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A highly efficient method for determination of some amino acids and glutathione by liquid chromatography

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ABSTRACT

High-performance liquid chromatography systems (I and II) with *pre*-column derivatization for separation of cyst(e)ine, selenium-cystine, homo-cystine, methionine, selenium-methionine, proline and glutathione (GSH) in biological materials are described. Biological samples were derivatizated with *o*-phthaldialdehyde (OPA) in the presence of ethanethiol. Prior to derivatization, cyst(e)ine, methionine, GSH and proline were oxidized using improved procedures. Performic acid and sodium hypochlorite were used as the oxidizing reagents. HPLC analyses of derivatives of all oxidized compounds were carried out using a C_{18} column (4 μm, 250 x 4.6 mm I.D., Nova Pak, Waters) and binary gradient elution program I (HPLC system I). HPLC system I with UV monitoring (at 336 nm) and fluorescence detection (excitation and emission wavelengths at $\lambda_{ex}/\lambda_{em}$ = 336/425 nm) was chosen as providing the optimum conditions for fractionation and quantification of all of the examined compounds. In comparison with the UV detection, fluorescence detection offers better sensitivity (limits of detection: 0.9-2.4 vs 0.3-0.8 $ng \cdot l^{-1}$). Clear separation of cyst(e)ine, GSH, methionine and proline was obtained in about 35 min. Separation of unoxidized GSH, cystine, seleno-cystine, methionine, seleno-methionine, homo-cystine from other free amino acids was achieved using HPLC system II with UV detection at 337 nm and/or fluorescence detection ($\lambda_{ex}/\lambda_{em}$=336/425 nm). Compared with fluorescence detection, UV monitoring offers better limits of detection (L_D) of cystine, seleno-cystine and homo-cystine, while worse L_D for GSH, methionine and seleno-methionine. The satisfactory purity of analytical peaks of the assayed compounds (near 100%), and the simplicity and precision of HPLC systems I and II render these methods suitable for routine analysis of these compounds in large numbers of biological samples.

KEY WORDS: amino acid determination, cyst(e)ine, Se-cystine, homo-cystine, methionine, Se-methionine, proline, glutathione, HPLC

INTRODUCTION

Proteins in foods contain some twenty common amino acids. All twenty amino acids are needed for protein biosynthesis, although about nine cannot be synthesized or adequately synthesized in the human body, and are referred to as essential amino acids (Lewis and Bayley, 1995; Ravindran and Bryden, 1999). In addition, some feed proteins usually contain hydroxyl-amino acids such as hydroxyl-proline or hydroxyl-lysine. Dietary requirements for protein and amino acids are based on demand for total amino nitrogen, essential amino acids and other important nitrogen compounds, such as neuro-transmitters and peptide hormones (Lindroth et al., 1985; Żebrowska and Buraczewski, 1998). Moreover, monitoring of sulphur- containing amino acids is important for nutritional and biochemical research, in pharmacodynamic studies of thiol drugs, or in the diagnosis of some diseases (Bald and Głowacki, 2001). In fact, cysteine is metabolically related to homo-cysteine and glutathione (GSH) (Bald et al., 2001). Homo-cysteine can be remethylated to methionine, important amino acids in protein biosynthesis, or converted to cysteine in the *trans*-sulphuration pathway (Chwatko and Bald, 2002). Therefore, there is ongoing interest in the improvement of chromatographic methods for determination of sulphur amino acids and GSH in biological samples for regulatory purposes and examination of amino acid profiles in foods. Traditionally, amino acids are separated by ion-exchange chromatography (IEC) with ninhydrin derivatization (Ng et al., 1991; Moller, 1993; Sarwar and Botting, 1993). However, in the last year HPLC methods have replaced IEC. Indeed, due to the use of simpler instrumentation in *pre*-column derivatization, small-bore HPLC columns, small particle size (3-5 μm) resins, high-pressure pumps coupled with high-sensitivity detectors, HPLC methods offer reduced analysis time and improved limits of detection of about 1 pmol (Sarwar and Botting, 1993; Williams, 1994; Cohen and Michaud, 1998; Czauderna and Kowalczyk, 1998; Peter et al., 1998; Polak and Golkiewicz, 2000; Czauderna et al., 2002). The most widely used reagent for derivatization was *o*-phthaldialdehyde (OPA) in the presence of a thiol (Kutlan and Molnar-Perl, 2001; Molnar-Perl, 2001; Czauderna et al., 2002). Unfortunately, due to the lack of OPA reactivity with imino acids, instability of methionine during hydrolysis of proteins and poor fluorescence of cyst(e)ine OPA derivatives (Lindroth et al., 1985; Czauderna et al., 2002), these amino acids should be oxidized prior to derivatization (Bech-Andersen et al., 1980; Czauderna and Kowalczyk, 1998) and then separated as OPA derivatives using reversed-phase HPLC systems. Therefore, the main purpose of the current study was to develop and improve oxidation procedures and HPLC methods with OPA-*pre*-column derivatization for simultaneous determination of cyst(e)ine, methionine, GSH and proline in biological materials. Furthermore, attention has also been paid to omitting the oxidation steps and simultaneous quantification of OPA-derivatized GSH, homo-cystine, cystine, methionine, seleno-cystine and seleno-methionine in the presence of other amino acids.

MATERIAL AND METHODS

All amino acids, glutathione (GSH) and *o*-phthaldialdehyde (OPA) were obtained from Sigma (St. Louis, MO, USA) while ethanethiol (ESH), tetrahydrofuran (THF) and sodium hypochlorite water solution (4% available Cl) were from Flucka. All other reagents were of analytical grade (POCh, Poland), whereas methanol was gradient HPLC grade (Becker). 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). The amino acid protein hydrolysate standard kit was obtained from Waters Corporation (AccQ·Tag™, Part No. WATO52875, USA).

Chromatographic equipment

An alliance separation module (model 2690, Waters) with a Waters 996 photodiode array detector (DAD) and Waters 474 fluorescence detector (FD) was used for development of HPLC methods. An autosampler was thermostated at 4°C. The OPA derivatized compounds in the effluent were simultaneously detected by DAD and FD. DAD was operated in a UV range from 195 to 390 nm with a measurement frequency of 1 spectrum per sec and a spectral resolution of 1.2 nm. The OPA-derivatized amino acids and GSH were UV detected at 336 or 337 nm, while fluorescence detection was at an excitation wavelength of 336 nm and an emission cut-off filter of 425 nm. An analytical Nova Pak C_{18} column (4 μm, 250x4.6 mm, I.D., Waters) was fitted with a *pre*-column of 10x6 mm I.D. (Nova Pak, Waters) containing C_{18} pellicular packing material (30-40 μm).

Analytical mobile phases and gradient elution programs

Two gradient elution programs were used for fractionation and quantification of the assayed compounds in the examined samples. In HPLC system I the following gradient elution program I was used (Table 1). Solvent A was THF - 0.05 M sodium acetate (pH was adjusted to 6.6 with phosphoric acid) (1:99, v/v), while solvent B was methanol. For analysis of unoxidized analytes, gradient elution program II was applied (Table 2). The following elution mobile phases were used: solvent C was THF - buffer C (1:99, v/v). Buffer C was prepared from 0.02 M Na_2HPO_4 adjusted to pH 3.5 with 10% phosphoric acid. Solvent D was THF - buffer B (1:99, v/v). Buffer B was prepared from 0.04 M Na_2HPO_4 adjusted to pH 6.6 with 10% phosphoric acid. Solvent B was methanol, while the last solvent used in this HPLC system was water (solvent E). The minimal system pressure was 20.1±0.1 MPa; the maximal pressure was 37.4±0.2 MPa. Injection volumes were 5-30 μl. Amino acids were identified by the retention time of processed standards injected separately

TABLE 1

Binary gradient elution program I[1] used for analysis of oxidized assayed species (*pre*-column OPA HPLC system I)

Time	Composition[2], %	
min	solvent A	solvent B (methanol)
0	85	15
3	72	28
20	56	44
26	44	56
34	20	80
54	79	21

[1] the initial flow-rate was 1.3 ml/ml, then 1.5 ml/min from 20 to 60 min

[2] all changes of solvents composition were linear

TABLE 2

Quaternary gradient elution program II[1] used for analysis of OPA-derivatizated free amino acids and GSH in biological materials (*pre*-column OPA HPLC system II)

Time	Composition[2], %			
min	solvent B	solvent C	solvent D	solvent E
0	15	0	85	0
1.8	15	0	85	0
3.0	28	0	72	0
19.0	28	0	72	0
19.5	36	0	64	0
22.0	36	0	64	0
28.0	46	0	54	0
30.2	45	55	0	0
30.5	56	44	0	0
35.5	57	43	0	0
37.0	56	0	44	0
42.0	63	0	37	0
45.0	60	0	40	0
50.0	80	0	0	20
55.0[3]	85	0	0	15

[1] flow-rate: 1.8 ml/min; column temperature: 37ºC

[2] all changes of solvent composition were linear

[3] 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

and by adding standard solution to biological samples. The limit of detection (L_D) was calculated as a signal-to-noise ratio of 3, while the limit of quantification (L_Q) 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 HPLC grade 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 oxidizing solutions

NaClO (*oxidizing reagent 1*): to 0.995 ml of deionised water, 5 µl of sodium hypochlorite solution (4% available Cl) were added.

Performic acid (*oxidizing reagent 2)*: 0.1 ml of 40% hydrogen peroxide and 1.125 ml of 85% formic acid were allowed to incubate at 50ºC for 3 min.

It is recommended to protect the oxidation reagents from light and to store refrigerated at -79ºC when not in use. As a guide, fresh reagent solutions should be used.

Preparation of derivatizing reagent (OPA/ESH)

Seventy mg of OPA were dissolved in 4.5 ml of methanol and 0.5 ml of the borate buffer. Next, 0.08 ml of ESH was added and the resulting solution was mixed. The reagent solution was prepared at least 3 h before use. It is recommended to protect the derivatizing solution from light and to store refrigerated (-18ºC) 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 materials

Samples of rumen bacteria (*Lachnospira multiparus 685*) were frozen, freeze-dried and the obtained homogeneous materials (~500 mg) were hydrolysed with 50 ml of 6 M HCl at 104±2ºC for 20 h in sealed tubes. After cooling the hydrolysates were filtered through filter paper and washed three times with water. Hydrochloric acid was removed from the filtrates in a vacuum rotary evaporator. Ten ml of deionized 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 lyophilized residues were stored at -18ºC 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 the resulting solution were used for the OPA/ESH derivatization procedures as below.

Derivatization procedure I with improved oxidation step 1

To autosampler vial I were added 10-20 µl of an assayed sample, 0.2 ml of oxidizing reagent 1 (NaClO) and 20 µl of 2 M NaOH. The content was mixed and

reacted for 24 min at 25ºC. At the end of the 24 min period, 0.45 ml of the OPA/ESH reagent were added to the first vial and allowed to react for 2 min. Mixture I was protected from light.

Derivatization procedure II with improved oxidation step 2

To autosampler vial II were added 10-20 µl of an assayed sample and 20 µl of oxidizing reagent 2 (performic acid). The content was mixed and reacted for 24 min at 40ºC. Oxidation was terminated by adding ca. 20-30 mg of sodium pyrosulphide ($Na_2S_2O_5$). After cooling (to 0-1ºC), the pH of the resulting solution was adjusted to 9-10 by adding 0.04 ml 10 M NaOH. Next 0.45 ml of the OPA/ESH reagent was added to vial II and allowed to react for 2 min. Mixture II was protected from light.

HPLC analysis

Mixtures I and II were then combined and after mixing, 10-40 µl of the resulting solution were injected onto the HPLC column. The separation and quantification of OPA derivatives of oxidised compounds was performed using HPLC system I. The derivatizing procedures for standards were the same as for biological samples.

Procedure for OPA derivatization of free amino acids

To blood plasma samples *(*20-50 µl) or other physiological fluids (30-50 µl) (Czauderna and Kowalczyk, 1998; Czauderna et al., 2002), immediately after collection, was added 0.9 ml of the OPA/ESH reagent and then the pH was adjusted to 9-10 with 10-20 µl of 2 M NaOH. At the end of the 2 min period, 10-40 µl of the resulting solution were injected onto the column. The standards were processed in the same way as the biological samples. Separation of derivatized unoxidised compounds was carried out using HPLC system II.

RESULTS AND DISCUSSION

The goal of elucidating the physiological functions of sulphur, selenium amino acids, GSH (the major cellular thiol) and proline provided the impetus for the development of accurate and sensitive OPA-HPLC methods for simultaneous determination of these compounds in the presence of other amino acids. Moreover, HPLC analyses of this type are performed at research centres conducting nutritional studies. Therefore, to achieve optimal conditions for assays of biological samples, a long column (250 mm) packed with strongly hydrophobic silica bonded phase (C_{18}) with photodiode array detection should be used.

Because proline (i.e. an imino acid) lacks OPA reactivity, it was first oxidised with sodium hypochloride in an alkaline medium (Czauderna and Kowalczyk, 1998) and then separated after *pre*-column OPA-derivatization. However, in order to improve the purity of the obtained oxidation products we applied a more diluted solution of NaClO (i.e. 0.02% available Cl) than used in our previous studies (i.e. 0.3% available Cl) (Czauderna and Kowalczyk, 1998). As the objective of the presented work was to develop a new HPLC method for simultaneous determination of other important compounds, we combined the improved NaClO oxidation of proline (step 1) with improved oxidation of cyst(e)ine, methionine and GSH with using performic acid (step 2). Next, the oxidized compounds reacted with the OPA/ESH reagent (in ~pH 9-10) to form derivatives possessing very high molar absorption in the long UV range (i.e. from 320 to 360 nm). Indeed, these OPA-derivatives having a high quantum yield ensured high sensitivity of UV and fluorescence detection. Recent work (Czauderna and Kowalczyk, 1998; Czauderna et al., 2002) has demonstrated that OPA amino acid derivatives can be successfully quantificated by reversed-phase C_{18} columns using a gradient elution program and UV or fluorescence detection. Thus, for real biological samples it is necessary to optimise the gradient program, UV and fluorescence detection. Fortunately, binary gradient elution program I (Table 1) composed of sodium acetate buffer (pH 6.6) in methanol was found to provide excellent baseline stability and a wide range of solvent strength. By manipulating the percentage of buffer in methanol, the new OPA- HPLC system I enabled satisfactory fractionation of derivatives of oxidized cyst(e)ine, methionine, GSH and proline in bacterial samples and in amino acid protein hydrolysate standard. All analytes were substantially retained on the Nova Pak C_{18} column. Satisfactory peak shapes, nearly symmetrical, were obtained with sample elution times from 5 to 36 min. In our HPLC system I cyst(e)ine was eluted at 5.8±0.2 and 6.6±0.2 min, GSH at 20.4 ±0.2 min, methionine at 24.2 ±0.3 and 25.3 ±0.3 min, while proline had a retention time of 33.9 ±0.3 min (Figure 1). As expected, the peaks of all assayed compounds were absent from the blank when the presented HPLC system I was used. Although fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm) is usually applied (Lindroth et al., 1985; Czauderna and Kowalczyk, 1998; Czauderna et al., 2002), UV detection at 336 nm also provided adequate sensitivity when compared with the original concentration of these species in biological materials. The reliability of the proposed HPLC system I was evaluated by simultaneous use of UV-photodiode and fluorescence detection. The accuracy of HPLC system I with UV detection was documented by comparing UV spectra (from 195 to 390 nm) of assayed analytes in standards and ones in examined biological materials or the amino acid protein hydrolysate standard. No co-elution of assayed compounds with unidentified species present in the examined materials was observed for UV detection in the spectral ranges of 215-245 and 300-360 nm. Subsequently, attempts were made to compare the results as dependent on the detection mode used. As can be seen from HPLC

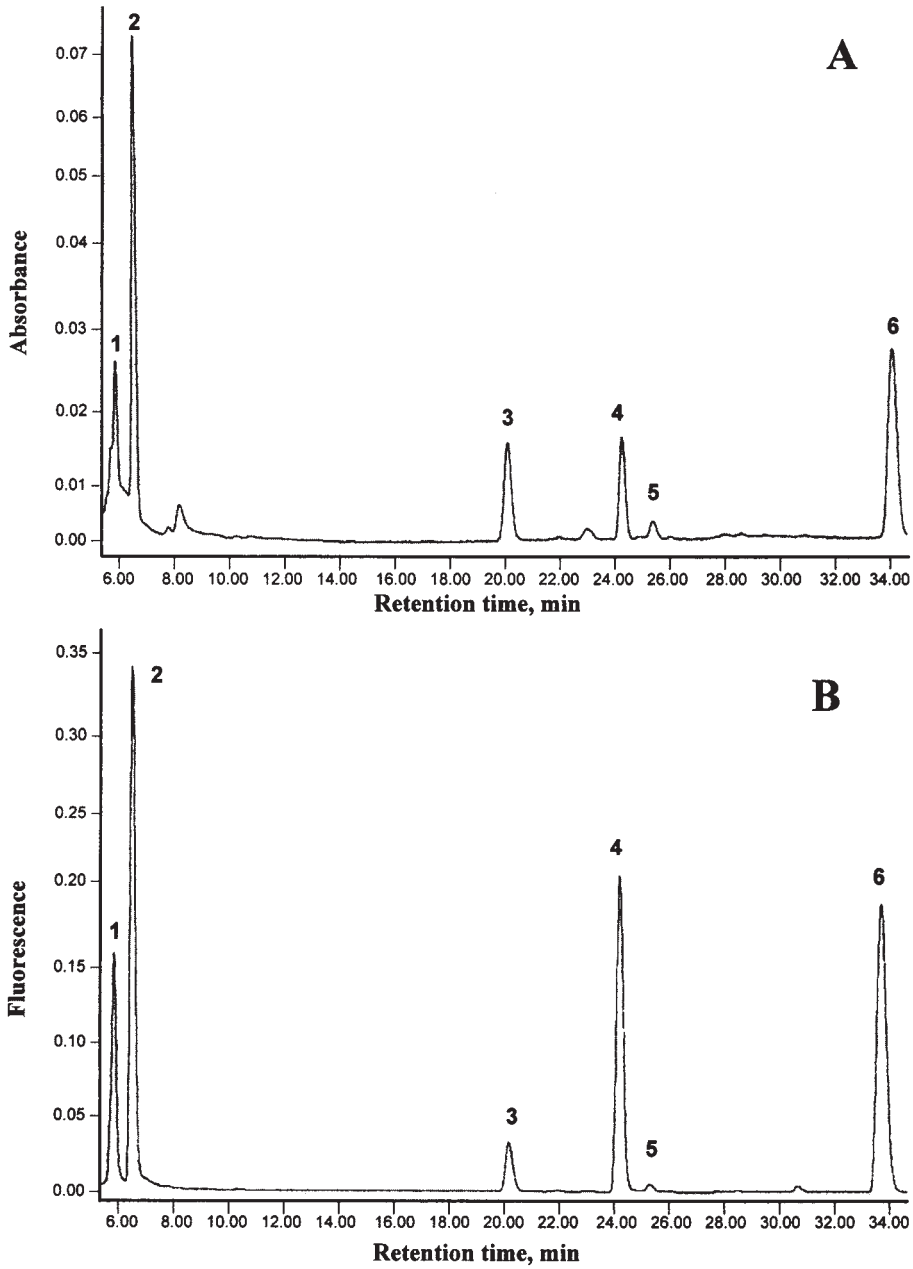

Figure 1. Part of typical HPLC chromatogram for oxidized compounds as OPA/ESH derivatives obtained by the binary gradient elution program I (the HPLC system I) with UV detection at 336 nm (a chromatogram A) and the fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm) (a chromatogram B). Injection volume was 20 µl. Peaks: 1 and 2 - cyst(e)ine; 3 - glutathione (GSH); 4 and 5 - methionine; 6 - proline

runs of processed samples, the response ratios of the photodiode detector (DAD) to the fluorescence detector were practically identical for all examined compounds in all assayed standards and biological samples. Furthermore, the same relations were obtained when another binary gradient elution program (not presented in the current study) with the same detection modes was applied. As expected, excitation at 230 nm ($\lambda_{ex}/\lambda_{em}$ = 230/470 nm) provided a greater response of the fluorescence detector as compared to excitation at 336 nm ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm) and UV detection at 336 nm, however, the noise and background fluctuations are larger for fluorescence detection using 230 nm excitation. Consequently, the limits of detection (L_D) for the assayed compounds were worse and, especially for biological samples, the analytical peaks were not clearly resolved from interfering impurities (i.e. decreased precision of analyses). The results of GSH determination were particularly interesting and important (Table 3) because UV detection at 336 nm produced a greater signal than fluorescence detection applying a 336 nm excitation wavelength. Detailed analysis of chromatograms (Figure 1) and results summarized in Table 3 evidenced that the second cyst(e)ine peak and the first methionine peak (i.e. larger peaks) are most suitable for routine analysis.

Attempts were made to evaluate the accuracy of HPLC system I by analysing recovery (R, %) of a known quantity of analysed compounds from bacteria (from 1 to 2 mg of lyophilized bacterial hydrolyzate). The obtained average recovery for assayed compounds evidenced that the examined HPLC system I provided satisfactory accuracy when UV monitoring at 336 nm (i.e. 96-101%) and fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 336/425 nm) (practically 100%) were applied. Comparison of UV spectra of the examined species in standards and ones in biological samples also proved the accuracy of the presented HPLC system. Furthermore, we investigated the relationships between the UV detection wavelength (λ_{nm}) and ratios (R^{nm}_{sample}) of peak areas of the examined compounds in rumen bacteria and calibration standards ($R^{nm}_{standard}$) (for abbreviations see Table 3). As can be seen from results obtained from detection in the long UV range (300-360 nm), all average values of R^{nm} of cyst(e)ine, GSH, methionine and proline were nearly 1. Therefore, these compounds can be determined using long UV-wavelengths. As expected, in the examined short UV range (from 215 to 245 nm) all values of relative standard deviation (RSD) were also small, as well as average values of R^{nm} were practically equal to 1. However, we suggest that the current HPLC system I based on longer wavelength UV detection (300-360 nm) is more suitable for analysis of the assayed compounds since other endogenous species, in general, possess relatively high absorbance in the short UV range (λ<240 nm). Indeed, the presented data (Table 3) documented that detection at the longer UV range (300-360 nm) provides better purity of analytical peaks (compare values of RSD and R^{nm} for applied short and longer UV range). Moreover, the high molar absorptivity of OPA derivatives and the close proximity to the absorbance maximum at 335-336 nm was almost ideally suited for analysis

TABLE 3

Average ± SD of R^{nm} values[1] obtained in the UV monitoring ranges of 215 - 245 and 300 - 360 nm and limit of detection (L_D) derived from oxidized compounds in examined samples obtained by UV monitoring and fluorescence detection (FD) (OPA-HPLC system I)

Compounds	Retention time min	Value[2] of S_n^{DAD}/S_n^{FD}	$R^{nm} \pm SD$ monitoring UV range				Limit of detection (L_D)[3]	
			215-245 nm	RSD, %[4]	300-360 nm	RSD,%	UV detection ng·l^{-1}	FD ng·l^{-1}
Cyst(e)ine[5]								
Peak 1	5.80 ± 0.17	0.45	0.96 ± 0.06	6.2	1.01 ± 0.25	25.0	0.94	$5.47 \cdot 10^{-2}$
Peak 2	6.58 ± 0.19	0.55	1.03 ± 0.03	3.3	1.04 ± 0.07	6.7	0.87	$3.33 \cdot 10^{-2}$
GSH	20.4 ± 0.2	1.17	0.99 ± 0.03	3.5	0.99 ± 0.01	1.1	2.42	1.35
Methionine[6]								
Peak 1	24.2 ± 0.3	0.35	0.84 ± 0.15	18.0	0.99 ± 0.08	8.3	1.01	0.22
Peak 2	25.3 ± 0.3	0.31	1.04 ± 0.10	1.5	1.01 ± 0.06	6.4	1.23	0.76
Proline	33.9 ± 0.3	0.33	0.92 ± 0.19	21.0	1.02 ± 0.06	5.6	2.35	0.26

[1] 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 300 to 360 nm: at $\lambda_{maximum}$ = 336 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 wavelengths (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 examined wavelengths (i.e. $S^{nm}_{sarmple}$): $R^{nm}_{sample} = S^{nm}_{sample}/S^{maximum}_{sample}$.

[2] response ratio of the photodiode array detector and the fluorescence detector

[3] UV detection at 336 nm; fluorescence detection (excitation and emission wavelengths: 336/425 nm)

[4] relative standard deviation of values R^{nm}

[5] area ratio of peak 1 to peak 2: 0.44 (UV detection at 336 nm)

[6] area ratio of peak 1 to peak 2: 5.2 (UV detection at 336 nm)

of these compounds. Consequently, all analytical peaks can be integrated using the total peak area method.

The stability of oxidized compounds was investigated in detail at -18ºC with respect to storage time of processed standards and biological samples. Our studies indicated that the areas of the assayed compounds were practically the same when processed samples were protected from light and stored for 1 h at -18ºC. Unfortunately, the main disadvantage of oxidised cysteine derivatives is their high instability; in fact, 24 h of storage at -18ºC of derivatives of oxidized cyst(e)ine exhibited ~50% degradation.

As can be seen from the limit of detection (L_D) summarized in Table 3, the proposed HPLC system I with UV or fluorescence detection offers satisfactory sensitivity permitting detection and quantification of a relatively low level of these compounds compared with the original contents of free cyst(e)ine, methionine, proline and GSH in biological materials. As expected, the obtained values of L_D evidenced that fluorescence detection used at 336 nm excitation offers the highest sensitivity for all assayed compounds.

Determination of free amino acids and GSH

In order to minimize the contribution of sample preparation, the oxidation steps were omitted. Therefore, immediately after collection the assayed samples were derivatized using OPA in the presence of ESH. The key step in OPA-derivatized free amino acid analysis is complete separation from numerous unidentified endogenous species present in biological materials. The results from previous studies (Czauderna and Kowalczyk, 1998; Czauderna et al., 2002) and the current exhaustive investigation have demonstrated that a long C_{18} column(25 cm, Nova Pak) and UV monitoring (at 337 nm) or fluorescence detection ($\lambda_{ex}/\lambda_{em}$=336/425 nm) provide suitable HPLC conditions for simultaneous determination of cystine, seleno-cystine, homo-cystine, methionine and seleno-methionine in the presence of other amino acids (Figure 2). Moreover, as can be seen in Figure 2, HPLC system II also resulted in satisfactory separation of GSH from all assayed amino acids. Satisfactory chromatographic fractionation in ca 53 min of an HPLC run was achieved due to the use of quaternary gradient reversed-gradient HPLC system II with UV detection in a relatively clean UV region (i.e. 337 nm) or selective fluorescence detection ($\lambda_{ex}/\lambda_{em}$=336/425 nm). Unidentified endogenous species present in rumen bacteria, intestinal digesta, faeces, meat and milk therefore do not interfere with all assayed compounds (Czauderna et al., 2002). Obviously all examined compounds were absent from the blank when HPLC system II was used. The accuracy of the described HPLC system II was assessed by the response ratios of assayed analytes of UV and fluorescence detector for standards and biological samples. As expected, the response ratios of DAD to the fluorescence detector

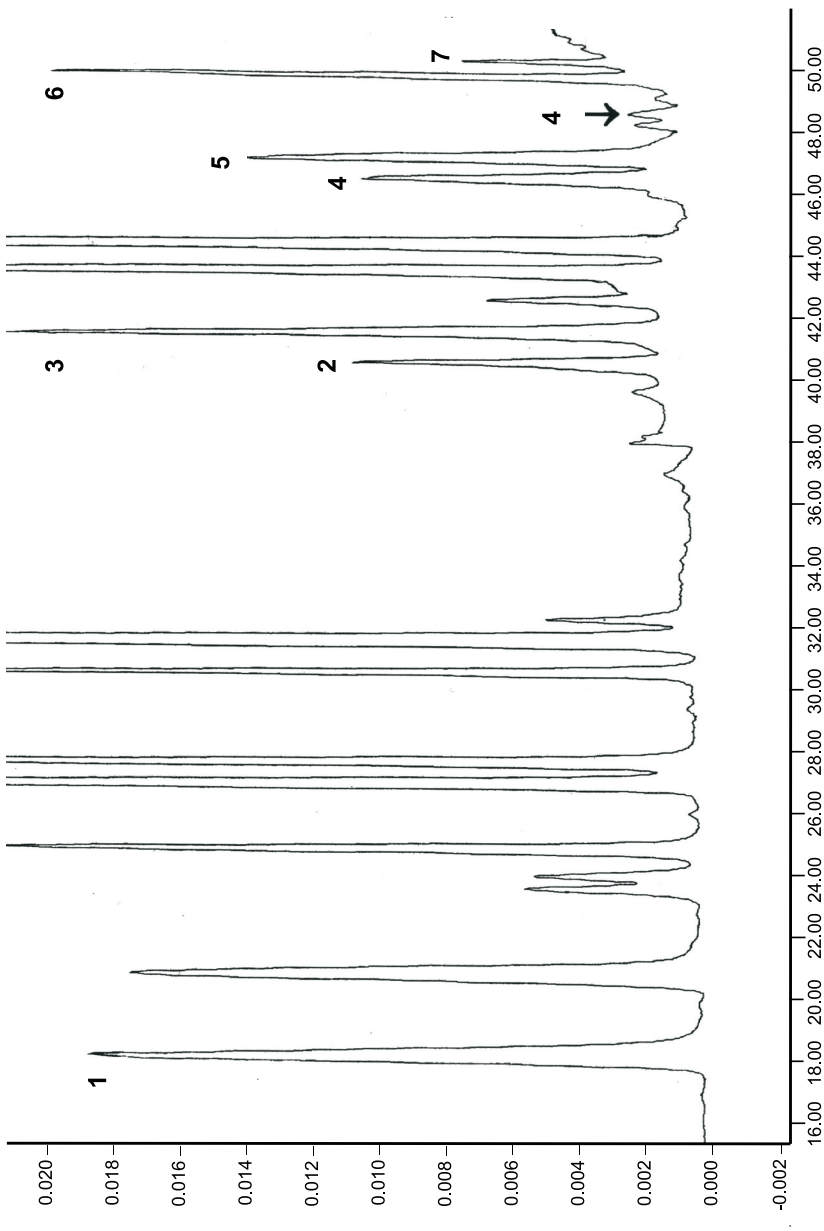

Figure 2. Part of elution profiles of unoxidized compounds using the quaternary gradient elution program II (the HPLC system II) with UV detection at 337 nm. Peaks: 1 - GSH; 2 - methionine; 3 - seleno-methionine; 4 - seleno-cystine (46.0 ± 0.3 and 48.1 ± 0.4 min); 5 - cystine; 6 - homo-cystine (49.0 ± 0.3 min); 7- homo-cystine (49.6 ± 0.3 min)

were practically identical for all assayed compounds. As can be seen from previous studies and the data summarized in Table 4, cystine (Czauderna at al., 2002), seleno-cystine and homo-cystine derivatives gave higher UV responses in comparison with fluorescence (Table 4). Surprisingly, seleno-cystine yields the lowest fluorescent properties, while homo-cystine showed the highest fluorescence responses. The obtained results are sensible since the sulphur atom in the homo-cystine molecule is separated from the amino group by one more carbon atom than in the cystine molecule. Our results indicate that the selenium atom in seleno-cystine most efficiently reduced the quantum emission yield of the fluorescent OPA selenium-product. On the other hand, the thiol group of GSH decreased the quantum yield of the fluorescent derivative the least. The accuracy of HPLC system II was also proved by determining the relationships between detecting wavelength (λ_{nm}) and ratios (R^{nm}) of an area of assayed compounds in biological samples (R^{nm}_{sample}) and standards ($R^{nm}_{standard}$) (for abbreviations see in Table 3). As can be seen from the results displayed in Table 4, all of the examined compounds can be determined using longer wavelengths. The absorbance maximum near 337 nm clearly makes these OPA derivatives almost ideally suited for analysis in various types of biological materials. Further detailed analysis of peak purity revealed that ratios (R^{nm}) of seleno-methionine, seleno-cystine and GSH were nearly 1 in the short UV range (Table 4). However, detailed results indicated that, generally, in the short UV range integrated analytical seleno-methionine and seleno-cystine peaks are less pure than in the longer UV range. In particular, poor purity can be found when monitoring homo-cystine *via* the second peak in the short UV range (Table 4). Considering the above results, it can be concluded that all analytical peaks can be integrated applying the total peak area method as devoid of substantial co-eluting impurities with peaks absorbing in the longer UV range (300-360 nm). HPLC system II based on UV detection at 337 nm is seen to be the most accurate and precise.

The sensitivity of HPLC system II was assessed by determining the limit of detection (L_D) values for the examined analytes. As expected, all values of L_D for UV detection at 337 nm are low. Therefore, this detection mode offers satisfactory sensitivity permitting detection and quantification ($L_Q=3\cdot L_D$) of relatively low levels of assayed compounds when compared with original levels of these species in biological materials. The results of GSH, seleno-methionine and methionine (Czauderna et al., 2002) are particularly important (Table 4) because fluorescence detection provides better sensitivity than UV detection, while worse L_D for seleno-cystine, homo-cystine and cystine (Czauderna et al., 2002). Considering the results summarized in Table 4 and HPLC chromatograms (Figure 2), both seleno-cystine and homo-cystine in biological materials should be quantified using the first (larger) peak of OPA derivatives.

TABLE 4

Average ± SD of R^{nm} values[1] obtained in the UV monitoring ranges of 215 - 245 and 300 - 360 nm and limit of detection (L_D) derived from compounds in examined samples obtained by UV monitoring and fluorescence detection (FD) (OPA-HPLC system II)

Compounds	Retention time (min)	Value[2] of S_n^{DAD}/S_n^{FD}	$R^{nm} \pm SD$ monitoring UV range				Limit of detection (L_D)[3] UV detection	FD
			215-245 nm	RSD, %[4]	300-360 nm	RSD,%	ng·l^{-1}	ng·l^{-1}
GSH	18.2 ± 0.3	1.23	0.999 ± 0.003	0.34	1.000 ± 0.005	0.47	0.41	0. 32
Seleno-methionine	41.1 ± 0.4	0.35	1.014 ± 0.024	2.4	1.003 ± 0.003	0.26	0.48	0.12
Seleno-cystine[5]								
Peak 1	46.0 ± 0.3	69	0.99 ± 0.04	4.2	1.01 ± 0.01	0.94	0.51	ND
Peak 2	48.1 ± 0.4	120	1.06 ± 0.15	14	0.98 ± 0.10	9.7	2.9	ND
Homo-cystine[6]								
Peak 1	49.0 ± 0.3	1.6	1.06 ± 0.12	11	1.07 ± 0.19	18	0.61	0.87
Peak 2	49.6 ± 0.3	2.2	1.60 ± 0.78	-49	1.07 ± 0.16	16	1.21	1.20

[1] abbreviations as in Table 3

[2] response ratio of the photodiode array detector and the fluorescence detector

[3] UV detection at 337 nm; fluorescence detection (excitation and emission wavelengths: 336/425 nm)

[4] relative standard deviation of values R^{nm}

[5] area ratio of peak 1 to peak 2: 14.5 (UV detection at 337 nm)

[6] area ratio of peak 1 to peak 2: 4.9 (UV detection at 337 nm)

CONCLUSIONS

The HPLC methods presented are highly accurate, selective and sensitive for simultaneous determination of proline, GSH, and some sulphur and selenium amino acids. Proline, cyst(e)ine, methionine and GSH prior to OPA-HPLC analysis are oxidized using the two improved procedures. Due to the use of gentle oxidation conditions, we always obtained pure products. Next, OPA-derivatives of oxidized compounds were separated using widely available and relatively inexpensive C_{18} columns and typical UV or fluorescence detectors. Fortunately, all assayed compounds can be monitored using a UV detector only because the limits of detection are low compared with the original content of cyst(e)ine, methionine, proline and GSH in biological samples. The combination of *pre*-column OPA derivatization and gradient elution program II (HPLC system II) provides quantitative information on concentrations of free GSH, seleno-cystine, homo-cystine, methionine and seleno-methionine in the presence of other amino acids (Czauderna et al., 2002). The free cysteine concentration can be calculated by subtraction of the results based on the method using OPA derivatization (HPLC system II) from the results based on the method including two oxidation procedures followed by OPA derivatization (HPLC system I). The presented HPLC methods with selective UV detection, based on simple and rapid preparation, can be possible alternative methods to other methods with fluorescence detection. Our HPLC procedures should find application especially in routine analysis of GSH, proline, and sulphur and selenium amino acids in the presence of other amino acids in nutritional and clinical laboratories.

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STRESZCZENIE

Oznaczanie niektórych aminokwasów i glutationu wysokosprawną metodą chromatografii cieczowej

Opisano metody HPLC (I i II) oznaczania cysteiny, seleno-cysteiny, seleno-metioniny, metioniny, homo-cysteiny, glutationu (GSH) i proliny w próbkach biologicznych. Analizowane związki przeprowadzono w pochodne używając *o*-dialdehydu ftalowego (OPA) w obecności etanotiolu. Przed przeprowadzeniem w pochodne, cysteinę, metioninę, GSH utleniano kwasem nadmrówkowym, natomiast prolinę podchlorynem sodowym. Pochodne OPA utlenionych związków rozdzielano wykorzystując kolumę C_{18} z odwróconą fazą (4 μm, 250 x 4.6 nm, Nova Pak, Waters) poprzez binarną elucję gradientową (I metoda HPLC). Pochodne tych związków oznaczano stosując detekcję UV przy długości fali 336 nm oraz detekcję fluorescencyjną (wzbudzanie λ_{ex}=336 nm, pomiar λ_{em}=425 nm). Stosując detekcję fluorescencyjną otrzymuje się niższe wartości granicy detekcji (L_D) (0.3-0.8 ng·l^{-1}) niż wykorzystując monitorowanie UV (0.9-2.4 ng·l^{-1}). Czas rozdzielenia wszystkich analitów wynosił ok. 35 min. Oznaczanie pochodnych OPA nieutlenionego GSH, metioniny, seleno-metioniny, cystyny, seleno-cystyny i homo-cystyny, przeprowadzono poprzez poczwórną elucję gradientową (II metoda HPLC) wykorzystując detekcję UV przy 337 nm oraz detekcję fluorescencyjną ($\lambda_{ex}/\lambda_{em}$=336/425 nm). Stosując detekcję UV otrzymuje się niższe wartości L_D dla seleno-cystyny i homo-cystyny, natomiast detekcja flurescencyjna zapewnia lepsze granice oznaczalności GSH, seleno-metioniny i metioniny. „Czystość" analitycznych pików (~100%) oznaczanych związków oraz precyzja i prostota metody HPLC I i II jest wystarczająca do rutynowego oznaczania badanych związków w próbkach biologicznych.