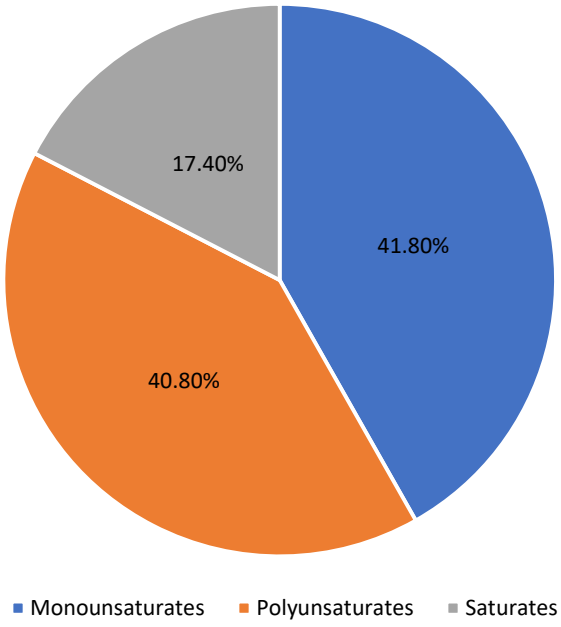

Figure 1 Ratio between fatty acids and fatty part of sesame seeds, %.

To determine the appropriate dose of SS, the cheese products were produced with a seeds weight fraction of 1%, 3% and 5%. For this purpose, after being standardised by fat, milk was pasteurised at 72 – 75 °C for 2 – 3 min and cooled to 32 – 34 °C. Then, a culture (its composition contained *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis biovar diacetylactis*), $CaCl_2$ and rennet were added. After thorough mixing for 15 min, the mixture was fermented at 32 ±2 °C for 40 – 45 min until a dense curd formed and the whey separated. After cutting, the curd was gently kneaded, the whey was removed, and various doses of SS were added to the cheese mass. Then, the cheese heads were moulded by filling and self-pressing for 1.5 – 2.0 h, with turning every 15 – 20 min at 12 – 14 °C. Afterwards, the cheese heads were held in 10% salt brine at the same temperature for 10 – 12 h, and dried. The results are presented in Table 5.

According to the data obtained, it was found that Sample I and III did not have appropriate sensory characteristics. Addition of Sample I (1% SS) to the cheese mass led to a filler with weak flavour. Sample III (5% SS) had a cheese flavour with a predominant and strong specific seed flavour that not everyone would like. Sample II, with 3% weight fraction of SS, was established to have the most balanced taste and smell. This sample had a clean cheese taste with a pronounced SS flavour that complemented the cheese base. Its sweetish, nutty flavour and aroma perfectly complemented the taste of the cheese base. Known studies of the dose of sesame in the production of dairy products **(Titov et al., 2018)**. The authors of the studies also believe that with an increase in concentration of 3%, sesame flavor prevails, which was evaluated as extraneous and undesirable based on the objectives of the experiment.

CONCLUSION

The study found that SS are a highly effective functional ingredient with high nutritional value and high content of essential amino acids and unsaturated fatty acids. The appropriate method of the heat treatment and technological stage of addition involved holding the SS in milk at 73 ±2 °C for 25 min, cooling to 30 ±2 °C and adding to the cheese mass before moulding the head of cheese. The dose of SS was established as 3% of the filler. The cheese product enriched with SS can be recommended as a functional product for systematic consumption without restrictions for all groups of a healthy population.

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ANTIBACTERIAL AND ANTIOXIDANT POTENTIAL OF LEAVE EXTRACTS OF *HELIANTHUS ANNUUS*

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ABSTRACT

Helianthus annuus has been widely used for its medicinal and nutritional properties. This study was aimed at assessing the ethyl acetate, n-hexane and methanol extracts of *Helianthus annuus* for antibacterial and antioxidant potentials. The phytochemical screening, total phenols, DPPH radical scavenging assay and nitric oxide radical scavenging activity were carried out following standard procedures. Preliminary screening of the antibacterial activities of the extracts was carried out on five bacterial species (*Bacillus subtilis, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*), using the agar-diffusion method. Growth rate studies in presence of the extract was investigated on two bacterial species (*Bacillus subtilis* and *E. coli*). The methanol extract was observed to inhibit the growth of the five bacterial species while ethyl acetate and N-hexane extracts showed inhibition against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa.* Extended lag periods of 5 – 6 h were observed when the *Bacillus subtilis* and *Escherichia coli* were grown in broth medium that contained the respective extracts. In broth medium with mixture of extract and ascorbic acid, there was no observed growth of the *Bacillus subtilis* and *Escherichia coli* throughout the 7 h incubation period. The total phenolics content of the extracts revealed concentrations of 6.66 ±0.45, 5.58 ±0.11 and 6.06±0.41 mg TAE.g^{-1}) for the methanol, N-hexane and ethyl acetate extracts respectively. The DPPH radical scavenging assay results displayed gradual increase in percentage inhibition from the lowest to the highest concentration across all the standard groups, a similar trend was observed with the extracts, the ethyl acetate extract showed highest percentage inhibition amongst the other extracts. All the extracts showed high reducing power ability. The nitric oxide scavenging ability of the extracts showed constant increase with increase in concentration. *Helianthus annus*, it could be a good source of antimicrobial and antioxidant especially in a world where resistance to antibiotic has increasingly become a global concern.

Keywords: Antibacterial; antioxidant; growth inhibition; *Helinathus annuus*

INTRODUCTION

Plants are of great economic value all over the world as it has been as raw materials in the industries especially the pharmaceuticals, nutraceuticals and cosmetics **(Eze et al., 2015; Ibrahim et al.,2014)**. The use of plants and its products as medicines is an age long pattern. Despite the advent of modern medicine, plant extracts and its constituents are being used traditionally as therapy in 70 – 80% of the world population to meet their health needs, most especially the rural dwellers in developing countries **(Ibrahim et al.,2014; Subashini and Rakshitha, 2012)**. Most of these claims has not been scientifically proven as only few plants have been evaluated of their therapeutic effects.

Helianthus annuus has been noted for its medicinal and nutritional usage worldwide. *H. annuus* L. (*Asteraceae*) commonly referred to as sunflower is cultivated in America, Africa, Asia and Australia. Its seed is an important source of edible oil all over the world **(Ibrahim et al.,2014; Al-Snafi, 2018)**. The plant has a characteristic tap rooted plant with coarse toothed leaves, yellowish flower, rough-hairy stem and 1.5 – 3.5 m high **(Eze et al., 2015; Al-Snafi, 2018)**. The seeds, roots, flowers and bark of *H. annuus* have been used for medicinal purposes **(Al-Snafi, 2018)**. Analysis of the phytochemical analysis present in *Helianthus annuus* reveals that it contained phenolics, flavonoids, steroids, alkaloids, saponins, carbohydrates, tannins, phytosterols, triterpenoids and fixed oils. *Helianthus annuus* seed can be used as salad garnish, snacks and some bakery goods **(Guo et al., 2017)**.

The seed oil and shoot have been used as stimulant, anti-oxidant, anti-inflammatory, cathartic, diuretic, anti-hypoglycemic, antitumor, anti-asthmatic, antipyretic, antimicrobial and for stomach problem purposes. The seed

is essential for diuretic, cough, throat and lung infections treatment **(Eze et al., 2015; Subashini and Rakshitha, 2012)**. In Venezuela, *H. annuus* flower and seed serve as cancer treatment. Reports has also shown crushed leaves serve as medicinal covering on swelling, sores, snakebites and spiderbites **(Jiraungkoorskul, 2016; Al-Snafi, 2018)**. This study was therefore aimed at assessing the antimicrobial and antioxidant properties of ethyl acetate, hexane and methanol extracts of *Helianthus annuus* leaves.

Scientific hypothesis

Helianthus annuus extracts has antibacterial and antioxidant properties.

MATERIAL AND METHODOLOGY

For preparation of the plant extracts, the leaves of the *Helianthus annuus* were collected from the Teaching and Research Farm of Landmark University, Omu Aran, Kwara state. After collection, the leaves were washed with clean tap water to remove dirts and other unwanted materials, before sun-drying for five days. Following drying, the leaves were pulverized, and known quantities were placed in beakers containing the respective extracts. Extraction and concentration of the extracts were carried out as describes by earlier workers **(Eze et al., 2015; Ibrahim et al.,2014; Subashini and Rakshitha, 2012)**.

For preliminary evaluation of the antibacterial potential and determination of the minimum inhibitor concentrations (MIC) of the extracts, the agar diffusion method. The bacterial species used were *Staphylococcus aureus*, (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* and *Klebsiella* sp. The *Escherichia coli* and *Klebsiella* sp were laboratory stock cultures of the Department of Microbiology, Landmark University, Omu-Aran, Nigeria. Prior to use, the respective isolates were streaked onsterile nutrient agar plates to ascertain their purity, before subculturing in sterile peptone water. Two bacterial species (*Bacillus subtilis* and *Escherichia coli*) were used for the growth rate studies. Growth rate studies was carried out liquid medium, with four different composition, which were: nutrient broth only, nutrient broth + ascorbic acid, nutrient broth + extract, nutrient broth + extract + ascorbic acid. In 100 mL sterile nutrient broth, 5 mL of MIC of the respective extracts against a known bacterial species was added and incubated in an orbital shaker (S15200) at 37 ^{o}C at 120 rpm. Prior inoculation and every 1 h interval for 7 h incubation period, 5 mL of sample was aseptically withdrawn from each flask for determination of optical density at wavelength of 720 nm.

Growth rate was calculated as:

$$\textbf{Growth rate } (d^{-1}) = \frac{\ln(C1) - \ln(C0)}{t1 - t0}$$

Where:

C0 and C1 represent initial and final absorbance, respectively; t0 and t1 represent initial and final time, respectively.

All experimental setups were carried out in duplicates.

The phytochemical screening methods used were protocols of **Trease and Evans (2002)** and **Sofowora (1993)**. Total phenols content was determined by the method of **McDonald, Prenzler and Antolovich (2001)**, DPPH radical scavenging assay was done using the protocol of **Shimada et al., (1992)**. Nitric oxide radical scavenging activity was carried out by the method of **Panda et al., (2009**).

All chemicals and reagents used for the study were of analytical grade. Also, all experiments were carried out in duplicate.

Statistic analysis

Statistical analysis was carried out using the SPSS Staistical Software. Data were presented as means of duplicates analysis and error bars calculated as standard errors of means. The One-Way Analysis of Variance (ANOVA) was used in the determination of comparison of means. Statistical significance was determined at *p*-value of 0.005.

RESULTS AND DISCUSSION

Phytochemical profile of the extracts

Ten phytochemical tests were carried out on the ethyl acetate, methanol and N-hexane extracts, only alkaloids, flavonoids, saponins, tannins, phenols, cardiac glycosides and terpenoids were detected out of the ten while anthraquinones, phlobatannins and steroids were not detected. Flavonoids, tannins and phenols were detected in all the extracts while terpenoids and saponins were only detected in the methanol extract (Table 1).

Antioxidant properties of the extracts

The total phenolics content of the extracts revealed concentrations of 6.66 ±0.45, 5.58 ±0.11 and 6.06±0.41 mg TAE.g^{-1}) for the methanol, N-hexane and ethyl acetate extracts respectively. These concentrations were calculated using the equation of calibration curve for tannic acid, y = 0.1471x, R^2 = 0.9922 and expressed as mg/g tannic acid equivalent TAE (Figure 1).

For the DPPH radical scavenging assay, gradual increase in percentage inhibition from the lowest to the highest concentration was observed across all the standard groups (rutin, vitamin E and vitamin C).

Table 1 Phytochemical profile of the extracts.

Extracts	Phytochemicals									
	Alk.	**Fla.**	**Sap.**	**Tan.**	**Phe.**	**Car.**	**Ter.**	**Anthr.**	**Phlo.**	**Ste.**
Ethyl acetate	+	+	-	+	+	-	-	-	-	-
Methanol	-	+	+	+	+	+	+	-	-	-
N-hexane	+	+	-	+	+	+	-	-	-	-

Note: Alk., Fla., Sap., Tan., Phe., Car., Ter., Anthr, Phlo and Ste. Represent Alkaloids, flavonoids, saponins, tannins, phenols, cardiac glycoside, terpenoids, anthraquinones, phlobatannins and steroids, respectively. '+' and '-'indicate detected and undetected, respectively.

A similar trend was observed with the extracts, with the ethyl acetate extract showing highest percentage inhibition amongst the other extracts (Figure 2). With respect to the reducing power ability of the extracts, while there was consistent increase with increase in concentration of the standard rutin, the extracts did not reveal that trend. Although all the extracts showed high reducing power ability, it was not dose-dependent (Figure 3).

The nitric oxide scavenging ability of the extracts showed constant increase with increase in concentration. This observation was irrespective of the extracts and standards. When compared with the standards, none of the extracts was observed to show substantial nitric oxide scavenging ability (Figure 4).

Antimicrobial potential of the extracts

The antibacterial potential of the extracts revealed the methanol extract showing inhibition against the five isolates tested. The hexane extract was not observed to inhibit growth of *Staphylococcus* and *Klebsiella pneumoniae.* Only *Staphylococcus* showed inhibition to the ethyl acetate extract (Table 2).

In presence of the ethyl acetate extract, gowth of the *E. coli* was observed to experience an extended lag period of 6 h in broth medium that contained the *Helianthus annuus* extract only. In medium that contained both the extract and ascorbic acid, the *E. coli* showed no growth throughout the period of incubation. However, in the control broth medium (contained neither extract nor ascorbic acid), and broth medium that contained ascorbic acid, *E. coli* growth was observed from 1 h incubation period.

Table 2 Zone of inhibition and minimum inhibitory concentrations of the extracts against selected bacteria species.

Test bacteria	Ethyl acetate	Hexane	Methanol
Bacillus subtilis	25 mm (1000 mg.L^{-1})	10 mm (3000 mg.L^{-1})	20 mm (3000 mg.L^{-1})
Escherichia coli	10 mm (1000 mg.L^{-1})	20 mm (2000 mg.L^{-1})	10 mm (3000 mg.L^{-1})
Pseudomonas aeruginosa	15 mm (2000 mg.L^{-1})	21 mm (2000 mg.L^{-1})	12 mm (1000 mg.L^{-1})
Staphylococcus aureus	-	-	10 mm (2000 mg.L^{-1})
Klebsiella pneumonia	10 mm (3000 mg.L^{-1})	-	12 mm (2000 mg.L^{-1})

Note: Values in mm represent zones of inhibition while values in brackets indicate minimum inhibitory concentration of the extracts. '- 'represents resistance to the extract.

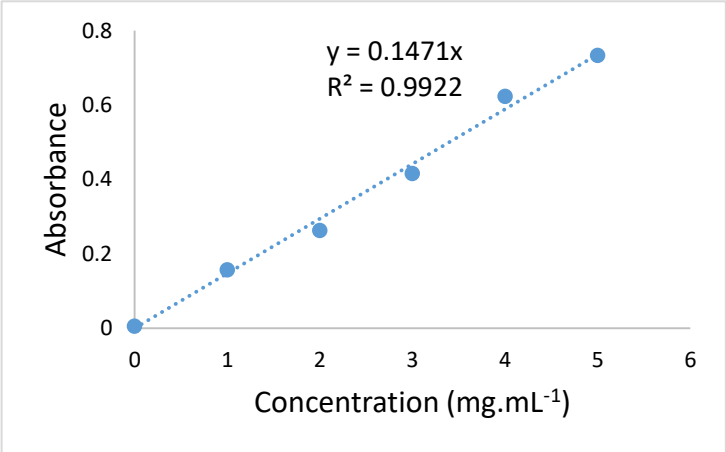

Figure 1 Calibration curve for total phenols.

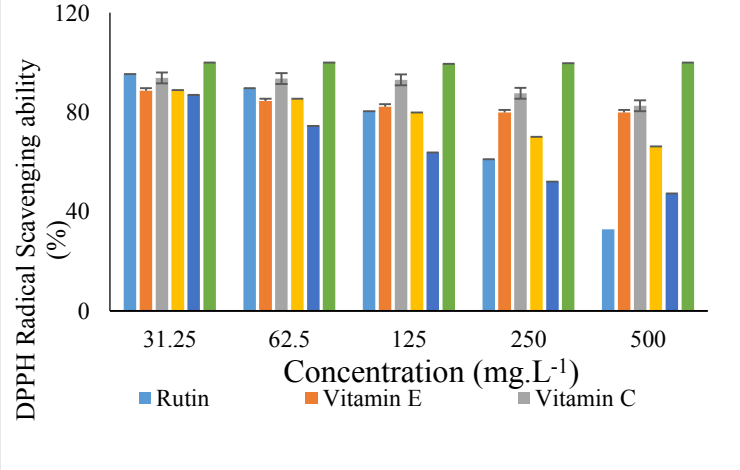

Figure 2 Free radical scavenging ability of extracts using DPPH.

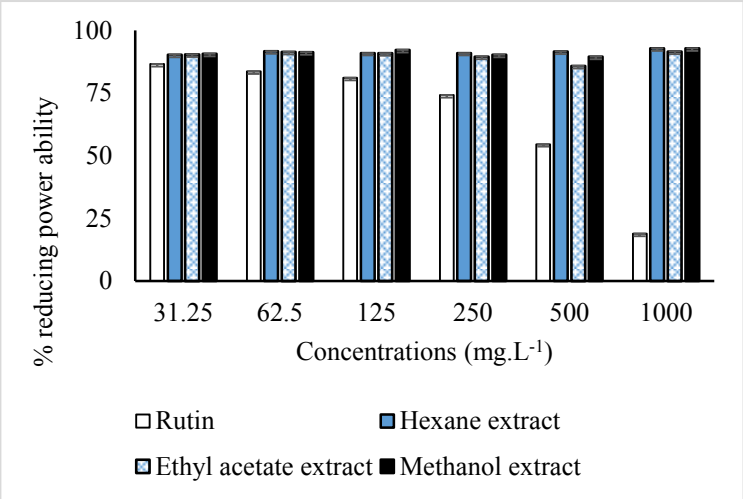

Figure 3 Reducing power assay of the extracts.

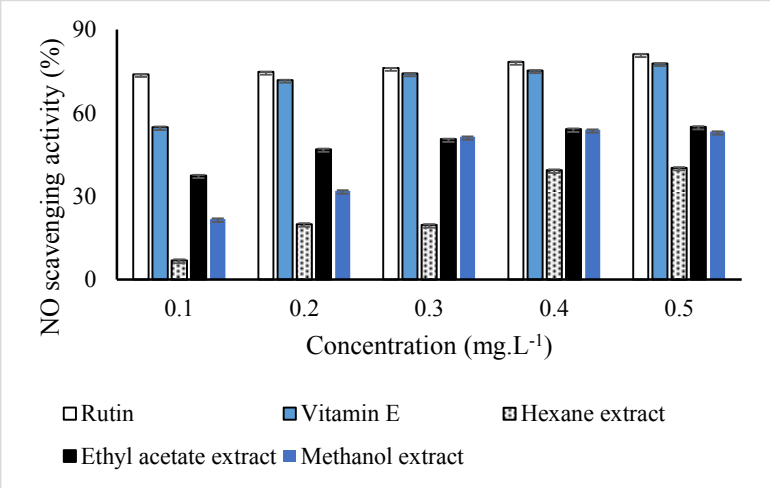

Figure 4 Nitric oxide scavenging assay of the extracts of the methanol extract.

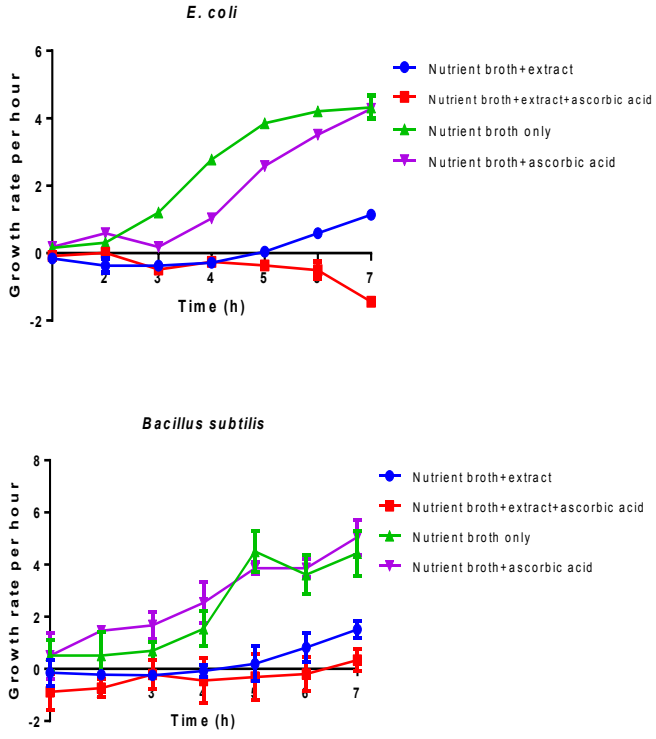

Figure 5 Growth profile of the bacteria species in presence of the ethyl acetate extract.

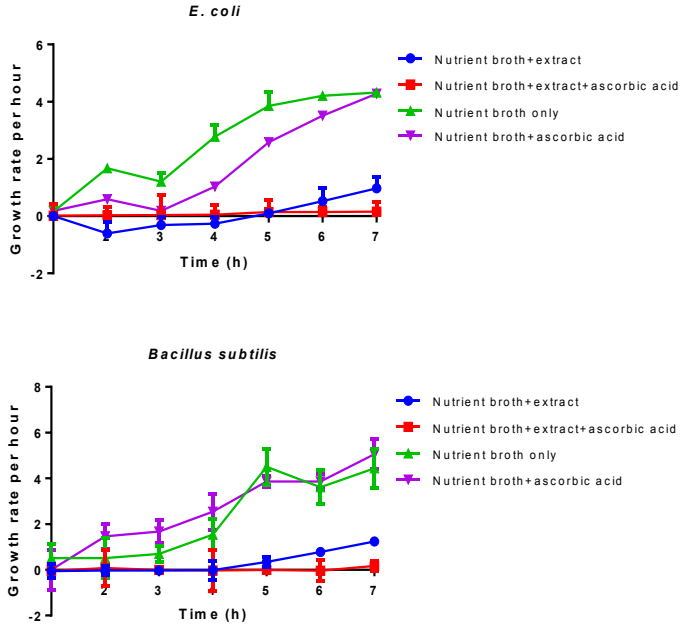

Figure 6 Growth profile of the bacteria species in presence of the hexane extract.

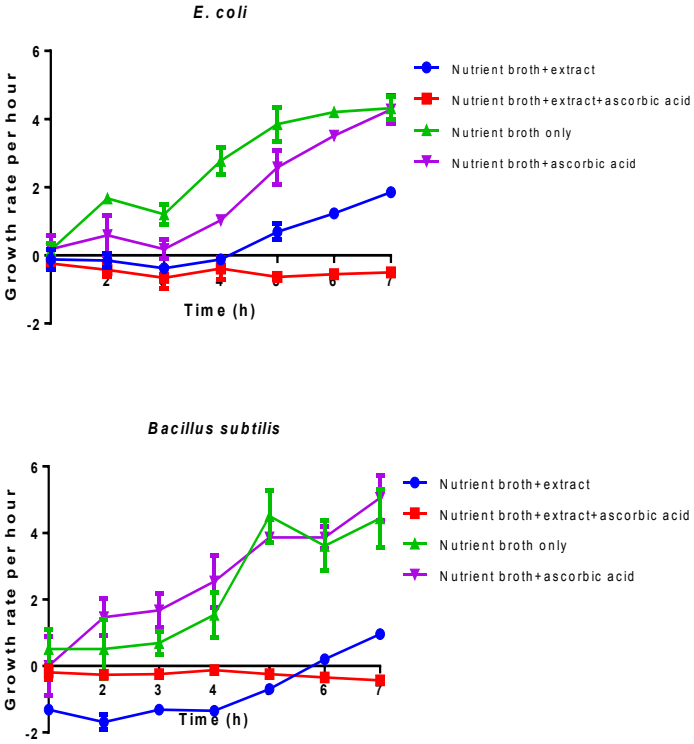

Figure 7 Growth profile of the bacteria species in presence Phytochemical components of the extracts.

Generally, growth rates of 1.14, 1.44, 4.32 and 4.27 were observed for the *E. coli* in media with nutrient broth + extract, nutrient broth + extract + ascorbic acid, nutrient broth only and nutrient broth + ascorbic acid, respectively (Figure 5). In the case of the *Bacillus subtilis,* extended lag periods of 5 and 7 h were observed in media with nutrient broth + extract and nutrient broth + extract + ascorbic acid. In media with nutrient broth only and nutrient broth + ascorbic acid, growth was observed from the first hour of incubation. At the end of 7 h incubation, growth rate of the *Bacillus subtilis* was observed to be 1.51 and 0.34 in media with nutrient broth + extract and nutrient broth + extract + ascorbic acid, respectively (Fig. 5). Generally, growth rates of bacterial species were observed to be significantly higher in the absence of the ethyl acetate extract. No significant difference in growth rate was however observed in medium that contained only the extract or a combination of the extract and ascorbic acid ($p = 0.005$).

In presence of the hexane extract, growth rate of *E. coli* was observed to be minute at the end of incubation. This observation was irrespective of growth in media with nutrient broth + extract or nutrient broth + extract +

ascorbic acid. Extended lag of 4 h was observed when grown on nutrient both + extract but no lag period was observed in the other media compositions. Growth rates of 0.97, 0.15, 4.31 and 4.28 were observed for the *E. coli* in media with nutrient broth + extract, nutrient broth + extract + ascorbic acid, nutrient broth only and nutrient broth + ascorbic acid, respectively (Figure 6).

For the *Bacillus subtilis*, extended lag periods of lag period of 4 and 6 h, were observed in nutrient broth + extract and nutrient broth + extract + ascorbic acid. Growth was however observed in media with nutrient broth only and nutrient broth + ascorbic acid from the first hour of incubation. At the expiration of the 7 h incubation period, growth rates of 1.24 and 0.16 were observed in media with nutrient broth + extract and nutrient broth + extract + ascorbic acid, respectively (Figure 6). Significantly higher growth rates were observed for the test bacterial species in medium that did not conatin the hexane extract (p= 0.05).

In presence of the methanol extract of the *Helianthus annuus*, growth rates of 0.15, 4.31 and 4.24 were observed for the *E. coli* in media with nutrient broth + extract, nutrient broth only and nutrient broth + ascorbic acid, respectively. Throughout the 7 h period of incubation, growth was not observed in media with, nutrient broth + extract + ascorbic acid (Figure 7). For the *Bacillus subtilis,* as was observed for the *E. coli,* growth was not observed in media with nutrient broth + extract + ascorbic acid was observed throughout the incubation period. Minute growth rate of 0.96 was observed at the end of incubation in media with nutrient broth + extract (Figure 7). As was observed for the ethyl acetate and hexane extracts, significantly higher growth rates of the test bacterial species were observed in medium that did not contain the methanol extract
(p = 0.05).

This study revealed antimicrobial activity of the extracts against most of the test bacteria investigated, with the methanol extract showing inhibition against all the test bacteria in solid media. When the test bacteria were grown in liquid media containing the extracts, growth inhibition and extended lag periods were observed. The presence of vitamin C in the liquid media did not impact negatively on the inhibitory properties of the extracts on the test bacteria species. Minimum inhibitory concentrations (MIC) of 1000 – 3 000 $mg.L^{-1}$ were observed for the ethyl acetate and methanol extracts while 2000 – 3000 $mg.L^{-1}$ was observed for the hexane extract. In a related study, *Helianthus annus* seed oil, ethanolic stem extract have been established to possess anti-microbial properties against different microorganisms such as *Staphylococcus aureus* and *Candida albicans*. The MIC and MBC of the ethanol stem extract against *Staphylococcus aureus* and *Candida albicans* were 70 and 90 $mg.mL^{-1}$ and C. albicans were 50 and 70 $mg.mL^{-1}$ **(Al-Snafi, 2018**). In a report by **Dwivedi (2014)**, *Helianthus annus* extract have also been reported to inhibit the growth of bacteria, such as *Salmonella typhi*, *Staphylococcus aureus*, *vibrio cholera* and *Bacillus subtilis.* With respect to antifungal action, the extract has been indicated to display high antifungal action on *Rhizopus stolonifer*, *Aspergillus fumigates* and *Candida albicans* while *Fusarium oxysporum* is indicated to be resistant **(Subashini and Rakshitha, 2012; Dwivedi 2014; Eze et al., 2015).**

In this study, flavonoids, atannins and phenols were observed to be present in all the extracts. It is indicated that plants of rich medicinal potentials usually contain polyphenols as their primary and most abundant secondary metabolite. Phenols are reported to neutralize free radicals and prevent the breakdown of hydroperoxides to free radicals thereby contributing to plants' antioxidant potential **(Adawia, 2017)**. As observed in this study, when compared with the standard rutin, all the extracts showed similar abilities to reduce oxidized intermediates of lipid peroxidation processes. Reducing power ability of a sample signifies its potential antioxidant activity. A substance with strong antioxidant capacity can act as electron donor, hence can reduce oxidized intermediates of lipid peroxidation processes **(Benslama and Harrar, 2016)**.

The findings of this study revealed total phenolics content of 5.58 – 6.66 $mgTAE.g^{-1}$ using tannic acid as standard with higher value recorded in the methanol extract. A similar report on total phenol content and antioxidant potential of green and yellow beans of *Phaseolus vulgaris* varieties showed values that ranged between 2.27 and 4.55 $mg.g^{-1}$, when catechin was used as standard **(Weidner *et al.,* 2017)**. The high phenolic content observed in the methanol extract has been reported in similar studies. In a study on the total phenolic content and antioxidant and antibacterial activities of *Pereskia bleo,* highest phenolic content was recorded in the methanol extract that chloroform and hexane extracts **(Johari and Khong, 2019)**.

With respect to nitric oxide scavenging, all the extracts displayed ability to scavenge nitric oxide radical in a dose dependent manner. However, the standard rutin used inhibited the nitric oxide radical more than the extracts even at low concentrations when compared with the extracts. A similar observation has been reported by earlier investigators **(Boora et al., 2014).** Nitric oxide radical is a free radical that is obtained from the interaction of nitric oxide NO and oxygen or reactive oxygen species Its toxicity is greatly increased on its reaction with superoxide anion radical with the resultant formation of the highly reactive peroxynitrite anion radical $ONOO^-$ **(Bhaskar and Balakrishnan, 2009; Amaeze et al., 2011)**.

DPPH radical is a stable radical that accepts electron or donate hydrogen when reacting with antioxidant compounds and reduced to yellow-colored diphenyl picrylhydrazine radical **(Alara et al., 2017)**. The results showed that the different extracts had the ability to neutralize the free radicals' unpaired electron **(Pavithra and Vadivukkarasi, 2015)**. The DPPH scavenging activities of the extracts were effective in the order: Ethylacetate > Methanol > Hexane. An increase in the percentage of DPPH inhibition caused by antioxidant might be due to the scavenging ability of radicals by hydrogen donation. It can also be seen that the ethylacetate extract was more active than that of the standards used rutin, vitamin E and Vitamin C at the same concentrations. A similar observation was reported by **(Pavithra and Vadivukkarasi, 2015)**, where they showed that ethylacetate extract of *Helianthus annuus* possesses a strong antioxidant activity.

Resistance to antibiotic has increasingly become a global concern. The emergence of multidrug-resistant pathogens has threatened the clinical efficacy of many existing antibiotics **(Westh et al., 2004**). Therefore, many studies are carried out to discover novel antimicrobial compounds and their mechanisms of action for treating new and re-emerging diseases **(Ullah et al., 2017)**. In this current study, the methanol, ethylacetate and the hexane extract showed inhibitory effect against *Eschericha coli* and *Bacillus subtilis.* This was similar to the findings of **Al-Snafi (2018)** where *Helianthus annuus* displayed strong inhibitory effects against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis* and *Candida albicans*.

CONCLUSION

This study investigated the antibacterial and antioxidant activities of ethyl acetate, hexane and methanol leave extracts of *Helianthus annus*. The findings reveal that all extracts of *Helianthus annus* used in this current study contain flavonoids, tannins and phenols, displaying their rich phytochemical constituent. These extracts also displayed inhibitory activity against most test bacteria isolates used, as well as possess strong reducing power abilities. They also exhibited good free radical scavenging abilities (using DPPH) with ethyl acetate extract being the most effective in this regard. The result from this current study therefore shows the strong medicinal properties of *Helianthus annus*, it could be a good source of antimicrobial and antioxidant especially in a world where resistance to antibiotic has increasingly become a global concern.

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THE IMPACT OF INAPPROPRIATE FOOD ADVERTISING ON CONSUMER BEHAVIOR

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ABSTRACT

The impact of food advertising on consumer behavior is a matter of concern to psychologists, marketers, economists and the general public alike. It is well known that the consumer is not rational, and all the time does not carefully evaluate all available alternatives before purchase. There exist many stimuli influencing consumer behavior, which refers to the study of buying tendencies of consumers. There are several stages a consumer goes through before he finally picks up products available in the market. Various factors, be it cultural, social, personal or psychological, influence the buying decision of individuals. Submitted paper deals with the impact of inappropriate advertising on consumer behavior, namely the purchase of food. Related research was conducted at Department of Marketing and Trade, FEM SUA in Nitra, based on a questionnaire survey with a sample of 702 respondents from the Slovak Republic. We have used two research methods: Chi Square contingency test and Kolmogorov – Smirnov test. Obtained results proved that 38% of respondents have a personal experience with inappropriate food advertising and most respondents considered inappropriate food advertising as a deceptive, misleading and manipulative one. It was confirmed that the perception of inappropriate food advertising is not dependent on the age of the respondents and respondents do not avoid buying food previously seen in inappropriate advertising. They take into consideration more important characteristics and features of the food products such as quality, price, taste etc.

Keywords: food; advertising; consumer behavior; purchase; impact

INTRODUCTION

Advertising belongs to a group of marketing communication tools used to communicate with the companies as well as target consumers. Advertising can be used as a paid impersonal ability, moving from a seemingly simple task of conveying information to a lower level, potentially seeking to attract more and more sophisticated consumers **(Mokrý et al., 2016)**.

To be effective and influence human behavior, the advertisement has to pass the process of receiving and processing **(Nagyová et al., 2014)**. This means that the viewer must perceive and understand it by using the senses. That's why the creators of advertisements use the psychological aspects of perception and psychological tricks.

Rybanská (2015) points out the importance of psychological characteristics that need to be known because they influence individual decision making. When creating an advertisement, the lifestyle of the target group, personal attitudes, habits, etc. should be taken into account **(Géci, Nagyová and Rybanská, 2017)**. The impact of psychology on consumers is individual, but there are also general patterns that are used in advertising because they affect their perception **(Nagyová et al., 2018b)**.

The psychology of advertising is perceived by **Andocsová, Géci and Kubelaková (2017)** as an industry focused on emphasizing the merits of a product or service, which seeks to create the feeling that one needs this product urgently. The psychology of advertising addresses the recipient in an increasingly sophisticated way and seeks to motivate the recipient to buy the product.

Every human being manifests some behavior and responses to stimuli from the environment **(Nagyová et al., 2019)**. The perception, movement and manifestation of people can be described by a simple term as human behavior. In situations where an object for consumption is attached in any way to this behavior, we are talking about consumer behavior **(Kozelová et al., 2011; Kozelová, et al. 2014)**. According to **Kádek (2018)**, consumer behavior is predetermined by the inclusion of a person in society and his or her behavior in general.

Koprda (2014) refers to the manipulation of consumers with the offer of companies on the market as consumer behavior. If consumers are satisfied, they can generate interest from others through positive reviews. In the case of dissatisfaction and complaints, the overall interest in the product offerings decreases.

Consumer behavior according to **Džupina, Hodinková and Kiková (2016)** and **Ubrežiová et al. (2019)** reflects the whole of consumer decisions regarding the acquisition, consumption and disposition of goods, services, activities and experiences of human decision-making units.

Nagyová et al. (2018a) sees consumer psychology as a specialized area that explores how our thoughts, beliefs, feelings and perceptions affect how we buy goods and use services.

Experts in consumer psychology, respectively in the psychology of marketing, deal with these topics:

- the way consumers choose businesses, products and services;
- thought, processes and emotions in decision making;
- how variables such as friends, family, media and culture affect the decision;
- the reasons why consumers prefer one product over another;
- personal and individual reasons.

Advertising is a means that has a significant impact on the broader society and public opinion, gets everyone's awareness, influences our moods and behaviors, so it is essential that advertising adheres to ethical principles, and at the same time it is important that its compliance is controlled **(Polakevičová, 2015)**.

Scientific hypothesis

For a deeper analysis of the research objectives, the following hypotheses were formulated:

Hypothesis 1: The sample is representative in terms of respondents´ age.

Hypothesis 2: We assume that the perception of inappropriate food advertising is age-dependent.

Hypothesis 3: We assume that there is a link between the impact of inappropriate advertising on food and its subsequent purchase.

MATERIAL AND METHODOLOGY

The main aim of the paper was to find out how advertisements promoting food products influence consumer behavior.

As it was mentioned above, for the purposes of the questionnaire survey, hypotheses were established at the outset which, using statistical methods, can be confirmed or disproved on the basis of the survey results.

In order to ascertain the attitudes and opinions of consumers, a questionnaire survey was carried out via Internet by participating of 702 respondents from the Slovak Republic (Table 1) and was conducted at Department of Marketing and Trade, FEM SUA in Nitra.

Table 1 Respondents Divided by Age.

Age of respondents	Number
Up to 25 years	296
26 – 35	194
36 – 45	92
46 – 55	72
56 and more	48
TOTAL	**702**

Note:Source: Authors, own research.

Statistic analysis

The evaluation of the questionnaire consisted of comparing the individual answers of the questions in graphical form and by using the following statistical tests **(Matejková, Pietriková and Poláková, 2014)**:

Chi-square test of good compliance

The Chi -square of the test of compliance is calculated according to formula (1):

$$\chi^2 = \sum_{i=1}^{k} \frac{(E_i - T_i)^2}{T_i} \quad (1)$$

Where:
Ei – empirical abundance,
Ti – theoretical abundance,
r – number of rows.

The critical value is expressed as: x2 (α, (n – 1). (k – 1)). If the calculated value is greater than the critical value, the null hypothesis is rejected and an alternative hypothesis is accepted that says the sample at the selected significance level is not representative.

Chi Square contingency test

The Chi -square contingency test is calculated according to the formula (2):

$$\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{s} \frac{(E_{ij} - T_{ij})^2}{T_{ij}} \quad (2)$$

Where:
Ei – empirical abundance,
Ti – theoretical abundance,
r – number of rows,
s – number of columns.

The critical value is expressed as: x2 (α, (n – 1). (k – 1)). If the calculated value is greater than the critical value, the null hypothesis is rejected and an alternative hypothesis is accepted that says there is a dependency between the nominal data examined at the selected significance level.

Kolmogorov – Smirnov test

It is used when it is necessary to compare consumer preferences with theoretical or hypothetical preferences. It is used for nominal data for one sample.

The null hypothesis, H0, argues that there is no difference between consumer preferences and theoretical preferences.

An alternative hypothesis, H1 argues that there is a difference between consumer preferences and theoretical preferences.

The test characteristic is calculated using the formula (3):

$$D_{cal.} = \max [\text{abs} (F_i - G_i)] \quad (3)$$

Where:
Dcal. – calculated test characteristic,
Fi – cumulative relative abundance,
Gi – cumulative theoretical abundance.

The tabular value is calculated using the formula (4):

$$\frac{1,36}{\sqrt{n}} \text{ for } \alpha = 0.05 \quad (4)$$

Where:
n – number of respondents,
α – significance level.

RESULTS AND DISCUSSION

Consumer behavior is one of the constantly evolving and changing elements of today's world **(Carlucci et al., 2015)**. To understand it, it is necessary to examine the complex interaction of many influencing elements **(Moutinho, 1987)**, e.g. the impact of advertising as it was examined e.g. by **Story and French (2004)**, **Lobstein and Dibb (2005)**, **Chou et al. (2008)**, **Harris et al. (2009)**, **Kelly et al. (2010)**, **Andreyeva et al. (2011)**, **Vukmirovic (2015)** etc. who´s attention was mainly focused on children and the impact of the advertising on them.

Braun et al. (2002), **Hupp et al. (2008)**, **Lichtlé et al. (2014)**, **Šugrová et al. (2017)**, **Nagyová et al. (2018a)** and **Ubrežiová et al. (2019)** proved that advertising can lead to strong emotional arousal, marketers can use this method to engage with consumers regardless of the product they are promoting.

Another studies by different authors **(Horská and Orémus, 2008; Rybanská, 2015; Berčík et al. 2016; Andocsová, Géci and Kubelaková, 2017)** pointed out that, emotional branding strategy is a common advertising technique that many popular company's use to engage with consumers on a more personal level.

Respondents involved in a survey had been asked to answer questions related to their attitude to inappropriate food advertising and personal experience with such kind of advertising influencing their purchasing behavior. As seen in Table 1, we have analysed answers by total 702 respondents from the Slovak Republic (41% of respondnets were up to 25 years, 28% of respondnets between 26 and 35 years, 13% between 36 and 45 years, 10% between 46 and 55 years and 8% were 56 years and older).

In your opinion, what food advertising is inappropriate?

In the aswers on the above-mentioned question were listed several characteristics of the ad, that may be a reason for the inappropriate nature, while respondents were invited to indicate more options.

Under term "Inappropriate food advertising", most consumers imagine advertising that is deceptive (98%) and misleading (78%). The respondents made their decision mainly based on their own emotions (Figure 1).

Besides that, the current situation on the food market is influenced by various diet trends including eating healthy products **(Guziy, Šedík and Horská, 2017)**.

Do you have personal experience with an inappropriate food advertising?

Respondents consider as inappropriate or unethical advertisements presenting yogurts with plenty of fruit, while the reality is different. Also unethical are

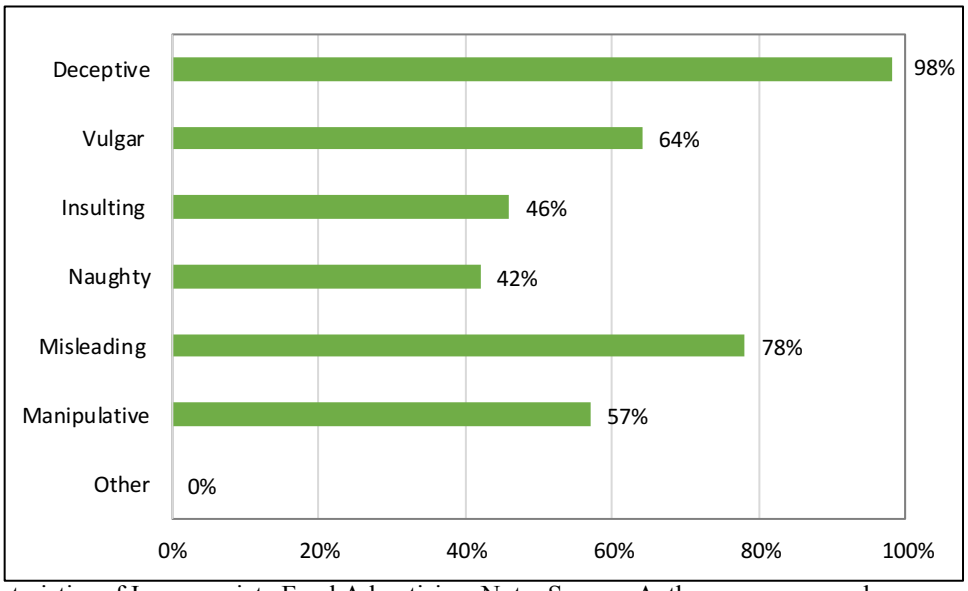

Figure 1 Characteristics of Inappropriate Food Advertising. Note: Source: Authors, own research.

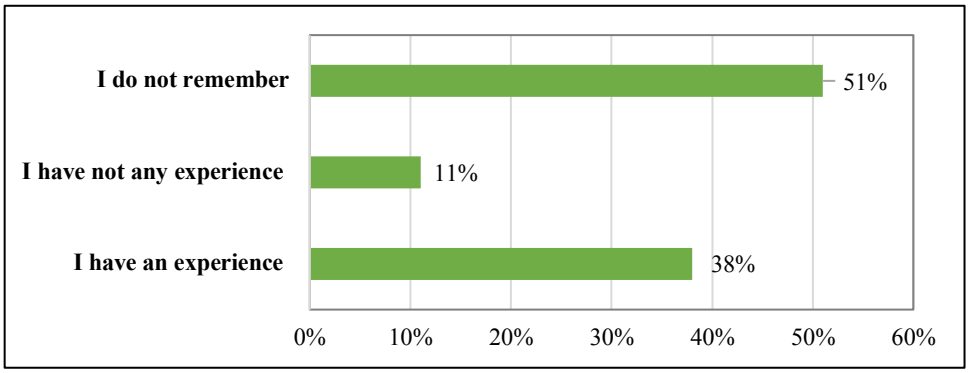

Figure 2 Personal Experience with an Inappropriate Food Advertising. Note: Source: Authors, own research.

Table 2 Results of Chi-square, Chi-square Contingency Test and Kolmogorov-Smirnov Test.

Chi-square Test			Chi-square Contingency Test			Kolmogorov-Smirnov test		
TCH		**TH**	**TCH**		**TH**	**TCH**		**TH**
0.81	<	9.47	15.507	>	0.49	0.11	>	0.10

Note: Source: Authors, own research.

Table 3 Results of Kolmogorov – Smirnov test.

Attitude	Absolute abundance	Relative abundance	Cumulative relative abundance	Theoretical abundance	Cumulative theoretical abundance	abs (F_i-G_i)
positive	148.00	0.21	0.22	0.33	0.33	**0.11**
negative	253.00	0.36	0.57	0.34	0.67	0.10
neutral	301.00	0.43	1.00	0.33	1.00	0.00
Total	702.00	1.00				

Note: Source: Authors, own research.

advertisements for nutritional supplements, e.g. for building the muscles without working out. Consumers also feel deceived when presenting healthy sticks and also butter, although the reality is different. 38% of respondents have the personal experience with an inappropriate advertising, while only 11% of respondents have not encountered such advertising. Up to 51% of respondents admit personal experience with the inappropriate food advertising, but they exactly do not remember (Figure 2). Anyway, it has to be stated that advertising has a strong influence and impact on consumer behavior even in the 21.st century **(Vivek, Beatty and Morgan, 2014; Nagyová et al., 2014)**.

Statistical hypothesis testing

Hypothesis 1

H0: The sample is representative in terms of respondents´ age.

H1: The sample is not representative in terms of respondents´ age.

Based on value 0.81 <9.47, we accept the hypothesis H0. With 95% probability can be said, that the sample is representative in terms of respondent age (Table 2).

Hypothesis 2

H0: We assume that the perception of inappropriate food advertising is age-dependent.

H1: We assume that the perception of inappropriate food advertising is not age-dependent.

Based on a value 15.507 >0.49 can be concluded that we reject the hypothesis H0 and accept the hypothesis H1. With 95% probability can be said that the perception of inappropriate food advertising is not dependent on the respondent's age (Table 2).

According the research by **APA (2019)**, there is proven association between advertising and age of respondents but mainly in most children under age 6, who cannot distinguish between programming and advertising and children under age 8 who do not understand the persuasive intent of advertising. Advertising directed at children this young is by its very nature exploitative. Children have a remarkable ability to recall content from the ads to which they have been exposed. Product preferences affect children's product purchase requests and these requests influence parents' purchasing decisions.

Hypothesis 3

H0: We assume that there is a link between the impact of inappropriate food advertising and its subsequent purchase.

H1: We assume that there is not a link between the impact of inappropriate food advertising and its subsequent purchase.

On the basis of the value 0.11 >0.10 we reject the hypothesis H0 and accept the hypothesis H1, which means that the relationship of the influence of inappropriate advertising on food on their subsequent purchase was not statistically proven (Table 3 and Table 2). Research by **Nagyová et al. (2014)**, **Rybanská (2015)** and **Šugrová et al. (2017)** also proved, that the advertising has only lower impact on consumer behavior, as the most important factors in condition of the Slovak Republic, influencing the consumers buying behavior are price, quality, taste, reliable references etc.

CONCLUSION

Our research was aimed at the impact of inappropriate advertising on consumer behavior when purchasing the food previously seen in advertisement. Results of the research conducted at FEM SUA in Nitra are on the basis of a questionnaire survey with a sample of 702 respondents from the Slovak Republic. It has been proven that 38% of respondents have a personal experience with inappropriate food advertising and most respondents considered inappropriate food advertising as a deceptive, misleading and manipulative one. We have tested three hypothesis:

H1: The sample is representative in terms of respondents´ age.

H2: We assume that the perception of inappropriate food advertising is age-dependent.

H3: We assume that there is a link between the impact of inappropriate advertising on food and its subsequent purchase.

We have obtained following results:

The research sample is representative in terms of respondent age (Hypothesis 1 was accepted).

Perception of inappropriate food advertising is not dependent on the respondent's age (Hypothesis 2 was rejected).

And the relationship of the influence of inappropriate advertising on food on their subsequent purchase was not statistically proven (Hypothesis 3 was rejected).

On the basis of above stated results can be concluded that respondents are not influenced by inappropriate food advertising when buying food previously seen in advertisement. They take into consideration more important characteristics and features of the food products such as quality, price, taste, reliable references etc.

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INVESTORS´ DECISION ON THE CONTEXT OF THE EFFECTIVE TAXATION OF AGRICULTURAL COMPANIES

Alena Andrejovská, Veronika Konečná

ABSTRACT

The analysis of the effective taxation combines two different effective tax rates which are crucial for placement and monitoring of the investment amount in the particular country. Both of these tax rates are important for investors who make a decision on the benefits, as well as the risks of corporate taxation in the country. The contribution deals with the problem of the effective taxation through effective average tax rates (EATR) and effective marginal tax rates (EMTR). Especially, it focuses on agricultural production companies. The effectivity of taxation was observed for selected intangible and tangible assets for a period of 2004 and 2018. Our analysis evaluated the influence of the change in the statutory tax rates (and the other taxes and indicators, as well) on the change in effective average tax rates on capital in the agricultural companies. Based on the results, the lowest EATR, ranging from 20.79% to 25.25%, reported agricultural lands in both reference periods and for both ways of financing. Analyzing EMTR we found out that the lowest value reported investments in intangible assets that have crucial significance for investors. Our results definitely made it clear that in the EATR ↔ EMTR relationship, a form of financing investments is decisive. This relationship is used when an investor decides between several mutually exclusive locations or types of investment in a given country. In equity financing, the most effective capital is investing in intangible assets, and when we consider financing from external sources it is investment into stocks. An increase in the statutory tax rate of 2% resulted in a 12% increase in effective average tax rates.

Keywords: tax; effectiveness; company; asset; agriculture

INTRODUCTION

The structure of tax systems is one of the factors that significantly affect the economic growth of countries. For this reason, it is important to look at individual taxes not only as a possible source of budget revenue, but also in terms of their impact on economic growth. As the structure of tax systems is diverse, it is appropriate to focus in particular on monitoring effective tax rates, which closely examine the tax bases and provide sufficient information not only for investors, but also for governments who create tax legislation and modify the structure of tax systems. In decision-making process, effective tax rates does not only serve investors, but also other entities such as politicians and economists who seek to create favorable conditions for foreign capital inflow into the economy. It is particularly effective tax rate that can increase the attractiveness of the country. **Castro and Ramírez Camarillo (2014)** and **Martin-Mayorales and Uribe (2010)** confirm that tax rates that aggregate the economic aspect which expresses the real rate of capital taxation is effective. Similarly, **Baker (1999)** and **Barrios, Nicodème and Sanchez Fuentes (2014)** claim that effective tax rates have a higher informative value than the statutory tax rates which are legally given. On the other hand, **De Laet and Wöhlbier (2008)** have evaluated that using the statutory tax rate to measure and compare the tax burden across countries is inaccurate and misleading. Since the first studies focusing on effective tax rates by **Jorgenson (1963)**, **Hall and Jorgenson (1967)**, **Mura et al. (2017)** was found, economists have become more interested in analyzing the impact of corporate income taxation on cost of capital. This approach is based on detailed data on the taxation of capital investments, taking into account the marginal revenue on the last unit invested of new investment projects at the same level as the project's marginal costs, including future taxation. **Jorgenson and Yun (1991)**, **Jorgenson and Gollop (1992)**, **Auerbach (1979)**, **King and Fullerton (1985)** in the empirical research have broadened the founding studies and in their analysis included other corporate and non-corporate tax rates, as well as source of financing and assets. This approach has led to the development of those indicators which define the placement and scale of investments.

The first indicator that has decisive role for location of the investment is effective average tax rate (EATR). The second one is an effective marginal tax rate (EMTR) which aims to capture the extent of the investment used in a given country. The effective corporate tax rates are used as a measurement of impulses, and they are obtained from various sources of corporate tax systems on a regular basis,

segmented by industry **(ZEW, 2018)**. A huge amount of empirical studies such as **McKenzie, Mintz and Scharf (1997)**, **Barrios, Nicodème and Sanchez Fuentes (2014)**, **Devereux and Griffith (1998)**, **(Devereux and Griffith, 2003)**, **Kubátová and Říhová (2009)**, **Devereux, Griffith and Klemm (2004)**, **Ključnikov, Mura and Sklenár (2019)** deal with the impact of effective corporate tax rates on the economic behaviour of enterprises, including their placement, choice of investment opportunity and profit spillover.

In the taxation of the agricultural sector, it is necessary to observe differences which are specific for this economic sector, mainly because of the use of the various elements of the tax base. **Felis (2015)**, **Darabos (2016)**, **Okanazu (2018)** considers that the effectiveness of agricultural tax also depends on the specific tax rate, which is set as the cash equivalent of the crop. The level of agricultural tax is a result of the change in specific tax rates, depending on the purchase price of the crop. However, this price was a subject to frequent fluctuations, leading to substantial changes in the level of the tax burden.

Severini, Tantari and Rocchi (2014) analyzed taxation of agricultural households and stated that the tax burden is not influenced by the level of actually produced income. Ironically, the taxation of agricultural income has a regressive effect, favoring farmer families where agriculture represents a large proportion of family income. In analyzed farmer families is relative average level of taxation (i.e. rate of corporate income tax to Gross Farm Income – GFI) approximately 13.3%. Therefore, agricultural incomes may be less tax burdened than non-agricultural incomes. The impact on agricultural investment, management and production decisions are often influenced by federal tax laws. Farmers benefit from a federal policy on income tax and real estate tax on agriculture. These provisions put pressure on the value of agricultural land and help to support an increasing number of very small and large farms. Tax credits for investments were used in large quantities, however the majority of these credits were eligible and included assets, such as machinery, equipment, livestock purchased for dairies, draught, breeding or sports animals, crop storage facilities and dedicated agricultural structures. The combined effect of tax credit on investment and Accelerated Cost Recovery System (ACRS) has led to negative tax rates in most agricultural machinery and equipment. Negative effective tax rates was an impulse for an increase of investment into agricultural capital and they occur for tax credits and deductions. However, the availability of investment with a negative effective tax rates is limited. As **Durst and Monke (2001)** state, negative effective tax rates can offset all income from a given investment along with additional income from other sources.

Scientific hypothesis

For a potential investor, the amount of the EATR is important to know in which country to place the investment. EMTR says about how much the investment should be. Basic hypothesis is: Is it more important for the investor to monitor EATR and EMTR or their relationship?

MATERIAL AND METHODOLOGY

The aim of this contribution is to analyze and evaluate the efficiency of taxation of selected types of intangible and tangible assets of agricultural companies on the basis of accounting and tax legislation in the Slovak Republic (SR). In the paper, we construct the EATR model and we take into account a period of the year 2004 (when the Slovak Republic became a Member State of the EU) and the year 2018.

In our model, the assets were classified into seven categories of intangible and tangible assets (i.e. intangible assets, agricultural buildings, machinery for agriculture and forestry, basic herd and the draught animals, permanent crops, land, and inventories). The basic herd and the machines have the identical results because there is the same classification in the depreciation, and the taxation is the same, as well. The design of the EATR model takes into account the discounted value of multiplying of the variability of tax discrimination, and the difference between the revenues and the costs of the investment project. The revenues were taxed at the required rate of return and accounting depreciations without the impact of inflation. The costs reflected the shareholder's discount rate, accounting depreciation and inflation, and they are expressed through the formula (1 – NPV tax depreciation shield), which expresses the tax savings from the depreciations.

The capital funding sources were divided into three groups, weighted by the OECD weights **(OECD, 1991)**, and processed according to the OECD long-term statistics averages as following:

1. undistributed profit (55%);
2. new deposit (10%);
3. debt (35%).

Table 1 below shows input data used in our analysis. The volume of corporate tax and the revenues from the interest deduction, that highlight the differences between the different ways of funding, are positively correlated.

Table 1 Input data for analysis.

Asset	Accounting depreciation ZEW (δ)	Recalculated life	Tax depreciation ($\emptyset$)
I. Intangible assets	15.35% = 0.1535	5 years	100/5 = 20%
II. Agricultural buildings	3.1% = 0.031	20 years	100/20 = 5%
III. Agricultural and forestry machinery	17.5% = 0.175	4 years	100/6 = 25%
IV. Basic herd and draught animals	17.5% = 0.175	4 years	100/6 = 25%
V. Growing units of permanent crops	4.5% = 0.045	12 years	100/12 = 8.33%
VI. Estates	x	x	x
VII. Inventory	x	x	x

Note: Source: own processing based on **ZEW (2018)**.

In the model, we also consider some additional input data, such as:

(r): Real rate of return determined as 5% of the alternative investment;

(p): Required rate of return before tax determined at the 20% level;

(π): Inflation rate (at the level of 2%);

(δ): Accounting depreciation rate determined by **ZEW (2018)**;

(τ): Effective statutory tax rate (22%);

(e): Effective real estate tax rate determined from the statutory real estate rate (n) 0.25%, reduced by the corporate tax rate (21%). Since the **ZEW** model **(2018)** considers a market value that does not share in all countries with a purchase price, it determines a uniform and optimal basis to capture the market value of 0.36%.

(v): Valuation of inventory loss which may use three methods:

- *FIFO method*: this method is used for valuation of inventory loss when the first inventory increase valuation price is used as the first price for inventory loss valuation (v = 1).
- *LIFO method*: is used for inventory valuation when the last inventory increase valuation price is used as first to evaluate the inventory loss. In the Slovak Republic (SR) this method is not allowed (v = 0). The weighted arithmetic mean is determined from actual purchase prices as the share of inventory in stock value, and the total inventory in stock state in the quantitative units, at least once per month (v = 0.5).
- *Predetermined Inventory Price*: this is the price for fast-moving inventories (mostly used in agriculture), in case of which we often do not yet know their price at the time of placing in storage (v = 2).

($\emptyset$): Tax depreciation for tangible assets. It will be used in a straightforward or accelerated manner in accordance with the **Law no. 595/2003**. Intangible assets are depreciated in accordance with this Act for a maximum of 5 years up to their acquisition price.

(i): Nominal interest rate that would increase with the increase of inflation rate and an increase in the real interest rate.

(ρ): Shareholder's discount rate.

(γ): Variability of the shareholder's tax discrimination, which reflects the ratio of the funds from the investment to the alternative investment funds. If we eliminate the personal income tax at this value, then a value of 1 is set, as the shareholder will not be discriminated against when deciding for the investment, but for the possibility of depositing of his funds in the bank.

Calculation methods that monitor the effective tax burden on hypothetical investment projects aim to estimate future burdens based on the existing legal framework. A marginal investment is expressed as an investment which the present value of the pre-tax return is zero. It means that the net present value of the investment income is equal to the present value of the investment cost **(Vítek, 2011)**. According to **ZEW (2018)**, the effective marginal tax rate, that expresses tax burden, is defined as a proportion of a difference of marginal investment pre-tax return $p^{\sim}$ and investment return rate after taxation s to marginal investment return $p^{\sim}$. In other words, we can describe it in the following form:

$$EMTR = \frac{p^{\sim} - s}{p^{\sim}} \qquad (1)$$

The $p^{\sim}$ value represents the real return rate before taxation that is necessary to achieve a nul economic income after taxation (capital cost is the initial investment). From a shareholder´s point of view, the s value represents the real rate of return after taxation. Based on the facts stated above, the effective marginal tax rate includes a wide range of indicators, which go beyond the statutory corporate tax rate, such as the elements of tax base, the method of financing the investment (i.e. through debt, undistributed accounting profit, or new capital funds) and the depreciation rules or level of inflation rates. However, when we speculate about taxation, the return on investment changes. The optimal return on investment requires the same return on different investments at the given margin. The $p^{\sim}$ value called also capital costs is necessary calculate for each investment type depending on a form and investment funding source.

The intangible assets, agricultural buildings, agricultural and forestry machinery, basic herd and draught animals, growing units of permanent crops, because it is depreciated assets, there have to be included depreciation rate in the formula. Also, we have to add to formula coefficient e, which expresses a property tax and which will increase the amount of investment in buildings. The property tax on buildings is calculated as following: *Tax rate + (number of floors * charge for floor) * building area*

Since it is a direct cost, the formula is in the following form:

$$\tilde{p}_{1-5} = \frac{(1-A)}{(1-\pi)*(1-\tau)}\{\rho + \delta * (1+\pi) - \pi\} - \delta * e \qquad (2)$$

The next tangible assets in our model is estate, which belongs to non-depreciated assets. When we adjust the formula (2) to reduce tax depreciation, where $\delta = 0$, and add the property tax on estates e (calculating as *tax rate* * *estate area* * *estate value*), we can write the formula as:

$$\tilde{p}_6 = \frac{1}{(1-\pi)*(1-\tau)}\{\rho - \pi + 1 * \tau * \pi * e\} \quad (3)$$

For inventories, *e*: the property tax is excluded from the equation (2), and the whole formula is reduced by the multiplication of the tax rate, the inflation rate, and the inventory valuation method. If the company decides for the *LIFO* method (the cost also includes the increase in the price level) we will insert 0 instead of $v$and so it will reset the whole expression. In case of the *FIFO* method, $v = 1$, while in the method of the weighted arithmetic mean $v = 0.5$. Last, in case of the method of predetermined inventory price, we will use the $v = 2$ (which we have set as the basis, since it represents the agricultural fast-moving inventories).

$$\tilde{p}_7 = \frac{1}{(1-\pi)*(1-\tau)}\{\rho - \pi + 1 * \tau * \pi * v\} \quad (4)$$

It is also important to deal with the investment financing sources. In practice, there are three basic ways to finance investment: financing from own sources, i.e. through undistributed profit or new shareholders´ contributions, and from external sources such as debt financing. In a case, there is no personal income taxation $\gamma = 1$, then debt financing will always be nul and capital costs for investments financed by new contributions and investments financed by undistributed profit will be equal. However, in the case of debt financing, companies optimize capital structure in order to have costs as low as possible. Corporate tax is the cost of equity financing and often exceeds equity cost in the form of tax-deductible interest, and thus causes tax shield (i.e. reduction of the tax base).

If we choose debt financing, the formula for individual asset types need to be added as follows:

(i) Intangible assets, agricultural and forestry machinery, basic herd and draught animals, growing units of permanent crops, estates and inventories:

$$\tilde{p}^{DE} = -\frac{(\rho - i(1-\tau))}{(1+\pi)(1-\tau)} \quad (5)$$

(ii) Agricultural buildings: firstly, it is necessary to adjust the formula (5) and add expression $(1 + e)$, where e expresses the property tax:

$$\tilde{p}^{DE} = -\frac{(1+e)(\rho - i(1-\tau))}{(1+\pi)(1-\tau)} \quad (6)$$

If we choose financing from own sources through new shareholders´ contribution or from undistributed profit, we will use the following formula:

$$\tilde{p}^{NE} = \frac{\rho(1-\gamma)(1+e)}{\gamma(1+\pi)(1-\tau)} \quad (7)$$

;As we can see, the effective marginal tax rate includes a wide range of elements. If we compare the impact of taxation on expected return on investments while the investment would be realized in various countries, then we can conclude that those countries which have higher capital costs or higher level of EMTR are less attractive for investors.

The second indicator used to assess the attractiveness of the location or economic industry and simultaneously calculates rates for hypothetical investment projects, is the effective average tax rate (EATR). The EATR is defined by the ratio of the present value of taxes paid to the net present value of income flows. However, we have to emphasize that EATR does not include the initial cost of investment. Usually, the procedure for setting EATR is in proportionate reduction of the economic income generated by the investment as a result of taxation. In other words, it has the following form:

$$EATR = (R^* - R)/R^* \quad (8)$$

The main drawback of this method is that it does not determine investment projects without taxation $R^* = 0$. Therefore, **Devereux a Griffith (1998)** suggested an approach, which calculates the difference between R^*, and they define coefficient R as a proportion of the net present value of return on investment before taxation $p/(1 + r)$. Simultaneously, there is also included the influence of personal effective marginal tax rates from capital revenues (defined by King and Fullerton) **(King and Fullerton, 1985)**. This variable has the following formula:

$$EATR = \frac{\frac{R^* - R}{p}}{\frac{}{(1+r)^t}}, \quad (9)$$

where R* is the economic income flows from the project without tax and expresses the difference between the required rate of return before tax and the real interest rate from the next investment. To determine the present value of the project's profit, it is necessary to discount it with the real interest rate:

$$R^* = \frac{p-r}{1+r} \quad (10)$$

The evaluation tracks the different assets and the equation is adjusted (i.e. reduced or increased) by the individual indicators. Intangible assets, machinery for agriculture and forestry, basic herd, and the draught animals were calculated using the equation in the basic form:

$$R_{1,3,4} = \frac{\gamma}{1+\rho} * \{[(p + \delta) * (1 + \pi) * (1 - \tau)] - [\rho + \delta * (1 + \pi) - \pi] * (1 - A)\} \quad (11)$$

For agricultural buildings and permanent crops, equation (10) was reduced by e − the property tax (on buildings and on land), and this tax is the direct cost to this type of asset:

$$R_{2,5} = \frac{\gamma}{1+\rho} * \{[(p + \delta) * (1 + \pi) * (1 - \tau)] - [\rho + \delta * (1 + \pi) - \pi] * (1 - A)\} - e \quad (12)$$

In case of the land, accounting and tax depreciations are excluded from equation (5), i.e. δ = 0, (1 - A) = 0 (the land constitutes a specific group of undepreciated assets):

$$R_6 = \frac{\gamma}{1+\rho} * \{[p * (1+\pi) * (1-\tau)] - [\rho - \pi]\} - e \quad (13)$$

For inventories, the property tax e is excluded from the equation (12), and the whole formula is reduced by the multiplication of the tax rate, the inflation rate, and the inventory valuation method. If the company decides for the *LIFO* method, we will insert 0 instead of v, that will reset the whole expression). In case of the *FIFO* method v = 1, while in the method of the weighted arithmetic mean v = 0.5, and in case of the method of predetermined inventory price we will use the v = 2 (which we have set as the basis, since it represents the agricultural fast-moving inventories).

The equation has the form:

$$R_6 = \frac{\gamma}{1+\rho} * \{[p * (1+\pi) * (1-\tau)] - [\rho - \pi]\} - v * \tau * \pi \quad (14)$$

The investment was financed from own funds (i.e. through undistributed profit and the new contributions) and from the external resources (i.e. debt financing). In the absence of personal taxes, γ = 1, the last indicator will always be zero, and capital costs for investments funded by new capital and investments funded by undistributed profit will be equal. The difference is only in financing in the form of debt. To keep costs as low as possible, the companies optimize their capital structure. Corporate tax is the cost of equity financing, and often this cost is higher than other costs, such as this in form of interests is a tax-deductible item, what cause a reduction in the tax base, so called interest tax shield. Therefore, the economic rent of the project with taxation should be increased by the ratio of the discounted value of the difference between the discount rate of the shareholder and the nominal interest rate, and by the interest tax shield. It is necessary not to forget the effective rate of property tax paid in the period of direct investment activity $(1 + e)$. Formula for debt financing has the form:

$$F^{DE} = \frac{\gamma * (1+e) * (\rho - i + i * \tau)}{1+\rho} \quad (15)$$

Formula for financing through a new capital contributions has the form:

$$F^{NE} = -\frac{\rho(1-\gamma)(1+e)}{1+\rho} \quad (16)$$

When we adjust formulas above, we can write a relationship between EATR ↔ EMTR. This correlation is used to investment decision making and it assesses location as well as the amount of the investment. EATR expresses the proportion of effective average level of taxation to level of investment rentability. It also reflects the real cash-flows and tax burden. However, the more appropriate indicator for investment decision making is EMTR because it better explains savings impulses and investing. The relationship between marginal and average effective tax rate can be then written as follows:

$$EATR = \frac{\tilde{p}}{p} EMTR + \frac{p - \tilde{p}}{p} \tau, \quad (17)$$

where τrepresents statutory corporate tax rate.

The result of investment selecting depends on tax rate of marginal investment, which expresses effective average tax rate. As **Kubátová (2011)** states, EMTR and EATR are tax wedge, expressing return rate of investment before and after taxation. Both tax rates are used to evaluate the impact of taxation on investment decision making.

Statistic analysis

For the calculation of EATR, EMTR and their relationship for agricultural companies in the Slovak Republic we use the method from **Devereux and Griffith (2003)**. Calculations were based on this methodology and modified for the conditions of Slovakia. The calculation is extensive and the individual sub-calculations were given in the previous paragraph.

RESULTS AND DISCUSSION

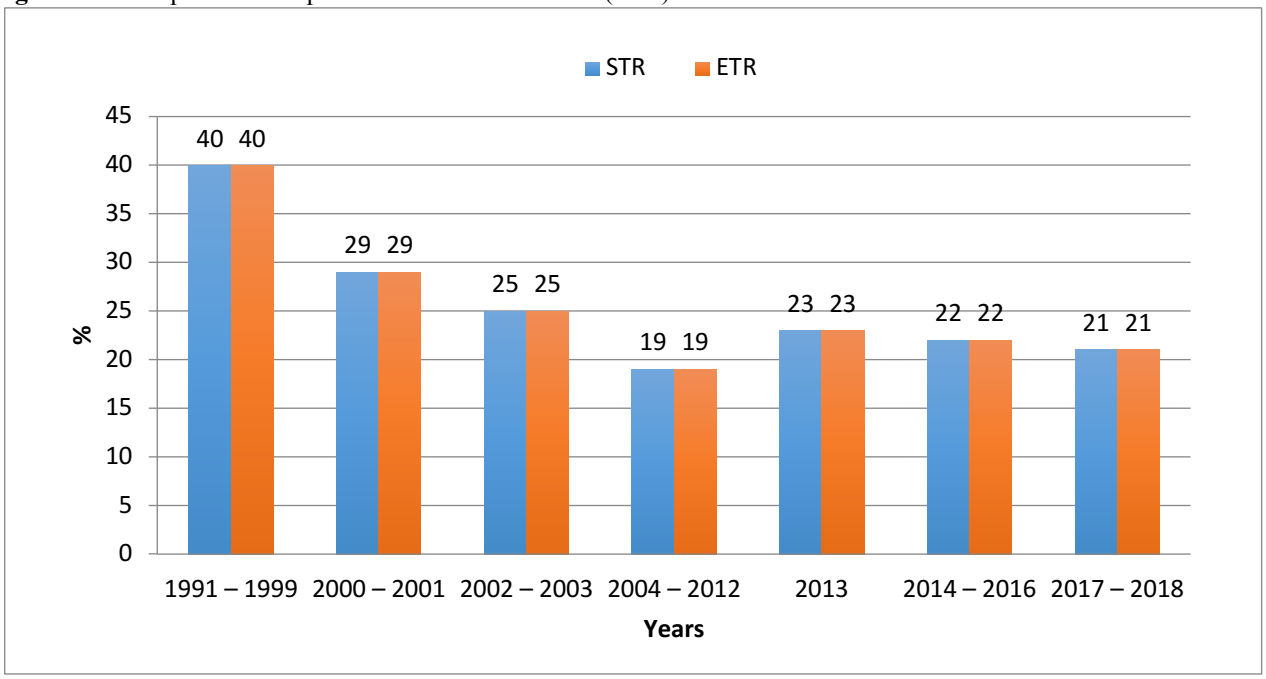
In our analysis, EATR for agricultural companies was monitored within the years 2004 and 2018. We selected these two years because in 2004 Slovakia became a Member State of the European Union, and in that time EATR was at the level of 19%. Since this period, the statutory rate has increased up to the current 22% (which we used in our analysis). Note that 23% tax rate in 2013 represents the only change since 2004. Figure 1 shows the development of corporate tax rate since 1991, which is connected to the formation of the Slovak Republic.

To determine the tax base, it is necessary to estimate tax depreciation. In 2015, there was a significant change in Tax law, which increased depreciation groups from 4 to 6 and extended the depreciation period for individual groups. Table 2 shows depreciation groups in detail.

For the straight-line method of depreciation, the share of the entry price and depreciation period was used. This method takes only a fraction of the annual depreciation, depending on the number of months since the property was put into use. In the last year, the remaining months of the year are counted. The tax and accounting depreciation rates for the monitored assets are mentioned in the methodology of the work. The property tax (on land and buildings) is a local tax and is imposed by a city or municipality.

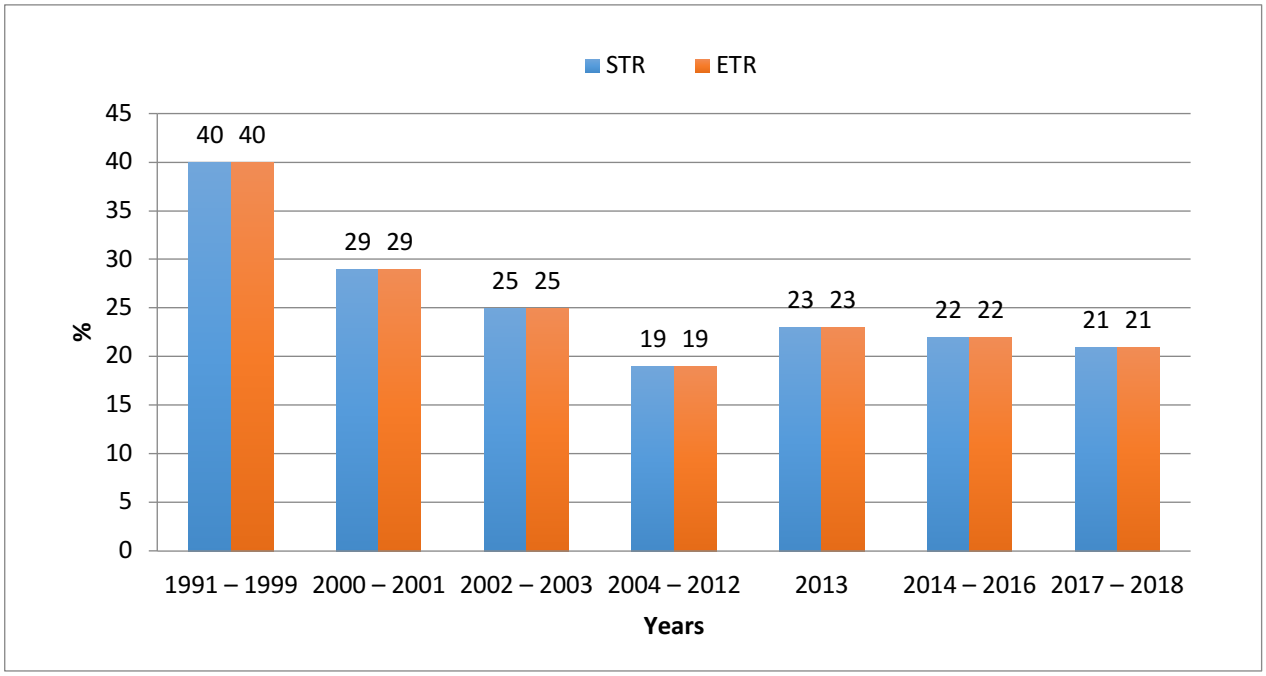
The property tax on land was determined by multiplying the land area in m^2 and the corresponding value per 1 m^2. The property tax on buildings was determined by the area of the built-up area in m^2 and the tax rate determined in the generally binding regulations. **ZEW (2018)** calculates the tax on invested capital in buildings (real estate) by an indirect method. Figure 2 shows a four-fold increase in the level of the nominal property tax base since 2005. In the effective property tax, the amount has been distributed with the direct correlation since 1991, when it increased by 0.01% up to the year 2005. After this period, there was also a single four-fold increase.

Figure 1 Development of corporate tax rates in Slovakia (in %).



Note: Source: own processing according to **(ZEW 2018)**.

Figure 2 Development of property tax rates in Slovakia (in %).



Note: Source: own processing based on **ZEW (2018)**.

Table 2 Depreciation period for tangible assets.

Group	Years	Assets
1.	4	agricultural and forestry machinery, basic herd and draught animals
2.	6	-
3.	8	-
4.	12	basic herd and draught animals
5.	20	agricultural buildings
6.	40	-

Note: Source: own processing based on **ZEW (2018)**.

Table 3 The values of EATR and EMTR from our model (2004 – 2018).

Title	Values	
	Tax depreciation rate	Accounting depreciation rates
Intangible assets	20%	15.3%
Agricultural buildings	5%	3.1%
Agricultural and forestry machinery	25%	17.5%
Basic herd and draught animals	25%	17.5%
Growing units of permanent crops	8.33%	4.5%
Estates	-	-
Inventories	-	-

Table 4 Relationship between EATR and EMTR.

Economic rent after tax	Retained earnings		Equity contribution		Debt	
	2004	2018	2004	2018	2004	2018
Intangible assets	0.0666	0.0564	0.0666	0.0564	0.0792	0.0710
Agricultural buildings	0.0874	0.0794	0.0874	0.0794	0.1000	0.0940
Agricultural and forestry machinery	0.0630	0.0518	0.0630	0.0518	0.0756	0.0664
Basic herd and draught animals	0.0630	0.0518	0.0630	0.0518	0.0756	0.0664
Growing units of permanent crops	0.0826	0.0741	0.0826	0.0741	0.0952	0.0887
Estates	0.1033	0.0976	0.1033	0.0976	0.1159	0.1122
Inventories	0.0996	0.0924	0.0996	0.0924	0.1122	0.1070
EATR (in %)	**Retained earnings**		**Equity contribution**		**Debt**	
	2004	**2018**	**2004**	**2018**	**2004**	**2018**
Intangible assets	40.06	45.41	40.06	45.41	41.32	46.87
Agricultural buildings	29.14	33.34	29.14	33.34	30.40	34.08
Agricultural and forestry machinery	41.95	47.83	41.95	47.83	43.21	49.29
Basic herd and draught animals	41.95	47.83	41.95	47.83	43.21	49.29
Growing units of permanent crops	31.66	36.12	31.66	36.12	32.92	37.58
Estates	20.79	23.78	20.79	23.78	22.05	25.25
Inventories	22.73	26.51	22.73	26.51	24.00	27.98
EMTR (in %)	**Retained earnings**		**Equity contribution**		**Debt**	
	2004	**2018**	**2004**	**2018**	**2004**	**2018**
Intangible assets	10.49	12.70	10.49	12.70	-26.48	-28.96
Agricultural buildings	18.45	20.46	1845	20.46	-11.15	-12.72
Agricultural and forestry machinery	12.56	14.72	12.56	14.72	-22.38	-24.60
Basic herd and draught animals	12.56	14.72	12.56	14.72	-22.38	-24.60
Growing units of permanent crops	16.10	18.18	16.10	18.18	-15.55	-17.36
Estates	22.19	24.11	22.19	24.11	-4.31	-5.54
Inventories	24.97	27.10	24.97	27.10	0.62	0.17
EATR ↔EMTR (in %)	**Retained earnings**		**Equity contribution**		**Debt**	
	2004	**2018**	**2004**	**2018**	**2004**	**2018**
Intangible assets	16.62	18.75	16.62	18.75	20.48	22.54
Agricultural buildings	18.83	20.44	18.83	20.44	17.24	19.16
Agricultural and forestry machinery	17.16	19.31	17.16	19.31	19.69	21.72
Basic herd and draught animals	17.16	19.31	17.16	19.31	16.96	21.72
Growing units of permanent crops	18.14	20.22	18.14	20.22	18.25	20.22
Estates	20.03	22.19	20.03	22.19	15.48	17.33
Inventories	20.99	23.09	20.99	23.09	14.06	15.70

Note: Source: own processing.

The funding methods that were processed during the analysis were oriented to financing from undistributed profit, new shareholders´ contributions and debt financing. However, in the analysis, there is an absence of personal taxes because our analysis follows commercial companies and their dividends are not taxed in Slovakian conditions. Capital costs for investments financed by a new contributions and investments financed by undistributed profits will be equal.

Thanks to our analysis, we found out the values of effective average tax rate and effective marginal tax rate, their differences and the economic income of the project including taxation, which means financial benefit from the investment project.

The analysis combines two effective tax rates (Table 3 and Table 4). The first one was EATR, which has a decisive impact on the placement of the investment (i.e. which country is appropriate for investing), and the second one was EMTR, which measures the extent of the investment in the country (i.e. the investment value). When we combine these two indicators, we got the relationship between EATR ↔ EMTR, which is crucial to make a decision on the investment realization, as well as evaluates the most advantageous relationship between the location, size and way of investment financing. EATR indicator includes the economic income of the project with taxation and expresses the size of the financial benefit of the project with the taxation aspect. The highest value of economic income is simultaneously the lowest level of EATR. The highest value of economic income in compared period reported estates (0.0976 in 2018 financed through own sources; 0.1159 in 2004 financed from external sources). For land, the level of EATR was at 20.79% in 2004, respectively 25.25% in 2018. On the other hand, the lowest value of economic income was reported by agricultural and forestry machinery, basic herd and draught animals in both analyzed years (values ranged from 0.0518 to 0.0756 that was financed through external sources). EATR for these assets was 41.95% in 2004 and 47.83% in 2018 for financing from own sources, and 43.21% and 49.29% for external financing. When deciding on the location of investments in Slovakia, the best option would be investment in land and inventories. On the other hand, the worst decision for an investor would be to invest in tangible assets in agricultural and forestry machinery, basic herd and draught animals and in intangible assets. Differences occurred in the assessment of individual periods, as it was a 2% increase in the statutory rate for reference period. In addition to the tax rate increase, the depreciation period for tangible assets changed, while intangible assets change did not affect. For buildings, the depreciation period increased from 20 years to 40 years, and for machinery and equipment from 4 years to 6 years. This change increased the tax base, and also had an impact on the overall 12% increase in EATR over the reporting period.

The second analyzed tax rate is EMTR. It expresses conventional way of measuring the impact of corporate tax on the level of investment capital. The basic idea of EMTR is that the investor will invest financial sources until the marginal capital value is equal to the cost of capital. It is clear that the marginal product is declining, resulting in a profit-maximizing income level. The higher EMTR, the higher cost of capital, and so it reduces capital inflow (or increases capital outflow).

When compared to the reference years, EMTRs increased by approximately 11.7%. The lowest rates and hence the most efficient investment options were at intangible assets, at 10.49% and for 2004 and slightly increased to 12.70% (2018), with own funds. The second effective investment appeared in tangible assets (agricultural and forestry machinery and basic herd and draught animals), which were between 12.56% and 14.72%. The most critical were investments of stocks, with rates exceeding 20% of the optimum value and reaching 24.97% and 27.10% for 2018 in 2004. The land plots were similar (22.19% and 24.11% respectively). The negative values reported by EMTRs in external financing (debt) were due to a 5% real rate of return on alternative investment, which reduced the cost of capital to negative rates. In other words, from the point of view of an investor wishing to carry out an investment project at the cost of capital considered, these negative rates are advantageous as they express savings over the optimal rate of return on an alternative investment, which is determined on average. Debt financing was most beneficial for intangible assets, with rates ranging from -26.48% to -28.96%. During this period, the cost of capital was 4.1% for 2004 and 3.8% for 2018, while the rate of return on alternative investment was 5%. The last measured variable is the relation EATR ↔ EMTR, by which we determine the impact of the tax on a hypothetical investment project. Given the specific structure of tax-legislative and business-policy conditions in individual countries, it is not easy for an investor to make the right decision to ensure the highest profit for him. It is by comparing these relations of the two rates that we find out to what extent the pre-tax profit is reduced by taxation. The results have shown that in this relationship the way of financing the investments is decisive. Although the most efficient placement and scale of investment appeared for intangible assets, but only for own-funded financing, rates in this item were 16.62% in 2004 and increased by 12% by 2018. What is interesting, however, when financing from external sources, these rates are the highest in the range of 20.48% to 22.54%. On the other hand, inventories (20.99% to 23.09%) showed the highest rates of financing from their own resources, while in financing from external sources they fell by 15% and appeared to be the most effective. Therefore, it is crucial for the investor to make these investments definitely from his own resources. Compared to the reference years 2004 and 2018, EMTR increased by approximately 11.7%. The lowest and simultaneously the most effective tax rates for investment conditions were reported in intangible assets (10.49% in 2004, 12.70% in 2018), while it was investments financed through own sources. The second most attractive investment, according to our analysis, was investment in tangible assets (i.e. agricultural and forestry machinery, and basic herd and draught animals) with values range from 12.56% to 14.72%. The most discerning investment represents inventories where effective tax rate exceeded 20% (24.19% in 2004, respectively 27.10% in 2018). Similarly, estates also had effective tax rate above 20% (22.19% in 2004, 22.11% in 2018). Negative values of EMTR in external debt financing were due to a 5% real rate of return

on alternative investment. It means that if an investor wants to realize investment project at the level of considered costs of capital, negative rates are favourable as they represent savings over the optimal rate of return on an alternative investment. The most beneficial for intangible assets was debt financing, with rates ranging from -26.48% to -28.96%. During the period, cost of capital was 4.1% for 2004 and 3.8% for 2018, while the rate of return on alternative investment was 5%.

The last analyzed variable, which can explain the impact of taxation from a hypothetical point of view, is a relationship EATR ↔ EMTR. Since a specific structure of tax-legislative and business-political conditions in individual countries, for investor is very hard to make a decision about investment. Comparing this relationship, we found out how much pre-tax profit is reduced by tax. Results showed that there is a decisive way of investment financing in this relationship, as well. The most effective investment location from tax rate view was in intangible assets (16.62% in 2004, and until 2018 an increase by 12%), however only if investment is financed by own sources. We found interesting that when external financing is used, tax rates are the highest and range from 20.48% to 22.54%. On the other hand, inventories reported also the highest tax rates in financing through own sources, while in financing from external sources there was reported a reduction in tax rate by 15%. Therefore, it is crucial for investors to make a decision about investment financing through own sources.

In summary, we can state that overall the lowest tax rate have location and investment amount. It is tax rate which takes into account economic conditions, as well as costs of capital, accounting and tax depreciations, inflation rate and nominal interest rate (so called "shareholder discount rate"). When investors take into consideration all of these tax rates, then the investment decision is the most effective and most optimal.

In literature, the effect and impact of differences in tax rates on investment decisions was analyzed by **Arachi and Biagi (2005)**, **Hanlon and Heitzman (2010)**, **Feld and Heckemeyer (2011)**. Also **Devereux, Griffith and Klemm (2002)** states that with different forms of EATR and EMTR monitoring, capital can be financed from different sources, including debt financing, as our analysis showed.

According to **Blechová (2015)**, the impact of the taxes on the return of planned investments (in case of their implementation in different countries) was negatively correlated. It means the higher was the indicator of effective average taxation, the less attractive were these countries for potential investors. In our case, the rate was based on the type of capital, and the land and inventories were the most attractive investments for investors. In case of capital location and investment amount, the most attractive investments were in agricultural and forestry machinery and basic herd and draught animals.

Devereux, Griffith and Klemm (2004) claim that the placement of investments is clearly affected by differences in tax rates. EATR and EMTR as well as statutory tax base are crucial indicators to make a decision about placement and amount of investment. **Vegh and Vuletin (2012)** states that it is also decisive to take into account various specifics of tax politics of individual countries. In general, an increase in statutory tax rate will lead to lower investment, and thus to a reduction in the returns from production factors other than capital.

Reduction of agricultural taxable income is possible due to various reliefs and specificities of the country's tax system, which governments provide mainly to small farmers **(Andersen et. al., 2002)**. The fair taxation in agriculture is lacking, especially in developing countries. in these rural areas, poverty reduction through support for agriculture is very challenging **(Khan, 2005)**. The amount of the agricultural tax is closely associated with the specific tax rate, which is linked to specific crops. However, its changes depend on the purchase price of the crop and, therefore, there are often changes at the level of the agricultural tax burden **(Felis, 2015)**. In comparison to non-agricultural incomes, agricultural income may be less burdened by taxation, as the tax burden is not affected by the amount of income actually generated. **Severini, Tantari and Rocchi (2014)** claim that regressive effects of agricultural taxation are mainly felt by households. **Durst and Monke (2001)** point out the occurrence of negative effective tax rates is only temporary and it should not be relied on by farmers in the long-term period. The negative effective tax rates in the agricultural sector are influenced mainly tax credits and deductible items that compensate for investment income. In the analysis, negative tax rates for effective taxation were found only in external financing, which was affected by a 5% real rate of return on alternative investment.

CONCLUSION

Effective tax rates play a crucial role in the allocation of investments, and also in determining the amount of investments that will generate future profits. In our analysis, we focused on the impact of effective taxation on costs of capital in determining the net present value of the specified investment project. Based on the level of effective marginal tax rate and effective average tax rate, we identified the optimal selection of investment projects and investor´s support in decision making about the amount and location of investment. EATR included economic income that reported the lowest value of agricultural and forestry machinery and basic herd and draught animals during the period, while the value ranged from 0.0518 to 0.0756, with a higher value calculated on external financing. The lowest value of economic income shows the highest value of EATR, and the rate of these assets was at the level of 41.95% in 2004, respectively 47.83% in 2018 for own funds, and 43.21% in 2004 and 49.29% in 2018 for external financing.

According to the analysis we found that the lowest EATR was reported by land in both reporting periods and for both financing methods. When deciding on the location of investments in Slovakia, the most advantageous option is investment in land, followed by stocks. Over the reference period, EMTR increase by approximately 12%. The lowest and the most effective tax rate for investment conditions were reported in intangible assets (10.49% in 2004, 12.70% in 2018), while it was investments financed through own sources. Negative values of EMTR in external debt financing were due to a 5% real rate of return on alternative investment. It means that if an investor wants to realize investment project at the level of

considered costs of capital, negative rates are favourable as they represent savings over the optimal rate of return on an alternative investment. Differences also occurred in the assessment of individual periods, as this was a 2% increase in the statutory rate for the reference period. In addition to the rate increase, the depreciation period for tangible assets changed, while the intangible assets did not affect.

The last monitored variable was the relation EATR ↔ EMTR, which is decisive on the location and investment amount. Our results proved and made it clear that in this relationship the way of financing investments is also very crucial. Intangible assets, whose rate was 16.62% (2004) and increased by 12% by 2018, were decisive for the investor's own funding. In financing from external sources, stocks were most effective, with rates ranging from 14.06% to 15.70% over the reporting period. Our results definitely confirm that the relationship EATR ↔ EMTR is decisive for investors. In summary, we can conclude that Slovakia is certainly an interesting and challenging country from the tax point of view and has a lot to offer to foreign investors.

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AUTHENTICATION AND PREFERENCE MAPPING OF HAM

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ABSTRACT

Effective connection between the food industry and consumer demands are specific needs of consumers whitch were monitored in this study by using a preferential mapping method. Preference mapping is based on Principal Component Analysis (PCA), which is performed on preferences ratings given for each product and preferences of each consumer through an online questionnaire. Key features for the consumer choice were colour, odour, consistency, total flavour and overall appearance. We verified the composition and mapped the preferences of 10 hams purchased in Slovakia. In view of the persistence of identified cases of food counterfeiting and meat fraud, intensive monitoring and scrutiny is required through effective and accurate analytical methods, which are crucial for maintaining consumer confidence and ensuring compliance with local legislation and labeling. The reference approach for identifying animal species in food is the PCR method, which is however limited to several animal species, meat types. The use of microarray technology enables the identification of a wider range of animal species and greater user comfort, especially the speed of obtaining the results. It allows 24 animal species to be identified in one analysis in 8 samples at a time. Detection was performed using Chipron LCD Aarray Kit Meat 5.0. In all analyzed samples, components of animal origin were identified in accordance on the packaging of the products. The Meat 5.0 LCD chip, which was used for analysis, has detected the presence of other animal species.

Keywords: ham; consumer preference; sensory; PCA; DNA; animal species

INTRODUCTION

Consumer perception of food quality is different and strongly dependent on personal preferences such as level of experience, cultural influences, demographic and physiological characteristics, product perception and quality expectations. It may be affected by several factors, e.g. brand origin, price, nutritional information and traditional technological processes **(Supeková, 2008)**.

In the overall quality of the meat, taste plays a major role, and therefore the presence of sensory defects and/or lack of typical taste significantly reduces its quality, causing financial losses for the dairy industry **(Engel et al., 2001)**. The evaluation of sensory attributes makes it possible to define the taste profile and consumer preferences for dairy products with innovative properties. Meat sensory profile analysis allows to identify specific attributes that could be preferential properties and evaluate the impact of health information on consumer preferences, expectations and choices **(Santillo and Albenzio, 2015)**. Large data sets are becoming increasingly common and often difficult to interpret. Principal Component Analysis (PCA) is a technique to reduce the size of such data sets, thereby increasing interpretability but at the same time information loss is minimized **(Jolliffe and Cadima, 2016)**.

Preferential mapping is a set of statistical methods aimed to detecting consumer preferences of the products being compared using sensory profiles. This method is used in the food industry to develop new products, especially according to consumer requirements **(Meullenet et al., 2007)**. Preferential mapping is carried out by trained evaluators to better understand consumer acceptability of products. Product placement on the market determines their usability **(MacFie, 2007)**. It is also a key management tool that is often used to optimize products by combining consumer and sensory data **(Greenhoff and MacFie, 1994)**. Consumer segmentation strategies aim to identify sectors of the population of consumers with different criteria of preferences **(Carbonell et al., 2007)**. Using a preferential map, it is possible to describe consumer preferences for a set of competing products from the sensory profiles of these products, based on a trained panel of evaluators. Preferential mapping refers to a group of multivariate statistical techniques that provide a comprehensive overview of both external and internal mapping. External preference mapping is based on multidimensional product display based on their

sensory profile or a set of other external data, such as instrumental analysis using electronic tongue, nose and eye. This result is usually obtained through Principal Component Analysis (PCA). However, with this technique, it is possible to reduce each consumer's hedonic assessment to a set of descriptive attributes **(Cadena et al., 2012)**. External mapping approaches are limited by the fact that the sensory space (i.e., multidimensional representation) is obtained only from external data without the preference of attributes based on their importance to consumers **(Meullenet et al., 2007)**. Internal preference mapping brings a multidimensional depiction of products and consumers. This representation is obtained by means of a PCA data matrix with products such as rows and consumers as columns. For that consumer, the data is based on from the hedonic score **(Greenhoff, MacFie, 1994)**.

Meat and meat products have a profound impact on human nutrition and hence on consumer health. Meat is a rich source of protein, containing all essential amino acids is a good source of iron, phosphorus, zinc, selenium, riboflavin, niacin, vitamin B6, vitamin B12, choline and others. Red meats such as beef, pork and mutton contain many essential nutrients essential for healthy growth and development in children. Red meat is one of the best sources of iron and zinc that is well absorbed in the body. Meat is also used as part of many foods and meat products **(Mansoor et al., 2015)**. Given the high commercial value, meat has attracted the attention of counterfeiters for centuries **(Barai et al., 1992)**. Identifying the origin of meat and meat products is an important issue for the prevention and detection of fraud that could have economic, ethical and health implications **(Bertolini et al., 2015)**. Currently, there are several methods capable of recognizing chicken, pork and beef, which are among the most consumed meats in the world. Various analytical techniques have been proposed that identify these meats either alone or in mixtures **(Hsieh, 2006; Günssen et al. 2006)**.

Hygiene and proper labeling on the label of food products are very important aspects in particular for public health. Food safety covers all precautions for food supply and ensuring health and hygiene conditions for consumers **(Özpinar et al., 2013)**. Adulteration detection is a demanding industry in the food industry. Active development of additives, as well as novel foods that have undergone significant changes in the food matrix, increase the demands for accuracy and reliability of analytical methods based on the identification of sensory, anatomical, morphological and histological differences in the detection of counterfeiting **(Yosef, 2014)**. Testing techniques are becoming faster, more accurate, more sensitive, more user-friendly, capable of detecting more than one species in one reaction **(İlhak and Arslan, 2007)**. These tests are also available as commercial test kits, are suitable for routine analysis due to their ease of use, speed and relatively low cost, but limited use for highly processed foods **(Montowska et al., 2014)**.

Scientific hypothesis

Hypothesis 1: To map the preferences of consumers of hams produced in Slovakia using the preferential mapping method.

Hypothesis 2: Verified the composition of 10 hams.

Hypothesis 3: Determine whether there is a link between consumer preferences and the composition of the product.

MATERIAL AND METHODOLOGY

Preference mapping

Internal mapping

Samples were prepared from 10 hams produced in Slovakia. Samples were served on white ceramic plates, coded with three-digit random numbers and served at temperature of consumption 20 ±2 °C. The evaluation was carried out in a standardized sensory laboratory **(ISO 8589, 2007)** built in the Slovak University of Agriculture in Nitra on Department of Food Safety and Hygiene. 13 assesors participated in the analyzes, who evaluated 10 ham samples. They evaluated the following sensory qualities: colour, odour, consistency, total flavour and overall appearance, which they could assign 1 – 9 points (1 – very bad and 9 – very good).

External mapping

External mapping (consumer survey) was conducted through an electronic questionnaire in which 140 respondents were addressed, from whom a statement on the products was requested. Their task was to organize the individual products according to their personal preferences (from 1 – best to 9 – worst).

Species identification

Ten samples were purchased in various retail networks. The identified animal species were compared to the product composition of the manufacturer. In the first step of the analysis, we used Maxwell 16 DNA Purification kit (Promega, Wisconsin, USA) to achieve optimal DNA purity obtained from the purchased products. A PCR product was generated from the isolated DNA using the Aarray Kit Meat 5.0 (Chipron, Berlin, Germany), which was verified by gel electrophoresis followed by the protocol of the Chipron LCD manufacturer Aarray Kit Meat 5.0, allowing 24 species (cattle, sheep, equine, goat, camel, water buffalo, pork, kangaroo, hare, rabbit, reindeer, roe deer, red deer, fallow deer, springbok, canine, cat, chicken, turkey, goose, ostrich, mallard duck, muscovy duck and pheasant) to be identified. We used instrumentation, scanner and software (SlideReader V12) designed and recommended by the manufacturer for evaluation.

Statistic analysis

XLSTAT statistical software (2019.1.1, Addinsoft) was used to process data from both internal, external evaluation and authentication. PCA (Principal Component Analysis) was used for internal data from sensory analysis and AHC (Agglomerative Hierarchical Clustering) for external data (questionaire). Preferential map was created by combining these two outputs. ANOVA was used to determine if there was a statistically significant difference between the samples.

RESULTS AND DISCUSSION

Table 1 show summary evaluation of sensory analysis. From the PCA (Figure 1) we can observe that three groups of samples have been specified. Samples 2, 4, 8, 9, and 10 show obtained a higher rating from samples in colou, odour, consistency, total flavor and overall appearance.

Table 1 Summary evaluation of sensory analysis.

Samples	Sensory qualities									
	Color		Odour		Consistency		Total flavour		Overall appearance	
	Average	Variance	Average	Variance	Average	Variance	Average	Variance	Average	Variance
1	6.2	2.40	5.7	5.57	6.1	5.43	5.3	6.23	5.4	5.82
2	6.8	2.18	7.1	0.77	6.7	2.68	6.4	2.71	6	4.89
3	5.8	2.84	4.8	5.29	5.7	6.01	5.1	4.99	5.2	4.40
4	7.1	1.88	6.8	5.29	6.9	2.99	7.5	2.06	7.1	3.21
5	6.3	3.12	5.5	0.72	6.4	5.82	5.8	2.40	5.4	2.71
6	6.1	4.32	5.3	2.68	5.7	5.79	4.8	3.73	5.5	3.61
7	6.3	2.23	5.8	3.07	6.5	2.28	5.8	1.51	6	2.22
8	6.7	2.68	6.5	1.61	6.8	3.73	6.7	1.79	6.6	2.49
9	6.8	2.62	6.3	5.12	7.1	3.66	7	3.33	7.2	2.62
10	7.3	1.57	7	3.33	7	1.33	6.6	4.27	6.5	4.94

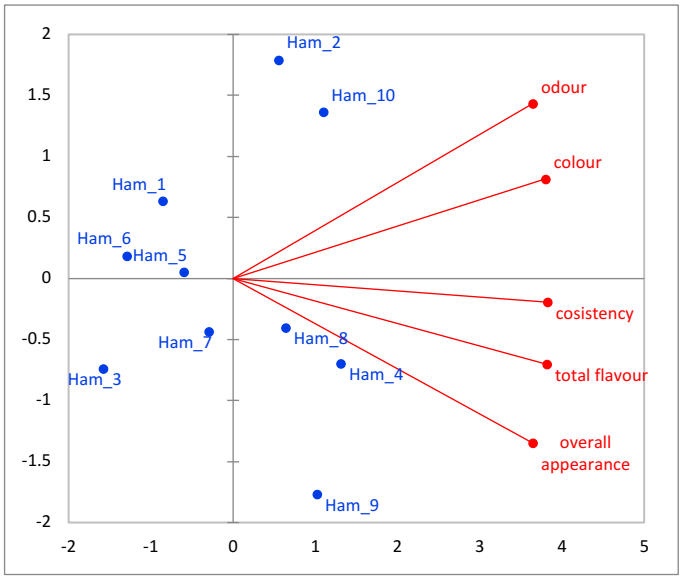

Figure 1 Evaluation using PCA.

The resulting processing of internal and external data is a map of preferences (Figure 2).

From the graphical representation of the results, we can conclude that sample 10 was placed in the highest consumer preference zone (80 – 100%) and samples 2, 4, 8 and 9 in the preference zone from 60 – 80% based on the characteristics of the surveyed products. Samples placed in the lowest consumer preference zone (0 – 40%) were 1, 3, 5, 6, 7 and recorded also lowest scores for overall appearance, odour, consistency, flavour and colour. The ANOVA test results show that there is a statistically significant difference between the samples (*p*-value = 0.033).

Our results are consistent with studies done abroad. These techniques have recently been applied to dulce de leche **(Gaze et al., 2015)**, ice cream **(Cadena, et al., 2012)**, apples **(Bonany et al., 2014)**, raspberries **(Villamor, et al., 2013)**, tomatoes **(Oltman, Yates and Drake, 2016)** and have shown that they provide a very good understanding of the attributes that lead to popularity among consumers.

DNA Microarray and Real Time PCR methods differentiate from each other in simultaneously detection of animal species in one reaction. The only common similarity between them is the step of DNA isolation. Microarray Analysis can enable us for detecting more than one species in one reaction only whereas Real Time PCR requires specially designed primers and probes needed to simultaneously amplify the specially selected regions of DNAs belonging to different species. This difference means longer time needed in the optimization step of primers and probes **(Myers et al., 2010)**. DNA Microarray can deliver the results faster and more sensitive using amplified DNA by conventional PCR technique **(Azuka et al., 2011)**. DNA Microarray makes possible the whole genome to be displayed on a chip and to express the interaction of thousands of genes with each other simultaneously **(Pereira, Carneiro and Amorim, 2008; Miller and Tang 2009)**.

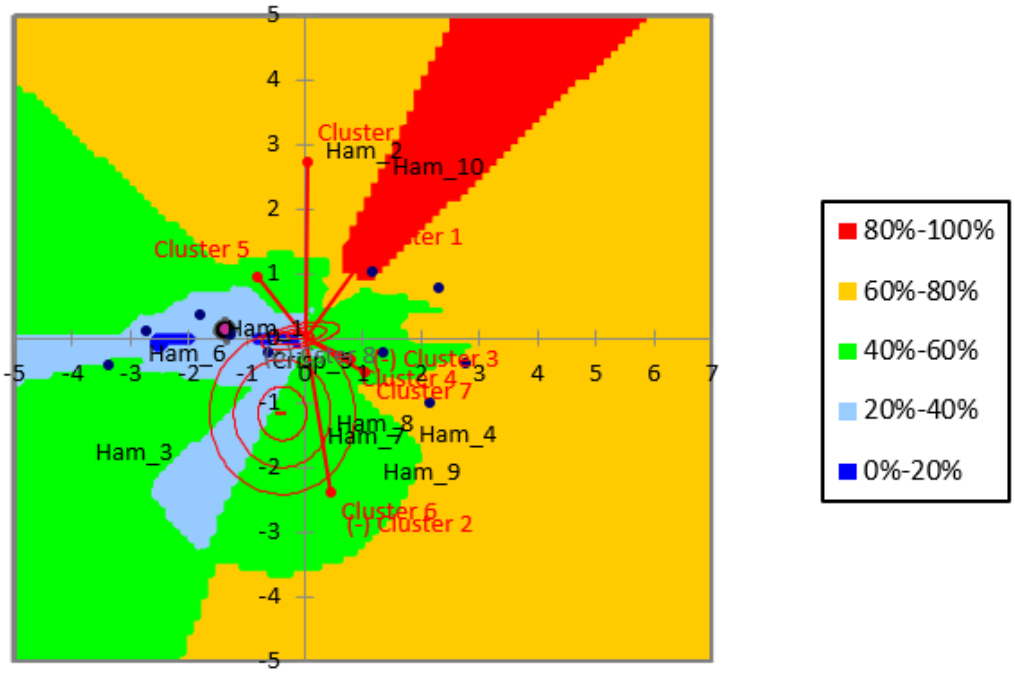

Figure 2 Preference mapping of hams.

Table 2 Composition of samples and species identification.

Sample	Fat (g)	Carbohydrates (g)	Protein (g)	Salt (g)	Meat content %	Cattle	Pork	Chicken	Turkey
1	9.0	1.0	20.0	2.75	96	-	X	-	-
2	2.0	1.0	20.0	2.5	96	-	X	-	-
3	3.0	1.0	20.0	2.5	96	-	X	-	-
4	4.1	<0.5	19.5	2.22	96	-	X	-	-
5	3.0	0.5	19.5	1.9	94	-	X	-	-
6	3.2	1.2	15.6	2.1	85	-	X	-	-
7	3.2	1.2	15.6	2.1	85	-	X	X	X
8	4.0	0.3	14.3	2.1	75	-	X	-	-
9	2.0	1.0	20.0	2.25	95	-	X	-	-
10	8.0	1.4	19.1	2.0	90	X	X	-	-

Note: X – Declared, - undeclared.

Table 2 shows the composition of the individual samples including the animal species identified in the samples. The analyzed sample set consisting of hams included samples with a declared single animal species on the product label. Based on the results obtained from ten samples, we collected three samples which contained DNA of another animal species. Eight products were in line with labeling and identified animal species.

Based on the EU recommendation **(European Commission, 2013)**, a detection threshold of 1% (w/w) was targeted. Our results are consistent with studies done abroad. In a study carried out in Turkey, 73 samples of meat and meat products sold in shops, markets and public bazaars located in different urban areas in Istanbul were analyzed. The study pointed to a number of disagreements with the label on the product label **(Özpinar et al., 2013)**. A study on meat processing revealed that the DNA fragment size was progressively degraded into smaller fragments with increase in duration of heating and temperature **(Şakalar et al., 2012)**. Species identification is important for legal authorities to detect undeclared ingredients in food products. When an undeclared species is detected, the next step is to discriminate between intentional substitution with cheaper meat or unintentional contamination during food preparation **(Cravero et al., 2019)**. DNA Microarray as a method has been widely preferred for understanding mechanisms, detection of foodborne microbial pathogens and food safety studies, nutreaceuticals and functional foods as well as following up the different expression levels of DNA in bacteria, yeasts, plants and human; genetic and mutation analyses; environmental studies; identification of antimicrobial genes, proteomics, protein-nucleic acids, protein-protein interactions, biochemical analysis of protein functions and drug development **(Bottero and Dalmasso, 2010; Kostrzynska and Bachand, 2006)**.

CONCLUSION

From the obtained results we can conclude that it is necessary to put emphasis on intensive control and management of technological steps in the production of meat products. In the analyzed samples, we captured 2 samples that did not conform to the label on the product label. DNA of other species was also detected in the samples. The presence of bovine and poultry DNA is explained by the fact that some manufacturers may have added bovine haemoglobin or poultry globin to improve product colour or it may be contamination. The results obtained are an incentive for further investigation and analysis.

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AUTHENTICATION AND PREFERENCE MAPPING OF HAM

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