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Validation of an HPLC method for the determination of amino acids in feed

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Abstract: The subject of this study was the validation of a high-performance liquid chromatography method for the analysis of amino acids in fodder. The contents of amino acids were determined in maize, soybean, soybean meal, as well as in their mixtures enriched with different amounts of methionine, threonine and lysine. The method involved the acid hydrolysis of the sample (6 h at 150 °C), automated derivatisation of the amino acids with the aid of *o*-phthaldialdehyde and 9-fluorenylmethyl chloroformate reagents, separation on a ZORBAX Eclipse-AAA column and detection using a diode-array detector. The method is characterized by high specificity (the difference between the retention times of the fodder samples and standard mixtures were below 1.7 %), wide linear range (from 10 to 1000 nmol cm^{-3}, r^2 = 0.9999), high accuracy (recovery 93.3–109.4 %), and the precision of the results (*RSD* below 4.14 % in the case of repeatability and below 4.57 % in the case of intermediate precision). The limit of detection and the limit of quantification were in the ranges 0.004–1.258 μg cm^{-3} and 0.011–5.272 μg cm^{-3}, respectively. The results demonstrated that the procedure could be used as a method for the determination of the composition of primary amino acids of fodder proteins.

Keywords: amino acids; feed; liquid chromatography; method validation.

INTRODUCTION

The determination of the amino acid composition of the proteins in food is of great importance.[1] Namely, the amino acid level is an indicator of the nutritional value of food and fodder proteins.[2] As a laboratory technique, the analysis of amino acid plays an important role in biochemical, pharmaceutical and biomedical fields.[3] Hitherto, several different methods have been developed for the

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determination of amino acids.[4–26] Mostly, the methods were based on the technology developed by Moore and Stein,[4] which includes post-column derivatisation and detection in the visible region on an amino acid analyser. These analyses are reliable, but costly and time-consuming.[9] The HPLC technique, combined with pre-column derivatisation of amino acids, has become a very important method for the analysis of amino acids.[9] It should be emphasized that pre-column derivatisation has gained wide acceptance and a number of different derivatisation reagents have been used.[10–18] One of the most popular derivatisation reagents is *o*-phthaldialdehyde (OPA), which ensures relatively easy derivatisation and rapid reaction in aqueous solution at room temperature.[3,18–20] Nevertheless, during the manual procedure of derivatisation by OPA reagent, due to the time differences between the reaction and injection, significant errors in quantification may occur. By applying an automated procedure with an autosampler, the exact time and sample volume of each step can be controlled according to the injector program, which prevents human error inherent in the manual procedure.[21] Furthermore, OPA derivatisation is suitable for the analysis of primary amines only; hence, secondary amino acids need to be derivatised by another reagent.[22] 9-Fluorenylmethyl chloroformate (FMOC) is such a reagent used for the derivatisation of secondary amino acids, including hydroxyproline, sarcosine and proline.[23] Liu[3] managed to bring together OPA and FMOC in an automated derivatisation procedure to enable the simultaneous detection of both primary and secondary amino acids. In addition to derivatisation, protein hydrolysis is also a very important procedure in the analysis of amino acids.[8] The first acid hydrolysis was performed in 1820 by Baconnot, who used sulphuric acid to hydrolyse gelatine, wool and muscle fibres.[8] This was demonstrated in a collaborative study performed by the Association of Biomolecular Resource Facilities,[7] which indicated that many laboratories obtained satisfactory results by performing the hydrolysis in 6 mol dm^{-3} HCl at 150 °C under vacuum for 1 h. The traditional hydrolysis with 6 mol dm^{-3} HCl for 20–24 h at 110 °C under vacuum may lead to losses of serine, threonine, and tyrosine. On the other hand, during the acid hydrolysis, some amide bonds between aliphatic amino acids are more difficult to cleave. The Ala–Ala, Ile–Ile, Val–Val, Val–Ile, Ile–Val, and Ala–Val linkages are resistant to the hydrolysis, and may require a longer hydrolysis time of 48 or 72 h at 110 °C.[24]

The present paper compares two methods of sample hydrolysis for the determination of the amino acid composition of proteins in maize. In the first procedure, the samples were hydrolyzed traditionally with 6 mol dm^{-3} HCl at 110 °C for 24 h, and in the other with 6 mol dm^{-3} HCl (containing 0.1 % phenol) under vacuum at 150 °C for 6 h. In both cases, the samples were automatically derivatised with OPA and FMOC, and analyzed inline by HPLC with DAD detection, according to the method published in an Agilent application note.[25] Since

the second procedure appeared to be more suitable, it was applied for the determination of amino acids in maize, soybean, soybean meal, as well as in their mixture enriched with different amounts of methionine, threonine and lysine. The procedure was validated based on the specificity, linearity, accuracy, precision, limit of detection and limit of quantification for different fodder samples.

EXPERIMENTAL

Materials

Acetonitrile (LC grade), methanol (LC grade), and phenol (*p.a*. grade), were purchased from Sigma-Aldrich (St. Louis, MO). Borate buffer, OPA and FMOC reagents and standard solutions of mixture of 15 amino acids (10, 25, 100, 250 and 1000 nmol cm^{-3}) were obtained from Agilent Technologies (Waldbronn, Germany). Hydrochloric acid, used for the preparation of 6 mol dm^{-3} and 0.1 mol dm^{-3} HCl, was obtained from Lach-Ner (Neratovice, Czech Republic). Sodium phosphate monobasic was purchased from Acros Organics (New Jersey, USA). Nitrogen gas was purchased from Messer Technogas (Belgrade, Serbia). LC grade water was produced by a Heming ID-3 system (Belgrade, Serbia). The reference material of a complete fodder mixture for piglets was purchased from the National Reference Laboratory of the Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic).

Apparatus

Vacuum hydrolysis tubes (19 mm×100 mm) were obtained from Pierce (Rockfors, IL). Cellulose membrane syringe filter (0.22 μm pore size), screw cap vials and screw caps were purchased from Agilent Technologies (Waldbronn, Germany). Blue-labelled filter discs (quant.) grade: 391 were obtained from Munktell (Bärenstein, Germany).

The hydrolysis was performed using a Reacti-Therm™ heating/stirring module (Thermo Scientific, Rockford, IL), while the evaporation procedure also included a Reacti-Vap™ Evaporator (Thermo Scientific, Rockford, IL).

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), and a Zorbax Eclipse-AAA column (150 mm× 4.6 mm, i.d., particle size 5 μm) (Agilent Technologies, Santa Clara, CA).

Procedure

Samples. Maize, soybean, and soybean meal were analyzed for their amino acid content. Then, a mixture of maize, soybean, and soybean meal was made in the mass ratio 70:15:15. The mixture was divided into four parts, and one part, marked as “zero”, was used as such, while the other three parts were supplemented with methionine, threonine and lysine in different concentrations. The mixtures “one”, “two” and “five” contained 0.1, 0.2 and 0.5 % of each added amino acids, respectively.

Preparation of protein hydrolysates. The fodder samples and the mixtures were finely ground to pass through a 0.5 mm sieve. The samples were then hydrolyzed by two different procedures. First, 0.1–1.0 g was weighed (equivalent to 10 mg nitrogen content) into a screw-capped test tube and 2 cm^3 of 6 mol dm^{-3} HCl was added. The tubes were capped and the samples were hydrolyzed for 24 h at 110 °C. After the hydrolysis, the mixtures were evaporated to dryness under vacuum. The hydrolysates were reconstituted in 2 cm^3 of 0.1 mol dm^{-3} HCl.[27]

In the second procedure, samples of the same mass were weighed into vacuum hydrolysis tubes and 7 cm^3 of 6 mol dm^{-3} HCl with 0.1 % of phenol were added and mixed gently. The hydrolysis was realised in a Reacti-Therm™ heating/stirring module for 6 h at 150 °C. After the hydrolysis, the samples were cooled to room temperature and evaporated to dryness using a Reacti-Therm™ heating/stirring module and Reacti-Vap™ Evaporator, at 70 °C under a stream of nitrogen. The residues were quantitatively transferred into 50 cm^3 volumetric flasks using 0.1 mol dm^{-3} HCl. The solutions were filtered through quantitative filter paper into glass tubes and the filtrates were purified using 0.22 μm pore size, cellulose membrane syringe filters.[27]

HPLC Determination. The chromatographic conditions employed were in accordance with the Agilent method,[25] except for mobile phase A, which consisted of 5.678 g of Na_2HPO_4 per 1 dm^3 water, adjusted to the pH 7.8 with a 6 mol dm^{-3} HCl solution (buffer strength 40 mmol dm^{-3}). The mobile phase B was acetonitrile–methanol–water (45:45:10, vol. %). Briefly, the hydrolyzed samples or the solutions the standard amino acid mixture were automatically derivatised with OPA and FMOC by programming the autosampler (1. draw 2.5 μl from vial 1 (borate buffer), 2. draw 0.5 μl from sample (position X), 3. mix 3 μl in air, max. speed, 2×, 4. wait 0.5 min, 5. draw 0 μl from vial 2 (water, uncapped vial), 6. draw 0.5 μl from vial 3 (OPA), 7. mix 3.5 μl in air, max speed, 6×, 8. draw 0 μl from vial 2 (water, uncapped vial), 9. draw 0.5 μl from vial 4 (FMOC), 10. mix 4 μl in air, max speed, 6×, 11. draw 32 μl from vial 5 (water), 12. mix 18 μl in air, max speed, 2× and 13. inject). After derivatisation, 0.5 μl of each sample was injected into a Zorbax Eclipse-AAA column at 40 °C, with detection at λ_1 = 338 nm and λ_2 = 262 nm. The separation was performed at a flow rate of 2 cm^3 min^{-1} employing a solvent gradient (vol. %,) as follows: 0 min, 0 % B, 1.9 min, 0 % B, 18.1 min, 57 % B, 18.6 min, 100 % B, 22.3 min, 100 % B, 23.2 min, 0 % B and 26 min, 0 % B.

RESULTS AND DISCUSSION

The hydrolysis is an extremely important step in amino acid analysis because it significantly affects the amino acid recovery.[28] During traditional acid hydrolysis, cysteine and tryptophan are destroyed,[29] and losses of serine, threonine and tyrosine are observed.[7] Besides the losses of some amino acids, their quantification after traditional hydrolysis is quite hard and insufficiently precise. As can be seen in Fig. 1, the peaks of all amino acids are unsuitable for quantification. On the other hand, when the samples were hydrolyzed according to the second procedure, with a shorter hydrolysis time and hydrolysate evaporation under a stream of nitrogen, amino acid quantification was very good (Fig. 2). This is also supported by the chromatogram obtained for the standard amino acid mixture (Fig. 3). The shorter period of hydrolysis and better quantification clearly confirmed the advantage of the second hydrolysis procedure, which was then used for all samples.

Hence, the validation parameters were estimated for the amino acids that gave satisfactory results at the recovery level, which included aspartate (ASP), glutamate (GLU), serine (SER), glycine (GLY), threonine (THR), arginine (ARG), alanine (ALA), tyrosine (TYR), valine (VAL), methionine (MET), phenylalanine (PHE), isoleucine (ILE), leucine (LEU) and lysine (LYS). Unfortunately, none of

the secondary amino acids, derivatised by FMOC reagent, gave satisfactory results. In order to achieve a complete assessment of the validation parameters of the method, both the amino acid standard mixtures and fodder samples were analysed. This provided the validation of all steps in the amino acid analysis of the fodders.

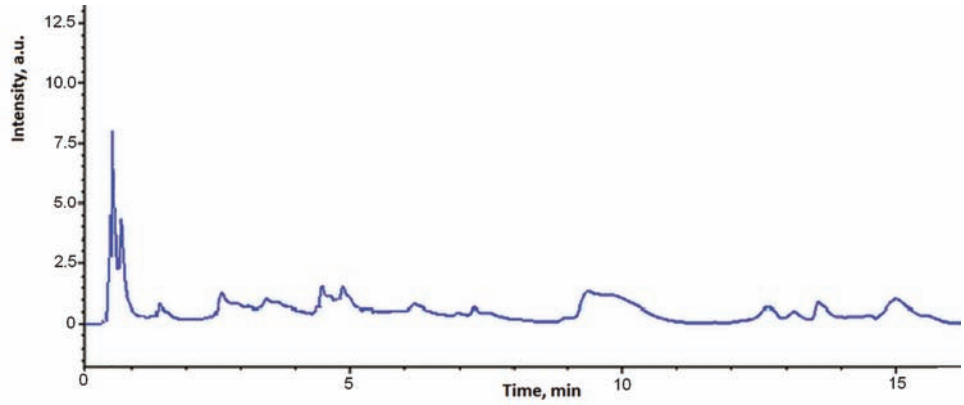

Fig. 1. Chromatogram of the maize hydrolysate obtained at 110 °C during 24 h.

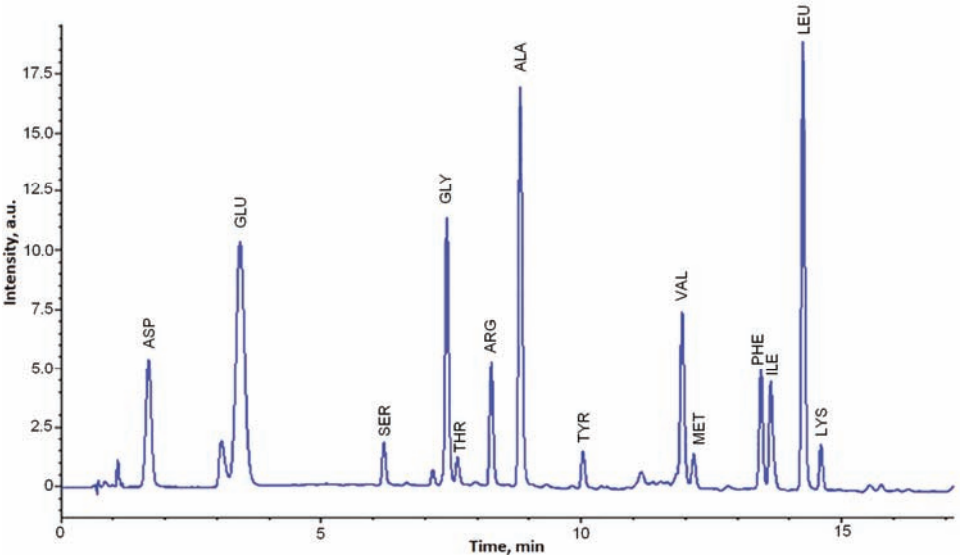

Fig. 2. Chromatogram of the maize hydrolysate obtained at 150 °C during 6 h.

Specificity. With the aim of studying the specificity of the method, a comparison was made of the retention times (t_R) of five different concentrations of standard solutions of the amino acid mixture (Table I) and seven different fodder samples (Table II). As can be seen from the Tables, the t_R values were not

significantly influenced either by the concentration of amino acids, or by the matrix. Namely, the relative standard deviation (*RSD*) of the t_R values of the standard amino acid mixtures was in the range from 0.02 to 0.52 % (Table I), and for the fodder samples, in the range from 0.02 to 0.46 % (Table II). As can be seen from the mean values of t_R for the standard amino acid mixtures and fodder samples, presented in Table III, there were slight variations between the t_R values, ranging from 0 to 1.7 %. According to Reason,[30] for specific methods, the difference between t_R values should be within ±3 %.

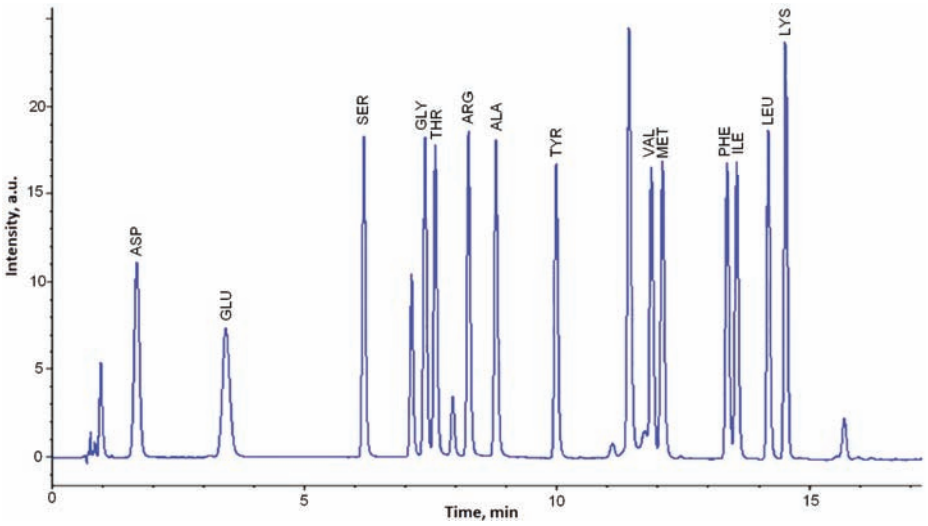

Fig. 3. Chromatogram of the standard amino acid mixture (1000 nmol cm^{-3}).

TABLE I. Specificity evaluation comparing retention times; retention times of the standard amino acid mixture (n = 3)

Amino acid	Concentration, nmol cm^{-3}					Average	*RSD* / %
	10	25	100	250	1000		
ASP	1.669	1.667	1.666	1.670	1.673	1.669	0.16
GLU	3.433	3.431	3.428	3.435	3.436	3.433	0.09
SER	6.170	6.165	6.166	6.166	6.167	6.167	0.03
GLY	7.384	7.373	7.374	7.373	7.378	7.376	0.06
THR	7.574	7.577	7.579	7.576	7.582	7.578	0.04
ARG	8.250	8.232	8.231	8.235	8.241	8.238	0.10
ALA	8.787	8.772	8.774	8.778	8.782	8.779	0.07
TYR	9.981	9.967	9.970	9.973	9.974	9.973	0.05
VAL	11.863	11.862	11.863	11.864	11.725	11.835	0.52
MET	12.084	12.078	12.079	12.080	12.078	12.080	0.02
PHE	13.367	13.370	13.365	13.367	13.362	13.366	0.02
ILE	13.558	13.554	13.553	13.544	13.549	13.552	0.04
LEU	14.180	14.184	14.179	14.179	14.175	14.179	0.02
LYS	14.512	14.513	14.509	14.521	14.519	14.515	0.03

TABLE II. Specificity evaluation comparing retention times; retention times of the amino acids in the fodder samples (n = 3)

Amino acid	Concentration, nmol cm^{-3}							Average	*RSD* %
	Maize soybean	Soybean Meal		Mixture 0	Mixture 1	Mixture 2	Mixture 5		
ASP	1.675	1.671	1.673	1.674	1.673	1.673	1.671	1.673	0.09
GLU	3.410	3.410	3.413	3.445	3.442	3.411	3.415	3.421	0.46
SER	6.168	6.161	6.164	6.168	6.170	6.170	6.167	6.167	0.05
GLY	7.370	7.367	7.372	7.374	7.370	7.373	7.375	7.372	0.04
THR	7.576	7.573	7.577	7.579	7.573	7.576	7.579	7.576	0.03
ARG	8.239	8.238	8.241	8.238	8.236	8.246	8.255	8.242	0.08
ALA	8.772	8.774	8.774	8.777	8.772	8.772	8.780	8.774	0.03
TYR	9.972	9.971	9.971	9.967	9.970	9.970	9.972	9.970	0.02
VAL	11.857	11.855	11.855	11.851	11.852	11.848	11.849	11.852	0.03
MET	12.076	12.075	12.073	12.069	12.071	12.068	12.067	12.071	0.03
PHE	13.359	13.357	13.355	13.351	13.351	13.345	13.342	13.351	0.05
ILE	13.548	13.544	13.544	13.539	13.538	13.531	13.530	13.539	0.05
LEU	14.171	14.168	14.170	14.163	14.160	14.152	14.152	14.162	0.06
LYS	14.520	14.515	14.517	14.511	14.509	14.499	14.500	14.510	0.06

TABLE III. The differences among the average retention times of the fodder samples and of the standard amino acids mixture

Amino acid	Average retention time, min		Difference, %
	Fodder samples	Standard mixtures	
ASP	1.673	1.669	0.4
GLU	3.421	3.433	–1.2
SER	6.167	6.167	0.0
GLY	7.372	7.376	–0.4
THR	7.576	7.578	–0.2
ARG	8.242	8.238	0.4
ALA	8.774	8.779	–0.5
TYR	9.970	9.973	–0.3
VAL	11.852	11.835	1.7
MET	12.071	12.080	–0.9
PHE	13.351	13.366	–1.5
ILE	13.539	13.552	–1.3
LEU	14.162	14.179	–1.7
LYS	14.510	14.515	–0.5

Linearity. The linearity was established using five standard solutions containing 10, 25, 100, 250 and 1000 nmol cm^{-3} of each amino acid. The data of peak area *vs.* amino acid concentration were treated by linear least squares regression analysis. The values of the slope, intercept and the coefficient of determination of the calibration curve for amino acids are given in Table IV. The linearity data obtained should obey the equation $y = bx + a$, where a is zero within the 95 % confidence limits, and the coefficient of determination (R^2) is greater

than 0.98.[30] In the present study, the area response obeyed the equation $y = bx + a$. The high value of the coefficient of determination indicates a good linearity, *i.e.*, R^2 in all cases was 0.9999 except for phenylalanine, where R^2 was 0.9986.

TABLE IV. Linearity data for the standard amino acids mixture

Amino acid	Slope×10^3	Intercept×10^4	R^2
ASP	1.584	0.60	0.9999
GLU	0.503	5.03	0.9999
SER	1.216	–0.11	0.9999
GLY	0.871	–0.12	0.9999
THR	1.393	–0.59	0.9999
ARG	1.997	–1.03	0.9999
ALA	1.004	–2.61	0.9999
TYR	2.195	–0.89	0.9999
VAL	1.226	0.28	0.9999
MET	1.676	0.99	0.9999
PHE	1.942	12.29	0.9986
ILE	1.533	1.19	0.9999
LEU	1.499	0.91	0.9999
LYS	1.585	17.32	0.9999

Accuracy. Accuracy may be defined as the agreement between the found value and the true value of the reference material provided by a reference laboratory, and can be presented as the percent recovery. This validation parameter was estimated by analyzing the reference material of the complete fodder mixture for piglets. The analysis was performed three times on three different days, by repeating the whole analytical procedure. After the analysis, the percent recovery was calculated for every well-recovered amino acid and the results are presented in Table V. As can be seen, the best percent recovery was obtained in the case of serine (99.4 %), while lysine had the highest (109.4 %) and methionine the lowest (93.3 %) percent recovery. The average percent recovery, considering all amino acids, was 104.6 %, which is within the range of 90–110 %, which can be considered acceptable.[30] Only in case of cystine and proline was no satisfactory recovery obtained.

*Precision***.** In this work, the precision was estimated by measuring the repeatability and intermediate precision. In the case of repeatability, the same sample of maize was derivatised and injected 6 times in a row and then the *RSD* was calculated for each amino acid. The obtained *RSD* values were in the range of 1.82–4.14 % (Table VI), which, being less than 5 %, could be considered acceptable.[30]

The intermediate precision was estimated by repeating the whole analytical procedure on three different days. The same maize sample was hydrolyzed three times, derivatised, and injected, separately on three different days. As can be seen

from Table VII, the *RSD* was less than 4.57 %, and, being below 10 %, it could be considered acceptable.[30]

TABLE V. Recovery for each amino acid in the reference material

Amino acid	Reference value, %	Found value, %				Recovery, %
		1	2	3	Average	
ASP	1.100	1.153	1.189	1.147	1.163	105.7
GLU	2.931	3.219	3.186	3.206	3.204	109.3
SER	0.654	0.634	0.650	0.666	0.650	99.4
GLY	0.592	0.634	0.642	0.652	0.643	108.6
THR	0.489	0.491	0.498	0.611	0.533	109.0
ARG	0.860	0.873	0.891	0.926	0.897	104.3
ALA	0.614	0.638	0.645	0.658	0.647	105.4
TYR	0.437	0.412	0.402	0.415	0.410	93.8
VAL	0.654	0.655	0.662	0.755	0.691	105.7
MET	0.326	0.298	0.305	0.308	0.304	93.3
PHE	0.697	0.732	0.742	0.796	0.757	108.6
ILE	0.517	0.541	0.553	0.548	0.547	105.8
LEU	1.044	1.097	1.111	1.127	1.112	106.5
LYS	0.790	0.853	0.877	0.862	0.864	109.4

TABLE VI. Repeatability of the determination of the amino acids in maize

Amino acid	Content of amino acids, %							*RSD* %
	1	2	3	4	5	6	Average	
ASP	0.629	0.614	0.661	0.602	0.620	0.603	0.625	3.56
GLU	1.998	1.944	2.099	1.914	1.958	1.946	1.983	3.62
SER	0.394	0.389	0.378	0.382	0.364	0.437	0.381	3.02
GLY	0.368	0.349	0.343	0.344	0.332	0.35	0.347	3.80
THR	0.350	0.324	0.350	0.332	0.341	0.335	0.339	3.36
ARG	0.414	0.402	0.398	0.396	0.375	0.398	0.397	3.56
ALA	0.701	0.694	0.687	0.671	0.635	0.682	0.678	3.88
TYR	0.224	0.217	0.215	0.210	0.201	0.214	0.213	4.01
VAL	0.463	0.449	0.438	0.438	0.420	0.426	0.442	3.59
MET	0.147	0.145	0.142	0.141	0.134	0.134	0.142	3.50
PHE	0.478	0.476	0.465	0.447	0.434	0.467	0.460	4.14
ILE	0.365	0.352	0.364	0.339	0.347	0.331	0.353	3.15
LEU	1.167	1.150	1.172	1.122	1.136	1.121	1.149	1.82
LYS	0.274	0.289	0.274	0.284	0.277	0.235	0.280	2.38

Limit of detection and limit of quantification. The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were determined based on the standard deviation of the response and the slope of the linearity plot. The *LOD* is calculated as 3.3α/b and *LOQ* as 10α/*b*, where α is the standard deviation of the *y*-intercept and *b* is the slope of the calibration curve.[30] The *LOD* and *LOQ* values calculated for each well-recovered amino acid are presented in Table VIII. The lowest *LOD* level was observed for arginine (0.004 μg cm^{-3}) and the highest

for leucine (1.707 μg cm^{-3}). The same observation was valid for the *LOQ* values, with the lowest (0.011 μg cm^{-3}) and the highest (5.172 μg cm^{-3}) values being obtained for arginine and leucine, respectively.

TABLE VII. Intermediate precision of the determination of the amino acids in maize

Amino acid	Content of amino acids, %				*RSD*
	1	2	3	Average	%
ASP	0.635	0.605	0.663	0.634	4.57
GLU	1.965	1.915	1.802	1.894	4.41
SER	0.412	0.398	0.385	0.398	3.39
GLY	0.365	0.353	0.368	0.362	2.19
THR	0.350	0.324	0.325	0.333	4.42
ARG	0.402	0.372	0.388	0.387	3.88
ALA	0.668	0.683	0.675	0.675	1.11
TYR	0.219	0.221	0.213	0.218	1.91
VAL	0.440	0.447	0.456	0.448	1.79
MET	0.139	0.143	0.135	0.139	2.88
PHE	0.431	0.458	0.442	0.444	3.06
ILE	0.361	0.349	0.358	0.356	1.75
LEU	1.162	1.148	1.131	1.147	1.35
LYS	0.286	0.298	0.305	0.296	3.24

TABLE VIII. *LOD* and *LOQ* values

Amino acid	*LOD*[a] μg cm^{-3}	*LOD*[b] mg g^{-1}	*LOQ*[a] μg cm^{-3}	*LOQ*[b] mg g^{-1}
ASP	0.118	0.0590	0.357	0.1785
GLU	0.150	0.0750	0.455	0.2275
SER	0.088	0.0440	0.267	0.1335
GLY	0.024	0.0120	0.074	0.0370
THR	0.069	0.0345	0.210	0.1050
ARG	0.004	0.0020	0.011	0.0055
ALA	0.028	0.0140	0.085	0.0425
TYR	0.023	0.0115	0.071	0.0355
VAL	0.044	0.0220	0.133	0.0665
MET	0.043	0.0215	0.129	0.0645
PHE	1.258	0.6290	3.812	1.9060
ILE	0.041	0.0205	0.123	0.0615
LEU	1.707	0.8535	5.172	2.5860
LYS	0.698	0.3490	2.115	1.0575

[a]μg of amino acid in cm^3 of solution injected into the HPLC; [b]mg of amino acid in g of fodder sample

CONCLUSIONS

The achievement of the study is a reliable and high throughput method for the separation and quantification of amino acids in the routine analysis of fodder. The method is based on the automated pre-column derivatisation of fodder samples using a combined OPA/FMOC reaction, which guarantees highly repro-

ducible reaction times and lack of degradation, and provides an important contribution to the results. The method appeared to be highly specific, accurate, precise, and linear across the analytical range. The *LOD* and *LOQ* values were in the range of 0.004–1.258 μg cm^{-3} and 0.011–5.272 μg cm^{-3}, respectively. The acid hydrolysis with 6 mol dm^{-3} HCl at 150 °C lasting 6 h, not only shortened the analysis time by 3 to 4 times, but also resulted in the chromatograms that were significantly more suitable the quantification of amino acids in fodder.

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И З В О Д

ВАЛИДАЦИЈА HPLC МЕТОДЕ ЗА ОДРЕЂИВАЊЕ АМИНОКИСЕЛИНА У ХРАНИ ЗА ЖИВОТИЊЕ

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Предмет овог истраживања је валидација методе за анализу аминокиселина у храни за животиње течном хроматографијом високе ефикасности. Одређен је аминокиселински састав кукуруза, соје и сојине сачме, као и њихове смеше, обогаћене различитим количинама метионина, треонина и лизина. Метода укључује киселинску хидролизу узорака (6 h на 150 °C), аутоматску дериватизацију аминокиселина помоћу реагенаса *о*-фталдиалдехида и 9-флуоренил-метил-хлороформата, раздвајање на колони ZORBAX Eclipse-AAA, и детектовање детектором са низом диода. Нађено је да је метода високо специфична (разлике између ретенционих времена узорака хранива и стандардних смеша су ниже од 1,7 %), са широким линеарним опсегом (од 10 до 1000 nmol cm^{-3}, r^2 = = 0,9999), високом тачношћу (ефикасност 93,3–109,4 %), и прецизношћу резултата (*RSD* испод 4,14% у случају поновљивости и испод 4,57 % у случају просечне прецизности). Граница детекције је у опсегу од 0,004 до 1,258 μg cm^{-3}, а граница одређивања од 0,011 до 5,272 μg cm^{-3}. На основу постигнутих резултата се може закључити да се метода може користити за квантитативно одређивање примарних аминокиселина протеина у храни за животиње.

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