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Determination of free- and protein primary amino acids in biological materials by high-performance liquid chromatography and photodiode array detection

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ABSTRACT

Work from this laboratory resulted in improved high-performance liquid chromatography methods for quantification of free- and protein primary amino acids in biological materials. Biological samples were hydrolyzed with 6 M HCl for 20 h at 104±2°C. Primary amino acids, with the exception of tryptophan, were separated after pre-column derivatization with o-phthaldialdehyde (OPA) in the presence of ethanethiol (ESH). Derivatized amino acids were analyzed using a Nova Pak C_{18} column (4 μm, 250 × 4.6 mm I.D., Waters) by quaternary gradient system I. Detection was carried out simultaneously using UV monitoring at 337 nm and fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm). Derivatized amino acids were completely resolved from all interfering species in about 50 min. HPLC system I with two detection modes can also be used for quantification of free primary amino acids in ovine blood plasma. A trace amount of cysteine as its OPA/ESH derivative can be quickly quantified (~ 3.5 min) using an isocratic HPLC system with UV detection at 274 nm. Separation and quantification of tryptophan in alkaline hydrolysates was achieved without derivatization using the same HPLC column by ternate gradient elution system II with UV monitoring at 219 nm or fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 280/360 nm). The obtained average recoveries of assayed amino acids added to biological samples were near 100% when UV and fluorescence detection were applied. Generally, fluorescence detection in comparison with UV monitoring offers lower limits of detection (0.04-1.25 vs 0.05-12.7 μmol·l^{-1}) and quantification (0.12-11.7 vs 0.18-42.0 μmol·l^{-1}), however, the sensitivity of the UV detection mode is satisfactory for accurate and precise quantification of free- and protein amino acids in biological materials. The HPLC systems with UV detection assured better resolution of amino acid peaks in comparison with fluorescence detection. Satisfactory purity of analytical amino acid peaks (near 100%) and precision of HPLC systems with both detection modes renders these procedures suitable for routine analysis of amino acid concentrations in large numbers of

biological samples. HPLC system I enabled quantification of 2,6-diaminopimelic acid, so the current chromatographic system can also be applied for the estimation of bacterial protein production in ruminants.

KEY WORDS: amino acids, pre-column derivatization, HPLC, UV detection, fluorescence detection

INTRODUCTION

The response of animals to feed protein (PN) and non-protein nitrogen sources (NPN) basically depends on the amount and nature of feed protein and amino acids absorbed from the small intestine. In recent years much interest has focused on covering amino acid requirements of animals for growth, development and productivity (Batterham, 1992; Lewis and Bayley, 1995; Żebrowska and Buraczewska, 1998; Ravindran and Bryden, 1999). Thus, because amino acid composition is so important, physiologists, geneticists or nutritionists should be interested in developing more accurate and precise amino acid analyses. Although there are many techniques for this analysis, the application of commercial amino acids analyzers using ion-exchange liquid chromatography is by far the most popular (Ng et al., 1991; Moller, 1993; Sarwar and Botting, 1993; Williams, 1994). However, many high performance liquid chromatography (HPLC) methods and gas chromatography techniques (GC) for quantification of amino acids have also been published in recent years (Sarwar and Botting, 1993; Cohen and Michaud, 1998; Czauderna and Kowalczyk, 1998; Peter et al., 1998; Albin et al., 2000; Polak and Golkiewicz, 2000; Kutlan and Molnar-Perl, 2001; Molnar-Perl, 2001).The major shortcomings of automatic amino acid analyzers for amino acids observed are long analysis time and inadequate detection limits, dedication of the analyzers to only amino acid analysis, and high cost of the instruments. On the other hand, the use of reversed-phase HPLC with pre-column derivatization for the analysis of protein- and free amino acids is becoming established as a cheaper alternative to commercial amino acid analyzers. Five main pre-column derivatization (o-phthaldialdehyde (OPA), phenylthiohydantoin, phenylthiocarbamyl, dansyl chloride and dabsyl chloride) HPLC methods were compared in terms of detection limit, precision, resolution, stability and duration of analysis (Sarwar and Botting, 1993). The rapid OPA derivatization procedure has become popular because the reagent itself does not fluoresce, the instrumentation in pre-column derivatization is simpler and the cost of such systems is lower compared with post-column derivatization or amino acid analyzers (Rattenbury, 1981; Sarwar and Botting, 1993; Williams, 1994). Moreover, fluorescent OPA derivatives were found to be most desirable in terms of quantitation limits and analysis time. Unfortunately, the main disadvantage of the OPA method lies in the fact that OPA does not react with imino acids (i.e., secondary amino acids: proline and hydroxyproline).

As the OPA derivatization method is suitable for automation, this procedure can be used in routine determination of primary amino acids in biological samples. Therefore, the main purpose of our work was to find a more versatile HPLC method with UV detection than previously published for OPA amino acid assay in biological samples (Czauderna and Kowalczyk, 1998, 1999). Because OPA derivatization is known to be more sensitive than ninhydryn colorimetry, we also tried to improve a new HPLC system with fluorescence detetection.

MATERIAL AND METHODS

Reagents

All reagents were of analytical grade, whereas organic solvents were HPLC grade. Tetrahydrofuran and methanol were purchased from POCh Gliwice (Poland). Ethanethiol (ESH) and 2-mercaptoethanol (E(OH)SH) were obtained from Aldrich (Germany); o-phthaldialdehyde (OPA) and all amino acids used were from Sigma (St. Louis, MO, USA). Water used for the preparation of mobile phases and chemical reagents was prepared using an Elix™ water purification system (Millipore). The mobile phases were filtered through a 0.45 μm membrane filter (Millipore).

Chromatographic equipment

An Alliance separation module (model 2690, Waters) with a Waters 474 fluorescence detector and a Waters 996 photodiode array detector (PAD) was used for the gradient elution systems. An Alliance autosampler was thermostated at ~5°C. The OPA derivatives were simultaneously monitored using PAD and fluorescence detectors. The PAD was operated in a UV range from 190 to 400 nm with a spectral resolution of 1.2 nm and a measurement frequency of 1 spectrum per second. The fluorescence detections were taken at the optimum excitation and emission wavelengths: at $\lambda_{ex}/\lambda_{em}$ = 336/425 nm. Development of the gradient systems, collection and data integration were performed using Millennium 2001 software (version 2.15) and a Pentium III computer (Compaq). The ambient temperature was 20-22°C. The analytical column used was a Nova Pak C_{18} column (4 μm, 250 × 4.6 mm, I.D., Waters) in conjunction with a guard Nova Pak column (Waters) of 10 × 6 mm containing RP phase C_{18} (30-40 μm) pellicular packing material.

Analytical mobile phases and the gradient elution system

Two gradient elution systems were used for complete separation and detection of amino acids in the assayed biological samples. The following elution mobile phases

were used: solvent A was tetrahydrofuran - buffer A (1:99, v/v). The buffer A for mobile phase A was prepared from 0.02 M Na_2HPO_4 adjusted to pH 3.5 with ~10 % phosphoric acid. The solvent B was tetrahydrofuran - buffer B (1:99, v/v). The buffer B for mobile phase B was prepared from 0.04 M Na_2HPO_4 adjusted to pH 6.6 with ~10 % phosphoric acid. Solvent C was methanol, while solvent D was water. For analysis of derivatized amino acids in standards and biological samples, elution was carried out in quaternary gradient system I (Table 1); all separations were performed at a column temperature of 37°C. For direct analysis of tryptophan, ternate gradient system II (Table 2) using simultaneous UV detection (at 279 nm) and fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 280/360 nm) was applied. All direct assays of tryptophan were carried out at a column temperature of 31°C. The minimal system pressure was 25.5±0.1 MPa, the maximal pressure was 36.3±0.2 MPa. Injection volumes were 5-40 µl. Amino acids were identified by the retention time of processed standards injected separately and by adding standard solutions to bio-

TABLE I

Quaternary gradient elution[a] system I used for analysis of OPA/ESH amino acids derivatives in biological samples hydrolysed with 6 M HCl (column temperature 37°C)

Time	Composition[b], %			
min	Solvent A	Solvent B	Solvent C	Solvent D
0	0	85	15	0
1.8	0	85	15	0
3.0	0	72	28	0
19.0	0	72	28	0
19.5	0	64	36	0
22.0	0	64	36	0
28.0	0	54	46	0
30.2	55	0	45	0
30.5	44	0	56	0
35.5	43	0	57	0
37.0	0	44	56	0
42.0	0	37	63	0
45.0[c]	0	40	60	0
46.5	0	0	85	15
53.0[d]	0	0	85	15

[a] – flow-rate: 1.8 ml/min

[b] – all changes of solvents composition were linear

[c] – after 45 min, the gradient composition: solvent C (methanol)/solvent D (water) - 69:31 (v/v) was chosen as the optimum separation of lysine from interfering endogenous species in blood plasma (modification for fractionation of free amino acids in ovine blood plasma)

[d] – after 53 min, the column was re-equilibrated for 10 min in 85% solvent B and 15% solvent C at a flow-rate of 1.8 ml/min

TABLE 2

Ternate gradient system II used for direct analysis of tryptophan (column temperature 31°C)

Time	Flow rate	Composition[a], %		
min	ml/min	Solvent B	Solvent C	Solvent D
0	1.9	100.0	0	0
5.0	1.9	100.0	0	0
8.5	1.9	94.2	5.8	0
9.0	2.0	0	95.0	5.0
19.5	2.1	0	95.0	5.0
19.8	1.9	0	75.0	25.0
20.0	1.9	100.0	0	0
30.0	1.9	100.0	0	0

[a] – all changes of solvents composition were linear

logical samples. The limits of detection (LOD) were calculated as a signal-to-noise ratio of 3, while the limit of quantification (LOQ) was defined as 10 times the noise under a peak (Gratzfeld-Husgen and Schuster, 1996; Meyer 1999).

Preparation of the borate buffer

Boric acid, 2.474 g, was dissolved in 80 ml of water and the pH was adjusted to 9.8-9.9 with 5 M KOH. The resulting solution was filtered through filter paper and then diluted to a volume of 100 ml to make 0.4 M borate buffer.

Preparation of derivatizing reagent (OPA/ESH)

Seventy-five mg of OPA were dissolved in 4.5 ml methanol and 0.5 ml borate buffer. Next, 70 µl of ethanetiol (ESH) were added and the resulting solution was mixed. The reagent solution was prepared at least 2 h before use. It is recommended to protect the derivatizing solution from light and to store refrigerated (-18°C) when not in use. This solution was retained no longer than two weeks. The reagent strength was maintained by addition of 10 µl of ESH every 2-3 days.

Preparation and hydrolysis of biological samples with 6 M HCl

Samples of biological materials as rumen bacteria (*Lachnospira multiparus 685*), ovine duodenal digesta, faeces, meat and milk were frozen, freeze-dried and the obtained homogeneous materials (about 400-500 mg) were hydrolyzed with 50 ml of 6 M HCl at 104±2°C for 20 h in sealed tubes. After cooling the hydrolysates were filtered through filter paper and washed three times with water. Hy-

drochloric acid was removed from the filtrates in a vacuum rotary evaporator. Ten milliliters of water were added to the residue and then evaporated to dryness again in vacuum to remove residues of HCl. This evaporating procedure was repeated two times. The residues were stored at -18°C when not in use. The residue was re-dissolved in 1 ml of the borate buffer (pH 9.8-9.9). 10-40 µl of resulting solution was used for the OPA/ESH derivatization procedure as below.

Procedure for preparation of the hydrolysate for tryptophan determination

Lyophilized homogeneous pea seeds (i.e. ~500 mg) was weighed (sample corresponding to ~20 mg N) into 50 ml plastic bottles and mixed with 14 g $Ba(OH)_2 \cdot H_2O$ and 15 ml H_2O (Buraczewska and Buraczewski, 1984). Then 3 drops of octyl alcohol were added and the bottles were covered with glass stoppers. Hydrolysis lasted 16 h in an autoclave at 123°C. After the hydrolysis the content of the bottle was transferred with hot water into beakers, cooled in an ice-water bath and treated with 3 to 3.5 ml conc. H_2SO_4 for barium precipitation. The sample was then mixed occasionally and kept in the bath about 25 min. Afterwards the sample was transferred into 100 ml tubes for centrifugation. The supernatant was checked for complete barium precipitation and the precipitate washed twice with hot water followed by centrifugation. The collected supernatant was adjusted to pH ~ 7 with 2 M NaOH and the volume was made up to 90 ml with water. The appropriate volumes (10-20 µl) of resulting solution were directly introduced into the HPLC system just after preparation. It is recommended to protect the hydrolysates from light and to store refrigerated (-18°C) when not in use.

Preparation of ovine blood plasma for free amino acid HPLC analysis

Blood samples from the jugular vein of sheep were collected into heparinized tubes (kept in an ice bath) and centrifuged at 2000 g for 15 min (at 0-4°C). The plasma was stored at -18°C. On the day of analysis, 1 ml of plasma (0-1°C) was deproteinized with 1 ml of 7 % cooled solution (0-1°C) of trichloroacetic acid and centrifuged at 2000 g for 15 min (at 0-4°C).

The obtained supernatant was filtered through a 0.2-µm nylon filter (Cole Parlmers) into an autosampler vial. 100-150 µl of supernatant was used for the OPA/ESH derivatization procedure as below.

Derivatization procedure

To an autosampler reacti-vial were added an appropriate volume (20-50 µl) of assayed biological sample, 1 ml of OPA/ESA derivatizing reagent and 10 µl of 1 M NaOH. The contents were mixed and reacted for 3 min at room temperature. The

pH of the resulting solution should be from 9 to 10. At the end of the 3 min derivatization period, the processed samples were injected on to the HPLC column. Thus, the total time of the reaction was constant: ca 4 min. The derivatizing procedure for standards was the same as for biological samples. It is recommended to protect all derivatized samples from the light and to store them at - 18°C until analyzed.

RESULTS AND DISCUSSION

The aim of this work was to extend our earlier investigations (Czauderna and Kowalczyk, 1998, 1999) in order to determine the possibility of free- and protein amino acid assays in biological materials. Interest in amino acid analysis has grown because information on the amino acid requirements, especially essential and semi-essential amino acids, in animal nutrition has great economic value, permitting the formulation of diets to meet amino acid requirements without useless excess. Therefore, recently many methods for amino acid quantification have been rapidly developed. In fact, in the last several years some characteristics of OPA/ESH and OPA/E(OH)SH amino acid derivatives have been identified (Czauderna and Kowalczyk, 1998, 1999; Molnar-Perl, 2001). Exhaustive investigations have demonstrated that OPA/E(OH)SH and OPA/ESH amino acid derivatives can be successfully resolved by gradient RP-HPLC with fluorescence detection (Sarwar and Botting, 1993; Czauderna and Kowalczyk, 1998, 1999; Kutlan and Molnar-Perl, 2001; Molnar-Perl, 2001). Therefore in the presented paper, on the basis of our earlier experience (Czauderna and Kowalczyk, 1998, 1999), a new gradient HPLC system with UV detection for primary amino acids has been developed. Our earlier work (Czauderna and Kowalczyk, 1998) showed that clear resolution of OPA/E(OH)SH amino acid derivatives can be obtained using a Nova Pak C_{18} column (4 μm, 250 × 4.6 mm I.D., Waters) and fluorescence detection. Thus, after detailed investigations of the influence of column temperature, mobile phase composition and the pH of buffers on resolution of derivatized amino acids, the use of a Nova Pak C_{18} column, the OPA/ESH derivatizing reagent and 37°C elution temperature were chosen as optimum separation conditions of all assayed primary amino acids using UV detection at 337 nm. As shown in Figures 1A and B, in the quaternary gradient system (Table 1) developed in the presented study, OPA/ESH amino acid derivatives are substantially retained on the column (in contrast to OPA/E(OH)SH amino acid derivatives) and clearly distinct from unidentified species, background interference and endogenous substances present in all assayed samples. To enhance the resolution of amino acids eluted between ~6 and ~31 min, buffer B should be prepared from 0.04 M Na_2HPO_4 and adjusted to pH 6.6, while to increase the resolution between methionine and valine, buffer A should be prepared from 0.02 M Na_2HPO_4 and adjusted to pH 3.5. The water used in the last gradient

step (Table 1) as the mobile phase D, resulted in low system pressure (especially after 46 min of HPLC run) and excellent peak shapes, close to symmetrical even with analyte elution times to ~50 min. As can be seen from the chromatograms (Figure 1 A and B), the gradient system developed in this study and applied UV detection mode were found to provide excellent baseline stability (like in the fluorescence detection mode). Obviously, all assayed amino acid peaks were absent from the blank, when the developed gradient program was used. So, all fractionate derivatized primary amino acids can be easily quantified using Millennium software. Based on UV spectra of OPA/ESH amino acid derivative spectra (Figure 1C) and blank chromatographic measurements, UV detection at 337 nm was found to produce the greatest signal (i.e. a peak area) and low background under analytical peaks. Subsequently, attempts were made to compare the results of amino acid quantification depending on the detection modes used. Table 3 summarizes the comparison of responses of the PAD and fluorescence detectors and shows that derivative measurements at 337 nm can provide a second satisfactory alternative UV detecting mode. As expected, the responses of the PAD detector to the concentration of the assayed amino acids are linear functions. In fact, the correlation coefficients (r) and standard error in slopes (SES) evidenced that monitoring at 337 nm provided generally better linearity of amino acid derivative responses than fluorescence detection (at $\lambda_{ex}/\lambda_{em}$ = 336/425 nm). Detailed analysis of chromatograms revealed that, also, better resolution of amino acid derivatives was obtained using UV detection in comparison with fluorescence detection. Moreover, UV detection at 337 nm produced generally only 2-3 smaller signals than the fluorescence emission signal (λ_{ex} = 425 nm) obtained by applying the 336 nm excitation wavelength. The results of 2,6-diaminopimelic acid (DAPA) quantification are particularly important (Table 3) because the UV and fluorescence detection produced practically the same signals (i.e. peak areas). In other words, the presented method with UV monitoring produced a new valuable analytical tool for determining trace amounts of DAPA (important as a marker of bacterial protein production in ruminants) (Robinson et al., 1996; Czauderna and Kowalczyk, 1999; McKerrow et al., 2000) in the presence of a large excess of other amino acids. Consequently, HPLC system I with UV detection can be simultaneously applied for primary amino acid assays and for the estimation of ruminal bacterial protein supply to ruminants. Furthermore, our quaternary gradient program fractionates cysteine and cystine from other primary amino acid derivatives and background fluctuations. These sulphur-containing amino acids can be easily quantified by UV monitoring at 337 nm. As shown in Figure 1A (chromatogram A) a cysteine peak had a retention time of 4.56±0.03 min (mean±SD of 8 HPLC runs), while a cystine peak had a retention time of 45.40±0.12 min (mean±SD of 9 HPLC runs). Moreover, the cysteine derivative could be differentiated from other amino acid derivatives by the use of a photodiode array detector (PAD). As can be seen from UV spectra (Figure 1 C), derivatized

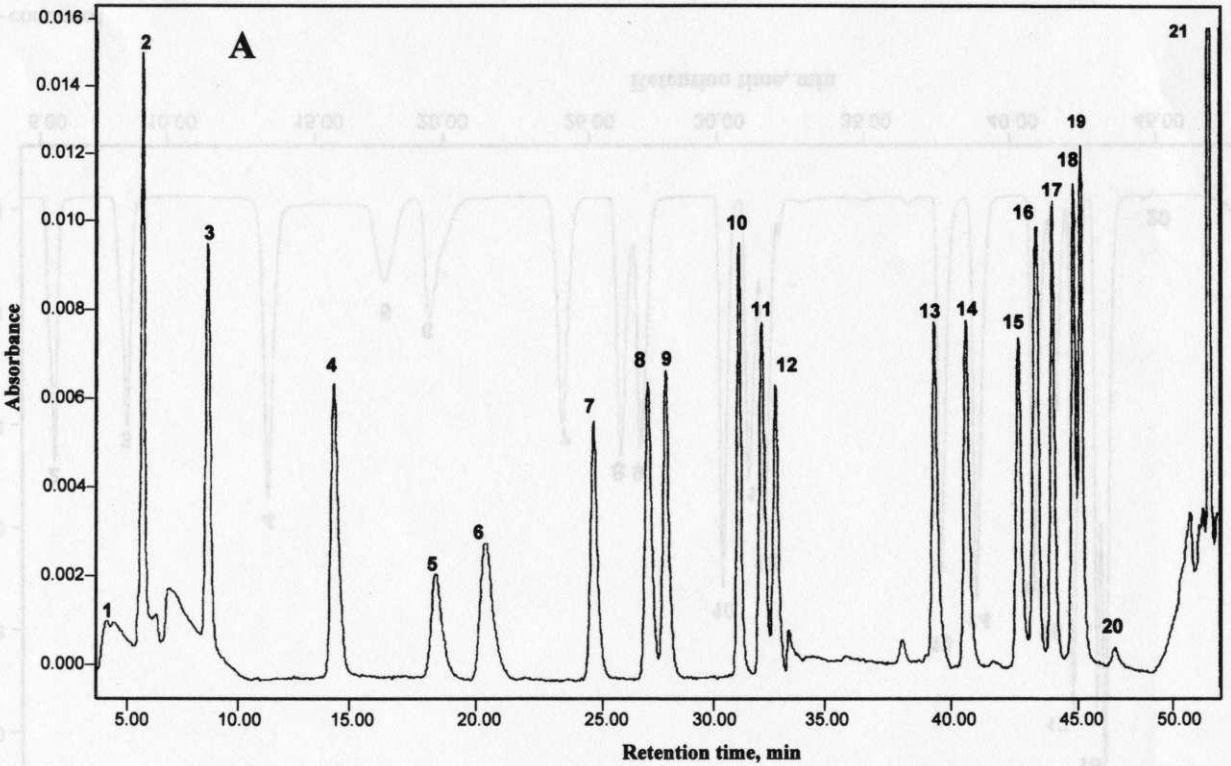

Figure 1. Part of typical HPLC chromatograms for standard of OPA/ESH primary amino acid derivatives using quaternary gradient system I and UV detection (A) at 337 nm (a chromatogram A) and (B) – fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm) (a chromatogram B). C – typical UV spectra of the OPA/ESH primary amino acid derivatives (including derivatized cystine) (line 1) and derivatized cysteine (line 2). Injection volumes were 20 µl. Peaks: 1 – cysteine; 2 – aspartic acid; 3 – glutamic acid; 4 – asparagine; 5 – histidine; 6 – serine; 7 – arginine; 8 – glycine; 9 – threonine; 10 – tyrosine; 11 – alanine; 12 – taurine; 13 – methionine; 14 – valine; 15 – phenylalanine; 16 – 2,6-diaminopimelic acid; 17 – isoleucine; 18 – norleucine (an internal standard); 19 – leucine; 20 – cystine; 21 – lysine

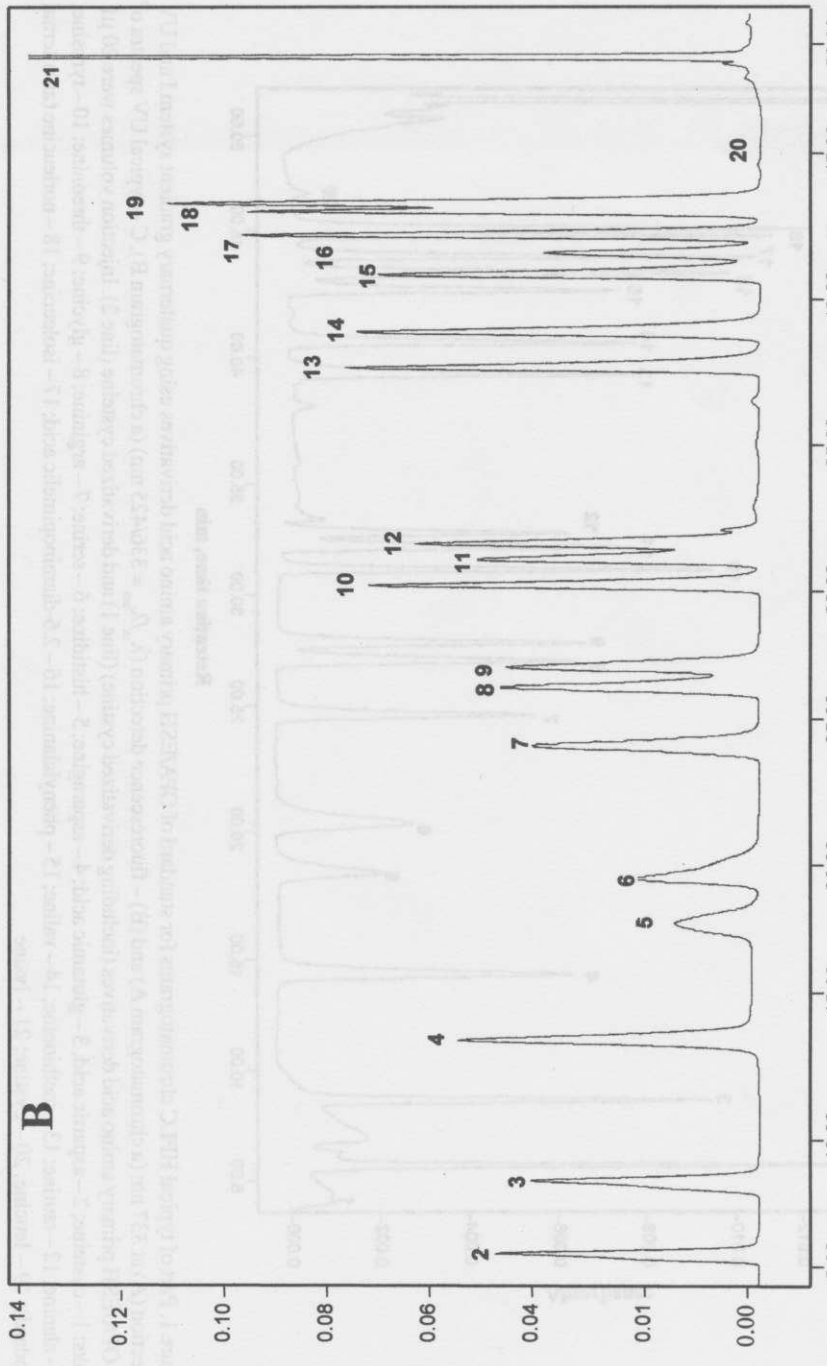

Figure 1 – continued

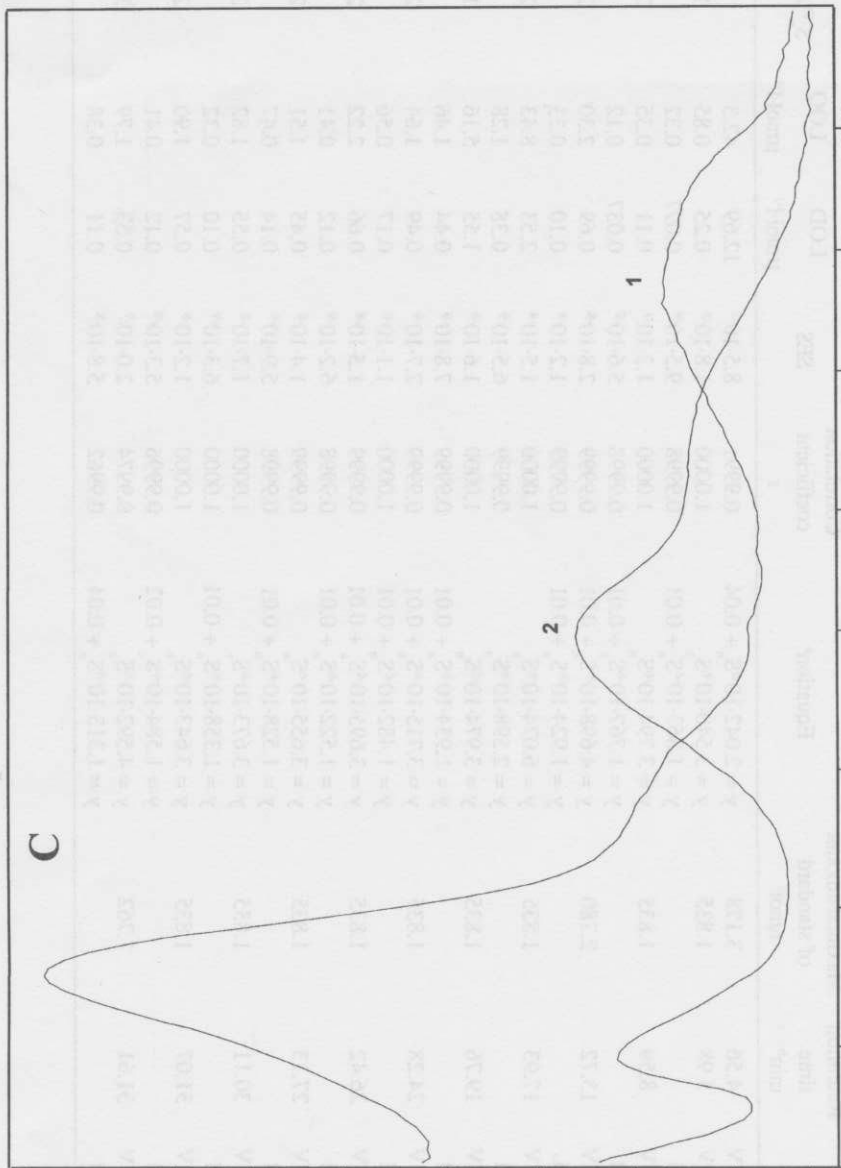

Figure 1 – continued

TABLE 3

Linear regression curves of amino acids derivatives, correlation coefficients (r), standard error in slope (SES), limits of detection (LOD), and limits of quantification (LOQ) derived from determination of OPA amino acids in standards obtained by UV and fluorescence (Fl) detection

Amino acid		Retention time min[b]	Maximal amount of standard nmol[c]	Equation[a]	Correlation coefficient r	SES	LOD µmol·l^{-1}	LOQ µmol·l^{-1}	S_n^{Fl} / S_n^{UV}
Cysteine	UV	4.56	3.178	$y = 2.042 \cdot 10^{-5} S_n + 0.04$	0.9991	$8.5 \cdot 10^{-7}$	12.69	42.3	-
Aspartic acid	UV	5.98	1.835	$y = 3.540 \cdot 10^{-6} S_n$	1.0000	$1.8 \cdot 10^{-8}$	0.25	0.85	1.84[d]
	Fl			$y = 1.863 \cdot 10^{-6} S_n + 0.01$	0.9998	$9.5 \cdot 10^{-9}$	0.097	0.32	
Glutamic acid	UV	8.59	1.835	$y = 3.794 \cdot 10^{-6} S_n$	1.0000	$1.2 \cdot 10^{-8}$	0.11	0.35	2.12
	Fl			$y = 1.767 \cdot 10^{-6} S_n + 0.01$	0.9998	$5.6 \cdot 10^{-9}$	0.037	0.12	
Aspargine	UV	13.72	2.780	$y = 4.698 \cdot 10^{-6} S_n + 0.01$	0.9999	$2.8 \cdot 10^{-8}$	0.69	2.30	2.38
	FL			$y = 1.924 \cdot 10^{-6} S_n + 0.01$	0.9999	$1.2 \cdot 10^{-8}$	0.10	0.33	
Histidine	UV	17.93	1.835	$y = 6.074 \cdot 10^{-6} S_n$	1.0000	$1.5 \cdot 10^{-8}$	2.53	8.43	2.36
	Fl			$y = 2.598 \cdot 10^{-6} S_n$	0.9999	$6.5 \cdot 10^{-9}$	0.38	1.28	
Serine	UV	19.76	1.835	$y = 3.974 \cdot 10^{-6} S_n$	1.0000	$1.6 \cdot 10^{-8}$	1.55	5.16	1.95
	Fl			$y = 1.934 \cdot 10^{-6} S_n + 0.01$	0.9999	$7.8 \cdot 10^{-9}$	0.44	1.46	
Arginine	UV	24.28	1.835	$y = 3.715 \cdot 10^{-6} S_n + 0.01$	0.9999	$2.7 \cdot 10^{-8}$	0.49	1.64	2.52
	Fl			$y = 1.482 \cdot 10^{-6} S_n + 0.01$	1.0000	$1.1 \cdot 10^{-8}$	0.17	0.56	
Glycine	UV	26.42	1.835	$y = 3.695 \cdot 10^{-6} S_n + 0.01$	0.9999	$1.5 \cdot 10^{-8}$	0.66	2.22	2.40
	Fl			$y = 1.522 \cdot 10^{-6} S_n + 0.01$	0.9998	$6.2 \cdot 10^{-9}$	0.12	0.41	
Threonine	UV	27.13	1.835	$y = 3.655 \cdot 10^{-6} S_n$	0.9999	$1.4 \cdot 10^{-8}$	0.45	1.51	2.37
	Fl			$y = 1.528 \cdot 10^{-6} S_n + 0.01$	0.9998	$5.9 \cdot 10^{-9}$	0.14	0.47	
Tyrosine	UV	30.11	1.835	$y = 3.673 \cdot 10^{-6} S_n$	1.0000	$1.7 \cdot 10^{-8}$	0.55	1.82	2.66
	Fl			$y = 1.358 \cdot 10^{-6} S_n + 0.01$	1.0000	$6.3 \cdot 10^{-9}$	0.10	0.32	
Alanine	UV	31.07	1.835	$y = 3.643 \cdot 10^{-6} S_n$	1.0000	$1.2 \cdot 10^{-8}$	0.57	1.90	2.26
	Fl			$y = 1.584 \cdot 10^{-6} S_n + 0.02$	0.9996	$5.3 \cdot 10^{-9}$	0.12	0.41	
Taurine	UV	31.61	1.762	$y = 4.592 \cdot 10^{-6} S_n$	0.9974	$2.0 \cdot 10^{-7}$	0.53	1.79	3.78
	Fl			$y = 1.313 \cdot 10^{-6} S_n + 0.04$	0.9962	$5.8 \cdot 10^{-8}$	0.11	0.38	

TABLE 3 continued

Methionine	UV	37.93	1.835	$y = 3.463 \cdot 10^{-6} S_n + 0.01$	0.9999	$2.7 \cdot 10^{-8}$	2.80	9.35	2.95
	Fl			$y = 1.157 \cdot 10^{-6} S_n + 0.01$	1.0000	$9.0 \cdot 10^{-9}$	0.58	1.93	
Valine	UV	38.26	1.835	$y = 3.553 \cdot 10^{-6} S_n$	1.0000	$1.9 \cdot 10^{-8}$	0.81	2.71	3.01
	Fl			$y = 1.152 \cdot 10^{-6} S_n + 0$	1.0000	$6.2 \cdot 10^{-9}$	0.17	0.56	
Phenylalanine	UV	41.30	1.835	$y = 3.691 \cdot 10^{-6} S_n + 0.01$	0.9999	$3.0 \cdot 10^{-8}$	0.53	1.75	2.92
	Fl			$y = 1.258 \cdot 10^{-6} S_n + 0.01$	1.0000	$1.0 \cdot 10^{-8}$	0.11	0.38	
2,6-diamino-pimelic acid	UV	42.08	1.545	$y = 2.074 \cdot 10^{-6} S_n$	1.0000	$1.0 \cdot 10^{-8}$	0.18	0.59	1.12
	Fl			$y = 1.907 \cdot 10^{-6} S_n$	1.0000	$9.2 \cdot 10^{-9}$	0.17	0.56	
Isoleucine	UV	42.75	1.835	$y = 3.250 \cdot 10^{-6} S_n + 0.01$	0.9998	$1.6 \cdot 10^{-8}$	0.32	1.08	2.96
	Fl			$y = 1.985 \cdot 10^{-6} S_n + 0.03$	0.9993	$9.9 \cdot 10^{-9}$	0.09	0.31	
Norleucine	UV	43.52	1.835	$y = 3.951 \cdot 10^{-6} S_n + 0.03$	0.9982	$1.7 \cdot 10^{-7}$	0.52	1.75	2.84
	Fl			$y = 1.371 \cdot 10^{-6} S_n + 0.01$	0.9955	$6.0 \cdot 10^{-8}$	0.23	0.78	
Leucine	UV	43.89	1.835	$y = 2.666 \cdot 10^{-6} S_n$	0.9995	$1.9 \cdot 10^{-8}$	0.26	0.86	2.77
	Fl			$y = 0.900 \cdot 10^{-6} S_n$	0.9984	$6.5 \cdot 10^{-9}$	0.07	0.25	
Cystine	UV	45.26	0.917	$y = 1.580 \cdot 10^{-5} S_n + 0.01$	0.9996	$8.1 \cdot 10^{-7}$	1.89	6.30	0.14
	Fl			-	-	-	3.50	11.70	
Lysine	UV	49.73	1.835	$y = 1.910 \cdot 10^{-6} S_n$	1.0000	$1.0 \cdot 10^{-8}$	4.60[d]	15.40	1.91
	Fl			$y = 0.977 \cdot 10^{-6} S_n$	1.0000	$5.1 \cdot 10^{-9}$	1.25	4.20	

[a] – S_n and y are the peak areas and amino acids amounts (nmol) injected on to the column, respectively. Multilevel forced through zero option for generation of linear calibration curve fit. Number of points used in the calibration curves: 4

[b] – mean of 3 standards

[c] – maximal amount of amino acids standard injected on to the column

[d] – the lysine peak eluted near significant background fluctuations caused by a significant change of composition of mobile phases

cysteine showed a very high and broad band in the spectral range from 220 to 360 nm with the maximum at 282-284 nm. However, UV detection at 337 nm should be used for the clear distinction of the derivatized cysteine peak from background interference and endogenous substances present in ovine samples. Obviously, these amino acids cannot be quantified using a fluorescence detector (Figure 1 B) because fluorescence detection of cysteine and cystine derivatives gave very low responses in comparison with UV detection (Table 3). Unfortunately, main disadvantage of derivatized cysteine is its serious instability; after 2 h of storage at -18°C, the OPA/ESH cysteine derivative exhibited ca. 85% degradation. On the other hand, the cystine derivative is more stable than converted cysteine. Other primary amino acids, however, formed more stable derivatives in comparison with cystine.

The low values of the limit of detection (LOD) and limit of quantification (LOQ) for derivatized amino acids point to the satisfactory sensitivity of the proposed UV detection mode. As the presented method was applied to lyophilized samples (feeds, bacteria, intestinal digesta, faeces, blood plasma, meat and milk), sensitivity can be reduced several times, however, it seems clear from the LOD and LOQ values that the proposed method offers satisfactory sensitivity permitting detection and quantification of a relatively low level of amino acids when compared with the original amino acid contents in all assayed biological samples.

Direct determination of tryptophan

Tryptophan possess a relatively high absorbance band in the short UV range (absorbance maximum at 219 nm) and a lower one (a chromophore) in the spectral range from 245 to 290 nm with the absorbance maximum at ~279 nm (Figure 2A). Therefore, a simple gradient elution program (Table 2) with fluorescence detection (λ_{ex} /λ_{em} = 280/360 nm) and UV monitoring at 219 and 279 nm can be used for direct determination of tryptophan in biological samples. Fortunately, tryptophan can be differentiated from tyrosine and phenylalanine by the use of the photodiode array detector, because they possess different UV spectra (Figure 2A). Obviously, all other amino acids present in alkaline hydrolysates are transparent in the applied UV range. As shown in Figure 2A and B, in the proposed elution system II, underivatized tryptophan was substantially retained on the C_{18} Nova Pak column, and was completely separated from tyrosine (the average retention time: 1.97 min), phenylalanine (average retention time: 2.89 min), background interference and unidentified species. Thus, basing on above results it can be suggested that short-UV monitoring (at 219 nm) should also be used for direct determination of tryptophan. As expected, the tryptophan peak having a retention time of 5.71±0.11 min (mean±SD of 9 samples) was absent from the blank when the HPLC system II with UV (at 279 and 219 nm) and fluorescence detection was used. As can be

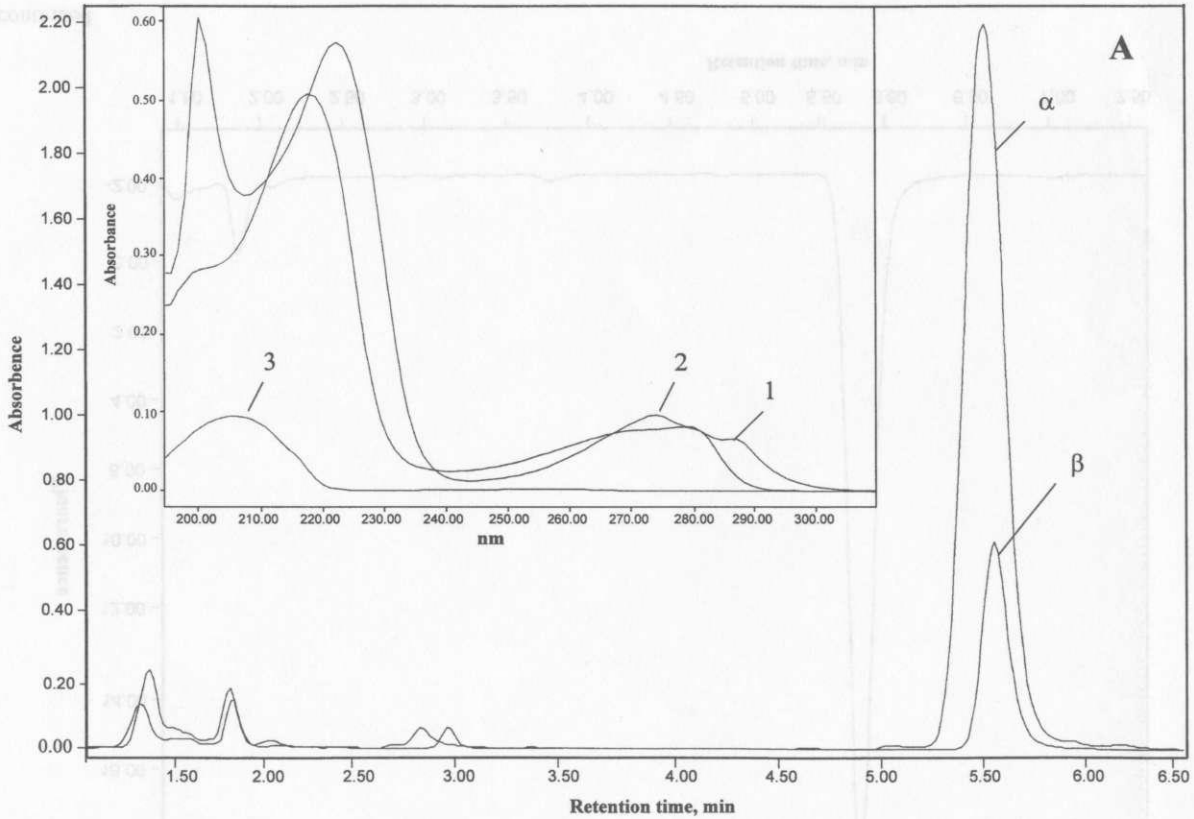

Figure 2. Part of elution profiles of underivatized tryptophan (the retention time: 5.71±0.11 min) for the standard (8.9 nmol/injection; line α) and a pea sample (line β) hydrolysed with $Ba(OH)_2$ (Buraczewska and Buraczewski, 1984). Chromatograms A – the HPLC system II with UV detection at 219 nm. In the left upper corner – UV spectra of tryptophan (line 1), tyrosine (line 2) and phenylalanine (line 3). Chromatogram B – the HPLC system II with fluorescence detection (λ_{ex} /λ_{em} = 280/360 nm). Injections volumes were 20 µl

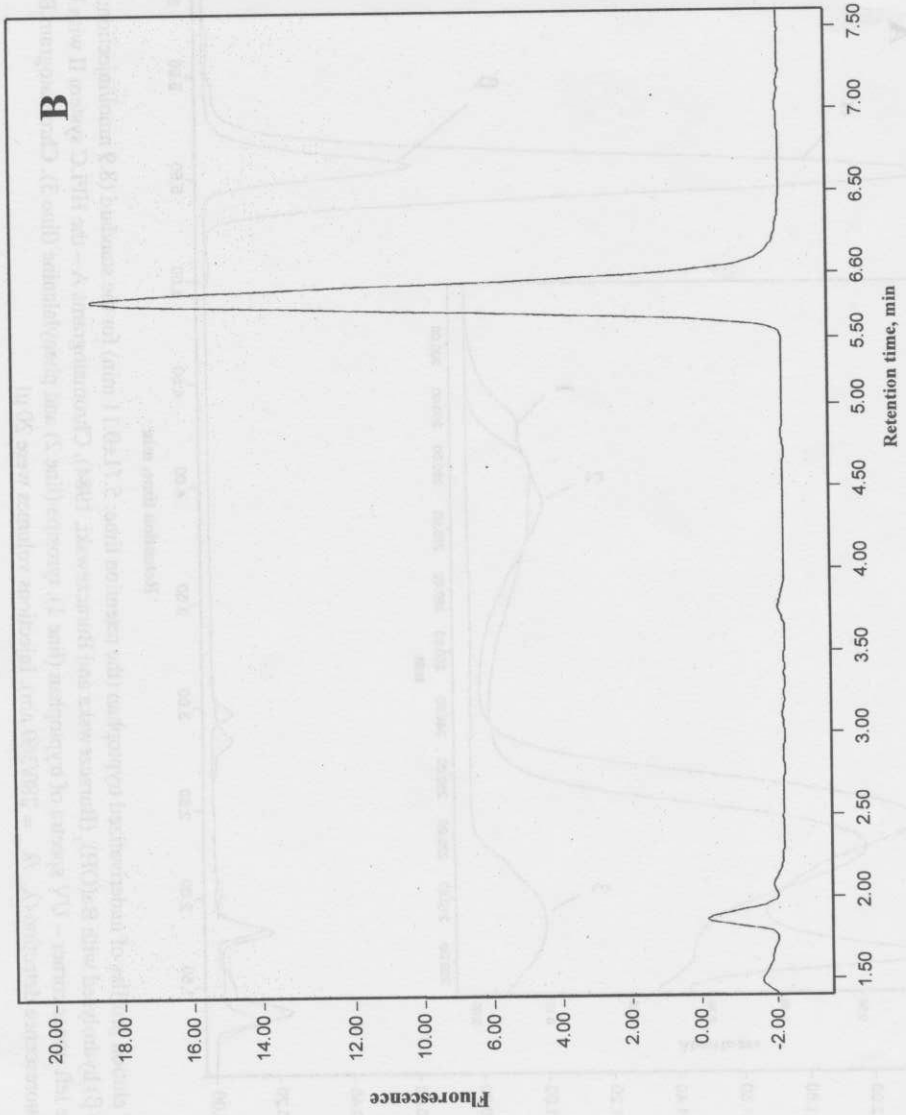

Figure 2 – continued

TABLE 4

Summary of HPLC analyses of underivatized tryptophan standard: comparison of three detection modes (gradient elution system II)

Parameter	UV detection		Fluorescence detection
	at 219 nm	at 279 nm	$\lambda_{ex}/\lambda_{em}$ = 280/360 nm
Linear regression curves[a]	$y=2.51\times10^{-7}S_n+0.08$	$y=1.70\times10^{-6}S_n+0.3$	$y=1.14\times10^{-6}S_n+0.02$
Correlation coefficient (r)	0.99963	0.99977	0.99967
Standard error in slope (SES)	4.0×10^{-9}	2.6×10^{-8}	2.5×10^{-8}
Limit of detection (LOD), μmol·l^{-1}	0.054	0.304	0.210
Limit of quantification (LOQ), μmol·l^{-1}	0.180	1.010	0.699
Values of peak areas ratio[b]	$S_n^{219nm}/S_n = 4.5$	$S_n^{279nm}/S_n = 0.73$	-

[a] – S_n and y are the peak areas and tryptophan amount (μg) in standard, respectively. Multilevel forced through zero option for generation of linear calibration curve fit. Number of points used in the calibration curves: 5. Maximal amount of tryptophan injected onto column: 7.76 μg

[b] – S_n^{219}, S_n^{279} and S_n: tryptophan peak areas obtained by the use of UV monitoring at 219 and 279 nm, and fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 280/360 nm), respectively

seen from the data summarized in Table 4, the responses of detectors to the concentrations of tryptophan were linear functions for all monitoring modes. It seems clear from the LOD and LOQ values, fluorescent and UV responses that HPLC system II with UV detection at 219 nm is the most suitable for direct quantification of tryptophan. Moreover, the correlation coefficient and standard error in slope evidence that this short-UV detection is fully suitable for routine tryptophan analysis. The accuracy of the direct method was also assessed by examining the UV spectra (190-430 nm) of tryptophan in assayed biological samples and by determining relationships between the monitoring wavelength (λ_{nm}) and the ratios (R^{nm}) (i.e. $R^{nm} = R^{nm}_{sample}/R^{nm}_{standard}$, for abbreviations see Table 5) (Czauderna and Kowalczyk, 2001). The ratios (R^{nm}) of tryptophan for alkaline hydrolysate of pea seeds were nearly 1 in the UV range from 200 to 305 nm (i.e. the average±SD ratio: 1.029±0.068). Similarly, for rumen bacteria *Lachnospira multiparus 685* (200 μl of bacterial hydrolysate in 400 μl of water) spiked with tryptophan (18.7 and 149.6 μg), the ratios (R^{nm}) were practically one (i.e. the average±SD ratios were 0.891±0.206 and 0.996±0.208, respectively). Accuracy of the method was also assessed by examining the recovery of known quantities (from 18.7 to 150 μg) of tryptophan added to rumen bacteria samples (200 μl of bacterial hydrolysate in 400 μl of water). The average±SD recovery was 96.3±0.7%, the correlation coefficient (r): 0.9996, while the ratio of responses (slopes of regression calibration lines) of tryp-

tophan peak area to the quantity of tryptophan added to water (standard) and to bacterial hydrolysate was close to 1 (i.e. in the two applied UV detection modes the average ratio was 0.96). It seems clear from the LOD and LOQ values that the proposed HPLC procedure (especially with detection at 219 nm) offers satisfactory sensitivity permitting quantification very low levels of tryptophan (i.e. 0.8 ng *per* HPLC analysis) when compared with original tryptophan contents in biological samples (e.g., in a pea seeds: 2.2 mg/g DM).

TABLE 5

Average ± SD of R^{nm} values[a] obtained in the UV monitoring range of 314-360 nm for assayed biological samples

Amino acids	Rumen bacteria	Duodenal digesta	Faeces	Blood plasma[b]	Meat	Milk
Cysteine	-[c]	-[c]	-[c]	-[c]	-[c]	0.97 ± .20
Aspatic acid	0.95 ± .06	0.96±.11	0.98±.02	-[c,d]	0.93 ± .11	0.93 ± .12
Glutamic acid	1.01 ± .01	1.09±.11	1.08±.16	0.94 ± .11	1.07 ± .18	1.02 ± .08
Aspargine	-[c]	-[c]	-[c]	1.00 ± .28	-[c]	0.98 ± .15
Histidine	1.02 ± .11	1.02 ± .02	1.03 ± .04	1.02 ± .23	0.99 ± .06	0.98 ± .06
Serine	1.01 ± .01	1.01 ± .01	1.00 ± .01	1.04 ± .21	1.01 ± .05	1.00 ±. 05
Arginine	1.00 ± .01	1.00 ± .01	0.99 ± .02	1.01 ± .05	0.99 ± .06	1.00 ±. 40
Glycine	1.00 ± .01	1.00 ± .09	1.01 ± .01	1.02 ± .05	0.99 ± .10	1.00 ±. 06
Threonine	1.01 ± .01	1.00 ± .01	1.06 ± .01	1.06 ± .10	1.00 ± .05	1.00 ± .05
Tyrosine	1.00 ± .01	1.01 ± .02	1.01 ± .01	1.17 ± .22	1.00 ± .06	1.00 ± .05
Alanine	1.01 ± .01	1.00 ± .01	1.00 ± .01	1.06 ± .05	1.00 ± .03	1.06 ± .17
Taurine	1.01 ± .11	1.11 ± .02	1.06 ± .04	-[c]	1.02 ± .05	-[c]
Methionine	0.98 ± .05	1.01 ± .02	1.02 ± .05	1.01 ± .09	1.04 ± .16	1.00 ± .06
Valine	1.00 ± .01	1.00 ± .02	1.00 ± .01	1.01 ± .04	0.99 ± .06	0.99 ± .06
Phenylalanine	1.00 ± .01	1.01 ± .01	1.00 ± .01	0.98 ± .19	1.00 ± .06	1.01 ± .06
DAPA[e]	1.00 ± .01	1.02 ± .03	1.02 ± .03	-[c]	-[c]	-[c]
Isoleucine	1.00 ± 01	1.00 ± .01	1.00 ± .02	1.06 ±.11	1.00 ± .05	1.01 ±.05
Cystine	0.94 ±.08	1.02 ± .08	1.04 ± .05	-[c]	0.96 ± .07	1.00 ±.06
Leucine	0.99 ±.01	1.00 ± .01	1.00 ± .01	0.99 ± .12	0.99 ± .05	1.00 ±. 05
Lysine	1.03 ±.04	1.01 ± .04	1.04 ± .04	0.99 ± .02	0.99 ± .05	0.99 ± .29

[a] – values (R^{nm}) of ratio R^{nm}_{sample} and $R^{nm}_{standard}$: $R^{nm} = R^{nm}_{sample}/R^{nm}_{standard}$. Absorption maximum in the UV range of 314 to 360 nm: at $\lambda_{maximum} = 337$ nm. Values ($R^{nm}_{standard}$) of ratio of amino acid peak area in a standard monitored at $\lambda_{maximum}$ (i.e. $S^{maximum}_{standard}$) and other examined wavelength (i.e. $S^{nm}_{standard}$): $R^{nm}_{standard} = S^{nm}_{standard}/S^{maximum}_{standard}$. Values ($R^{nm}_{sample}$) of ratio of amino acid peak area in biological samples monitored at $\lambda_{maximum}$ (i.e. $S^{maximum}_{sample}$) and other wavelength (i.e. S^{nm}_{sample}): $R^{nm}_{sample} = S^{nm}_{sample}/S^{maximum}_{sample}$.

[b] – determination of free amino acids in ovine blood plasma

[c] – below LOQ in examined UV range from 314 to 360 nm (above LOQ only in the proximity of 337 nm, i.e. ± ~10 nm)

[d] – quntificated by fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm)

[e] – 2,6-diaminopimelic acid

Reliability of the HPLC system I

The development of a new HPLC method with UV detection for determining primary amino acids provided the impetus for its application to biological samples such as rumen bacteria, intestinal digesta, faeces, ovine meat, milk and blood plasma. In fact, amino acid analysis of these samples is of ongoing interest to physiological and nutritional research centres conducting nutritional studies. Thus, different detection modes were applied to check the resolution efficiency of amino acid peaks. Moreover, no co-elutions of amino acid peaks with unidentified species present in the examined samples were observed for UV detection in the spectral range of 300-370 nm. The accuracy of gradient elution system I with PAD detection was proved by comparing UV spectra (from 190 to 400 nm) of amino acids in standards and ones detected in assayed biological samples. The accuracy of the presented method was also investigated in detail by determining relationships between detecting wavelength (λ_m) and ratios (R^{nm}) of the area of amino acid peaks in all assayed samples (R^{nm}_{sample}) and a calibration standard ($R^{nm}_{standard}$) (for abbreviations see in Table 5). As can be seen from data summarized in Table 5, all values for detected amino acids were practically equal to 1. Considering the above results it is reasonable to conclude that all peaks corresponding to amino acids in all assayed biological samples were "pure" in the UV range of 314-360 nm, i.e., devoid of interference due to co-eluting peaks of endogenous species absorbing in the UV range used. Further detailed analysis of "peak purity" of amino acids of cow and goat milk revealed that all peaks corresponding to the assayed primary amino acids to be also pure in the UV range of 320-355 nm. Thus, all amino acid peaks in the assayed samples can be integrated using the total peak area method. HPLC system I was also evaluated by analyzing recoveries of amino acid standards (4.5, 9.0 and 13.5 nmol) added to 10 µl of bacterial hydrolysates. Poor precision of integration of some small amino acid peaks caused deviations of recovery values from 100%. However, the obtained average recoveries of added amino acids were satisfactory (near ~ 100 %), when UV and fluorescence detection were used.

As can be seen from the data summarized in Table 5, HPLC system I enables analysis of free amino acids in blood plasma. However, to enhance the separation of the lysine peak from unidentified species the water content needed to be increased. On increasing the water content of the eluent (in the last gradient step; see in Table 1), 31% proved to be optimum of separation of lysine from interfering endogenous species present in plasma. Moreover, the presented HPLC system I with fluorescence detection assured better separation of glycine from threonine and significantly reduced the background under the lysine peak in comparison with the chromatographic conditions offered in our previously published HPLC method with fluorescence detection (Czauderna and Kowalczyk, 1998).

No significant decreases (up to 5%) of amino acid contents (with the exception of cysteine, cystine) in any assayed biological samples were observed when the derivatization solutions were protected from light and stored for 8-9 days at -18°C. On the other hand, detailed investigations of the stability of the cysteine derivative proved that the cysteine peak disappeared practically after 1 day of storage at -18°C, while the cystine derivative was more stable, therefore, even after 8 days of storage at -18°C, about 1/5 of the initial amount of derivatized cystine was detected. For all assayed biological samples stored for 8-9 days, HPLC chromatograms obtained using systems I and II with UV and fluorescence detection revealed that new unknown species formed but did not affect precise integration of analytical amino acid peaks. The reproducibility and reliability of the current HPLC systems were evaluated by repeat analysis of biological materials and blank samples. The low values of relative variations of amino acid peak areas (below ±2%) obtained for UV and fluorescence detection render HPLC systems I and II suitable for evaluating the primary amino acid profile in such important for nutritionists samples as meat, milk, blood plasma or intestinal digesta.

To enhance the resolution between derivatized cysteine and unidentified species present in biological materials, the methanol content of the mobile phase needed to be significantly reduced. Therefore, in order to obtain acceptable separation for OPA/ESH cysteine derivative the isocratic elution system (9 min in 100% solvent A at a flow-rate of 2.2 ml/min) with UV detection at 274 nm should be used (see Table 6). As shown in Figure 3 derivatized cysteine appeared in chromatograms as two peaks. Detailed analysis revealed that the optimum detection of the first larger cysteine peak was obtained using UV monitoring at 274 nm, while the second smaller one was at 284 nm (i.e., measured at their maxims - see Figure 3). As expected, both OPA/ESH cysteine peaks cannot be identified applying fluorescence detection. Obviously, both derivatives were absent from the blank. As can be seen from results summarized in Table 6, the response of the UV detector to concentrations of cysteine in the standard was a linear function. Moreover, the correlation coefficient, the standard error in slope, very low values of LOD and LOQ evidenced that the first cysteine peak and UV detection at 274 nm are the most suitable for routine cysteine analysis. The accuracy of cysteine quantification was also investigated by determining relationships between the monitoring wavelength (λ_{nm}) and ratios (R^{nm}) of areas of both OPA/ESH cysteine peaks in biological samples (R^{nm}_{sample}) and a calibration standard ($R^{nm}_{standard}$). The obtained results (Table 6) demonstrated that the first largest peak was purer in comparison with the second smaller peak corresponding to derivatized cysteine, i.e., devoid of interference due to co-eluting peaks of endogenous species in the UV range of 260-305 nm. Probably, poor precision of integration of the first cysteine peak beyond the UV range of 260-305 nm (see UV spectra in Figure 3) caused deviation of peak "purity" from 1. In summary,

TABLE 6

Summary of the quantification of OPA/ESH cysteine derivatives obtained at 39°C elution temperature, in solvent A at a flow-rate of 2.2 ml/min (the isocratic HPLC system)[a]

Parameter	UV detection	
	at 274 nm	at 284 nm
	The first peak (2.73±0.12 min)[b]	The second peak (8.43±0.07 min)[b]
Linear regression curves[c]	$y = 1.1036 \cdot 10^{-6} S_n + 0.31$	$y = 2.3368 \cdot 10^{-5} S_n + 0.31$
Correlation coefficicient (r)	0.9987	0.9970
Standard error in slope (SES)	$1.32 \cdot 10^{-8}$	$1.05 \cdot 10^{-6}$
Limit of detection (LOD) nmol·l^{-1}	0.8	1.2
Limit of quantification (LOQ) nmol·l^{-1}	2.4	3.9
Average±SD of R^{nm} values for[d]		
milk	0.93 ± 0.08	1.11 ± 1.00
meat	0.93 ± 0.07	1.05 ± 0.15
ruminal bacteria	1.01 ± 0.09	the concentration < LOQ

[a] – after 4 or 10 min (depending on used cysteine peak), the column was cleaned for 10 min in 95% methanol and 5 % water at a flow-rate of 3 ml. Next the column should be re-equilibrated for 8 min in 100 % solvent A at a flow-rate of 2.5 min (the column temperature: 39°C)

[b] – the retention times of derivatized cystene peaks: mean±SD of 8 HPLC samples

[c] – S_n and y are the peak area and derivatized cysteine amounts (µmol) injected on to column, respectively. Multilevel forced through zero option for generation of linear calibration curve fit Number of points used in the calibration curves: 5

[d] – average±SD of R^{nm} values for the first peak in the UV range of 260-305, while for the second peak in the UV range of 260-316 nm

trace amounts of cysteine in biological samples should be quantified using the isocratic HPLC system and the larger peak of OPA/ESH derivatized cysteine as the basis of cysteine detection at 274 nm.

CONCLUSIONS

The presented HPLC methods based on widely available C_{18} Nova Pak column and UV detection allow accurate and precise quantification of primary amino acids in biological materials such as feeds, bacteria produced in the rumen, intestinal digesta, meat and milk. In contrast with previous methods, OPA/ESH cysteine and cystine derivatives can be differentiated from other primary amino acids and simply quantified using HPLC system I with UV monitoring of the effluent at 337 nm.

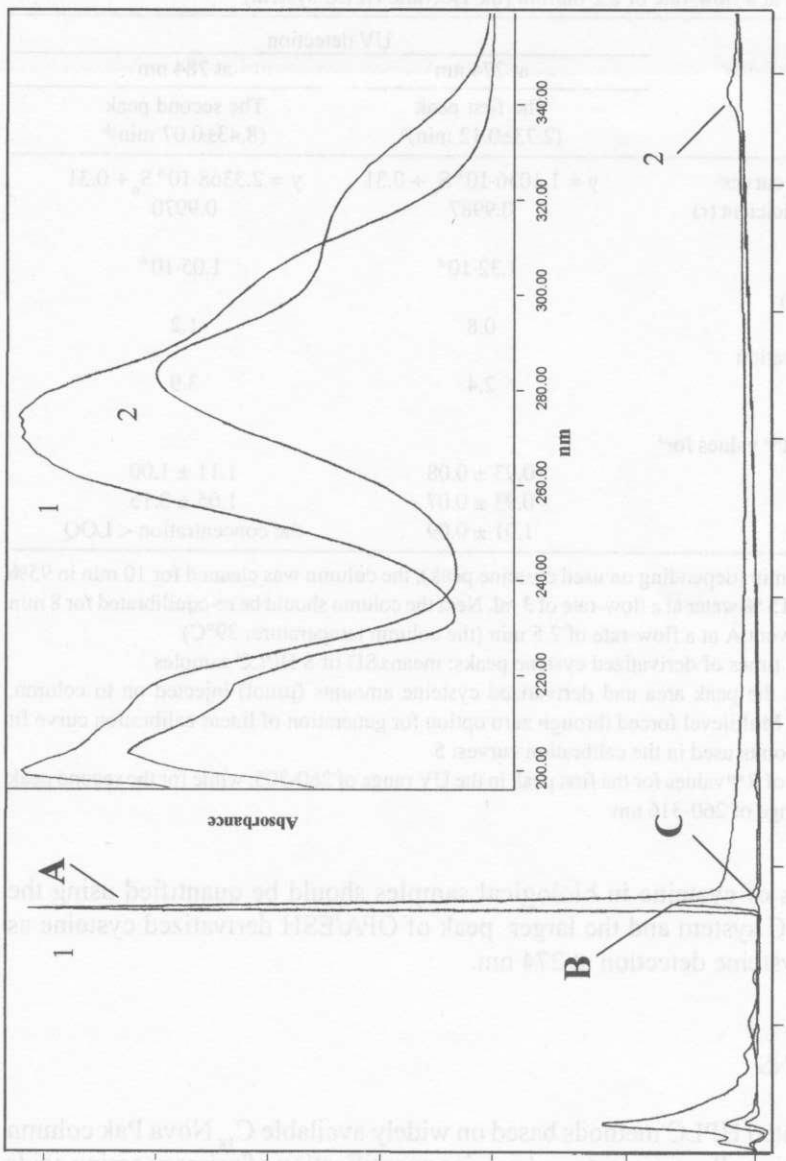

Figure 3. Separation of cysteine (peaks 1 and 2) as OPA/ESH derivatives obtained by the isocratic elution system and UV detection at 274 nm. Line A – processed cysteine standard (1.3 µmol/injection). Line B – a meat sample. Line C – a rumen bacteria (*Lachnospira multiparus 685*) sample. Injection volumes were 20 µl. In the right upper corner – UV spectra of OPA/ESH cysteine derivatives (peaks 1 and 2)

Obviously, these amino acid derivatives cannot be detected using fluorescence detection. We found many species in assayed biological samples with the characteristic spectra of OPA/ESH amino acids of unknown origin. Thus, in this respect, the quaternary gradient system I with UV detection was chosen as the optimum condition for fractionation of all assayed OPA/ESH amino acids in examined biological samples hydrolyzed with 6 M HCl. Consequently, our system I with UV detection gives satisfactory separation and sensitivity of free primary amino acids analysis in blood plasma samples.

Due to lack of direct OPA reactivity with imino acids (secondary amino acids) like proline or hydroxyproline, these amino acids should be oxidized with sodium hypochlorite (Czauderna and Kowalczyk, 1998) and next separated as OPA derivatives using RP-HPLC systems. Moreover, this oxidizing agent oxidized cyst(e)ine to cysteic acid that reacts with OPA/ESH to form an OPA-cysteic acid derivative possessing, like other primary amino acids, relatively high absorbance band in the UV spectral range from 310 to 360 nm. The separation and quantification of oxidized proline, hydroxyproline and cyst(e)ine, as their OPA/ESH derivatives, can be achieved applying a Symmetry C_{18} column (5 μm, 250 × 4.6 mm, I.D., Waters) and UV detection at ~337 nm (Czauderna and Kowalczyk, 1998). Obviously, secondary amino acids may be derivatized with other reagents (e.g., 4-chloro-7-nitrobenzofurazan or 9-fluorenylmethyl chloroformate), and then separated using the HPLC systems reported by Umagat (1982) or by Sarwar (1993). Moreover, methionine and cyst(e)ine estimation in biological materials hydrolysed with hydrochloric acid show that certain amount of cyst(e)ine and methionine can be oxidized. Therefore, the preparation procedure should be incorporated an oxidation step to convert these amino acids to respectively cysteic acid and methionine sulphone prior to hydrolysis and derivatization (Rattenbury, 1981; Buraczewska and Buraczewski, 1984).

The presented HPLC systems with UV detection can be possible reliable alternatives to earlier chromatographic methods with fluorescence detection.

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STRESZCZENIE

Oznaczanie wolnych i białkowych aminokwasów w materiałach biologicznych metodą wysokosprawnej chromatografii cieczowej z detekcją fotodiodową

W pracy opisano metodę oznaczania wolnych i białkowych pierwszorzędowych aminokwasów w materiałach biologicznych, które hydrolizowano w 6 M HCl przez 20 godzin w temperaturze 104±2°C. Po odparowaniu hydrolizatu aminokwasy przeprowadzano w pochodne używając o-dialdehyd ftalowy w obecności etanotiolu. Pochodne aminokwasów rozdzielano na kolumnie C_{18} z odwróconą fazą (Nova-Pak, 4 µm, 250 × 4.6 mm) poprzez poczwórną elucję gradientową. Pochodne oznaczano stosując monitorowanie UV przy 337 nm lub detekcję fluorescencyjną (wzbudzenie 336 nm, pomiar 425 nm). Czas rozdzielenia wszystkich aminokwasów wynosił po 50 min. Prezentowana metoda (system I) może być również użyta do oznaczania wolnych aminokwasów we krwi owiec. Oznaczanie śladowych ilości cysteiny można przeprowadzić po 3.5 min. izokratycznej elucji wykorzystując monitorowanie UV przy 274 nm. Tryptofan w badanych próbach oznaczono po 16 godz. hydrolizie alkalicznej w $Ba(OH)_2$. Po usunięciu jonów baru i doprowadzeniu pH hydrolizatu do wartości ~7, tryptofan oznaczano bezpośrednio wykorzystując tę samą kolumnę, potrójną elucję gradientową oraz monitorowanie UV przy 219 nm lub detekcję fluorescencyjną (wzbudzenie: 280 nm; pomiar: 360 nm). Średni odzysk standardów aminokwasów dodanych do próbek biologicznych był bliski 100% zarówno przy monitorowaniu UV jak i przy detekcji fluorescencyjnej. Detekcja fluorescencyjna pozwala na uzyskanie lepszych wartości granic jakościowej i ilościowej detekcji w porównaniu z detekcją UV. Czułość detekcji UV oraz „czystość analitycznych pików" jest wystarczająca do rutynowego oznaczania pierwszorzędowych aminokwasów w masie bakteryjnej, treści przewodu pokarmowego owiec, kale, mięsie oraz mleku. Prezentowana metoda HPLC może być wykorzystana do oceny rozmiaru biosyntezy białka bakteryjnego.