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EVALUATION OF THE DIETARY PROTEIN USING AN OPTIMISED HPLC METHOD

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Abstract: Protein quality is determined by the type and concentration of the essential amino acids and by their bioavailability. Hence, the content of dietary essential amino acids from a protein or mixture of proteins is a factor which determines the feeding quality of the protein. The purpose of the study was to evaluate the quality of the protein from a high protein raw ingredient (corn gluten) using a chromatographic method (HPLC) under optimised experimental conditions. In this study, we used pre-column derivatization, separation by high-performance liquid chromatography (HPLC) and UV detection. The optimised method was used to determine the amino acids from high-protein raw ingredients commonly used in animal feeding (corn gluten, 61%CP). A set of 6 amino acids analyses has been performed in 6 different days, each sample being prepared in double. For characterization of data strings we used quality parameters: average, standard deviation, standard error, confidence level, precision, accuracy. For verifying the Gaussian shape of the strings we used the Kernel Density. For identifying and rejecting the outliers from the data strings we used the Q test. The very low values of the accuracy for several amino acids (glutamic acid, threonine, alanine, valine, phenylalanine, isoleucine, leucine) determined us to introduce the dilution stage (1:2) for the hydrolysed samples. The method can be considered as repeatable (precision in different days) and accurate (evaluation by tracing yields) for all the determined amino acids.

Key words: dietery protein, HPLC method

Introduction

The physiological role of the protein is to support the synthesis of the protein body and the production of other nitrogenous compounds such as hormones and neurotransmitters involved in a range of physiological functions *(Gilani et al., 2008)*. Protein quality is determined by the type and concentration of the essential amino acids and by their bioavailability. Hence, the content of dietary essential

amino acids from a protein or mixture of proteins is a factor which determines the feeding quality of the protein. Therefore, knowing the amino acid composition of the proteins is used to assess the feeding value of the dietary proteins. It may also allow evaluating the modification of the feeding value during the preparation, processing or storage of the feed *(Young et al., 1984)*. In order to meet the protein requirements, thus the amino acids requirements, the diet must include enough essential amino acids (EAA) in the right proportions and enough nitrogen for the synthesis if the semi-essential and non-essential amino acids. The fast and accurate determination of the amino acids from the raw materials used in animal feeding is necessary for a correct estimation of the feeding value of the protein.

The modern methods for the separation and quantification of the free amino acids include chromatographic methods (ion exchange chromatography – (IEC), high performance liquid chromatography (HPLC) *(Gilani, 2008; Fontaine, 2003)*, gas chromatography (GC) *(Conkerton, 1974)*, capillary electrophoresis *(Peace et al., 2005)*, near infra-red spectrometry (NIRS) *(Fontaine, 2003; Kryeziu et al., 2007)* and others. The feeding importance of the amino acids prompted the European scientists to join forces and develop new, reliable, fast and accessible methods and equipment of determination. Reference methods have been developed both in Europe and in the USA *(Fontaine, 2003)*. CE Regulation no. 152/2009 recommends for the amino acids, ion exchange chromatography with protein hydrolysis, post-column derivation with ninhydrine and detection in the visible spectrum. The chromatographic techniques and the detection systems must be improved continuously, however, by reducing the amounts of sample used for determination, by reducing the amount of reagents, by improving the identification process, the resolution and sensitiveness of the methods for amino acids analysis. *(Peace et al., 2005)*.

The purpose of the study was to determine the concentration of amino acids in a high protein raw ingredient (corn gluten) using a chromatographic method (HPLC) under optimised experimental conditions.

Materials and Methods

The optimised method was used to determine the amino acids from high-protein raw ingredients commonly used in animal feeding. We used corn gluten as sample which was manufactured in Romanian by Roquette S.A. According to the trading license, the sample had 61% protein

Principle of the method. The chain of amino acids from the protein molecule is broken by acid hydrolysis with HCl 6N. The sulphur amino acids cysteine and methionine, are oxidised with performic acid into cysteic acid and methionine-sulphone before the acid hydrolysis. The excess of performic acid is decomposed by adding sodium metabisulphite. After hydrolysis, the sample is derivatized with OPA (ortho-phtaldehyde), AMP (mercaptopropionic acid) and FMOC (9-fluorenylmethyl chloroformate). The amino acids are separated on a C18

column with silicagel, by reversed chromatography, eluted with pH7 buffer solution; reading is done in ultra-violet (λ = 338 and 262 nm, respectively). The concentration is calculated by relating the peak area of the sample to a calibration curve.

Reagents:
- hydrogen peroxide (H_2O_2), 30% (Merck);
- formic acid (CH_2O_2), 98-100% (Merck);
- thiodiethanol ($C_4H_{10}O_2S$), 98% Merck);
- DL-norleucine ($C_6H_{13}NO_2$), (Sigma);
- monohydrated L- cystic acid ($C_3H_7NO_5S \cdot H_2O$), 99% (Sigma);
- DL-methionine sulphone ($C_5H_{11}NO_4S$), 99% (Aldrich);
- disodium phosphate (Na_2HPO_4), (GPR Rectapur);
- sodium citrate ($C_6H_5O_7Na_3 \cdot 5_{1/2}H_2O$) 99%, (Chimopar);
- phenol (C_6H_6O), puriss (Riedel-de Haën);
- hydrochloric acid (HCl) 37% (Sharlau);
- methanol (CH_3OH) chromatographic purity 99.9% (Sigma);
- acetonitrile (C_2H_3N chromatographic purity 99.9% (Sigma);
- sodium hydroid (NaOH), 99% (Merck);
- ultrapure distilled water (H_2O) (Rompack);
- OPA – ortho-phthalaldehyde ($C_8H_6O_2$), (Merck);
- AMP – mercaptopropionic acid ($C_3H_6O_2S$), (Merck);
- boric acid (H_3BO_3), 99.5% (Merck);
- sodium bisulphite ($NaHSO_3$), 100.2% (Fisher Chemicals);
- FMOC - 9-fluorenylmethyl chloroformate (C15H11ClO2), (Merck).

Equipment:
- HPLC Surveyor – Thermo Electron, Waltham, Massachusetts, United States.
- column HyperSil BDS C18, with silicagel, dimensions 250×4.6 mm, particle size 5 μm
- analytical scale Sartorius, Germany.
- adjustable drying stove 103±2 ^{0}C, ITM 100, Memmert, Germany;
- pH-metre, WTW Wissenschaflich, Germany.
- centrifuge 5430 R, Eppendorf Germania,
- rotating evaporator, Buchi, Zurich, Switzerland.
- vacuum pump, Buchi Vac V-500, Zurich, Switzerland.
- filters with 0.2 μm pores.
- class A laboratory glassware, Schott Duran;
- glass tubes closing in fire, Termodensirom, Romania.

Sample preparation and chromatographic procedure. A set of 6 amino acids analyses has been performed in 6 different days, each sample being prepared in double. The samples were prepared using the same reagents, the same glassware, the same work hand and the determination was done with the same equipment.

This type of procedure was applied in order to evaluate the method in terms of accuracy, precision and repeatability.

The gluten sample was dried in the stove at 65^0C and ground. Amino acid determination was done on a sample of 0.3 g, weighed with analytical accuracy, in a glass tube. For the determination of the sulphur amino-acids – methionine and cysteine, the weighed sample was oxidized by adding 5 mL oxidizing mixture (performic acid, formic acid phenol ; hydrogen peroxide 4.5:0.5 (v:v)). The oxidized sample was kept for 16 h at 0^0C; afterwards, the surplus of performic acid was removed with sodium metabisulphite. The methods recommended by AOAC propose the use of hydro bromic acid as variant of removing the performic acid. The oxidized sample was hydrolysed with 15-20 mL HCl 6 M. The glass tubes are sealed at the flame of a gas bulb and they are introduced in the stove at 110^0C, for 24 hours. Another amount of sample (0.3 g), weighed with analytical accuracy, in glass tubes, was hydrolysed with 15-20 mL HCl 6M. The glass tubes are sealed at the flame of a gas bulb and they are introduced in the stove at 110^0C, for 24 hours and are used to determine the other amino acids (except for the sulphur amino acids and tryptophan); this is the most used method of hydrolysis which releases the amino acids from the protein molecule *(Gilani and Rutherfurd, 2009; Moughan, 2008)*. The hydrolysed samples were concentrated in a Rotavapour to dryness and transferred quantitatively in marked flasks of 10 mL with pH7 disodium phosphate buffer. The oxidized and hydrolysed samples were concentrated in a Rotavapour to a volume of 5 mL and transferred quantitatively in marked flasks of 25 mL with pH2.2 sodium citrate. From these solutions, 50 µL samples are derivatized with 50 µL OPA, 250 µL borate buffer, pH 10.2, 50 µL FMOC, 400 µL distilled water. 50 µL of the oxidized and hydrolysed samples were derivatized with 50 µL OPA, 300 µL borate buffer, pH 10.2, 400 µL distilled water. These solutions have been injected in the separation column from the HPLC. For some amino acids (aspartic acid, glutamic acid, valine, phenylalanine, isoleucine, leucine, threonine, alanine), we introduced an additional operation (dilution of the hydrolysed samples, before derivatization) besides the usual methods (AOAC, CE Regulation no. 152/2009), to make sure that the results will have high yields of amino acids tracing.

Statistical analyses. For characterization of data strings we used quality parameters: average, standard deviation, standard error, confidence level, precision, accuracy. For verifying the Gaussian shape of the strings we used the Kernel Density.

It was used the Q test for identifying and rejecting the outliers from the data strings. This test is indicated when we intend to reject a value from a small series of measurements. The values are ranged in increasing order, thus the diverging value will be at one of the ends of the string.

The calculation formula for Q is:

$$Q = \frac{|x_1 - x_2|}{x_1 - x_n}$$

where: x1 = unsure value; x2 = closest value to x1;

x1 - xn = difference between the largest value and the smallest value of the data string

Q value from experimental data (Qexp) and is compared with the table value (Qtable), considering 90% probability level. Q test interpretation is the following: if Qexp > Qtable, the unsure result is rejected; if Qexp < Qtable, the unsure result is accepted.

Results and Discussion

The determination of the total amino acids content of the forages by a chromatographic method requires protein hydrolysis, which must consider the stability of the individual amino acids and the resistance of the peptide bonds to hydrolysis.

The chromatographic separation was done by gradient elution of the amino acids, stage also described by Sherwood, 1990. This stage is accompanied by a stage of derivatization which can be done either before (pre-column), or after (post-column) separation *(Peace et al., 2005). Deyl et al. (1986)* have shown that the detection with ninhydrine is preferred when there is no need for high sensitiveness. In this study, the post-column derivatization with ninhydrine followed by cation-exchange chromatography and detection in the visible spectrum (recommended by AOAC and by CE Regulation no. 152/2009), was replaced by the pre-column derivatization, separation by high-performance liquid chromatography (HPLC) and UV detection. The samples were derivatized with ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl) also used by *Bütikofer et al. (1992), Fontaine (2003), Henderson et al. (2000)*. Table 1 shows the data strings obtained with the undiluted hydrolysed samples.

Table 1. Data strings obtained after the first run on the chromatographic column

Sample no.	Determined amino acids (g % g sample)											
	cys	meth	lyz	argin	glu. ac	gly	threo	ala	val	phenilala	isoleu	leu
1	0.75	0.94	0.82	1.59	5.191	1.54	0.738	2.595	0.909	1.632	0.874	4.990
2	0.72	0.98	0.91	1.55	5.621	1.58	0.745	2.654	0.944	1.676	0.917	5.210
3	0.74	0.94	0.99	1.50	6.539	1.65	0.726	2.751	0.884	1.569	0.895	5.309
4	0.75	0.97	0.99	1.46	5.343	1.39	0.702	2.710	0.900	1.559	0.890	5.310
5	0.77	0.95	1.08	1.69	5.104	1.56	0.863	2.987	0.984	0.023	0.998	5.788
6	0.76	0.92	0.91	1.27	5.197	1.57	0.625	2.744	0.787	1.286	1.059	5.876

Calculating the repeatability, regarded as precision of the determinations performed in different days and the accuracy considered as tracing yield compared to reference values *(Burlacu, 2002)* we obtained the data shown in Table 2..

Table 2. Quality parameters obtained by using the proposed method with undiluted hydrolysed samples

Quality parameters	cys	meth	lyz	argin	glu. ac	gly	threo	ala	val	phenilala	isoleu	leu
Average (g % g sample)	0.75	0.95	0.95	1.51	5.36	1.55	0.73	2.74	0.90	1.29	0.94	5.41
Reference value	0.73	1.13	0.88	1.53	12.01	1.65	1.56	4.83	2.44	3.12	2.46	8.08
Precision (%)	2.45	2.30	9.50	9.43	9.84	5.58	10.5	4.91	7.37	9.85	7.82	6.38
accuracy (%)	102.7	84.2	107.9	98.6	44.64	93.9	46.99	56.73	36.93	41.38	38.17	67.68

Table 2 shows precision percentages of maximum 10.5%, but very low values of the accuracy for several amino acids (glutamic acid, threonine, alanine, valine, phenylalanine, isoleucine, leucine). This is the reason why we introduced the dilution stage (1:2) for the hydrolysed samples. The dilution was performed with a buffering disodium phosphate solution (pH = 7). Table 3 shows the results obtained with the hydrolysed and diluted samples.

Table 3. Data strings obtained using samples as diluted hydrolysed

Sample no.	Determined amino acids (g % g sample)						
	ac glu	treo	ala	val	fenilala	izoleu	leu
1	11.60	1.65	4.70	2.23	3.13	2.00	8.95
2	11.85	1.81	4.83	2.32	3.31	2.08	10.60
3	12.23	1.64	5.03	2.39	3.54	2.12	9.76
4	10.82	1.62	4.39	2.15	3.16	1.99	8.50
5	11.71	1.66	4.82	2.25	3.42	2.05	9.43
6	12.38	1.60	5.00	2.34	3.59	2.15	9.84

The quality parameters were used to evaluate the data strings obtained by analysing groups of 6 corn gluten samples (Table 4)

Table 4. Quality parameters obtained by using the proposed methos with dilution of the samples

Quality parameters	ac glu	treon	ala	val	fenilala	izoleu	leu
average (g % g sample)	11.77	1,67	4.79	2.28	3.36	2.06	9.51
standard deviation	0.55	0,07	0.23	0.09	0.19	0.06	0.73
standard error	0.23	0,04	0.09	0.03	0.08	0.03	0.30
confidence level (95 %)	0.58	0,09	0.24	0.09	0.20	0.07	0.77

The above data allow us to evaluate the strings as homogenous, the standard deviations being below 10% of the average. Figure 1 show scientific data spreading.

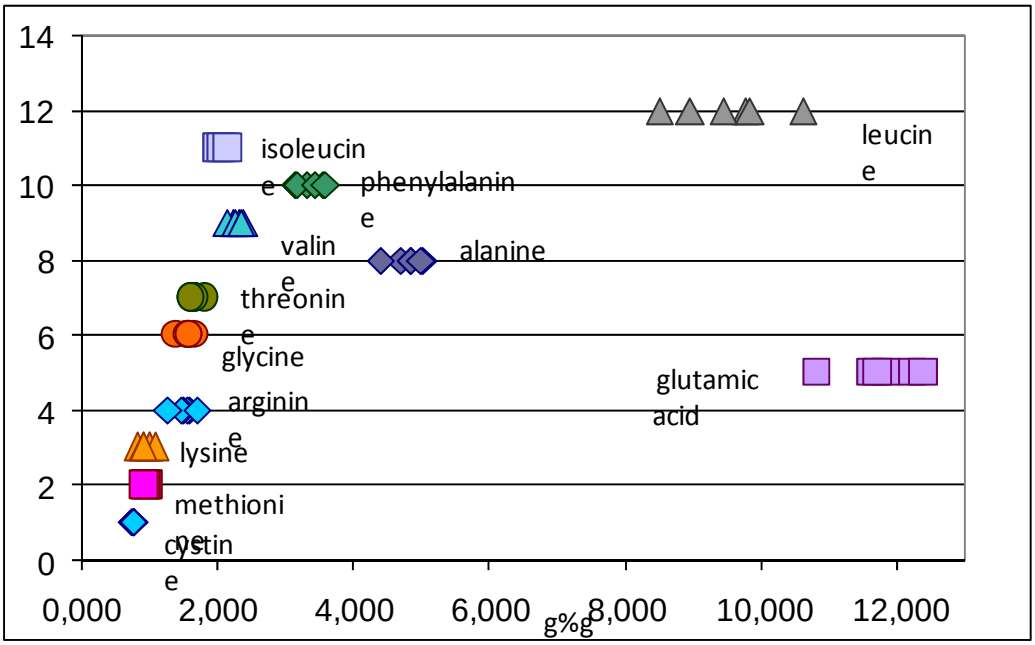

Figure 1. Spreading of the scientific data showing amino acid concentration in the corn gluten sample

In Figure 1 we can see a rather broad range of values for leucine and glutamic acid. For these amino acids, we verified whether these data strings observed the Gaussian distribution or they were contaminated with values belonging to a different family of analytical data. Fig. 2 shows the Kernel distributions for the analysed amino acids.

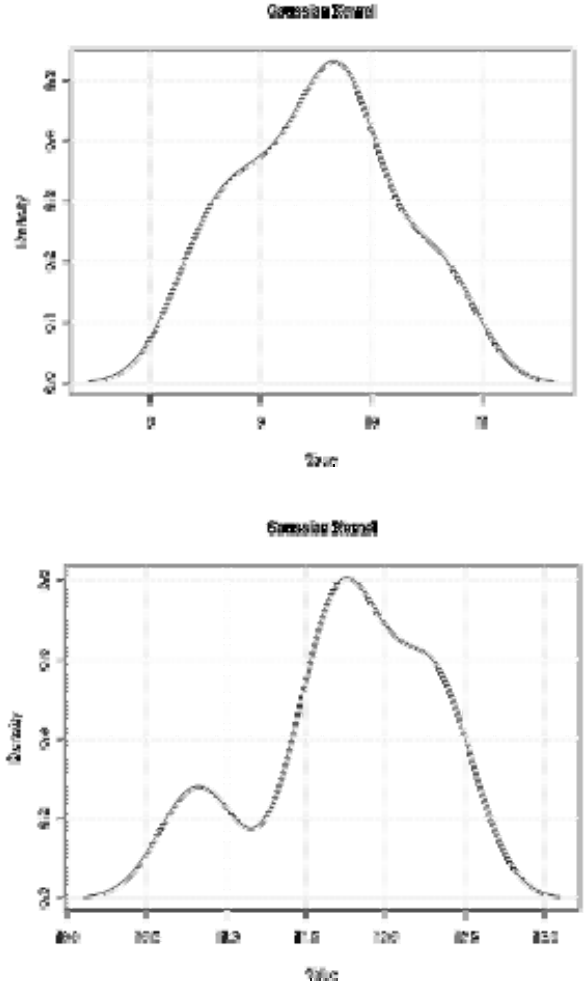

Figure 2. Kernel distribution for leucine and glutamic acid from the gluten sample

The figures show that the data of the 2 strings cannot be distributed perfectly on a Gaussian curve, which makes us believe that there are data contaminating the strings. It was applied the Q test *(Jatschi and Nascu, 2009)* to determine the values which don't belong to the string. After this, it was considered the extremes of the data strings. Table 4 shows Q value from experimental data (Qexp) and its comparison with the table value (Qtable), considering 90% probability level. For a string of 6 determinations with 90% probability, Qtable = 0.48.

Table 4. The results obtained by using Q test

GLUTEN					
leucine			glutamic acid		
String extreme	Qexp	Qtable	String extreme	Qexp	Qtable
8.50	0.22	< 0.48	10.82	0.50	> 0.48
10.60	-0.36	< 0.48	12.38	-0.098	< 0.48

The lower extreme of the data string for the glutamic acid must be removed, which is confirmed by the shape of the Kernel curve. The new average of the data string corresponding to the glutamic acid is 11.95 %.

The new data strings are then used to calculate the quality parameters of the method, as shown in Table 5.

Table 5. Quality parameters of the methods

Quality parameters	ac glu	treo	ala	val	fenala	izoleu	leu
average (g % g sample)	11.96	1.66	4.80	2.28	3.36	2.07	9.51
reference value	12.01	1.56	4.83	2.44	3.12	2.46	8.08
precision (%)	2.81	4.58	4.86	3.83	5.67	3.00	7.71
accuracy (%)	99.5	106.6	99.3	93.4	107.6	83.9	117.7

The evaluation of the performance parameters of the data strings show percentages ranging between 80 and 120 for accuracy and precision values lower than 10%.

Conclusion

A chromatographic method (HPLC) has been elaborated for the determination of the amino acids from the high-protein feed ingredients, by optimising the experimental conditions, introducing sample dilution as essential stage of the working protocol. Thus, the tracing yield was improved for some amino acids (aspartic acid, glutamic acid, valine, phenylalanine, isoleucine, leucine, threonine, alanine). The method can be considered as repeatable (precision in different days) and accurate (evaluation by tracing yields) for all the determined amino acids.

Ocena proteina u obroku korišćenjem optimalne HPLC metode

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Rezime

Kvalitet proteina zavisi od vrste i koncentracije esencijalnih amino kiselina i njihove bioraspoloživosti. Stoga, sadržaj esencijalnih aminokiselina u ishrani iz proteina ili mešavine proteina je faktor koji određuje kvalitet ishrane proteinima.

Cilj studije je bio da se proceni kvalitet proteina iz visoko proteinskog sirovog sastojka (kukuruzni gluten) koristeći hromatografski metod (HPLC) u optimalnim eksperimentalnim uslovima. U ovoj studiji smo koristili pretkolonu derivatizaciju, razdvajanje tečnom hromatografijom sa visokim performansama (HPLC) i UV zračenje. Optimalan metod je korišćen da se odrede aminokiseline u visoko-proteinskim sirovinama koje se najčešće koriste u ishrani životinja (kukuruz glutena, 61% CP). Set od 6 analiza amino-kiselina se obavlja u 6 različitih dana, svaki uzorak se duplo priprema. Za karakterizaciju nizova podataka koristili smo pokazatelje kvaliteta: prosek, standardnu devijaciju, standardnu grešku, nivo značajnosti, preciznost, tačnost. Za proveru oblika Gausove krive koristili smo Kernelovu gustinu. Za identifikaciju i odbijanje podataka koji su izvan niza podataka koristili smo Q-test. Veoma niske vrednosti za tačnost nekoliko aminokiselina (glutaminska kiselina, treonin, alanin, valin, fenilalanin, izoleucin, leucin) su uticale da uvedemo novu fazu razblaženja (1:2) za hidrolizovane uzorke. Metod se može smatrati kao ponavljajući (preciznost u različitim danima) i tačan (procena praćenog prinosa) za sve utvrđene amino kiseline.

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