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Determination of amino acids in fodders and raw materials using capillary zone electrophoresis

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Abstract

Two schemes were offered for analysis of amino acid contents in fodders and raw materials for mixed fodders by capillary zone electrophoresis (CZE). The first variant provides express analysis of four technologically important amino acids (lysine, methionine, threonine, cystine) in borate buffer on characteristic absorption of aminogroup (190 nm), with limits of quantitation being on average 0.2%. The second scheme includes pre-capillary derivatization of amino acids using phenylisothiocyanate (PITC) and separation of phenylthiocarbamyl (PTC)-derivatives obtained by CZE with a detection on 254 nm, which allows to widen a list of detectable components up to 19 (without tryptophan) and significantly improve detection limits down to 0.01%. Acid hydrolysis was used for a sample preparation. The results of analysis of fodders were compared using such methods, as CZE, ion exchange chromatography (amino acid analyzer) and reversed-phase (RP)-HPLC (with gradient technique of elution).

Keywords: Derivatization, CE; Fodder; Amino acids

1. Introduction

Express quantitative analysis of amino acids in fodders is necessary for evaluation of their quality, stability and nutritive value. Amino acids are present in fodders both in free and bounded state (the latter are detected after hydrolysis, of acid kind as a rule, of proteins). Sample's nature (mixed fodder, fish flour, mill cake, nutrient yeast, premix) determines component parts and total content of amino acids, to take notice that lysine, methionine, threonine, and cystine are technologically important in the mixed fodder industry.

Chromatography is a traditional method for the control of amino acid content: ion exchange chromatography (IEC), using amino acid analyzer [1] and reversed-phase (RP)-HPLC [2,3], using usually a post- or a pre-column (pre-capillary) derivatization with various kinds of chromophores [4] or fluorophores [5,6]; the non-modified amino acids are detected in rare cases [7]. On the one hand, using of capillary electrophoresis (CE) is considered in numerous studies, for example, for analyzing of free amino acids in beer, juices, wines, and soybeans [8–10]. Most often, derivatives of amino acids are exposed to separation and detection [11], widespread reagents being: fluorescein isothiocyanate (FITC) [12], dansyl chloride (Dns) [13], phenylisothiocyanate (PITC) [14], *o*-phthaldialdehyde (OPA) [15]; infrequently a capability of direct measurement of amino acids is used, based on characteristic absorption of amino group (on 185 nm) [10]; and also an indirect UV-detection is used [16]. And the separation is made both by micellar electrokinetic chromatography (MEKC) technique and by capillary zone electrophoresis (CZE).

We have developed for the first time two application notes on detection of protein amino acids in fodders and raw materials for mixed fodders using simple, rapid analysis with UV-detection: simplified version of direct detection of underivatized amino acids (in particular lysine, methionine, threonine, and cystine) and one application note for analysis of 19 protein amino acids with pre-capillary derivatization by PITC. It was intended to carry out comparative experiments among offered electrophoretical schemes and chromatographic ones (ion exchange chromatography (amino acid analyzer) and RP-HPLC).

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2. Experimental

2.1. Reagents

Standards of amino acids, buffer components (sodium tetraborate (borax), sodium hydrophosphate, and sodium dihydrophosphate) were supplied by Reachim (Moscow, Russia); β-cyclodextrin (β-CD) was manufactured by Supelco (Bellefonte, PA, USA). For the synthesis of phenylthiocarbamyl (PTC)-derivatives of amino acids, ultra pure isopropyl alcohol and *n*-hexane were used, supplied by Vecton (St. Petersburg, Russia). PITC was produced by ICN Biomedicals (Irvine, CA, USA). Chemically pure sodium carbonate, hydrochloric acid, *o*-phosphoric acid, and sodium hydroxide were produced by Ekros (St. Petersburg, Russia). Solutions of cystine, tyrosine, and phenylalanine were prepared with 0.1 M HCl. All other solutions of amino acids, background electrolytes, as well as auxiliary solutions for synthesis of derivatives and capillary rinsing, were prepared on distilled water. Buffer solutions were filtered through 0.25 μm membrane filters (Sartorius, Goettingen, Germany) and degassed by centrifugation (4000 rpm, 5 min) before use.

2.2. Equipment

Capillary electrophoresis was carried out with the fully automated instruments (model Capel, Lumex, St. Petersburg, Russia) equipped with a liquid cooling system and UV-Vis detector (Capel-105 for determination of underivatized amino acids) or UV (only 254 nm) detector (Capel-104 for determination of PTC-derivatives). Collection and processing of data was performed on a PC with "Chrom&Spec" software (Ampersand, Moscow, Russia). Laboratory centrifuge, apparatus with air thermostating for fodder hydrolysis, and fan with temperature control were produced by Nauchpribor (Oryol, Russia).

2.3. Conditions of CE-separations

The separation was made in uncoated fused-silica capillary (Supelco), of 75 cm total length (65 cm effective length), 50 μm inner diameter. For analysis of non-modified amino acids the background electrolyte was prepared on 10 mM sodium tetraborate with addition of 10 mM β-cyclodextrin (β-CD), pH 9.18. Hydrodynamic injection of a sample (3000 Pa) during 15 s was used, working voltage of +20 kV was applied, detection wavelength was 190 nm. For the separation of PTC-derivatives of amino acids, background electrolyte was prepared of 30 mM phosphate buffer with addition of 4 mM β-cyclodextrin, pH 7.4. Hydrodynamic injection of a sample (3000 Pa) during 5 s was used, voltage of +25 kV was applied, detection wavelength was 254 nm.

The capillary was rinsed every day at the beginning of operation: by 0.5 M NaOH for 5 min, by water for 10 min, and then by background electrolyte for 10 min. Between analyses the capillary was rinsed for 2 min by suitable solution of the electrolyte. At the end of the working day the capillary was rinsed by HCl for 10 min and by water for 10 min.

2.4. Synthesis of PTC-derivatives of amino acids

Three hundred microliters of isopropyl alcohol, 5 μl of PITC, and 50 μl of solution of 19 amino acids, containing 100 mg/l of each of amino acids, were added to 100 μl of 0.1 M solution of sodium carbonate. The components were thoroughly mixed and stood for 35 min at room temperature. The mixture was then dried in the flow of warm air. The solid residual was dissolved in 500 μl of water, and then the excess of PITC and accompanying components was extracted by hexane (two times by 500 μl). Organic layer was thrown off, while lower water one was gathered into a dry Eppendorf vial avoiding ingress of hexane traces, centrifuged (4000 rpm for 5 min) and analyzed by CZE. Concentration of every amino acid in the final solution (in the form of PTC-derivative) was 10 mg/l.

2.5. Preparing samples of fodders (acidic hydrolysis of proteins)

Acidic hydrolysis of proteins, used on the stage of sample preparation, resulted in complete destruction of tryptophan. That is why electrophoretical behavior of this amino acid was not studied in the frames of this work. Besides, dimerization of cysteine up to cystine occurred during the hydrolysis process.

A weighed sample (100 mg) was put into a vial, and 10 ml of 6 M HCl were added. The mixture was tightly capped and put into the air thermostat ($T = 110\,^{\circ}C$) for 14–16 h. The received hydrolysate was filtered through an ash-free filter, and after taking 500 μl aliquote, it was evaporated to dryness in the flow of warm air. The solid residual was dissolved in 500 μl of water. Further activities were performed according to the final variant of detection.

For the direct detection of amino acids the received solution was centrifuged (4000 rpm, 5 min) and analyzed by CZE in a borate buffer.

For analysis of PTC-derivatives of amino acids a 100 μl aliquote was taken, and then 100 μl of 0.1 M sodium carbonate, 300 μl of isopropyl alcohol, 5 μl of PITC were added, and next operations followed according to Section 2.4.

If necessary, the final solutions were diluted by water in 5–10 times right before the analysis.

2.6. HPLC instruments and conditions

The RP-HPLC was used for comparison with two offered CE-schemes. Pre-column derivatization of amino acids using PITC (according to 2.4) and separation of PTC-derivatives by RP-HPLC were used.

Shimadzu liquid chromatograph LC-10Avp equipped with a UV-Vis detector. The column was a Spherisorb ODS (par-

ticle size 5 μm, 250 mm × 4.6 mm i.d.) from Waters. The injection volume was 20 μl. The measurements were carried out at a temperature of 45 °C. The detection wavelength was 254 nm.

The gradient elution with 0.05 M ammonium acetate (pH 6.8) as the eluent A and 0.1 M ammonium acetate- methanol (1:1, v/v) as the eluent B at flow rate of 1 ml/min was used: linear gradient of 1–50% eluent B in 65 min.

3. Results and discussion

3.1. Direct detection of amino acids by CZE

3.1.1. Selecting conditions for the separation

The protein amino acids have various chemical properties (basic, acidic, polar, hydrophobic and hydrophilic), that makes difficult their detection by CZE using the simple buffer systems. Nevertheless studies are known, describing the way to analyze five to nine amino acids in juices by CZE with the direct UV-detection in the 185–200 nm range [7,17,18], in rare cases with the indirect UV-detection [19] or with the preliminary obtaining of the derivatives [14]. Use of the methods with the direct detection is preferred for average and high concentrations of analytes, as a result of method's simplicity and absence of problems, caused by several factors: instability of the derivatives, interfering influence of a reagent, duration of the derivatization stage. Therefore to solve the problem of express control of lysine, methionine, threonine, and cystine the scheme was designed for separation of amino acids by CZE with UV-detection on 190 nm wavelength. Complexity of the matrix of the selected objects (fodder, mixed fodder, fish flour, mill cake) required a reliable identification of four amino acids, that were analyzed against a background of accompanying protein amino acids.

To optimize conditions of the separation of 19-component mixture of amino acids, and taking into account their zwitterionic nature, the separation was carried out in acidic (pH 2.7) and alkaline (pH 9.18) mediums. Both variants were not able to provide complete separation of the analytes, but the time of analysis in the acidic medium was appreciably higher. To increase resolution of the components, having a protonated primary amino group, a macrocyclic reagent 18-crown-6 was added, which had ability to cation selectivity in an acidic medium. Varying the concentration of the macrocycle from 1 to 10 mM, decrease of migration time of a series of amino acids and double growth of effectiveness were observed, due to formation of the "guest–host" inclusion complexes. Nevertheless, attempt to select the individual peaks of methionine and threonine was not successful.

Most of the amino acids have negative charge in the solution of sodium tetraborate and migrate as anions. Only two of them—lysine and arginine, that have isoelectric points at 9.59 and 11.15 correspondingly, behave as cations in that solution and reach the detector zone before EOF goes out. Though the selectivity of anion variant is higher than cation one, groups of inseparable components were formed during the analysis of 19 protein amino acids (Fig. 1). Thus, the next step to optimize the separation of the four technologically important amino acids against the background of accompanying components was to achieve a higher resolution in the group of peaks, which include methionine (Met) and aromatic amino acids (Tyr, Phe). Increased selectivity could be obtained by adding a complexation agent to the background electrolyte, which would selectively react with non-separated analytes. It is well known, that the size of β-cyclodextrin is very close to the size of the benzene ring. That is why we have studied addition of the very same macrocycle in the range of concentrations from 1 to 10 mM. The migration time of Phe and Met, in particular, decreased with the growth of β-CD concentration, while the behavior of aliphatic amino acids, of Met, specifically, did not change. The best result was achieved with the addition of 10 mM of β-CD; the electropherogram is shown in Fig. 2a.

As it is known, stability of inclusion complexes is affected by temperature [20], but the literature, referring to the studies of this fact, is extremely scanty. The separations of amino acids mixture were performed in the temperature range from +10 to +50 °C with a 10 °C step. It was ascertained, that the optimal temperature for detection of lysine, threonine and cystine was 20 °C (Fig. 2a), for methionine −40 °C (Fig. 2b), when the resolution of the mentioned components with neighboring amino acids had reached its maximum. At 10 °C the total time of analysis elongated to 25 min, while the effectiveness and the resolution of peaks were minimal. In spite of high separation factors at 50 °C, the detection of lysine at such conditions became impossible as a result of overlapping of its zone with arginine.

3.1.2. Analysis of lysine, methionine, threonine and cystine in fodders, mixed fodders and raw materials for mixed fodders

At the stage of a sample preparation a traditional hydrolysis was used in the medium of 6 M HCl [21]. The general scheme of receiving of a protein hydrolysate is presented in Section 2.5.

Linear concentration ranges of the amino acids, linear regression parameters (for three different concentrations) and limits of detection (LOD) and quantitation (LOQ) of four technologically important amino acids are given in Table 1.

In the selected optimal conditions the real samples have been analyzed (fish flour, sunflower mill cake, mixed fodders), Fig. 3. The comparative experiments were carried out by means of three methods: CZE, RP-HPLC (conditions are given in Section 2.6) and ion exchange chromatography (amino acid analyzer), presented in Table 2. The results of them allow to make a conclusion of opportunity to use CZE with a direct detection for the express detection of lysine, methionine, threonine and cystine in fodders and raw materials with high and low contents of proteins.

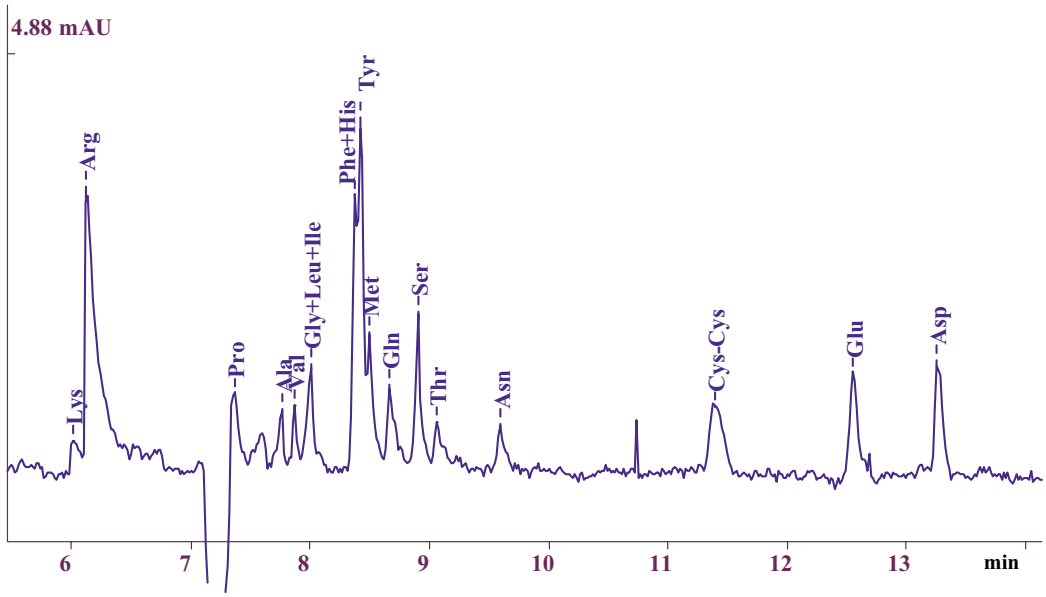

Fig. 1. CZE separation of the underivatized amino acids. Conditions: buffer, borate (10 mM, pH 9.18); fused-silica capillary, 75 cm (65 cm to detector) × 50 μm i.d.; voltage +20 kV; hydrodynamic injection (15 s at 3000 Pa); temperature 20 °C; UV-detection at 190 nm. Peaks: lysine (Lys, 40 mg/l), arginine (Arg, 40 mg/l), proline (Pro, 40 mg/l), alanine (Ala, 20 mg/l), valine (Val, 20 mg/l), glycine (Gly, 40 mg/l), leucine (Leu, 20 mg/l), isoleucine (Ile, 20 mg/l), phenylalanine (Phe, 4 mg/l), histidine (His, 10 mg/l), tyrosine (Tyr, 2 mg/l), methionine (Met, 20 mg/l), glutamine (Gln, 10 mg/l), serine (Ser, 40 mg/l), threonine (Thr, 20 mg/l), asparagine (Asn, 20 mg/l), cystine (Cys–Cys, 10 mg/l), glutamic acid (Glu, 40 mg/l), aspartic acid (Asp, 20 mg/l).

Table 1
Assay characteristics for underivatized amino acids

Amino acid	Parameters[a]			LOQ			LOD ($mg\,l^{-1}$)
	Range ($mg\,l^{-1}$)	b	r	$mg\,l^{-1}$	%[b]	R.S.D. (%) ($n = 9$)	
Lys	40–200	16.30	0.9982	40	0.40	9	20
Met	10–100	2.95	0.9998	10	0.10	4	5
Thr	20–100	6.65	0.9997	20	0.20	5	10
Cys–Cys	10–100	8.81	0.9988	10	0.10	7	5

[a] Calibration curve: $y = bx$, where x is the analyte concentration, expressed as $mg\,l^{-1}$ and y the peak area, expressed as arbitrary units; r is the linear correlation coefficient.
[b] Percentage (%) means amount of amino acid per 100 mg of sample.

Table 2
The data of the underivatized amino acids determination in the real samples using HPLC, CZE and IEC

Amino acid	Percentage of the amino acids in samples[a] (% ± S.D.)		
	CZE	RP-HPLC	IEC (arbitral method)
Fish flour			
Lys	0.85 ± 0.11	0.94 ± 0.16	0.84 ± 0.09
Met	1.42 ± 0.17	1.65 ± 0.20	1.35 ± 0.12
Thr	1.05 ± 0.13	1.10 ± 0.16	1.02 ± 0.11
Cys–Cys	0.56 ± 0.08	0.65 ± 0.13	0.69 ± 0.12
Mill cake			
Lys	1.15 ± 0.16	1.18 ± 0.19	1.09 ± 0.14
Met	0.75 ± 0.10	0.69 ± 0.10	0.69 ± 0.09
Thr	0.72 ± 0.12	0.80 ± 0.14	0.75 ± 0.16
Cys–Cys	0.24 ± 0.04	0.36 ± 0.07	0.23 ± 0.04
Mixed fodder			
Lys	1.52 ± 0.18	1.49 ± 0.13	1.27 ± 0.15
Met	0.45 ± 0.06	0.53 ± 0.06	0.52 ± 0.08
Thr	1.11 ± 0.14	0.98 ± 0.15	1.00 ± 0.15
Cys–Cys	0.33 ± 0.04	0.29 ± 0.04	0.41 ± 0.05

Each value is the mean of five independent assays.
[a] Percentage (%) means quantity of amino acid per 100 mg of sample.

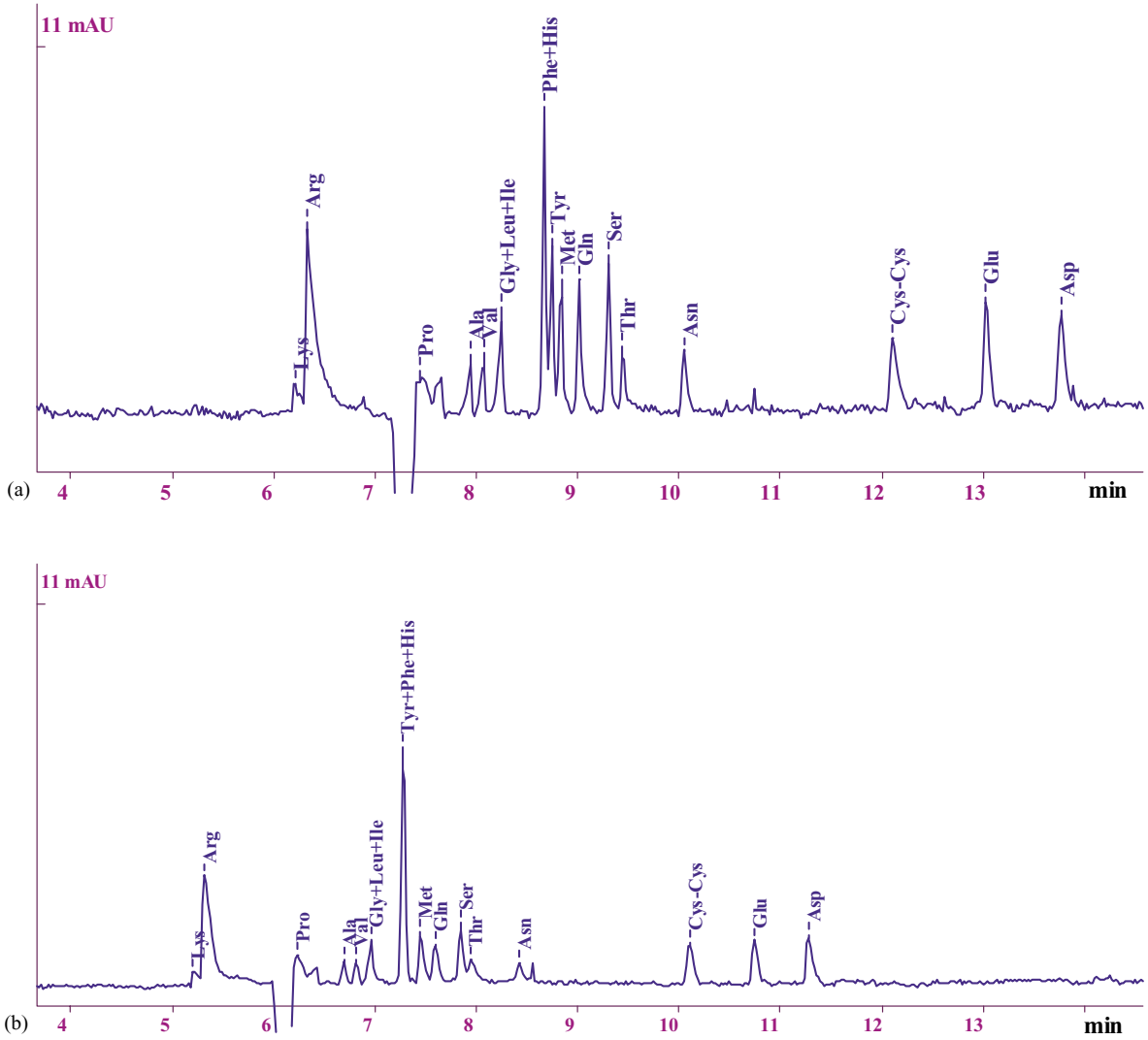

Fig. 2. Separation of the underivatized amino acids at different temperatures using borate buffer containing β-CD. Conditions: buffer, 10 mM borate with the addition 10 mM β-CD (pH 9.18). Temperature: 20 °C (a) and 40 °C (b). Other conditions as in Fig. 1.

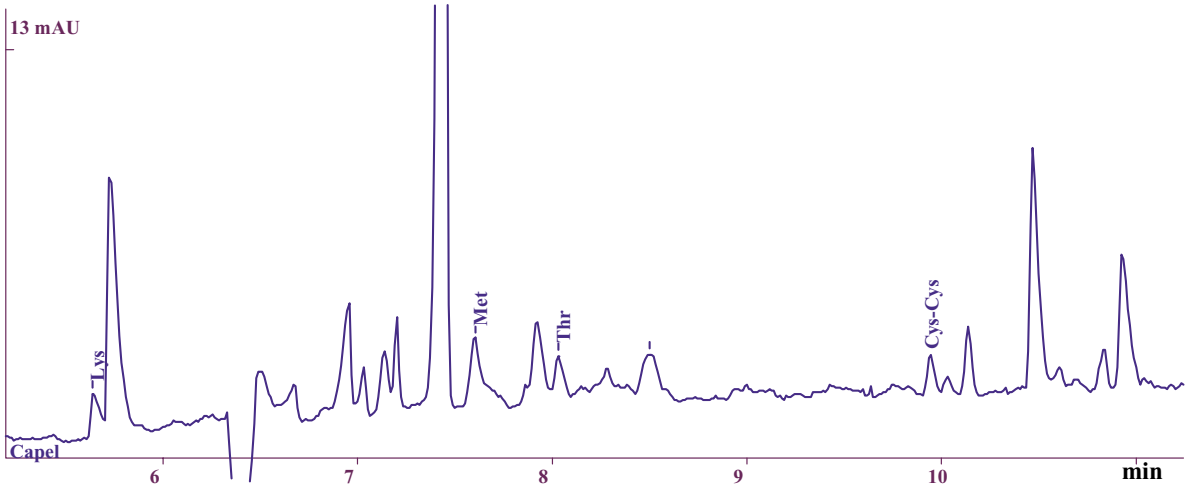

Fig. 3. Determination of Lys, Met, Thr and Cys–Cys in fish flour. Sample 100 mg, final solution was diluted 1:9. Conditions as in Fig. 2b. The results of the analysis: Lys (3.52%), Met (2.05%), Thr (0.97%), Cys–Cys (0.48%).

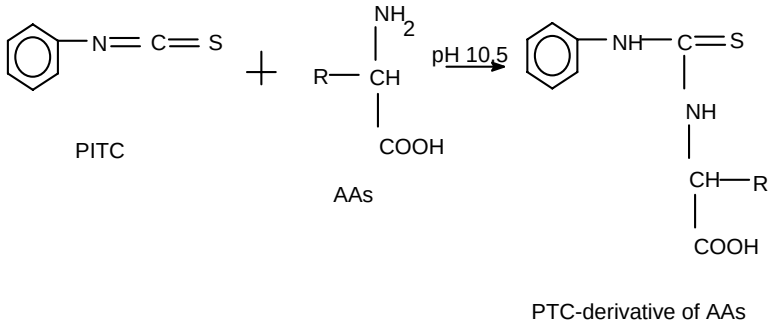

Fig. 4. Derivatization scheme for amino acids with the PITC.

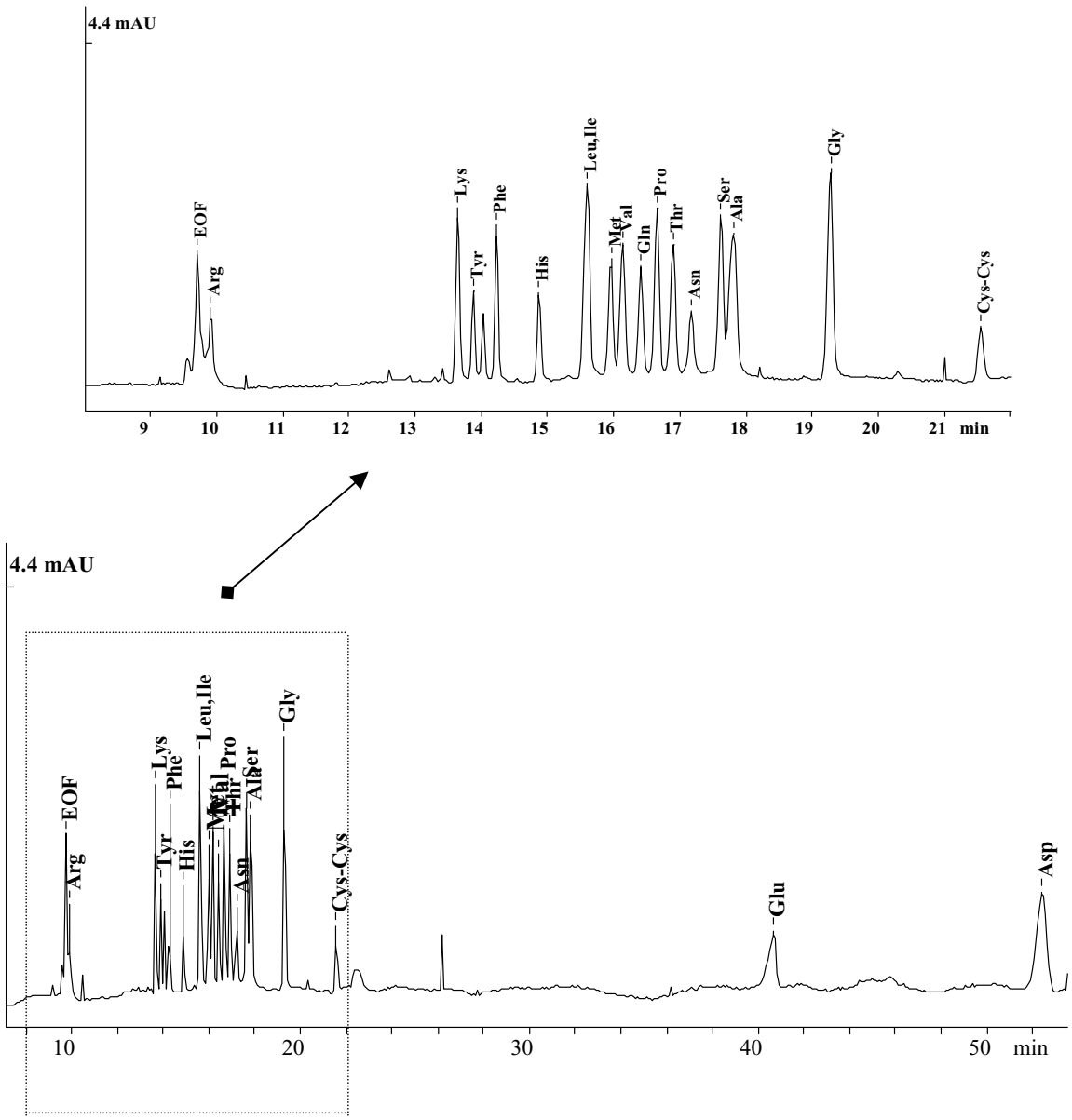

Fig. 5. Separation of a complex mixture of the PTC-derivatives of the amino acids. Conditions: buffer, phosphate (30 mM, pH 7.4) containing 4 mM β-CD; fused-silica capillary, 75 cm (65 cm to detector) × 50 μm i.d.; voltage +25 kV; hydrodynamic injection (5 s at 3000 Pa); temperature 20 °C; UV-detection at 254 nm. The starting concentration of each amino acid was 10 mg/l.

3.2. Separation and analysis of PTC-modified amino acids in fodders using CZE

Simplicity of use, effective separation of the received derivatives, sufficient reproducibility of the reaction conditions, good solubility of the derivatives in a water medium promote wide application of PITC for modification of amino acids in CE and also in HPLC. During the interaction of PITC with amino acids in an alkaline medium, PTC-derivatives are formed, which change into phenylthiohdation (PTH)-derivatives in an acidic medium. It should be noted, that PTC-derivatives exist in anion form in neutral and alkaline mediums, enabling the use of CZE method for their separation, while PTH-derivatives are neutral, therefore MEKC technique should be applied. Our preliminary experiments on synthesis of PTH-derivatives of amino acids have shown, that some by-products were formed along with the main product of the reaction, which could hardly be identified. As to the receiving of PTC-derivatives, there were no problems of that kind.

3.2.1. Optimization of separation conditions of PTC-derivatives by CZE

The scheme of the synthesis of PTC-derivatives of amino acids is shown in Fig. 4, and the procedure is described minutely in Section 2.4. For the separation of the received derivatives, which exist in anion form in neutral and alkaline solutions, the CZE was chosen. Buffer systems of different nature, pH and ionic strength were explored: (phosphate, pH 5.8–8.0; borate, pH 8.2–9.2; and carbonate, pH 10.0–10.8). Concentrations of every background electrolyte were varied from 10 to 50 mM. The influence was studied of β- and γ-cyclodextrins, added to the background electrolyte in the concentration range 1–5 mM, on selectivity of the separation. The selection of CDs was caused by the optimal steric coincidence between cavity size of macrocycle and ion radius of the analyte. Also, the parameters of the capillary were varied, as well as conditions of the sample injection and the voltage. As a result the conditions have been optimized, at which the maximum separation of PTC-derivatives of 19 amino acids was observed, shown in Fig. 5.

3.2.2. Quantitative determination of 19 modified protein amino acids in fodders, mixed fodders and raw materials for mixed fodders by CZE

The offered scheme of separation of PTC-derivatives of amino acids allows to analyze practically the whole list of protein amino acids, excluding triptophane. For leucine and isoleucine the total content is measured.

Linear concentration ranges of amino acids, parameters of linear regression (for seven different concentrations) and limits of detection (LOD) and quantitation (LOQ) of 19 amino acids in the samples are presented in Table 3. The real samples (fish and meat-bone flour, sunflower mill cake, mixed fodders) have been analyzed in the selected optimal conditions. The comparative experiments have been carried out by means of CZE method and arbitral one (ion exchange chromatography using an amino acid analyzer), which showed the satisfactory results. The electropherogram of the real sample is shown in Fig. 6.

Table 3
Assay characteristics for derivatized amino acids

Amino acid	Parameters[a]			LOQ			LOD (mg l^{-1})
	Range (mg l^{-1})	b	r	mg l^{-1}	%[b]	R.S.D. (%) ($n = 6$)	
Arg	0.50–40	3.90	0.9984	0.50	0.025	8.2	0.25
Lys	0.25–40	1.70	0.9844	0.25	0.013	9.5	0.13
Tyr	1.0–40	4.01	0.9976	1.0	0.050	6.9	0.50
Phe	1.0–40	9.12	0.9994	1.0	0.050	6.1	0.50
His	0.50–40	3.49	0.9987	0.50	0.025	6.7	0.25
Leu + Ile	0.50–40	0.84	0.9998	0.50	0.025	8.5	0.25
Met	0.40–40	2.04	0.9995	0.40	0.020	6.4	0.20
Val	0.40–40	1.59	0.9981	0.40	0.020	7.0	0.20
Gln	0.50–40	4.11	0.9976	0.50	0.025	6.4	0.25
Pro	0.25–40	1.14	0.9997	0.25	0.013	8.6	0.13
Thr	0.50–40	2.27	0.9993	0.50	0.025	10.1	0.25
Asn	1.0–40	5.06	0.9988	1.0	0.050	7.5	0.50
Ser	0.30–40	1.70	0.9996	0.30	0.015	9.2	0.15
Ala	0.30–40	1.44	0.9997	0.30	0.015	8.9	0.15
Gly	0.15–40	0.81	0.9997	0.15	0.008	11.5	0.08
Cys–Cys	1.0–40	4.46	0.9990	1.0	0.05	12.6	0.50
Glu	1.0–40	2.13	0.9982	1.0	0.05	12.2	0.50
Asp	0.5–40	0.98	0.9988	0.5	0.025	13.7	0.25

[a] Calibration curve: $y = bx$, where x is the analyte concentration, expressed as mg l^{-1} and y the peak area, expressed as arbitrary units; r is the linear correlation coefficient.

[b] Percentage (%) means amount of amino acid per 100 mg of sample.

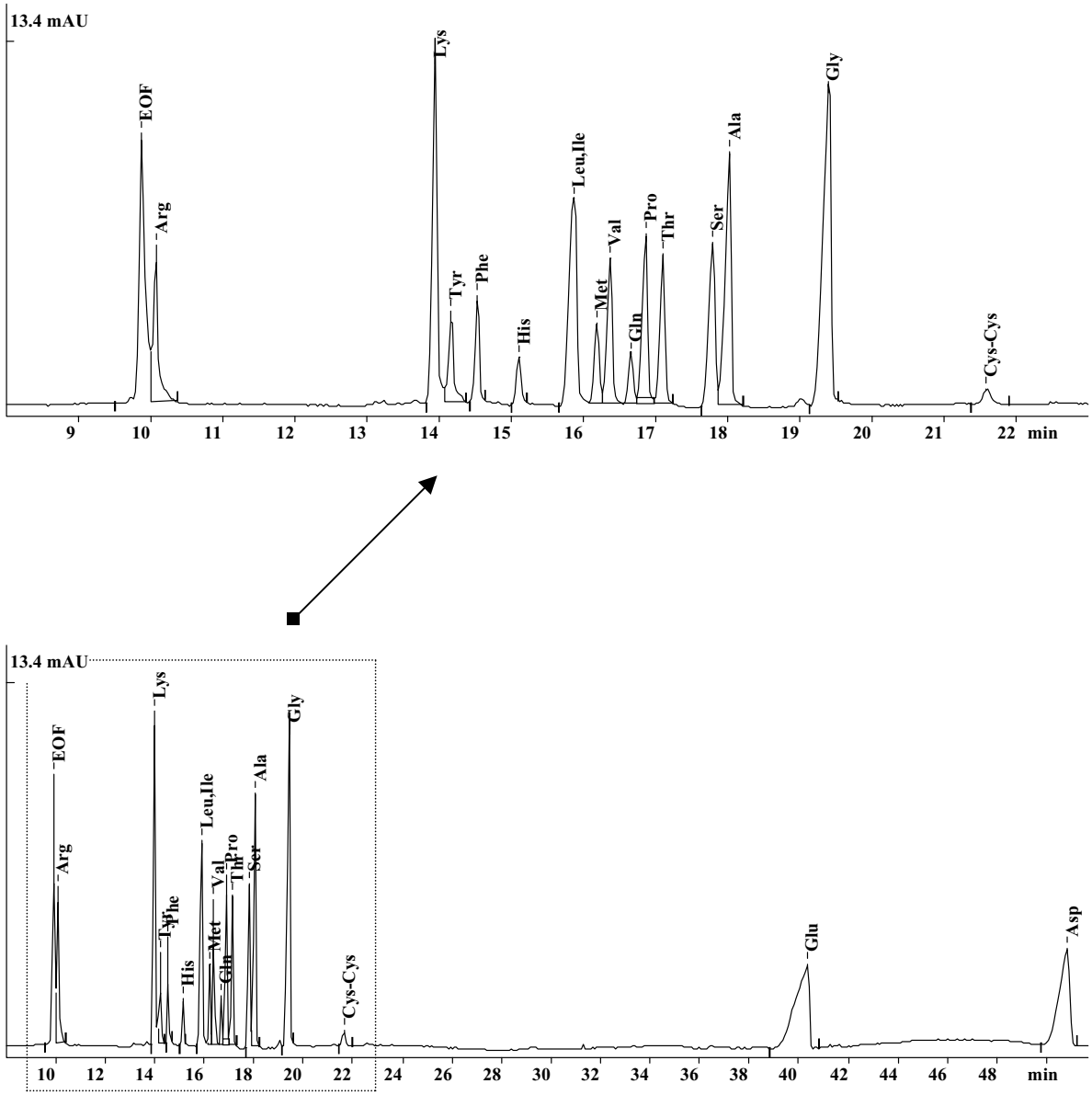

Fig. 6. Electropherogram of a fish flour. Sample 100 mg, final solution was diluted 1:9. Conditions as in Fig. 5. The results of the sample analysis, % (in parentheses are presented the results of arbitral method): Arg 3.63 (3.83), Lys 4.16 (4.28), Tyr 3.14 (2.04), Phe 2.03 (2.17), His 1.57 (1.44), Leu + Ile 6.64 (5.82), Met 1.66 (1.63), Val 2.60 (2.50), Gln 1.04 (not information), Pro 2.76 (3.04), Thr 2.51 (2.41), Ser 2.48 (2.65), Ala 3.57 (3.67), Gly 4.83 (4.34), Cys–Cys 0.63 (0.49), Glu 4.34 (4.66), Asp 7.48 (7.80).

4. Conclusions

A simple technique—CZE with direct UV-detection—was offered for the express analysis of lysine, methionine, threonine, and cystine contents, as the most important factors at fodder production and quality control of raw materials for mixed fodders. The complete analysis of protein amino acids in fodders (excluding tryptophan) was realized by CZE with preliminary obtaining of PTC-derivatives. The comparison of results received by both CE techniques has been performed with those, obtained by the arbitral method (ion exchange chromatography). It allows to recommend the CZE method (with or without derivatization) for analysis of amino acid contents in fodders, mixed fodders and raw materials for them. It should be mentioned, that CE features higher effectiveness, absence of any sorbents, less reagent consumption, and high rate of the analysis.

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