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Selenoamino Acid Speciation Using HPLC–ETAAS Following an Enzymic Hydrolysis of Selenoprotein

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A high-pressure liquid chromatography–electrothermal atomic absorption spectroscopy (HPLC–ETAAS) hyphenated technique was used for the determination of seleno compounds present in a selenium-enriched yeast. Conditions were optimized for the separation and quantification of the selenoamino acids, selenocystine and selenomethionine, in the presence of other compounds. The separation was achieved by ion-pairing chromatography using sodium heptanesulphonate as the anionic counterion. On-line detection was carried out using electrothermal atomic absorption with palladium(II) as a matrix modifier. Different extraction procedures were tested on a selenium-enriched yeast. A 92% recovery of the total selenium present in the material was obtained. Attempts to evaluate selenium speciation were carried out; selenomethionine and selenocystine were identified as the major components (42% and 35% respectively).

Keywords: analysis; speciation; HPLC–ETAAS; enzymic extraction; selenoamino acids

INTRODUCTION

Selenium is present in environmental samples at trace levels both as inorganic species, whose determination has been widely studied, and as organic compounds, which have recently become of growing interest. Selenium occurs naturally in several oxidation states: selenium(IV), selenium(VI), selenium(0) and selenium(-II). Some of these species are volatile, e.g. methyl selenides, resulting in terrestrial and marine ecosystem exhalation;[1] others, such as the anions selenite and selenate, are water-soluble and are produced essentially by soil leaching.[2] Selenium in the environment results mainly from biologial and geophysical processes but also from anthropogenic activities such as copper refining or glass production, which are therefore involved in the redistribution of the element.[2]

Selenium is an essential micronutrient for msot living organisms. For instance, it is present as four selenocysteine residues at the active site of the human enzyme glutathione peroxidase.[3] Therefore it plays an important role in inhibition of lipid peroxidation.[4] Recent studies have also been reported in its protective effect in chemically or virally induced cancers.[5]

Mankind is exposed to selenium mainly through the food chain and a wide concentration range has been reported.[2] Edible offal (liver, kidney) and seafood have a relatively high level of 0.4–1.5 mg Se kg^{-1}; the range for cereals and cereals products is 0.1–0.8 mg Se kg^{-1} while in fruit and vegetables the amount is less than 0.1 mg Se kg^{-1}.

Naturally occurring selenoproteins contain selenium as selenoamino acid fragments, the major forms being selenomethionine (found in plant tissues[6]) and selenocysteine (present in animal proteins[6]). Selenocystine was identified in corn and some methylated derivatives (selenocystathionine, selenohomocysteine) were reported in selenium-accumulating plants such as *Astragalus*.[7]

The metabolism of these organic compounds is different from that of the inorganic species, resulting in both toxic or beneficial effects. Concerning mammals, there are only a few studies on selenium lethal doses. Minimum lethal dose (MLD_{50}) values based on mice are 3.5 mg Se kg^{-1} for sodium selenite, 5.5 mg Se kg^{-1} for selenate,[8] 20.4 mg Se kg^{-1} for selenocystine[9] and 4.25 mg Se kg^{-1} for seleno-

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methionine.[9] The threshold between toxic and essential levels being only one order of magnitude,[10] the search for a better understanding of selenium metabolism, uptake and toxic effects is the reason why the study of selenium speciation in biological matrices is now of growing interest.

Organoselenium speciation involves separation of species and specific detection of the element. Both liquid and gas chromatography are commonly used. The latter implies a prior derivatization step; various reagents are available such as trimethylsilyl acetamide or cyanogen bromide.[11,12] Liquid chromatography is mainly carried out using an ion-exchange mechanism. Highly sensitive detectors are employed, e.g. mass spectrometry, neutron activation analysis or flame ionization detection (for a Review, see Ref. 13).

An analytical method previously developed for organotin compound speciation[14] was recently adapted to selenium, with application to the analysis of seleno compounds in a white clover sample.[15] Separation is achieved by HPLC which is hyphenated to a selenium-specific detection by ETAAS through a homemade interface consisting of a quartz flow-through cell.

MATERIALS AND METHODS

Reagents

DL-Selenocystine and DL-selenomethionine were purchased from Sigma. These products were used without further purification (90% purity for selenocystine). Stock solutions (1000 mg l^{-1}) in deionized water (Millipore 18 MΩ) were stored in the dark at 4 °C. 3% Hydrochloric acid (Merck Suprapur) was required to dissolve selenocystine. Working standards were prepared daily by dilution in deionized water. The mobile phase was prepared from sodium heptanesulphonate (Sigma) dissolved in a water/acetonitrile (Prolabo) mixture (90:10); the pH was adjusted to 2.4 by addition of nitric acid (Prolabo–Normapur).

The palladium(II) nitrate hydrate employed for matrix modification in ETAAS determinations was from Sigma, as well as Pronase E (Protease type XIV) which was used for extractions. The 30% perhydrol and 65% nitric acid employed for mineralization were Merck Suprapur products.

Enriched yeast

A sample of industrially produced selenium-enriched yeast was used. *Saccharomyces cerevisiae* was grown in the presence of sodium selenite out of which it naturally synthesizes organic seleno compounds. It was then pasteurized and dried.

Equipment

A Varian 5020 liquid chromatograph equipped with a 100 µl loop, a Hamilton PRP-1 column (styrene divinylbenzene; 250 mm × 4.1 mm) was coupled through a 300 µl interface[13–15] to a Varian ETAAS assembly (SpectrAA 30 GTA 96). The automatic sampling device of the spectrometer successively took the matrix modifier and the chromatographic effluent out of the flow-through cell, then the two portions were co-injected into the pyrolytic tube (Fig. 1). This technique is considered to be on-line because once the sample has been injected on the column every step is accomplished automatically until measurements by the detector are printed.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

Chromatographic separation of amino acids has been widely studied in the literature, the usual mechanisms being reverse-phase, ion-pair or ion-exchange separation. Following the works of Fialaire *et al.*,[16] we have chosen an ion-pair mechanism with an anionic counterion. The acid–base properties of amino acids are used to form an ion pair at pH 2.4 with alkyl or aryl sulphonate salts.[16] At this pH, corresponding to the first pK_a of the two amino acids, 50% of the compound is in an $R(COOH)NH_3^+$ form, the other part being a zwitterionic species $R(COO^-)NH_3^+$.

We have tested several reagents, such as sodium naphthalene sulphonate, that gave strong ETAAS interferences resulting in high degradation of the graphite tube, or octanesulphonate that led to a very large difference in retention times of the two amino acids. Heptanesulphonic acid, as sodium salt, gave the best results. First the separation was optimized by characterizing the effect of the acetonitrile content of the mobile phase on the retention of the two amino acids

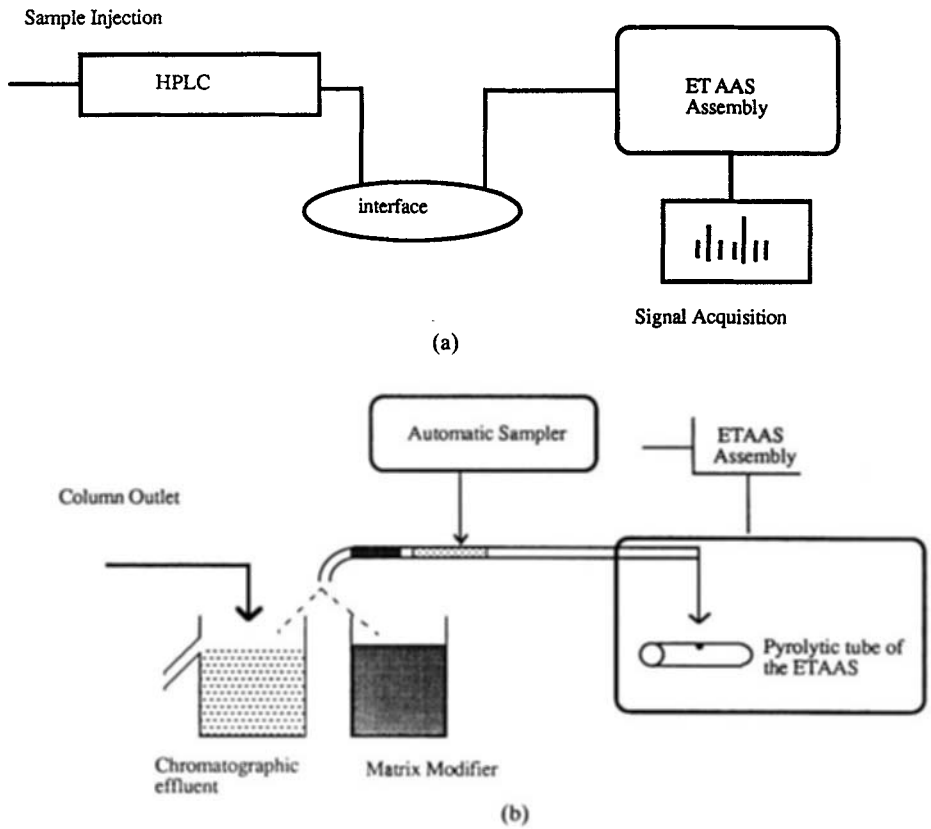

Figure 1 (a) Apparatus. (b) Interface.

(Fig. 2). A 10–90% acetonitrile–water solvent system was found to be efficient to separate selenocystine from selenomethionine at a 0.4 ml min^{-1} flow rate. Figure 3 presents the evolution of capacity factors with an increasing concentration of reagent in the mobile phase; a working concentration of 1.25 g l^{-1} was chosen as a compromise between sufficient retention of the selenocystine and a reasonable time of analysis. Inorganic species [selenium(VI) and selenium(IV)] are not retained on the column but they

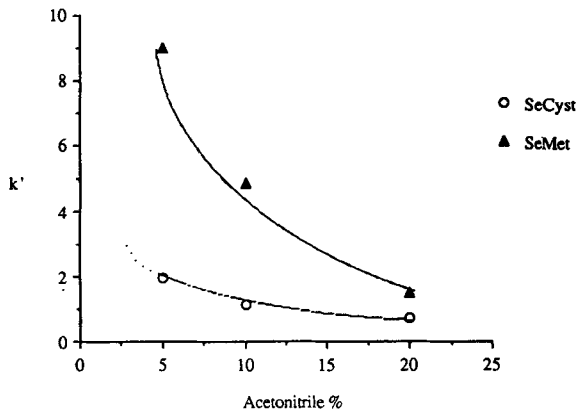

Figure 2 Effect of percentage of acetonitrile on capacity factor k'. Mobile phase: sodium heptanesulphonate, water–acetonitrile, pH = 2.4, flow rate 0.4 ml min^{-1}.

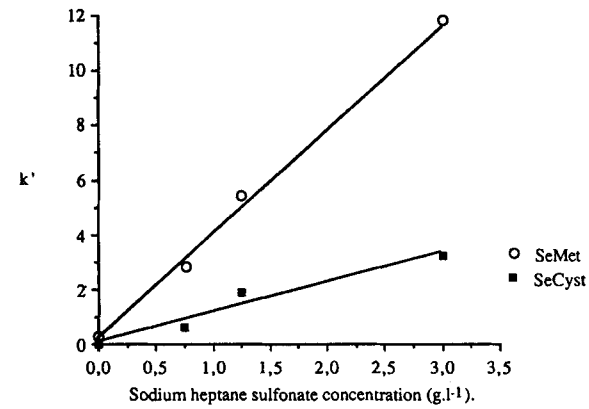

Figure 3 Effect of the concentration of ion-pairing reagent on capacity factor k'. Mobile phase: water–acetonitrile, 90:10, pH = 2.4, flow rate 0.4 ml min^{-1}.

Table 1 Furnace programme

Step	Temperature (°C)	Time (s)	Gas flow ($l\,min^{-1}$)	Read command
1	90	15	3.0	No
2	120	10	3.0	No
3	350	20	3.0	No
4	900	10	3.0	No
5	900	2	3.0	No
6	900	2	0.0	No
7	2400	2	0.0	Yes
8	2400	3	0.0	Yes
9	2600	1	3.0	No

interfere with the selenocystine peak when they are present in large excess.

Optimization of the atomic absorption detection

The atomic absorption conditions have to be adapted to the chromatographic effluent. The maximum sensitivity was obtained with a hollow-cathode lamp intensity of 8 mA at 196 nm. The best results occurred using pyrolytic tubes without a platform with palladium(II) as a matrix modifier, because of the possibility of reaching high ashing temperatures, while nickel(II) had been employed in preceding studies.[15] The optimum concentration of palladium(II) nitrate was in the region of 2.6 μg per ng Se as generally employed.[17,18] Soft drying or ashing temperatures slopes were found to be an important parameter to increase sensitivity (Table 1). The automatic sampling device on the spectrometer takes 5 μl of matrix modifier and then 15 μl of the effluent, and injects the two portions simultaneously into the graphite furnace. The overall time period between two measurements including cooling and sampling is 94 s.

Analysis of standard solutions

Standard solutions of each compound were analysed by HPLC–ETAAS, both in deionized water and enzymic extract. Peak integration was calculated as the sum of every ETAAS measurement. The calibration slopes were quite different, especially for selenomethionine and for the enzymic extract, as shown in Table 2.

The repeatability of the HPLC–ETAAS analysis was calculated from six consecutive injections of selenocystine ($1\,mg\,l^{-1}$) and was found to be 7.3%. Detection limits were evaluated by the IUPAC formula

Table 2 Slopes of the calibration curves expressed as DO μg^{-1}

	Inorganic Se	SeCys[a]	SeMet[b]
Water	0.80 ± 0.15	2.10 ± 0.16	1.70 ± 0.14
Enzymic extract	2.20 ± 0.4	1.3 ± 0.4	2.63 ± 0.04

[a] SeCys, selenocystine. [b] SeMet, selenomethionine.

$$DL = 3\sigma_d\, m^{-1}$$

where σ_d is the standard deviation of the blank based on 20 measurements and m is the slope of the calibration curve. They were 34 $\mu g\,kg^{-1}$ for selenocystine and 50 $\mu g\,kg^{-1}$ for selenomethionine.

Extraction procedures

Speciation analysis of biological material implies a preliminary extraction step which must not modify the speciation of the element. Prior to speciation, the total selenium content of the enriched yeast was determined after mineralization of the material (100 mg) with a mixture of 10 ml nitric acid and 5 ml of Perhydrol. A high level of $1038 \pm 101\,mg\,kg^{-1}$ was found, corresponding to the indicative range given by the industrial laboratory (less than $1050\,mg\,kg^{-1}$).

Protein hydrolysis to release free amino acids is commonly performed in hydrochloric acid at 110 °C,[18] but degradation of seleno compounds has already been noticed using this procedure.[19] A mixture of organic solvents (chloroform and alcohol) is also known to release selenoamino acids from materials such as plants.[7]

Forsyth and coworkers,[20,21] working on alkyllead species, have presented a new extraction procedure for organometallic compounds, i.e. enzymic hydrolysis performed on natural samples leading to high recoveries.

Table 3 summarizes the extraction procedures attempted on the selenium-enriched yeast in this study. Analysis of total selenium in extraction solutions was achieved for the first time by ERTAAS determination after digestion of the solutions. They are discussed in the following.

Acid hydrolysis

Dry yeast (50 mg) was placed into a sealed tube with 1.5 ml of 6 M hydrochloric acid and heated

Table 3 Summary of extraction yields

	Extraction procedure			
	Organic solvents	Water at 60 °C	Acid hydrolysis	Enzymic hydrolysis
Total Se concentration (mg g^{-1})	117 ± 8	200 ± 8	84 ± 2	955 ± 7
Yield (%)	11 ± 2	20 ± 1	8 ± 0.2	92 ± 1

for 5 h in a water bath; the resulting mixture was centrifuged (6000 rpm, 10 min) and analysed for its total selenium content (Table 3). The poor extraction yield probably resulted from selenium loss and sample degradation during the manipulations.

Organic solvent extraction

A portion of yeast (100 mg) was placed in a PTFE flask together with a mixture of deionized water, chloroform and methanol (2:3:5 by vol.) and shaken overnight. The suspension was then centrifuged (6000 rpm, 10 min). A 1 ml aliquot of the supernatant was mineralized and analysed for its total selenium content (Table 3). The extraction yield was very low.

Water extraction

Dry yeast (100 mg) was placed in a PTFE flask together with warm water, shaken for 5 h and centrifuged. This procedure released 20% of the total selenium present in the yeast. From this result and the preceding one it might be supposed that water or water–solvent mixtures solubilize only the selenium adsorbed on material surfaces.

Enzymic extraction

Protease (10 mg) was plced in a PTFE flask together with a 100 mg of the yeast and 4 ml of a phosphate–critic acid buffer with a pH of 7.5. It was magnetically stirred for 24 h in a water bath adjusted to 37 °C. After centrifugation, the solution was mineralized and analysed for its total selenium content. This procedure allowed a 92% recovery of total selenium. The protease was able to break specifically the peptide bonds of any protein present in the material. The use of a large excess of enzyme appeared to be efficient in cleaving the major part of these bonds.

Considering the two steps, extraction and speciation analysis, the RSD values were 22% for inorganic selenium, 25% for selenocystine and 9.6% for selenomethionine. The incomplete resolution of the peaks of inorganic species and selenocystine was probably responsible for the high RSD values.

Selenium speciation in the selenium-enriched yeast

The satisfactory solubilization of the material obtained by enzymic hydrolysis allowed a simple work-up. The enzymic extract prepared as reported was centrifuged at 600 rpm for 10 min. The supernatant was diluted (1/5) in the mobile phase and adjusted to pH 2.4 with nitric acid; 100 µl was then injected on the column.

The resulting chromatogram shows three peaks (Fig. 4). Seleno compounds were identified and quantified using the standard addition method. Selenomethionine and selenocystine appeared to be the main components with 42% and 35% respectively of the total selenium present (Table 4).

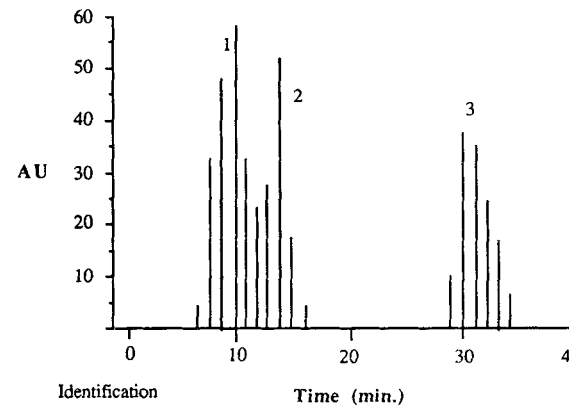

Figure 4 Chromatogram of the enzymic extract. Mobile phase: water–acetonitrile 90:10, sodium heptanesulphonate. 1.25 g l^{-1}, pH = 2.4, flow rate 0.4 ml min^{-1}. Identification of peaks: 1, inorganic selenium, t_R = 9.4 min; 2, selenocystine, t_R = 14.5 min; 3, selenomethionine, t_R = 29.7 min.

Table 4 Selenium speciation in the yeast

	Inorganic Se	SeCys	SeMet	Recovery
Concentration ($mg\ g^{-1}$)	76 ± 17	365 ± 91	436 ± 42	878 ± 101
Percentage of total Se	7 ± 1.5	35 ± 9	42 ± 4	92 ± 10

CONCLUSION

The identification of different selenium species in a single material is not surprising, considering the various studies found in the literature and especially concerning the identification of the major compound, selenomethionine, already reported to be the major seleno derivative present in yeast.[22] Selenomethionine was also found in soybean proteins[23] and plants.[7] The unstable selenocysteine was identified in proteins.[24] White clover was suspected to contain selenocystine[16] as the only selenated species present whereas numerous methylated derivatives were identified in seeds of different *Astragalus* species.[7]

In this study, most if not all of the selenium species present in yeast have been identified and determined. However further work on the stability of selenium species during enzymic hydrolysis is necessary, especially with regard to selenocysteine, which is a compound with a low stability. Further research on the chromatographic separation concerns the way to improve the resolution of inorganic forms and selenocystine.

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