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Determination of Amino Acids in Tunisian Animal Feedstuffs by HPLC-FLD

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The amino acids supplemented to animal feedstuffs cannot be determined directly. Thus, we need to optimize the sample preparation step as well as the analytical method. In this study, two hydrolysis mode were performed, one using 6 mol.L^{-1} hydrochloric acid at 105°C for 24 h and the other based on microwave radiation-induced hydrolysis. Moreover, a chromatographic method was developed for simultaneous determination of methionine and lysine in feedstuffs, using conventional High Performance Liquid Chromatography equipment equipped with an automated precolumn derivatization system using ortho-phthalaldehyde (OPA) / mercapto-2-ethanol (ME) reagents and the fluorescence detector. Reversed-phase chromatography appears to be the preferred method, with a C18 stationary phase and a gradient elution. Doehlert matrix was used to get the optimal separation for 18 amino acids. System Suitability Test (SST), Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), Repeatability and Specificity of the method were studied. The LOD calculated were 93 and 9.4 pmol.μL^{-1} and the LOQ were 173.5 and 36.2 pmol.μL^{-1} for methionine and lysine, respectively.

Keywords: amino acids, animal feedstuffs, HPLC-FLD, doehlert factorial design

INTRODUCTION

It is well known that animals require the amino acids to enable them to grow, reproduce and because they are the basic protein constituents and nutritionally important compounds. During digestion, proteins are broken down into amino acids and peptides which are absorbed in the organism and used to build new proteins, such as muscle [1]. Animal diets that are "balanced" with respect to amino acids contain a desirable level and ratio of the 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) required for maintenance, growth, reproduction and lactation [2]. It is noteworthy that the proteins of corn and other cereal grains used in feedstuffs are deficient in certain essential amino acids [3]. Consequently, these compounds were supplemented in diets formulation to correct this deficiency. The supplemented amino acids levels in feedstuffs used in animal diets must be

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determined to understand the nutritional value of these ingredients and to optimize diet formulations. Moreover, for economic and toxicological reasons it is necessary not to exceed their maximum recommended concentrations [4]. As lysine and methionine most often have been implicated as the most limited amino acids for the lactating dairy cows when they are fed a variety of corn-based diets [5, 6], this study was focused on these two amino acids.

In amino acid analysis the sample preparation step is of great concern. Usually, peptides and proteins hydrolysis is performed using 6 mol. L^{-1} hydrochloric acid at 105°C for 24 h [7]. However, another method based on microwave radiation-induced hydrolysis by a microwave oven is frequently used [7]. Moreover, developments in microwave equipment technology have enabled researchers to use dedicated apparatus for protein hydrolysis. These specific apparatus work on a single-mode or a multi-mode. Also, microwave radiation has been found to be very useful in protein hydrolysis, since this technique can generate high precision in breaking the peptide structure without causing any damage to the amino acid [8]. Furthermore, conventional hydrolysis methods take hours to break the peptides, whereas microwave protein hydrolysis reduces this to 10-30 min by processing samples at elevated temperatures (up to 200°C) [7] .

Generally, amino acid analysis techniques are based on ion-exchange separation coupled with post-column derivatization [7, 9]. Liming, W. et al. reported that the UPLC was a powerful tool for the analysis of amino acids in royal jelly [10]. Other techniques are based on pre-column derivatization and reversed-phase high-performance liquid chromatography [7, 11-14]. In the latter case, we report in the literature the instability of some pre-column derivatized amino acids occurring after few hours from derivatization reaction [15]. Some parameters of these techniques may influence the amino acids determination. It is then important to evaluate these parameters to see their level of influence. As many variables are involved throughout the chromatographic separation step in HPLC, experimental designs are powerful tools for the optimization of this technique. Among the different groups of designs, the Doehlert factorial design allows the optimization of the selected main variables with relatively few experiments [8-10]. Indeed, The choice of Doehlert design is justified by a number of advantages such as (1) its spherical experimental domain with an uniformity in space filling, (2) its ability to explore the whole of the domain, and (3) its potential for sequentially where the experiments can be reused when the boundaries have not been well chosen at first [16].

In this paper, the effect of 3 variables on amino acid chromatographic separation is studied. In order to improve the recovery of the extracted amino acids, we briefly compared two hydrolysis procedures of feedstuff proteins, a conventional acidic hydrolysis and a microwave radiation-induced hydrolysis. Moreover, we describe a fully automated system that enables the analysis of 18 amino acids using the commercially available ortho-phthalaldehyde (OPA) reagent, and mercapto-2-ethanol. The analytical system automatically derivatizes and injects the sample, allowing the routine amino acid analysis.

EXPERIMENTAL

Sources of feedstuff samples and preparation for analysis

Sources of feedstuff samples

The trade feedstuff samples type CF1 were obtained from an animal nutrition Tunisian manufacturer, they were used after grinding to fine particles and mixing. The formulated composition was as follows: 62 % corn, 26% soybeans, 8% $CaCO_3$ and 4% minerals and vitamins.

Sample preparation

Two different sample preparation methods were used. The first one was the conventional method with acid hydrolysis of feedstuff proteins with 6M hydrochloric acid for 24 h at 105 °C (figure 1) [7]. The second one was a method using microwave hydrolysis [7]. In this second method, we follow the same protocol used in the conventional method by acid hydrolysis except for the steps 3 and 4 which were replaced by microwave radiation at different energies and exposure time.

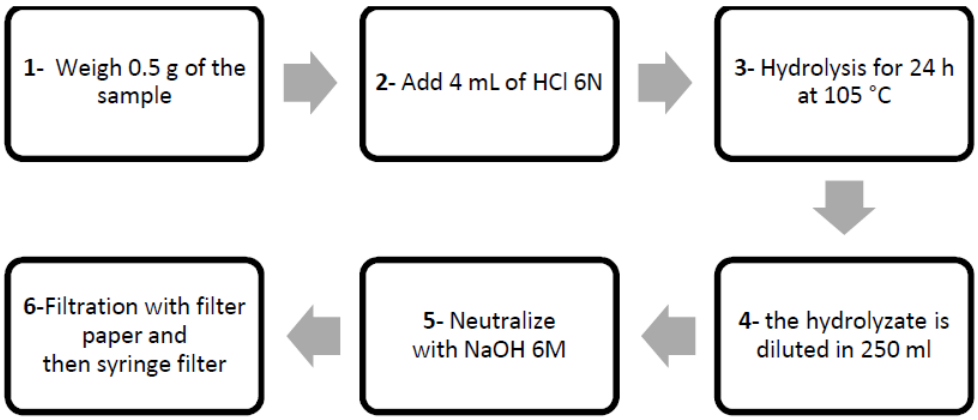


Figure 1: Sample treatment protocol

Reagents

Individual 18 amino acids standards were purchased from Sigma-Aldrich (USA). A volume of 1 mL standard solution (0.25 mM) of each amino acid was prepared in water and stored at -20 °C. The reagents used, like orthophosphoric acid, sodium phosphate dibasic (Na_2HPO_4), boric acid (H_3BO_3 99.5 %), sodium hydroxide (NaOH) and hydrochloric acid (34%) were of analytical grade and purchased from Carlo Erba (Italy). HPLC grade solvents were methanol (MeOH) (Fischer Scientific, Britain) and acetonitrile (ACN) (Sharlau, Spain). The *o*-phthalaldehyde (OPA, 99%) and the mercapto-2-ethanol (ME, 99%) were obtained from Sharlau (Spain) and Riedel-de Haën (Germany), respectively. The ultrapure water was coming from milli-Q system (Millipore, USA).

Instrumentation

The amino acid pre-column derivatization was performed using the OPA and the modified analytical procedure described previously in the literature [17, 18]. The HPLC separation of the amino acids was performed on a HP1100 Agilent chromatographer comprising a quaternary pump, programmable autosampler, solvent degasser, column oven, fluorescence detector (FLD) and photo-diode array detector (PDA) controlled by Agilent ChemStation software. The Hypersil ODS (C18) analytical column (250 x 4.0 mm, 5µm) with its respective precolumn were used and kept at 40°C for equilibration.

Automated derivatization procedure and chromatographic conditions

Derivatization reagents

The OPA stock solution was prepared by dissolving 10 mg of OPA in a mixture of 1 mL MeOH and 1 ml of 0.4 M borate buffer (0.4 M boric acid adjusted to pH 10 with

NaOH). Subsequently 10 µl of mercapto-2-ethanol was added to the mixture. The stock solution can be kept for one week at 4°C in the dark. Daily, a working OPA solution was performed by 1/10 dilution of the OPA stock solution in borate buffer.

Derivatization and injection procedure

Amino acids conversion to derivatives with OPA, according to the chemical reaction shown in Figure 2, should be using moderate excess of OPA relative to the substances in the mixture in order to avoid extensive formation of secondary products [19, 20]. This can be reach with the liquid autosampler by successively drawing from respective vials into the HPLC sample loop the following volumes: 2.5 µL borate buffer, 1.0 µL sample, 0.5 µL OPA reagent and 32 µL injection diluents (0.4 mL H_3P0_4 in 100 mL mobile phase). After drawing each volume, a mixing step was followed to homogenate the mixture. Finally, the mixture was automatically injected on the column.

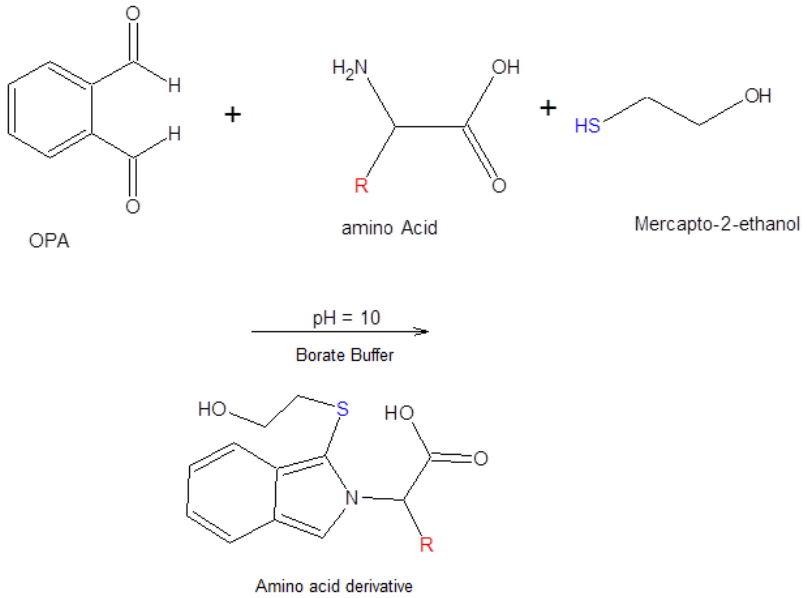


Figure 2: Scheme of the chemical reaction of derivatization of primary amino acids with OPA and mercapto-2- ethanol leading to amino acids fluorescent derivatives.

Chromatographic conditions

A binary gradient elution consisting of Na_2HPO_4 buffer (2.28 $g.L^{-1}$, pH 8) (eluent A) and a combination of MeOH and ACN (eluent B) was used at a flow-rate of 1 $mL.min^{-1}$. The gradient started with 70% A and 30% B and changed to 0% A and 100% B within 20 min (Table 1). So, in the Doehlert factorial design when we fixed the proportion of MeOH in eluent B, automatically we impose the percentage of ACN (%A + %MeOH + %ACN = 100).

According to the literature [14], the excitation and emission wavelengths were chosen to be 340 nm and 450 nm, respectively.

Table 1 Gradient elution program used for chromatographic separation of the amino acids

Time (min)	% Solvent (A)	% Solvent (B)
0	70	30
20	0	100

(A): Na_2HPO_4 buffer (2.28 $g.L^{-1}$, pH 8) ; (B): MeOH + ACN

Factors and experimental field

The objective of the experimental design is to find an optimal chromatographic separation between valine and methionine first and then between the remaining amino acids. The selection of the appropriate variables and responses was performed. Three variables, which could potentially affect the amino acid chromatographic separation in feedstuffs, were retained after a literature review of the various analytical protocols [17, 21, 22]. These parameters concern the combination of the organic modifiers, the water and the gradient elution method. It should be noted that we have chosen to study only the use of methanol (MeOH) and acetonitrile (ACN) as organic modifiers because tetrahydrofurane (THF) is unfavorable for the chromatographic separation [17]. Moreover, Georgy et al. reported that better reproducibility of the peak areas was observed when THF was not used [17]. The responses that we have chosen to study through this experimental design are by order of priority: R_s(valine, methionine), R_s(glutamine, histidine), R_s(glycine, threonine), peaks number and analysis time. Finally, variables and responses selected in this experimental design are presented in Table 2.

Doehlert factorial design

For the optimization a uniform shell design developed by Doehlert [23] was selected. A three variable Doehlert matrix design was selected which particularly is recommended for the HPLC analysis optimization [24]. The choice of this matrix relies on its modularity and reliability. It demands fewer experiments [23, 25, 26], which are flexible through the experimental domain [27]. In addition it allows the possibility of a free choice of the factors to be assigned to a large or a small number of levels [26]. Variables with stronger effect were chosen as mentioned above and four additional criteria were considered at a second level.

Then the number of experiments dictated by this kind of matrix was calculated according to the expression K^2+K+1 where K represents the number of variables to be studied or optimized. Hence, a Doehlert factorial design, with 13 experiments for 3 factors, was carried out as an optimization approach to find the best chromatographic conditions. The experiments, presented in Table 3, were executed in random order. For every experiment a blank reagent was also prepared. All measurements were run in triplicate for the sample and standard solutions. The average response value (n = 3) for every experiment is registered in Table 3, where R1, R2 and R3 were the responses representing the means of resolutions respectively for the pairs valine-methionine, glutamine-histidine and glycine-threonine. The response R4 corresponds to the number of peaks. Finally, R5 corresponds to the analysis time. The chosen range for the fraction of organic phase B varied between 10 and 90%. A reasonable range for the gradient time was selected to be between 10 and 30 min.

Table 2. Variables and responses selected in the Doehlert experimental design

	Variables (V) or Responses (R)
V1	Composition of the organic phase
V2	Percentage of the aqueous phase
V3	Gradient time
R1	R_s(valine, methionine)
R2	R_s(glutamine, histidine)
R3	R_s(glycine, threonine)
R4	peaks number
R5	analysis time

Software

All Doehlert factorial design data treatment has been performed using Nemrodw Software [28, 29] by LPRAI (Marseille, France)

RESULTS

Method Optimization

Optimization of the column temperature

Temperature has influence on all chromatographic techniques. For instance, retention time, peak shape, column efficiency, and total run time were affected by temperature because both the thermodynamics and kinetics of adsorption processes are functions of temperature (especially when using buffer solution) [30]. Thus, we studied the column temperature (T_c) required to enhance chromatographic separation of the 18 amino acids. Several experiences (T_c= 20, 30, 40, 45 and 50°C) showed that the best temperature was 40 °C (Figure 3).

Optimization of the feedstuff hydrolysis

Preliminary experiences with microwave-induced hydrolysis showed good selectivity toward some amino acids of feedstuffs samples. For optimization purposes several radiation time periods (5, 10, 15 and 25 min) were tested at constant radiation energy of 100 W. We observed an enhancement of the obtained amino acids concentration when the applied radiation exposure period increased from 5 min to 25 min. Then, we fixed this parameter at 25 min and we tried to optimize the microwave radiation energy by applying energy ranging from 100 W to 300 W. As the amino acids concentration increased proportionally to the radiation energy, we concluded that the optimum radiation energy was 300 W. Thus we have chosen 300W/25min as the optimum conditions of the microwave radiation–induced hydrolysis.

Table 3. The Doehlert experimental design (i) %A + %MeOH + % ACN =100

Exp, N°	V1 % buffer (A)	V2 % MeOH (i)	V3 Time (min)	R_s (Val, Meth)	R_s (Gln, His)	R_s (Gly, Thr)	Peaks Number	Analysis time (min)
1	90	5	20	0	0.63	1.18	17	14.25
2	10	45	20	0	0	0	2	3.92
3	***70***	***30***	***20***	***2.44***	***0.88***	***0.69***	***18***	***14.18***
4	30	0	20	0	0	0	3	1.75
5	70	0	20	0	0	0	7	5.48
6	30	70	20	0	0	0	4	1.55
7	**70**	**20.10**	**30**	**0**	**0**	**0.59**	**15**	**12.20**
8	30	23.30	10	0	0	0	4	1.90
9	70	10	10	0	0	0	11	8.83
10	**50**	**41.65**	**10**	**0**	**1.43**	**0.59**	**15**	**7.77**
11	30	46.70	30	0	0	0	4	1.63
12	50	8.35	30	0	0	0	3	1.96
13	50	25	20	0	0	0	7	4.44

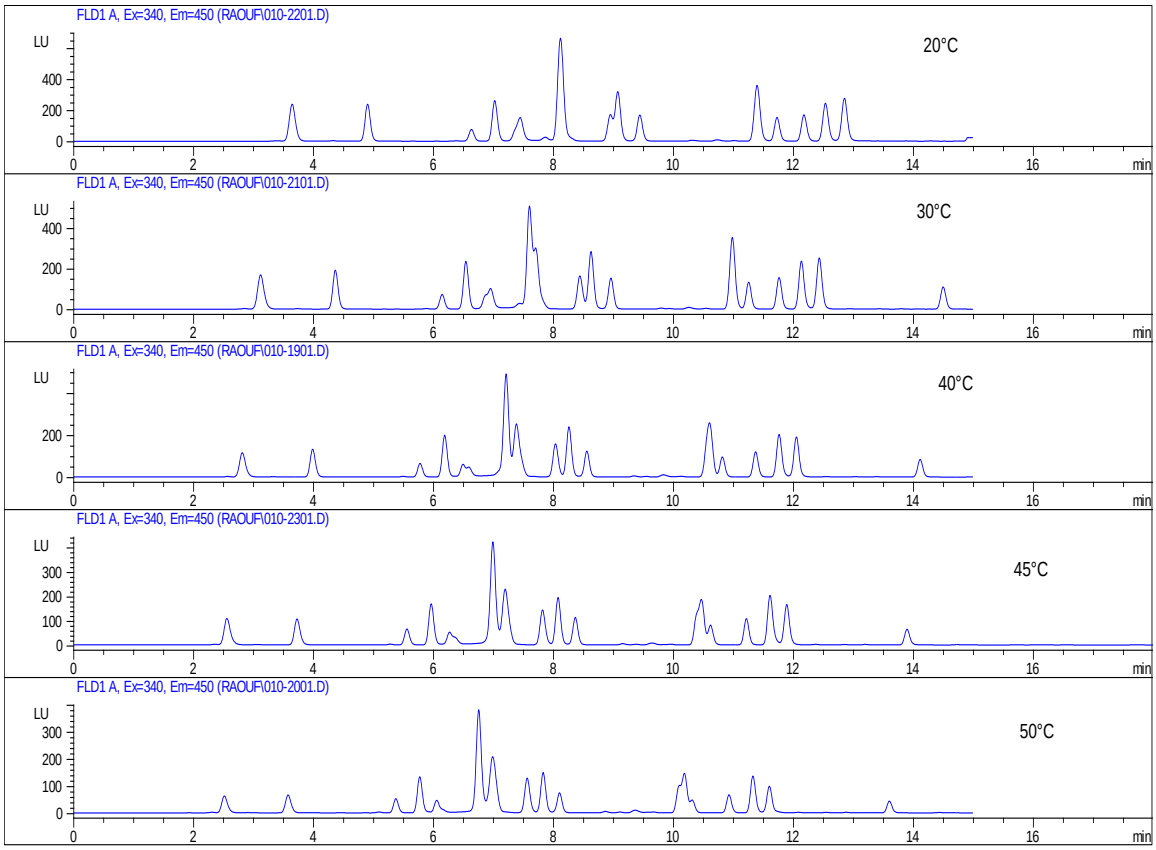


Figure 3: Optimization of the column temperature

A comparison between the obtained amino acids concentration at two different conditions of microwave radiation-induced hydrolysis (I) 100W/10min and (II) 300W/25min is presented in Figure 4. The results achieved showed that the condition (II) is more efficient than the condition (I).

To illustrate the difference between the conventional hydrolysis and microwave-induced hydrolysis we compared the amino acids concentrations obtained by the two methods. We remark that Hydrolysis efficiency is better with the conventional hydrolysis compared to microwave-induced hydrolysis (Figure 5), despite relatively high radiation time period (300W/25min). Thus, we decided to use the conventional hydrolysis method.

It's worth noting, that the used elution gradient in this optimization step was the same as that in section above, except that the eluent B is composed of a combination of ACN, MeOH and water in the proportions of (45/45/10).

Optimization of the separation

According to the Doehlert experimental design, the best conditions are obtained in the third experiment. Thus we maintain its elution gradient, which has started with 70% A and 30% B (MeOH) and changed to 0% A and 100% MeOH within a time period of 20 min. Under a column temperature of 40°C and the optimized elution gradient program the 18 amino acids could be almost separated (Figure 6)

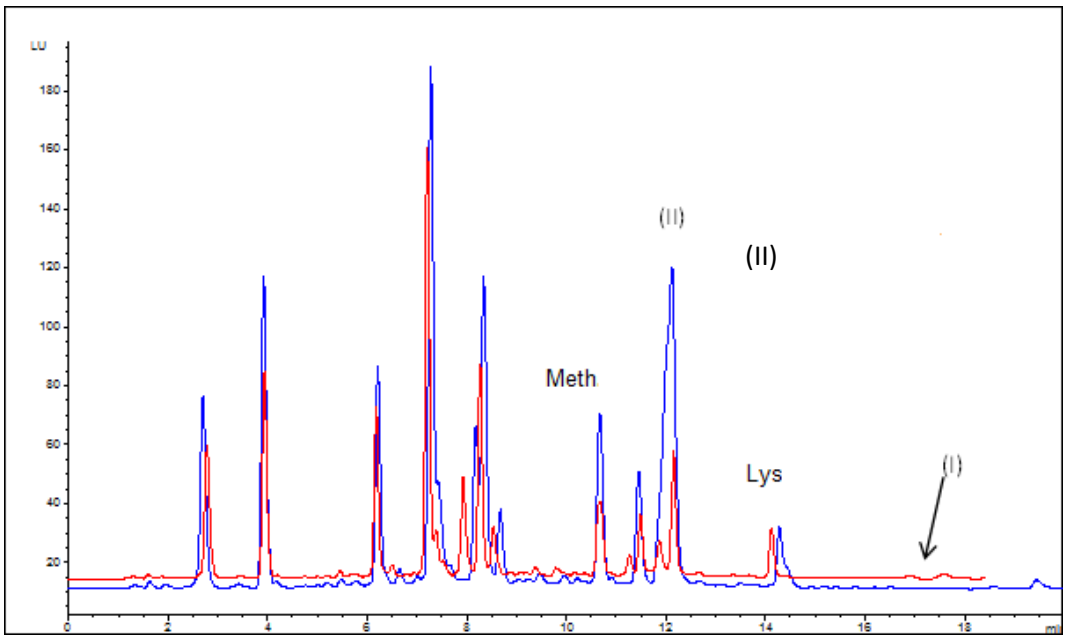


Figure 4: Overlay of chromatograms at two different conditions of microwave radiation-induced hydrolysis (I) 100W/10min and (II) 300W/25min

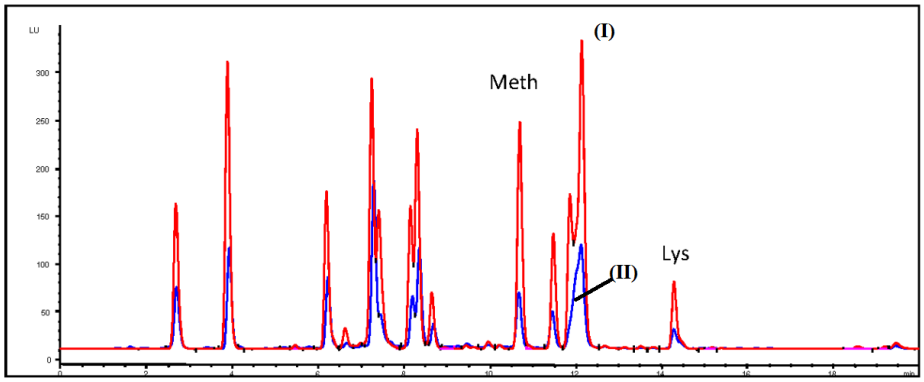
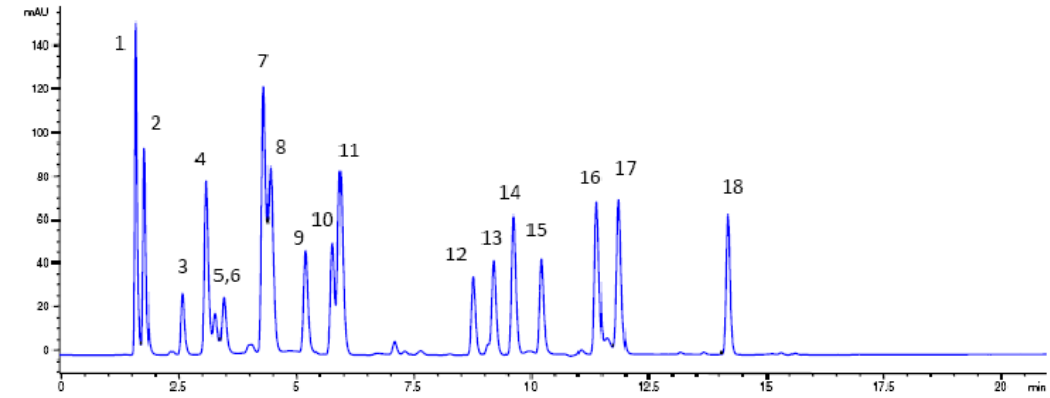


Figure 5

Figure 5: Overlay of chromatograms of the microwave-induced hydrolysis (I) and the conventional hydrolysis (II).

Method characteristics

Some essential validation parameters were evaluated. Indeed, as in the animal nutrition field a tolerance of +/-10% in amino acids concentrations in feedstuffs is allowed, we used the following validation parameters: System Suitability Test, linearity, limit of detection (LOD) and limit of quantification (LOQ), repeatability and specificity.



1. Aspartic acid (Asp)
2. Glutamicacid (Glu)
3. Asparagine (Asn)
4. Serine (Ser)
5. Glutamine (Gln)
6. Histidine (His)
7. Glycine (Gly)
8. Threonine (Thr)
9. Alanine (Ala)
10. Arginine (Arg)
11. Tyrosine (Tyr)
12. Valine (Val)
13. Methionine (Met)
14. Tryptophan (Trp)
15. Isoleucine (Ile)
16. Phenylalanine (Phe)
17. Leucine (Leu)
18. Lysine (Lys)

Fig. 6: Chromatogram of standard solution of 18 amino acids at a concentration of 500 pmol.µL^{-1} using Hypersil ODS (C18) column (250 x 4.0 mm, 5µm) operated at a column temperature of Tc=40°C.

System suitability test (SST)

The SST validation parameter allows evaluating the performance of both the analytical instrument before it is used for sample analysis and the analytical method before it is used routinely. This good analytical practice was performed immediately before the prepared feedstuffs samples in the laboratory are committed for analysis. Indeed, three injections of the working standard solution were performed and then the HP ChemStation software provides the tools to do automatically the SST. Thus several parameters were statistically controlled such as efficiency (N), tailing factor (Tf) and capacity factor (k') (Table 4).

Table 4 Capacity factors (K'), Efficiency (N) and Tailing Factor (Tf) of each amino acids

Amino acids	Capacity factor (K')	Efficiency (N)	Tailing Factor (Tf)
histidine	2.17	>3900	1.34
threonine	3.43	>7400	1.18
arginine	4.60	>11700	1.20
valine	7.57	>7600	1.21
methionine	8.38	>9000	1.32
tryptophan	8.45	>9100	1.23
isoleucine	9.03	>10100	1.24
phenylalanine	10.25	>12300	1.35
leucine	10.58	>13000	1.22
lysine	12.97	>16500	1.19

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity of the proposed method was assessed by estimating the linear dependence of the obtained peak area ratios on the concentrations of the standard solutions. The linearity of the FLD was tested by four injections of different standard solutions of the essential amino acids at concentrations of 10, 190, 390 and 550 pmol.µl^{-1}. The linear relationship between concentrations and peak area is given by a, b and r^2 (Table 5), where a and b are the coefficients of the regression equation y = ax + b, x being the concentration of the analyte, y the peak area and r^2 the coefficient of determination. The calibration curves have shown linearity from 10 to 550 pmol.µl^{-1} and a linear regression with r^2 of 0.991 and 0.983 for methionine and lysine (the compounds of interest), respectively. Obtained results showed good correlation between the peak area and the corresponding concentration of methionine. Whereas the correlation for lysine was only acceptable.

LOD and LOQ

Detection limit is the lowest concentration of the analyte that can be detected, but not necessarily quantitated as an exact value, under the stated experimental conditions. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1 and is confirmed by analyzing a number of samples near this value. While the limit of quantification is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy, it is frequently quoted as the concentration yielding a signal-to-noise ratio of 10:1 and is confirmed by analyzing a number of samples near this value.

In our study, the determination of the LOD and LOQ was based on the linearity study:

$$LOD = \frac{b_0 + 3s(b_0)}{b_1} \; ; \; LOQ = \frac{b_0 + 10s(b_0)}{b_1}$$

Where: S is the standard deviation; b_0 is the coefficient for the intercept that corresponds to the blank response and b_1 is the slope that corresponds to the sensitivity. Thus the LOQ was 173.5 and 36.2 pmol.µL^{-1}for methionine and lysine, respectively. While the LOD for methionine and lysine was 93 and 9.4 pmol.µL^{-1}, respectively.

Table 5 Retention times, calibration curves (y = ax + b) and correlation coefficient (r^2) of each amino acids

Amino acids	Retention times (min)	*a*	*b*	r^2
Histidine	3.23 ± 0.31	0.783	1.529	0.995
Threonine	4.52 ± 0.25	1.267	-3.134	0.994
Arginine	5.71 ± 0.23	1.123	12.65	0.992
Valine	8.74 ± 0.26	5.212	62.33	0.991
methionine	9.57 ± 0.29	4.482	85.93	0.983
Tryptophan	9.64 ± 0.35	0.769	-10.86	0.986
Isoleucine	10.23 ± 0.41	0.628	-2.354	0.995
phenylalanine	11.48 ± 0.39	0.609	-8.493	0.984
Leucine	11.81 ± 0.43	1.043	-7.537	0.991
Lysine	14.25 ± 0.38	2.612	4.236	0.996

Repeatability

The repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability was tested from six replications of one feedstuff sample on the same day and by the same operator using the same analytical method. The results showed that the method's repeatability was acceptable with a relative standard deviation (% RSD) of 1.28 % and 0.61% for methionine and lysine, respectively (Table 6).

Specificity

The specificity was performed to verify matrix interferences. Nine various types of feedstuff samples were fortified with appropriate methionine solutions and extracted following the same procedure. Figure 7 illustrates the superimposed calibration curves that of the standards added in water and that of the standards added in feedstuffs sample which is poor in methionine (Rye). We have found out that both curves were indistinguishable which demonstrates that there were no matrix interferences and do not influence recovery, as well. Thus, our method is specific for methionine (the same thing was observed for Lysine). It is worth noting that at the retention times of methionine and lysine there were no interference peaks which could influence our quantification.

Feedstuff samples analysis

Feeding stuff samples were obtained from an animal nutrition industry and the determination of Methionine and Lysine was performed using our validated analytical method. Methionine concentrations were found below the method's LOQ. Lysine could be accurately determined in feedstuffs samples due to concentrations that they were higher than that of the method's LOQ. The determined lysine concentration was 12.2 $g.Kg^{-1}$ which is in accordance with the theoretical concentration of 11.8 $g.Kg^{-1}$ announced by the industrial.

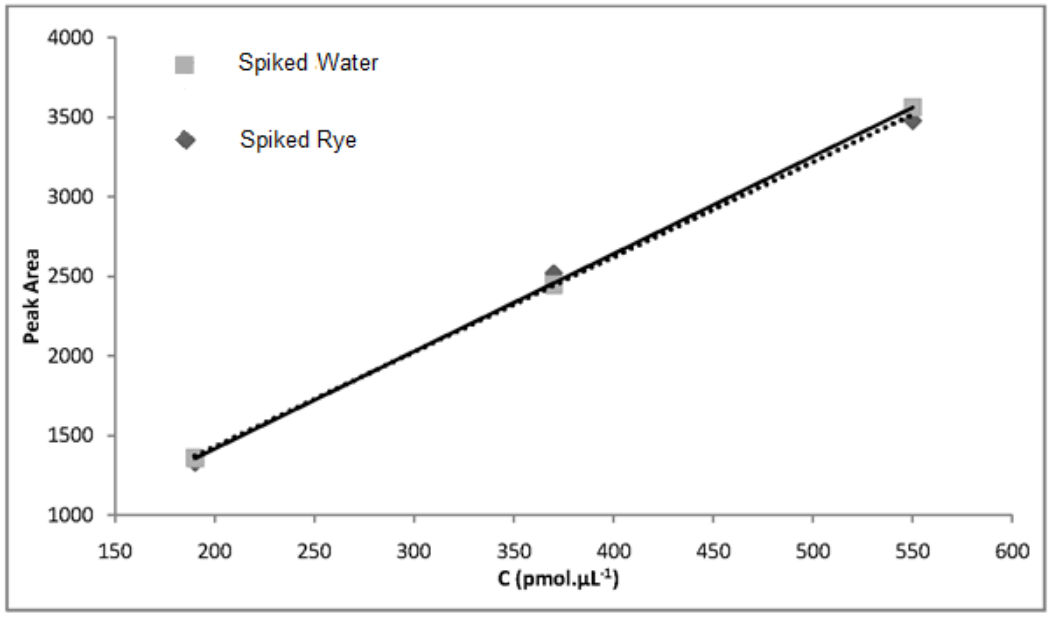


Figure 7: Specificity study of the analytical method by verifying matrices effects

Table 6 Repeatability (RSD %) study of the analytical method

	Peaks area		
	Mean value (n=6)	Standard deviation	RSD (%)
Methionine	79.93	1.02	1.28
Lysine	445.59	2.67	0.61

CONCLUSION

A chromatographic method, using conventional HPLC equipped with an automated precolumn OPA/ME derivatization and fluorescence detector, was developed, in order to determine methionine and lysine in animal feedstuffs. To resolve coelution of methionine and valine a Doehlert factorial design matrix was successfully used for the optimal separation of both amino acids (Rs = 2.44) and a mixture of 18 amino acids were satisfactorily separated in 14 min, as well. Some essential validation parameters of the method were studied. The developed method was revealed to be repeatable and specific for both amino acids. The linearity was studied for the targeted two compounds allowing to achieve a LOD of 93 and 9.4 pmol.µL^{-1} and a LOQ of 173.5 and 36.2 pmol.µL^{-1}for methionine and lysine, respectively.

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