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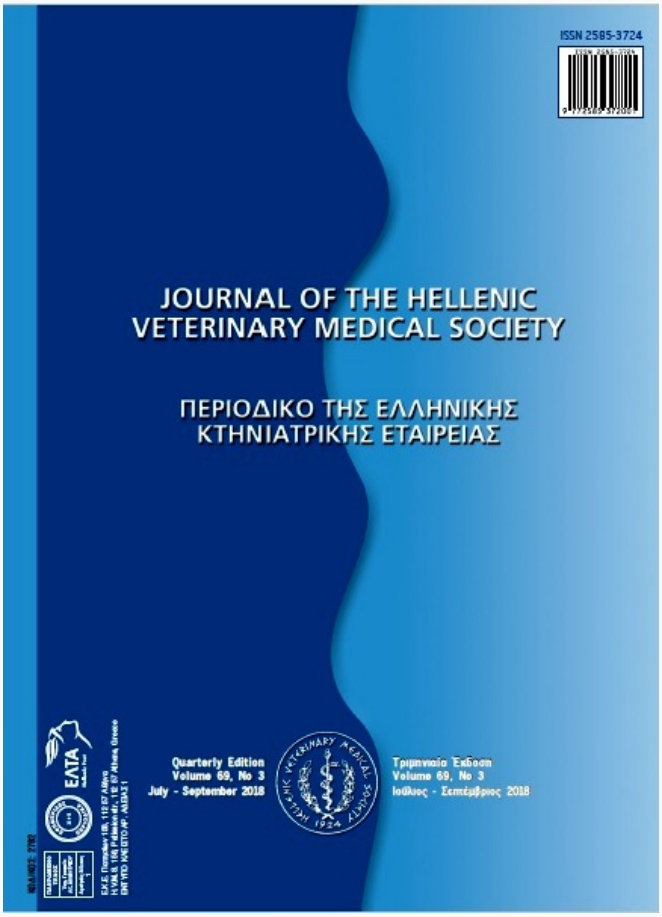

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S. JAKŠIĆ, I. JAJIĆ, S. KRSTOVIĆ, Ž. MIHALJEV

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Research article
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L-lysine determination in animal feed using microbiological microtiter plate assay

S. Jakšić[1], I. Jajić[2], S. Krstović[2*], Ž. Mihaljev[1]

[1] *Scientific Veterinary Institute, Rumenački put 20, 21000 Novi Sad, Serbia*

[2] *University of Novi Sad, Faculty of Agriculture, Trg D. Obradovića 8, 21000 Novi Sad, Serbia*

ABSTRACT. Chromatographic methods are most commonly used for the analysis of amino acids; however, there is growing need for faster, simpler and more price-effective assays. In this paper, the applicability of a rapid microbiological assay for quantification of the total content of L-lysine in feed samples was evaluated. The assay relies on the dependency of bacterial growth of *Pediococcus acidilactici* on the presence of L-lysine. Microbiological microtiter plate assay method for the quantitative determination of total (added and natural) L-lysine in feed samples has been verified, and parameters such as accuracy, precision, limit of detection, and limit of determination were evaluated. Results of total L-lysine determination in different feed samples have been compared with results of validated HPLC method. The microbiological microtiter plate assay method can be employed as a qualitative and quantification method for total L-lysine determination with detection and determination limit of 0.040 % and 0.085 %, respectively. However, further research on the influence of sample matrix on the determination of low lysine levels is required.

Keywords: L-lysine, determination, feed, microbiological assay, HPLC

Corresponding Author:
Saša Krstović,
University of Novi Sad, Faculty of Agriculture, Trg D. Obradovića 8,
21000 Novi Sad, Serbia;
e-mail: sasa.krstovic@stocarstvo.edu.rs;

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INTRODUCTION

Amino acids play very important role in animal nutrition. In modern animal nutrition, pure crystalline amino acids are replaced with trade products that contain amino acid mixtures. With the development of premixes and formulations, methods for amino acid determination have to change to be enough specific and selective for complex matrixes (Fontaine and Eudaimon, 2000). Lysine, as essential amino acid is not only a building block for proteins, but also a substrate for non-peptide molecules in animal bodies. Lysine can also affect the metabolism of other nutrients such as Ca and cholesterol (Liao et al., 2015). Deficiency of dietary lysine will impair animal immunity and elevate animal susceptibility to infectious diseases. Lysine in nutrition of monogastric meat animals can significantly increase body muscle protein accretion (Liao et al., 2015).

A range of analytic methods for amino acid analysis are used: spectrophotometry (Hasani et al., 2007), a whole cell green fluorescent sensor (Chalova et al., 2008), capillary electrophoresis (Latorre et al., 2001, Latorre et al., 2002), potentiometric sensor array (Garcia-Villar et al., 2001), and cyclic voltammetry (Saurin et al., 1999). Still, various chromatographic methods are mostly applied. The initial step involves protein hydrolysis, which can be acidic (Cottingham and Smalidge, 1988, Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006, Khan and Faiz, 2008, Jajić et al., 2013, Culea et al., 2015) alkaline (Fountoulakis and Lahm, 1998, Culea et al., 2015), or enzymatic (Fountoulakis and Lahm, 1998, Culea et al., 2015), followed by different chromatographic separations like ion exchange (Fontaine and Eudaimon, 2000, Khan and Faiz, 2008), reverse phase liquid chromatography (Cottingham and Smalidge, 1988, Bartolomeo and Maisano, 2006, Jajić et al., 2013), and gas chromatography (Culea et al., 2015). In addition to these methods, diverse derivatization reactions of amino acids such as pre- or post-column with nynhidrine (Fontaine and Eudaimon, 2000, Khan and Faiz, 2008), ortho-phthalaldehyde (OPA) (Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006), 9-fluorenylmethyl chloroformate (FMOC) (Cottingham and Smalidge, 1988, Jajić et al., 2013), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Fiechter and Mayer, 2014) are applied. Recently, methods using liquid chromatography–tandem mass spectrometry (LC–MS/MS) have been reported for amino acid analysis in physiological samples (Le et al., 2014). Many studies have been done to validate and compare different chromatographic methods, which were improved with development of instruments for chromatographic separations and spectrophotometric detections (Couch and Thomas, 1976, Cottingham and Smalidge, 1988, Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006).

Although chromatographic methods were most commonly used for separation and determination of a mixture of amino acids, there is a need for development of faster and cheaper methods. To that end, new enzymatic methods were designed for the selective assay of L-lysine in biological samples utilizing oxidase reaction and decarboxylation reaction by the L-lysine/specific decarboxylase/oxidase from *Burkholderia* sp. AIU 395 (Sugawara et al., 2015). Besides enzymatic techniques, novel microbiology methods for L-lysine determination were developed. Analytical microbiology technique relies on the fact that test microorganism and the medium used as reagents give the sensitivity and the specificity due to the metabolic process involved. Microbiological methods have a long history of development. First of all, selection of a suitable microbial culture, on which amino acids have impact, is of crucial importance, but avoidance of side effects and adverse reactions are equally significant. The development of methods for microbiological assay is comparative to the development of any analytical chemical procedure, test organism and the medium are used as reagents and the reaction should bring sensitivity, precision and selectivity of the analyte measurement (Loy and Wright, 1959). Microbiological methods involving different microorganisms and application of tube methods are described (Horn et al., 1946, Horn et al., 1947, Loy and Wright, 1959). The similar principle can be used in the form of microtiter plate assay, but instead of tube and titration, microtiter wells and spectrophotometric determination are used in form of commercial test kit (ifp, VitaFast® L-Lysin).

In this paper, we investigated possibility of use commercial set kit (VitaFast® L-Lysin) for total L-lysine determination in different feed samples. Also, we compared results obtained by using this

microbiological method with those obtained with HPLC method. To the best of our knowledge this is the only available test kit based on the microbiological method, and there are no studies about its comparison with the standard method for L-lysine determination in feed samples.

MATERIALS AND METHODS

Samples

For this study, different feed samples were used. Microbiological microtiter plate assay (MMPA) was applied for the determination of total L-lysine in maize, wheat, soy grits, wheat, and soybean meal, as well as in complete mixtures for laying hens, ducks and in dog and cat food.

First group of samples are presented in Table 1. Maize, soybean, and soybean meal were analyzed for natural L-lysine content. Then, a mixture of maize, soybean, and soybean meal samples was made in the ratio of 70:15:15 (w/w/w). The mixture was divided into four parts, and one part, marked "zero", was used as such, while the other three parts were supplemented with L-lysine in different concentrations. Thus, the mixture "one" contained 0.1%, "two" 0.2%, and "five" 0.5% of the added L-lysine.

The samples used for inter-laboratory studies, were also analyzed. Since robust average values for these samples were available based on analysis by a large number of laboratories, they were considered reference materials (RMs). The samples included: a total mixture for laying hens, dog food, wheat, two total mixtures for ducks, cat food, and soybean grits. Samples were purchased from National Reference Laboratory of Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic).

Microbiological microtiter plate assay

L-lysine microtiter plate assay is microbiological method for the quantitative determination of total L-lysine content (added and natural) in animal feed (VitaFast®, Art. No. P1012, R-Biopharm, Germany). Sample was treated by hot acid extraction with 2 M HCl in an autoclave (Colussi S.r.l. L 40E, Italy) 1 h at 121 ºC. After extraction of L-lysine from the sample, filtration and dilution of extract was done in sterile working conditions and by using sterile consumables.

The L-lysine assay was performed according to manufacturer's instruction. The preparation of samples for MMPA analysis was performed according to guidelines provided by the manufacturer of test kit for determination of total L-lysine (natural and added). After extraction of L-lysine from the sample and before assay implementation step, samples with expected L-lysine content higher than 0.40% were diluted 10 times in sterile conditions. Calibration curve for total L-lysine determination with MMPA was constructed in the working range of 0.020–0.400 g/100 g, by 4-parameter evaluation. Calibration curve was described by the following equation (Herman et al., 2008):

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}}$$

where x = mass concentration, y = turbidity, a = 0.073 (the minimum value that can be obtained, *i.e.*, value at 0 dose;); b = 1.127 (Hill's slope of the curve *i.e.*, b is related to the steepness of the curve at point c); c = 96501 (the point of inflection *i.e.* the point on the S shaped curve halfway between a and d), and d = 757,408 (the maximum value that can be obtained *i.e.*, value at infinite dose), with coefficient of correlation r = 0.9944.

The medium and diluted extracts are pipetted into the wells of a microtiter plate coated with *Pediococcus acidilactici* bacteria. The growth of *P. acidilactici* is dependent on the supply of L-lysine. Following the addition of L-lysine either as a standard or a compound of the sample, the bacteria grow until the L-lysine is consumed. The incubation is done in the dark at 37 °C for 44–48 h. The intensity of metabolism of growth of *P. acidilactici* in relation to the extracted L-lysine is measured as turbidity and compared with a standard curve (VitaFast®, ifp, Germany). The measurement is done using a microtiter plate reader at 620 nm (Multiscan MCC/340, Labsystem, Finland). Special software, the Rida®Soft Win (Art. No. Z9999, R-Biopharm, Germany), was used for the evaluation of the results.

HPLC method

The sample preparation for HPLC determination and the chromatographic conditions were in accord-

ance with Jajić et al. (2013). The samples were finely ground to pass through a 0.5 mm sieve. A sample weight, equivalent to 10 mg nitrogen content, was hydrolyzed using 6 M HCl (Lach-Ner, Neratovice, Czech Republic) containing 0.1 % (p.a. grade, Sigma-Aldrich, St. Louis, MO) of phenol for 6 h at 150 °C in vacuum. After the hydrolysis, the samples were cooled to room temperature and evaporated to dryness at 70 °C under a stream of nitrogen. The residues were quantitatively transferred into 50 cm3 volumetric flasks using 0.1 M HCl. The solutions were filtered through quantitative filter paper into glass tubes. The hydrolyzed samples and standard amino acid mixture solutions were automatically derivatized with OPA and FMOC (Agilent Technologies, Waldbronn, Germany) by programming the autosampler. The analysis was performed on an Agilent 1260 Infinity Liquid Chromatography system equipped with a μ-Degasser (G1379B), 1260 binary pump (G1312B), 1260 standard autosampler (G1329B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G1315C). After derivatization, 0.5 μl of each sample was injected into a Zorbax Eclipse-AAA column (150 × 4.6 mm, i.d., particle size 5 μm), at 40 °C, and detected on a DAD detector at 338 nm and 262 nm. The mobile phase A consisted of 5.678 g of Na_2HPO_4 per 1 L water, adjusted to the pH 7.8 with a 6 M HCl solution. The mobile phase B was acetonitrile–methanol–water (45:45:10, v/v). The separation was performed at a flow rate of 2 ml/min employing a solvent gradient. The calibration curve was constructed using five standard solutions containing 10, 25, 100, 250, and 1000 μM of each amino acid (Agilent Technologies, Waldbronn, Germany). The data of peak area vs. amino acid concentration were treated by linear least squares regression analysis.

Statistical Analysis

Statistically significant difference between the content obtained by the MMPA and the HPLC method, as well as the expected total L-lysine content in samples supplemented with L-lysine in different concentrations was analyzed using *t* test. The differences between the total L-lysine content obtained by the MMPA and the HPLC method in RMs, as well as difference between MMPA and reference value were analyzed using paired *t* test as well as linear regression analysis (PAST, Version 2.12, Oslo, Norway).

RESULTS AND DISCUSSION

The results of determination of total L-lysine in the first group of samples prepared by mixing of feeds and supplementation of L-lysine are presented in Table 1.

As it can be seen, in samples 1, 2, 4, and 7 there is no significant difference between MMPA and HPLC methods, as well as between the tested MMPA method and reference value at the 0.05 level for samples 1–4, and 7. For samples 3 and 5, there is no significant difference between tested and HPLC method at the 0.01 level. The *t* test revealed significant difference between the two methods, as well as between MMPA determined and declared content for total L-lysine only for maize sample No. 6 ($p<0.001$). Because of the low expected concentration of L-lysine in maize, which would be outside the range of the calibration curve after extract dilution, the extract was not diluted during preparation. Thus, potential interference of sample matrix with assay interpretation should be taken into consideration. Determination of total L-lysine in this sample has been repeated six times. The obtained high value of relative standard deviation (RSD) being 49% as compared to required 5% (Reason, 2003), points to the inaccuracy and imprecision in the determination of total L-lysine in this sample.

To the purpose of determining of intermediate precision, the same sample was extracted and assay was implemented in three different days (Table 2). The RSD values were 2.2% and 3.9% for samples with total L-lysine contents of some 1% and 3%, respectively. Since it was below 10%, which is maximum recommended value by both assay producer and international requirements (Reason, 2003), it can be considered acceptable. RSD is also comparable with values for coefficient of variation given in EC No152/2009 (2.1–2.8%) (European Commission, 2009), as well as the precision of the HPLC method (3.24%) (Jajić et al., 2013). Method specificity and selectivity is achieved by selecting suitable bacteria (*Pediococcus acidilactici*) which growth is conditioned by the presence of lysine (ifp, VitaFast® L-Lysin).

Table 1. Results of total L-lysine determination in different samples using two analytical methods

Sample No.	Sample type	Total L-lysine content, %		
		MMPA*	HPLC†	Reference value‡
1	Mixture „zero”	1.0	0.992	1.045
	p§		0.258	0.128
2	Mixture „one”	1.1	1.110	1.145
	p§		0.295	0.374
3	Mixture „two”	1.2	1.341	1.245
	p§		0.030	0.295
4	Mixture „five”	1.7	1.616	1.545
	p§		0.158	0.073
5	Soybean	2.8	2.462	1.81–2.59 (2.21)
	P§		0.046	0.027
6	Maize	0.03	0.304	0.14–0.37 (0.24)
	p§		<0.001	<0.001
7	Soybean meal	2.9	2.894	2.32–3.05 (2.73)
	p§		0.935	0.122

* samples 1, and 7, $n = 3$; samples 2–5, $n = 2$; sample 6, $n = 6$; † $n = 5$; ‡ For sample No. 1 reference value is obtained on the basis of HPLC analysis of each component (samples No 5–7), for samples No. 2–4, reference value was calculated from added L-lysine, and for samples No. 5–7 as reference L-lysine value is considered literature average value in parenthesis (Redshaw et al., 2010); § *t* test value (*p* values) comparison of data obtained using MMPA and HPLC, *i.e.*, reference value.

Table 2. Intermediate precision of total L-lysine determination by microbiological microtiter plate assay

	Total L-lysine content, %	
	Sample 1	Sample 7
Day 1	0.99	2.8
Day 2	1.0	2.8
Day 3	1.0	3.0
Average (%)	1.0	2.9
SD (%)	0.02	0.11
RSD (%)	2.2	3.9

MMPA has been applied also for the determination of total L-lysine in reference samples originating from international proficiency testing programs (Table 3). It is obvious that L-lysine content obtained in sample No. 10 is significantly lower as compared with the value obtained by HPLC method as well as the reference values. In this sample, the expected total L-lysine level was within the range of calibration curve, thus, sample extract was not additionally diluted. This also supported the likely matrix interference with assay results, so it can be concluded that the MMPA method is not suitable for every matrix. The analysis of paired *t* test revealed significant differences neither between the results for total L-lysine content in RMs obtained using MMPA and HPLC methods ($p = 0.396$), nor between contents determined by MMPA and reference values ($p = 0.274$), at the level 0.05.

The accuracy of the method was analyzed based on the results of determinations of L-lysine in all samples except in samples No. 6 and No. 10 (Table 4). As it can be seen, the obtained recovery (109.5%) was somewhat higher than indicated by assay producer (90–105%) but it was comparable to recovery recommended by international requirements (90–110%) (Reason, 2003) as well as these obtained using HPLC method (109.4%) (Jajić et al., 2013). However, investigation of correlation between total L-lysine content in all samples determined using two methods revealed high linear correlation coefficient ($r = 0.9942$; $p < 0.001$). Also, high correlation coefficient was recorded for total L-lysine content obtained by MMPA method and reference value ($r = 0.9905$; $p < 0.001$).

Table 3. Results of total L-lysine determination in different RMs using two analytical methods

Sample No.	Sample type	Total L-Lysine content, %		
		MMPA	HPLC	Reference value*
8	Total mixture for laying hens	0.80	0.882	0.745
9	Dog food	1.0	1.057	0.895
10	Wheat	0.05	0.307	0.311
11	Total mixture for ducks 1	0.9	0.918	0.904
12	Total mixture for ducks 2	1.0	1.157	0.904
13	Cat food	1.1	1.360	1.012
14	Soybean grits	3.9	3.614	3.088

* National Reference Laboratory of Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic)

Table 4. Recovery of total L-lysine by MMPA

Sample No.	Recovery, %	Bias, %
1	96.7	3.35
2	98.7	1.31
3	98.8	1.20
4	108.1	8.09
5	126.7	26.70
7	106.2	6.23
8	107.6	7.65
9	114.0	13.97
11	105.1	5.09
12	112.6	12.65
13	113.9	13.94
14	125.6	25.65
Average	109.5±9.7	

Limit of detection of MMPA method was calculated from average value ($n = 3$) of turbidity of standard solution mass concentration 0.020% and threefold standard deviation for turbidity being 0.040%. Based on the sum of average turbidity values for this standard solution and tenfold value of standard deviation for turbidity, the limit of quantification (LOQ) was calculated, being 0.085% (Thompson et al., 2002). However, analysis of real samples without additional dilution does not allow determination of lysine content, which is due to matrix interference. Recalculation of LOQ based on additional dilution of the sample reveals the LOQ 0.85%. Contrary to MMPA, the HPLC method yielded lower values for LOD and LOQ. Determination of LOD and LOQ of HPLC method for lysine quantification was also performed according to standard solution analysis (Jajić et al., 2013). LOQ of the HPLC method for lysine determination based on the standard deviation of the response and the slope of the linearity plot, calculated as 10α/b, where α is the standard deviation of the *y*-intercept and *b* is the slope of the calibration curve being 0.106% (Jajić et al., 2013).

According to test manufacturer, indicated standard range of the method is 0.02–0.40% (ifp, VitaFast® L-Lysin) however, the results revealed that this method can be used for total L-lysine quantitative determinations in samples of feed and total mixtures for animal feed with content of total L-lysine ≥ 0.085% after 10-fold dilution, meaning that the content should be ≥ 0.85%. Another shortcoming of this method is test kit format, because kit for 96 determinations must be used in three times. It means that 32 wells should be used at once. Further, in contrast to HPLC method which can be developed for the determination of several amino acids at a time, MMPA is designed for determination of single amino acid.

CONCLUSIONS

The applied MMPA method proved to be simple and fast for the determination of total L-lysine in animal feed samples. According to presented results, investigated MMPA gave results comparable to HPLC method. Even though some earlier reports suggested potential application of microbiological techniques for determination of amino acids, current scientific literature still does not provide any distinctly described method. This article offers for the first

time the results of the investigation and validation of commercial test relying upon this principle.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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