MICROWAVE-ASSISTED HYDROLYSIS FOR LYSINE DETERMINATION

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ABSTRACT

The Central Institute for Supervising and Testing in Agriculture (CISTA) ensures monitoring of amino acids in feedingstuffs. Sample preparation for amino acid analysis is a laborious and time consuming step which was significantly speeded up using microwave-assisted hydrolysis.

Keywords: feedingstuffs, lysine, microwave-assisted hydrolysis

INTRODUCTION

Lysine is an essential amino acid which plays an important role in pig nutrition **(Zalabák, 1980)**. Cereal protein pig fodder is usually enriched with lysine and the total content of lysine in various kinds of pig feeds is monitored to comply with the legislation limits.

Lysine determination is the most frequently performed analysis within the monitoring of amino acids in the CISTA laboratory in Brno. Lysine must be released from proteins prior to the final analysis. Peptide bonds are broken under acid and heat exposure. Feedinstuffs are conventionally hydrolyzed by the Stein and Moore procedure **(Stein, Moore, 1963, Šíma, 2001)**. Microwave-assisted hydrolysis was optimized and established in our laboratory with the goal to speed up the sample preparation for lysine determination in feedingstuffs.

MATERIAL AND METHODOLOGY

Origin of samples

Samples of feedingstuffs (complete feed mixtures, compound feedingstuffs and premixtures with lower content of added lysine) were collected within the CISTA proficiency testing.

Hydrolysis procedure

Microwave-assisted hydrolysis was carried out by a MWS-3 microwave oven (Berghof) in high pressure digestion vessels DAC 100 and DAC 100S (100 ml, 150 bar). Silica glass testing tubes (inserts) were used to ensure better handling and to protect digestion vessels.

Homogenized sample (0.06 g – 0.3 g, 0.5 mm mesh) was put into the insert. Hydrochloric acid (3 ml, c = 6 mol/l) was added gradually and simultaneously mixed. Oxygen absorbed in the suspension was blown out by an alternate application of inert gas and vacuum. A digestion vessel was filled with hydrochloric acid (18 ml, c = 6 mol/l) which served as a medium. The insert with the sample suspension was put into the digestion vessel, treated by the inert gas and finally the digestion vessel was closed. A hydrolysis program was used as follows: initial temperature of 110 ˚C (4 min) and 12.5 ˚C/min to 160 ˚C (30 min). Total run time (40 min) corresponded with the Berghof recommended parameters. After cooling down the hydrolyzate was buffered, sonicated (approx. 10 min) and finally filtered using a cellulose filter.

Final determination

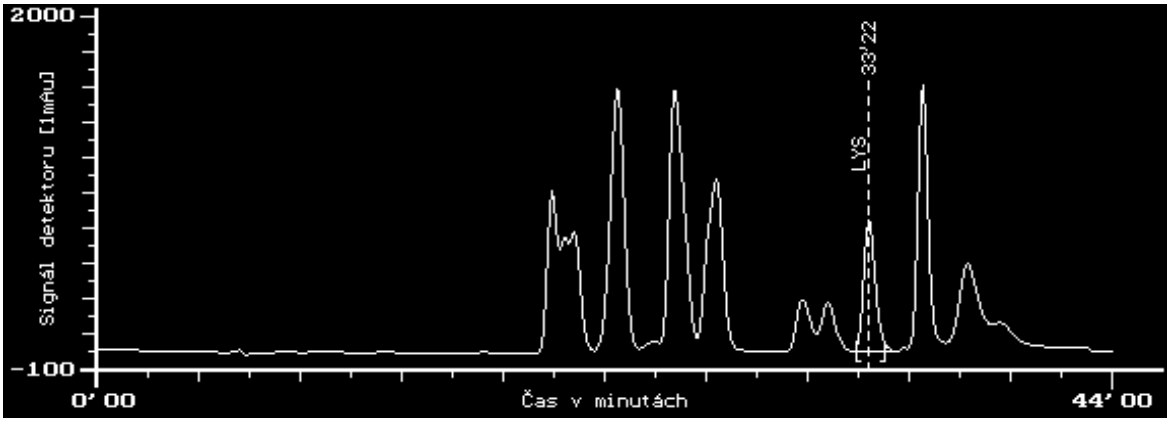
Lysine content in hydrolyzates was determined using an AAA 400 amino acid analyser (Ingos, Ltd.) with a ninhydrin post-column derivatization. Amino acids were separated on a Polymer 8 µm ion exchanger (Václav Havlíček ZMBD Chemik) filled in a glass column (45 cm × 0.37 cm id). The chromatographic column was prepared in the CISTA laboratory with a bed length of 30 cm. Other chromatographic conditions are stated in an operation manual **(Ingos, 2000)**.

RESULTS AND DISCUSION

Lysine determination

Lysine determination run time was shortened compared to a full amino acid spectrum analysis. The typical chromatographic profile of amino acids at lysine analysis is shown in **Figure 1**.

Figure 1. Amino acid profile at lysine analysis



This profile differed from the full amino acid spectrum, particularly in the front part of the chromatogram. Early eluting amino acids are missing because a derivatization

agent (ninhydrin) flow was cut off for 15 min. Lysine retention time (RT) in a calibration standard corresponded with RT in a hydrolyzed sample due to a sufficient capacity of the pH buffer. A slight difference in pH of the standard and the sample did not influence the retention behavior of lysine. No adverse influence of pH on the ion exchanger was observed.

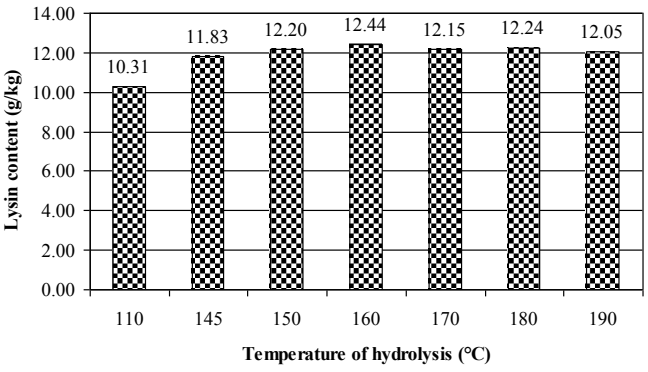
Microwave-assisted hydrolysis optimization

A complete feed mixture for pigs which is the most frequently inspected material for lysine content was chosen for hydrolysis optimization. Hydrolysis of feedingstuffs is conventionally carried out by the Stein and Moore procedure **(Stein, Moore, 1963)**. Microwave-assisted hydrolysis without optimization was firstly tested using a modified Stein and Moore procedure including an evaporation step. The recovery of lysine was about 90 % compared to the conventional method.

The following optimization of microwave-assisted hydrolysis included insert application and optimizing of hydrolysis temperature. The insert was introduced into the procedure to facilitate the sample and the hydrolyzate handling and to reduce the sample amount. However, the sample amount reduction (compared to the conventional sample weight) was possible provided that the sample was highly homogeneous. Moreover, microwave-assisted hydrolysis of the reduced sample amount yielded the reduced volume of the hydrolyzate which was only buffered without evaporation.

Hydrolysis temperature was optimized in order to improve the recovery of lysine from the selected feedingstuffs. The highest lysine content was determined in the hydrolyzate obtained at the temperature of 160 °C. Optimization data are shown in **Graph 1**.

Graph 1. Optimization of hydrolysis temperature



Temperature in the MWS-3 Berghof microwave oven is monitored by an infrared sensor which scans the temperature radiation of the hydrolyzed sample in the closed vessel. The temperature during the microwave process depends on the sample amount, homogenity and matrix. Various kinds of feedingstuffs produced different temperature radiations, therefore one kind of a feed material should only be processed within one hydrolysis cycle.

Despite above, this method speeded up the preparation step and improved the laboratory throughput compared to the conventional approach.

Microwave-assisted hydrolysis validation

Nine different feed materials were prepared in triplicates using both the conventional procedure according to Stein and Moore **(Stein, Moore, 1963)** and the optimized microwave-assisted hydrolysis. Lysine contents in the tested samples obtained using both the methods were evaluated by T-test ($t_{0.05}$, crit = 2.776). The statistical results (mean, standard deviation) are shown in **Table 1**. Furthermore, 50 samples prepared in duplicate were analyzed for the lysine content using the optimized microwave-assisted hydrolysis. The validation parameters of the method were calculated in the EffiValidation program. The repeatability of the validated method was 1.9 % and the extended uncertainty was 3.8 %.

Table 1. Statistical evaluation

Feed materials	Microwave assisted hydrolysis g/kg			Conventional hydrolysis g/kg			t0.05
	value	average	sd	value	average	sd	
Mineral supplement for pig	45.86	45.01	1.43	45.25	45.36	0.22	0.347
	43.35			45.62			
	45.81			45.22			
Mineral supplement for pig	58.67	60.04	1.96	63.18	62.67	1.52	1.502
	62.28			60.96			
	59.17			63.88			
Complete feed mixture for pig	9.75	10.18	0.37	9.63	10.10	0.44	0.190
	10.36			10.49			
	10.42			10.18			
Complete feed mixture for pig	9.72	9.79	0.12	10.16	9.99	0.26	0.996
	9.71			9.69			
	9.93			10.12			
Complete feed mixture for pig	30.61	31.75	1.09	32.22	32.14	0.23	0.490
	32.79			31.88			
	31.85			32.31			
Complete feed mixture for pig	9.37	9.54	0.17	10.31	10.25	0.49	1.917
	9.55			9.73			
	9.71			10.71			
Complete feed mixture for porket	9.23	9.20	0.03	9.66	9.90	0.58	1.728
	9.18			10.59			
	9.19			9.49			
Complete feed mixture for porket	13.82	13.91	0.34	15.49	14.68	0.75	1.313
	13.61			14.01			
	14.29			14.53			
Complete feed mixture for laying hen	6.33	6.56	0.45	6.78	6.64	0.13	0.265
	6.27			6.53			
	7.07			6.62			

CONCLUSION

Microwave-assisted hydrolysis was optimized for lysine determination in various feedingstuffs. The results described above indicated that, as far as lysine analysis is concerned, the microwave-assisted hydrolysis is a good alternative preparation technique to the conventional Stein and Moore procedure.

The National reference laboratory of CISTA (Regional Department in Brno) verified the optimized microwave-assisted hydrolysis by participating in a proficiency test for analysis of lysine in feedingstuffs in 2009.

In the future, the focus should lie on an increase in utilization of microwave-assisted hydrolysis. Moreover, the range of amino acids determined in one hydrolyzate will be extended.

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