Development and Validation of an RP-HPLC Method for Methionine, Cystine and Lysine Separation and Determination in Corn Samples

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In this paper a RP-HPLC method is developed and validated. Using a Hypersil BDS C18 column, a mobile phase: solvent A (phosphate buffer) and solvent B (water : acetonitrile : methanol, 20:20:60 v/v/v), a 45°C column temperature and a flow rate of 1.7 mL/min, the proposed method proved a good separation of the investigated amino acids, in 35 minutes.

Keywords: RP-HPLC method, methionine, cystine, lysine, corn

Corn (*Zea mays L.*) is a worldwide grown plant which has a high productive potential, twice as much compared to other cereals. It is a major component of the feed formulations for farm animals. Like in the other cereals, the feeding value of the corn protein is determined by the presence of essential amino acids (lysine, methionine, cystine and others). The determination of the essential amino acids concentration from corn is a key-instrument for the nutritionists, irrespective of the animal species. Hence, the necessity to develop more accurate and precise methods to determine the amino acids from the corn used in animal diets.

The high performance liquid chromatography (HPLC) has been used by many authors in order to analyse the amino acid content from all kind of feed matrices: sorghum grain [1], rice grain [2], peanut meal [1], fruits and vegetables [3-6], soybean [7], soybean meal [7], compound feeds [8], cotton [9]. EU Regulation no. 152/2009 [10], laying down the methods of sampling and analysis for the official control of feed, recommends for separation and quantification of amino acids the use of ion exchange chromatography (IEC) technique with post-column derivatization with ninhydrine and visible spectrum detection.

There are several methods describing the amino acid analysis in corn samples: IEC [11-15]; gas chromatography coupled with mass spectrometry (GC/MS) [16]; near infrared reflectance spectroscopy (NIRS) [17, 18] and HPLC [1, 7]. The reversed phase high performance liquid chromatography (RP-HPLC) was used for separation of the amino acids from corn, on a Superpac ODS-2 column coupled to an LKB dual pump HPLC system, controlled by a gradient generator with 50 minutes time of analyse [19].

The objective of our research was to develop and validate a fast and sensitive RP-HPLC method for methionine, lysine and cystine separation and determination. The proposed method was applied on several corn grain samples.

Experimental part

Equipment

- HPLC Finningan Surveyor Plus (Thermo-Electron Corporation, Waltham, MA);
- HyperSil BDS C18 column, with silica gel, dimensions 250 ×4.6 mm, particle size 5μm (Thermo-Electron Corporation, Waltham, MA);
- Rotary evaporator Buchi (Zurich, Switzerland);
- Drying stove BMT ECOCELL Blueline Comfort (Neuremberg, Germany);
- Sartorius analytical balance (Gottingen, Germany);
- Water purification system Mili-Q Ultrapure (Millipore, Billerica, MA);
- Laboratory mill, GRINDOMIX GM 200 Retsch (Haan, Germany);
- Glass vials closing in flame Termodensirom (Bucharest, Romania).

Class A glassware Schott Duran (Meinz, Germany) was used for transfer, dilution and storage of the working solutions.

Chemicals

Cysteic acid and methionine sulfone, reference materials for HPLC, were supplied by Sigma (Deisenhofer, Germany).

Lysine, aspartic acid, alanine and leucine reference materials for HPLC, were supplied by Merck (Darmstadt, Germany).

Stock solution of the standard amino acid mixture was prepared in hydrochloric acid (0.1 M) and contained 500 μg/ mL for each amino acid (cysteic acid, methionine sulfone, lysine, aspartic acid, alanine, leucine).

The reagents: hydrogen peroxide (30%), disodium phosphate, sodium citrate, phenol, hydrochloric acid (37%, d = 1.19 kg·L^{-1}), sodium hydroxide, boric acid, sodium disulphite (all analytical reagent grade), methanol and acetonitrile (HPLC grade) were supplied by Sigma (Deisenhofer, Germany).

Derivatization materials: orto-phtaldehyde (OPA), mercapto-propionic acid (AMP) and 9-Fluorenylmethyl chloroformate (FMOC), formic acid and thiodiethanol (all analytical reagent grade) were supplied by Merck (Darmstadt, Germany).

Buffer solutions: citrate (19.61 g/L, *p*H 2.2) and borate (33.35 g/L, pH 10.2) for the samples preparation, as well as phosphate (3.85 g/L, *p*H 7.8) for the mobile phase were prepared in our laboratory.

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Oxidation mixture: 889 g formic acid were mixed with 111 g water, and 4.73 g phenol were added; 4.5 mL from this solution were mixed with 0.5 mL hydrogen peroxide, and incubated at 20-30^{0}C for one hour in order to form performic acid, then was cooled for 15 min in a refrigerator before it was added to the sample.

Corn grain samples

For the amino acid determination, the corn grains samples were prepared according to Regulation 152/2009 [10], setting the sampling and analytical methods for the official control of the forages published by the Official Journal of the European Union. The official method [20, 21], also used by other authors, involves the acid hydrolysis for the release of amino acids from the protein molecules, preceded by oxidation with performic acid, for the sulphur amino acids.

The corn samples used in this study belong to 8 different hybrids produced by the National Agricultural Research Development Institute (INCDA – Fundulea, Romania) for multiple utilisations, among which that of raw feed ingredient. The sampling of the corn grains was done from collective samples (0.5 kg), reduced at 100 g sub-samples using the method of the sub-sampling quarters. The sub-samples were grounded using a laboratory mill and stored in plastic box, labelled.

For the determination of methionine and cystine, a quantity of 0.3 ± 2 . 10^{-4} g of corn samples (dried at 65°C and grounded), was weighed on a watch glass and quantitatively transferred into a glass tube with 5 mL of oxidation mixture. The oxidized sample was kept at 0°C for 16 h in a refrigerator; after that, the excess of oxidation reagents was decomposed by addition of 0.84 g sodium disulphite. In order to perform the hydrolysis, 20 mL 6 M HCl were added into the glass tube, closed in flame and introduced into the stove at 110°C, for 24 h. After full cooling at room temperature, the sample was concentrated using the rotary evaporator system and transferred quantitatively using sodium citrate buffer, *p*H 2.2 in volumetric flasks of 10 mL, filled to the mark with the same buffer.

For the determination of lysine, a quantity of 0.3 ± 2 . 10^{-4} g of corn sample (dried at 65°C and grounded), was weighed on a watch glass and quantitatively transferred into a glass tube with 20 mL 6 M HCl. The glass tube was closed in flame and introduced into the stove at 110°C, for 24 h. After complete cooling at room temperature, the sample was concentrated using the rotary evaporator system. The sample solution was transferred quantitatively using sodium citrate buffer, *p*H 2.2 in volumetric flasks of 10 mL, filled to the mark with sodium citrate buffer.

For methionine and cystine determination, 50 µL of final sample solution was derivatized with 50 µL OPA, 300 µL borate buffer *p*H 10.2 and 400 µL water. For lysine determination the final sample solution was derivatized with 50 µL FMOC, 50 µL OPA, 250µL borate buffer *p*H 10.2, 400 µL water. The derivatized samples were thereafter injected into the separation column of the HPLC.

Chromatographic separation

The chromatographic separation was performed by a RP-HPLC method using a Hypersil BDS column (C18), 250 x 4.6 mm, particle size 5 µm. During the separation, the column temperature was 45°C. Mobile phase used was a mixture of solvent A (phosphate buffer) and solvent B (water : acetonitrile : methanol (20:20:60 v/v/v)). The separation was carried out at a flow rate of 1.7 mL/min and at the solvent gradient (%, v/v) as follows: 2 min step at 0% solvent B; 23 min step that raised solvent B at 57%; 1 min step that raised solvent B at 100%; 3 min step at 100% solvent B; 1 min step that decreased solvent B at 0% and 5 min step at 0% solvent B; total analysis time of 35 min.

Validation of RP-HPLC method for the amino acids (methionine, lysine and cystine) separation and determination

The proposed RP-HPLC method has been validated according to the international regulations [22, 23]. The evaluated validation parameters were: accuracy, precision, linearity, detection and quantification limits, selectivity, recovery.

The accuracy was calculated with equation (1) and the bias interval was calculated with equation (2).

$$Accuracy\ \% = \frac{X}{\mu} \times 100 \quad (1)$$

$$Bias\ \% = \frac{X - \mu}{\mu} \times 100 \quad (2)$$

where: X is the average of the ten determinations; µ is the reference value.

Linearity was checked for all amino acids, using standard solutions having the concentrations between 25 and 200 µg/mL. According to the ICH [22], precision may be performed at different levels: repeatability, intermediate precision and reproducibility. In this study, the repeatability (intraday assay) was determined by ten consecutive injections of ten sample solution and then by calculating the relative standard deviation (RSD %) for the repeated measurements. Intermediate precision was studied by running the whole method on five different days. The precision expressed by the reproducibility (inter-days assay) was determined using five samples containing 100 µg/mL from each amino acid. The determinations were performed on 5 different days by two analysts, using different glassware and the same working method. Precision (RSD %) was calculated with equation (3).

$$RSD\ \% = \frac{S}{X} \times 100 \quad (3)$$

where: S is the standard deviation.

For precision, the required performance criterion is established by the Horwitz equation:

$$RSD = 2^{(1-0.5lgC)} \quad (4)$$

In order to check method accuracy, 10 standard samples with concentration of 100 µg/mL from each amino acid, were prepared from standard stock solutions. The limit of detection was calculated with equation (5) and the limit of quantification was calculated with equation (6).

$$LOD = 3.3 \times \frac{r}{S} \quad (5)$$

$$LOD = 10 \times \frac{r}{S} \quad (6)$$

where: r is the standard deviation of the response of the blank and S is the slope of the calibration curve.

The recovery was evaluated using five standard samples and another five standard samples to which 50 µg/mL from each amino acid were added. The percentage of analyte recovery was calculated with formula (7).

$$Recovery\ \% = \frac{X1 - X}{X1} \times 100 \quad (7)$$

where X is the average of the five sample concentrations without added analyte and $X1$ is the average of the five sample concentrations with added analyte.

Statistics

Data were acquired and processed with ChromQuest software, and the statistical analysis was done with STATVIEW for Windows (SAS, version 6.0).

Results and discussions

The feeding quality of the corn is influenced by the quality of its protein, which is the result of the ratio of the composing amino acids.

In order to develop a RP-HPLC method for the separation and determination of cysteine, methionine, lysine from corn samples, the method [24] was used as model. This method describes a procedure for separation and quantification of 24 amino acids from a standard mixture. This method is recommended by the authors for amino acid analysis of protein and peptide hydrolysates. Method [24] required the use of Zorbax Eclipse-AAA column, 150×4.6 mm, 5 μm particle size. During the separation of amino acids from standard mixture, the column temperature was 40°C. The used mobile phase was a mixture of solvent A (phosphate buffer) and solvent B (water : acetonitrile : methanol, 10:45:45, v/v/v). The separation was carried out at a flow rate of 2 mL/min and at the solvent gradient (%, v/v) as follows: 1.9 min at 0% solvent B; 16.2 min step that raised eluent B at 57%; 0.5 min step that raised solvent B at 100%; 3.7 min step at 100% solvent B; 0.9 min step that decreased solvent B at 0% and 2.8 min step at 0% solvent B; total analysis time was 26 min.

Using a Hypersil Gold column, 150×4.6 mm, 5μm particle size and the method [24] described above, the amino acids cysteine, methionine and lysine were separated but only from the standard mixtures. The method [24] applied on corn samples, showed a poor separation of lysine from leucine and also for the other amino acid pairs from corn samples.

In order to establish the optimal conditions for the separation and determination of amino acids from corn grain samples, the following parameters were varied: type of column, ratio of components of solvent B (water : ACN : MeOH), mobile phase flow rate, gradient program, chromatographic column temperature.

For a good separation of cysteine, methionine and lysine, the our method used a Hypersil BDS column (C18), 250 x 4.6 mm, particle size 5 μm. The method [24], recommends another type of column with a shorter length. Using a column with 250 x 4.6 mm and the working conditions described by method [24], the separation and quantification of amino acids were done in 45 min and the resolutions of separation of the three pairs of amino acids were: 1.15 for cysteine + aspartic acid, 1.60 for methionine + alanine and 0.85 for leucine + lysine.

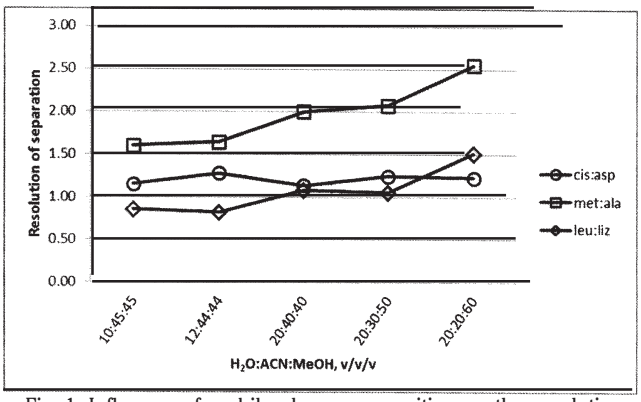

Fig. 1. Influence of mobile phase composition on the resolution

In order to establish the optimum composition of solvent B, a sample of standard mixture was analysed using the following components ratio: 10:45:45 (v/v/v); 12:44:44 (v/v/v); 20:40:40 (v/v/v); 20:30:50 (v/v/v); 20:20:60 (v/v/v), and the working conditions described in method [24] with a column with 250 x 4.6 mm. The resolution of separation for each of the three pairs of amino acids: cysteine + aspartic acid, methionine + alanine and leucine + lysine was improved by increasing the content of water and methanol in the mixture (fig. 1). The mixture of water : ACN : MeOH with 20:20:60 (v/v/v) ratio was chosen as optimal mobile phase composition due to the highest separation resolution values.

In a study of the influence of the mobile phase flow rate on the retention time and resolution of amino acids separation, the flow rate of the mobile phase was varied from 1 mL/min to 2 mL/min (table 1). A mobile phase flow rate of 1.7 mL/min was chosen to be optimal for a good peaks resolution in a reasonable time.

In order to optimize the total analysis time, the influence of the gradient program was investigated. Four gradient programs (further noted ***a***, ***b***, ***c***, ***d***) were designed. Gradients ***a***, ***b*** and ***c*** have 3 min at 0% solvent B at start, while gradient ***d*** has 2 min at 0% solvent B. Solvent B raised at 57% in 32 min for gradient ***a***, in 27 min for gradient ***b*** and ***c*** and in 23 min for gradient ***d***. After that, it followed a 1 min step that raised solvent B at 100% - for all the gradients, a 3 min step at 100% solvent B for gradient ***a***, ***c***, ***d*** and 7 min for gradient ***b***. The final steps are the same for all the gradients: 1 min step that decreased solvent B at 0% and 5 min step at 0% solvent B. By choosing the gradient ***d***, the total analysis time decreased from 45 min (using gradient ***a***), to

Flow rate mL/min	Amino acids	Resolution			Retention time min					
		Cys:Asp	Met:Ala	Leu:Lys	Cys	Asp	Met	Ala	Leu	Lys
1.0		1.22	3.04	1.00	6.18	7.01	22.76	24.68	39.12	39.58
1.2		1.26	3.22	1.84	5.19	5.90	21.34	23.15	37.70	38.68
1.5		0.98	2.94	1.84	4.20	4.78	19.86	21.58	36.07	37.45
1.7		1.02	2.59	2.25	3.74	4.27	19.04	20.78	35.09	36.53
2.0		0.98	2.32	2.04	3.19	3.67	18.07	19.65	34.25	35.84

Note: Cys – cysteic acid; Asp – aspartic acid; Met – methionine sulfone; Ala – alanine; Leu – leucine; Lys – lysine.

Table 1
INFLUENCE OF THE FLOW RATE ON THE RETENTION TIME VALUES AND ON THE RESOLUTION FOR THE SEPARATION OF CYSTEIC ACID, ASPARTIC ACID, METHIONINE SULFONE, ALANINE, LEUCINE AND LYSINE

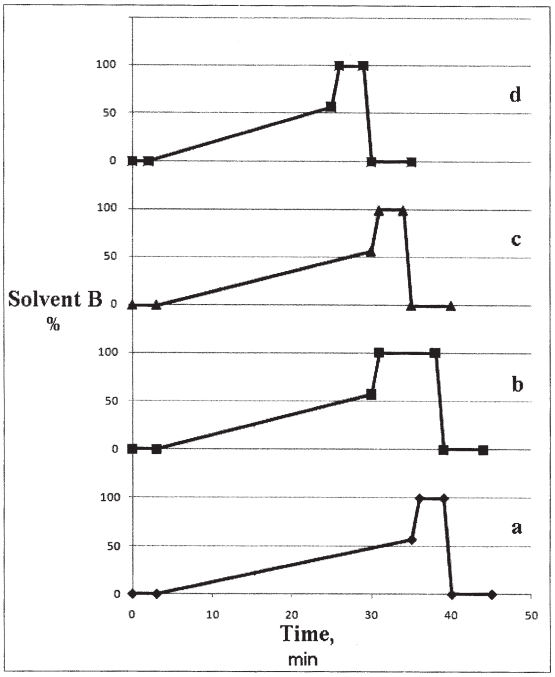

Fig. 2. Graphical representation of the gradient programs. a. 45 min gradient program; b. 44 min gradient program; c. 40 min gradient; d. 35 min gradient program

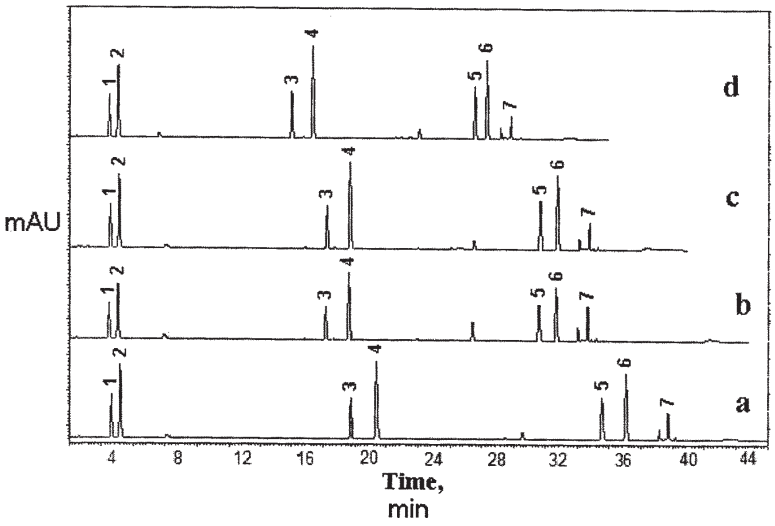

Fig. 3. The obtained chromatograms after applying the gradient programs: a. chromatogram corresponding to gradient **a**; b. chromatogram corresponding to gradient **b**; c. chromatogram corresponding to gradient **c**; d. chromatogram corresponding to gradient **d**. Note: 1-cysteic acid; 2-aspartic acid; 3-methionice; 4-alanice; 5-leucine; 6-lysine; 7-unknown compound

35 min. Figures 2 and 3 show the gradient programs profile and the corresponding chromatograms.

The influence of the column temperature on the retention time values and on the resolution of separation, was performed by testing different levels of temperature (30, 35, 40, 45^{0}C). As a result, the total analysis time decreased proportionally with the increase of column temperature. For 45^{0}C, the retention time of the last amino acid (lysine) on chromatogram decreased with 2.19 min. The resolution obtained for the 3 pairs of amino acids studied varied in the range 0.95 and 2.80 (fig. 4).

In conclusion, the amino acids: cysteic acid, methionine and lysine can be separated in 35 min using a Hypersil BDS column (C18), 250 x 4.6 mm, 5 μm particle size. Mobile phase consists in a mixture of solvent A (phosphate buffer 3.85 g/L) and solvent B (water : acetonitrile : methanol, 20:20:60, v/v/v), column temperature 45°C and a flow rate of 1.7 mL/min and the gradient program ***d***. Figure 4 shows a chromatogram obtained under these conditions, where the retention times are the following: 2.91 min for cysteic acid, 3.27 min for aspartic acid, 13.28 min for methionine, 14.53 min for alanine, 24.47 min for leucine and 25.17 min for lysine.

RP-HPLC Method Validation

The RP-HPLC method used for the separation and quantitative determination of methionine, cysteine and lysine was validated according to the international rules [22, 23].

The required performance criterion for accuracy is the bias in interval of -2 – 2%. For 10 repeated determinations and 9 degree of freedom [25], the value given in the table is t = 2.26. The obtained results for accuracy were evaluated using the Student's t test [26]. The calculated t values (between -2.06 and -0.80), were smaller, in all cases than the theoretical one. As shown in table 2 the data belong to the same population of values as the reference value, so the method can be considered validated for the accuracy parameter. The correlation coefficients, R^2 of the linear regression equations exceeded 0.9985, demonstrating a good correlation between the measured response (area of the peak) and the concentration of the analyte. The LOD value for the investigated amino acids ranged between 0.03 and 0.34 μg/mL, and the LOQ value between 0.10 and 1.04 μg/mL. The detection and quantification limits obtained for the proposed method are comparable with the values reported by other authors for

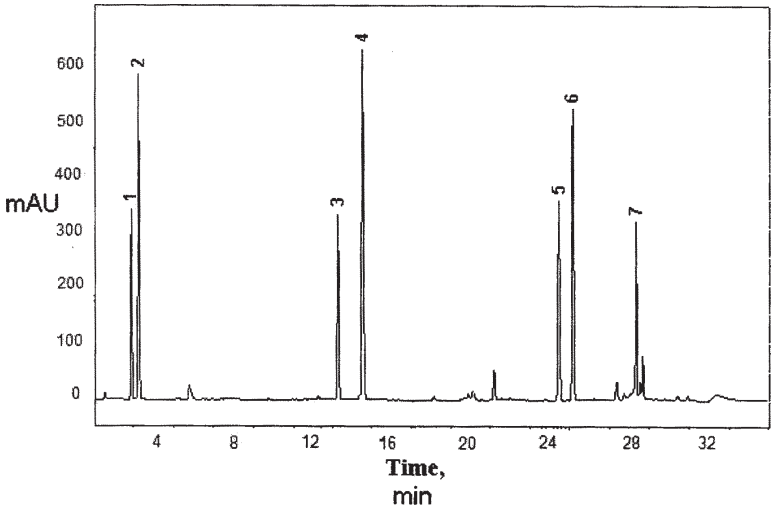

Fig. 4. Example of chromatographic separation of a standard mixture of aminoacids using the proposed method: 1-cysteine acid; 2-aspartic acid; 3-methionine; 4-alanine; 5-leucine; 6-lysine; 7-unknown compound

Table 2
OBTAINED RESULTS FOR THE VALIDATION OF THE RP-HPLC METHOD FOR THE DETERMINATION OF CYSTEINE, METHIONINE AND LYSINE

Parameters	Cystine	Methionine	Lysine
ACCURACY			
Accuracy	99.07	98.37	98.87
Bias	0.93	1.63	1.13
Calculated t	-0.80	-2.06	-1.33
PRECISION			
Repeatability – RSD (%)	3.71	4.16	2.71
Intermediate precision – RSD (%)	6.29	5.39	7.21
Reproducibility – RSD (%)	4.24	4.16	3.67
LOWER LIMITS OF DETECTION AND QUANTIFICATION			
LOD (μg/mL)	0.34	0.03	0.15
LOQ (μg/mL)	1.04	0.10	0.46
RECOVERY			
Value (%)	95.76 ± 9.58	102.90 ± 4.30	95.27 ± 7.12

Table 3
RESULTS OBTAINED FOR CYSTINE, METHIONINE AND LYSINE DETERMINATION IN CORN GRAIN SAMPLES USING THE RP-HPLC PROPOSED METHOD AND THE METHOD [24]

Sample no.	Cystine %		Methionine %		Lysine %	
	RP-HPLC proposed method	method [24]	RP-HPLC proposed method	method [24]	RP-HPLC proposed method	method [24]
1	0.193	0.211	0.179	0.189	0.169	0.211
2	0.204	0.203	0.162	0.179	0.218	0.248
3	0.183	0.204	0.163	0.165	0.237	0.263
4	0.164	0.172	0.153	0.163	0.232	0.250
5	0.281	0.263	0.195	0.205	0.216	0.234
6	0.220	0.216	0.161	0.180	0.224	0.238
7	0.183	0.194	0.164	0.189	0.205	0.240
8	0.182	0.191	0.205	0.219	0.252	0.254

the same type of analyte [27 - 29]. For a concentration of 1 mg / L, the maximum accepted RSD value is 10.72%, according to Horwitz equation [30]. The obtained values for RSD were between 2.71 - 6.29% for all the investigated amino acids. The range 80 - 120% is the required performance criteria for recovery. The values obtained for recovery varied from 95.27 to 102.90 for all the investigated amino acids. The results obtained from parameters evaluated were in the range of performance criteria required and this provided the validation of all steps in the amino acid analysis.

Amino acids determinations in corn grain samples

The proposed RP-HPLC method was applied to the determination of the essential amino acids: cysteine, methionine and lysine in real samples. Eight corn grain samples belonging to 8 hybrids, were collected for analytical determinations. The results obtained by the proposed method, were compared with those provided by application of the method [24] but using a 250 x 4.6 mm column dimensions. Table 3 shows the results obtained for cysteine, methionine and lysine determinations in the corn grain samples.

As shown in figure 5, there is a good correlation (R^2 = 0.9312 for cystine, 0.8620 for methionine, and 0.804 for

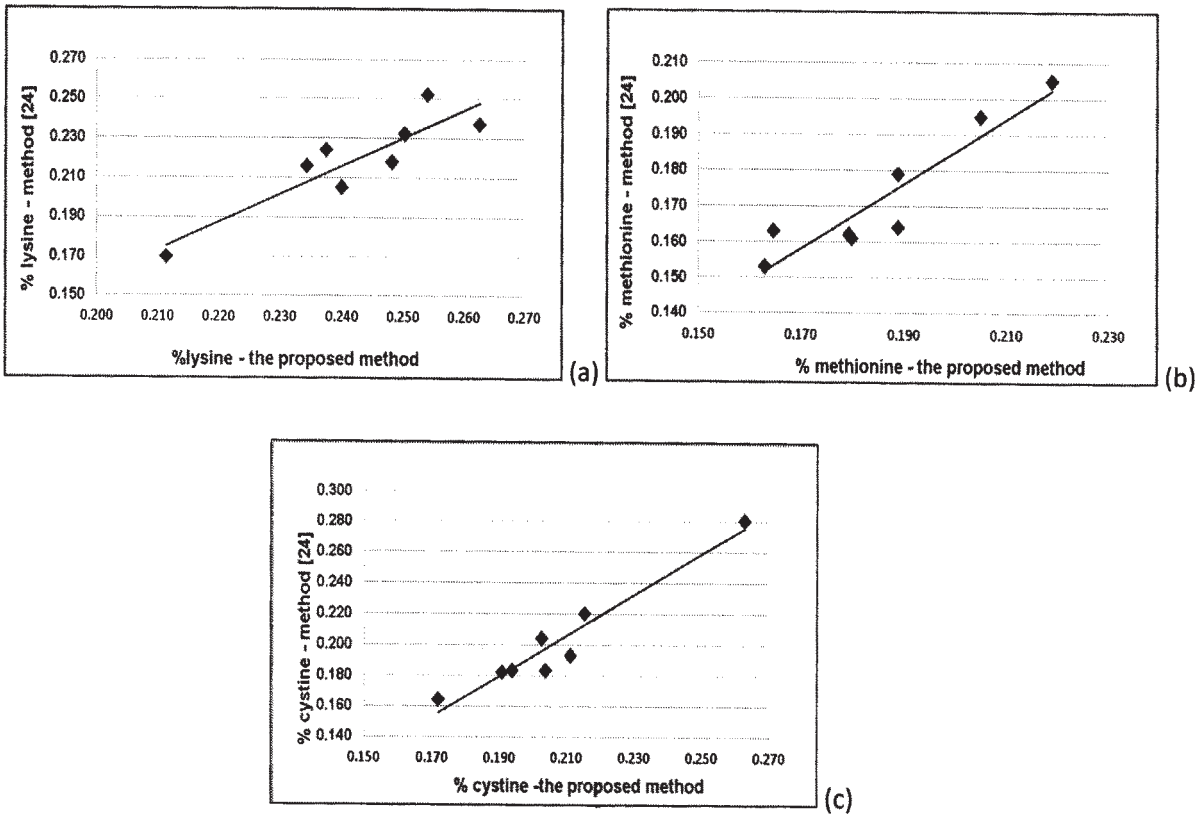

Fig.5.Correlation plot for amino acids concentrations determined by the proposed method (Hypersil BDS column (C18), 250 x 4.6 mm; 5μm particle size;45°C column temperature; mobile phase: solvent A - phosphate buffer, and solvent B - water: acetonitrile : methanol (20:20:60, v:v:v); 1.7 mL / min flow rate, non-linear gradient; time of analysis: 35 min), and by the method [24] (Hypersil BDS column (C18); 250 x 4.6 mm; 5 μm particle size; 30°C column temperature; mobile phase: solvent A - phosphate buffer, and solvent B - water acetonitrile : methanol (10:45:45, v:v:v); 1 mL / min flow; non-linear gradient; time of analysis: 45 min)

lysine, respectively) between the values determined with the proposed method and those obtained by application of the method [24] (using a 250 x 4.6 mm column dimensions).

In conclusion, the proposed RP-HPLC method enables a rapid and sensitive amino acids determination from corn grain samples.

Conclusions

This paper presents the development and validation of a RP-HPLC method for determination of cysteine, methionine and lysine from corn grain samples. These amino acids can be separated using a Hypersil BDS column (C18), 250 x 4.6 mm, 5 μm particle size. The separation and determination of cysteine, methionine and lysine were done in 35 min, with a column temperature of 45°C, a mobile phase composed by a mixture of two solvents (solvent A – phosphate buffer and solvent B – water : acetonitrile : methanol, 20:20:60, v/v/v), a flow rate of 1.7 mL/min and a non-linear gradient.

The proposed method proved to be accurate and precise as shown by the results of validation and of the corn grain samples analysis. The application of the RP-HPLC method on 8 corn grains samples showed good results for the investigated amino acids.

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