ELSEVIER

Journal of Chromatography A, 750 (1996) 327–334

JOURNAL OF CHROMATOGRAPHY A

Optimization of the determination of inorganic and organic selenium species using high-performance liquid chromatography–electrothermal atomic absorption spectrometry

N. Gilon*, M. Potin-Gautier, M. Astruc

Laboratoire de Chimie Analytique, Université de Pau et des Pays de L'Adour, 64000 Pau, France

Abstract

The liquid chromatographic simultaneous separation of selenite, selenate, selenocystine and selenomethionine was studied with a selenium-specific detector; electrothermal atomic absorption spectrometry. Three chromatographic modes were compared; two ion-pairing ones using either a cationic or an anionic reagent and ion-exchange. Separation parameters such as composition, pH and concentration of the mobile phase were investigated. The influence of chromatographic conditions on the sensitivity of the detector is studied. The ion-exchange method was determined to give the best results, with detection limits ranging from 8 to 17 $\mu g\ l^{-1}$ within a 30 min separation time. An application of this method to the analysis of seleno compounds present in a selenium-rich yeast after enzymic hydrolysis extraction is presented.

Keywords: Selenite; Selenate; Selenocystine; Selenomethionine; Seleno amino acids

1. Introduction

Selenium is an essential trace element present in all compartments of the environment and it occurs mainly under oxidation states: (VI), (IV), (0) and (−II). Se(VI) and Se(IV) are essentially present as selenite and selenate ions in water and soils. Inorganic selenides Se(−II) and elemental selenium Se(0) are mainly insoluble compounds. Se(−II) is present as organic selenides such as methylated volatile compounds. It occurs also as seleno amino acids, mainly selenocysteine and selenomethionine, covalently bound to proteins. Both the bioavailability and the toxicity of selenium are closely correlated to the species. With regard to humans, inorganic compounds would be less absorbed than selenomethionine and this latter form would serve for selenium storage in proteins for its eventual use in selenocysteine synthesis and its incorporation into selenium-specific enzymes [1–3].

Therefore, it is now important to be able to realize the separation and quantification of selenite, selenate and seleno amino acids. In the past years, there has been an increasing interest for the speciation of selenium in biological matrices, plants, soils, etc. The investigations were drawn towards whether the speciation of inorganic compounds Se(VI), Se(IV) or the determination of selenoaminoacids. A few authors reviewed their separation and analysis techniques [4,5].

Most chromatographic separations of selenite and selenate are carried out using liquid chromatography. The separation of the ionic species is achieved currently with ion-exchange chromatography [6,7].

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00484-0

Seleno amino acids are studied with both gas and liquid chromatography. Gas chromatographic (GC) separation is currently achieved after a derivatization step such as the selective complexation of selenium with 1,2-diaminobenzene ligand in acidic media to form piazselenol [8]. The presence of selenomethionine is characterized with GC flame ionization detection (FID) and with flame photometric detection (FPD) following a derivatization using either cyanogen bromide [9] or 2,4-dinitrophenyl derivative (DNP) [10], respectively.

Nevertheless, the most widely used method to separate organic selenocompounds is liquid chromatography and this is applied to many matrices. Cavalli and Cardellicchio [11] described the detection of seleno amino acids in dolphin liver with integrated pulsed amperometric detection (IPAD) [11]. Reversed-phase chromatography is employed as well, Jiang and Houk [12] have developed the HPLC separation inductively coupled plasma mass spectrometry (ICP-MS) detection of seleno amino acids with an ion-pair method [12].

Recently, the detection of selenite, selenate and seleno amino acids has been investigated. A DNP derivatization procedure followed by analysis with ion-exchange–thermochemical hydride generation–atomic absorption spectrometry (THG–AAS) has been developed [13]. Electrothermal atomic absorption spectrometry (ETAAS) has been studied as a specific detector using mainly ion-exchange chromatography by Kölb [14].

In all these studies the compatibility of the mobile phase with the detector strongly affects the detection limits. For instance THG–AAS requires a high methanol-containing eluent, whereas ICP-MS sensitivity is affected by saline solutions, ETAAS needs the use of a matrix modifier that enhances selenium detection. Thus, the investigation of separation has to be closely adapted to the detector.

We have previously described ion-pair chromatographic separation of seleno compounds using both anionic and cationic counter-ions [15,16]. In this work, a comparison of the way that chromatographic conditions (composition of the mobile phase, pH, concentration) influence capacity factors is discussed. The ETAAS detector used gives a discontinuous measurement of the chromatographic eluent and thus requires high selectivity. An application of the optimized conditions to the analysis of selenium speciation in a yeast is presented.

2. Experimental

2.1. Reagents

Seleno-D,L-cystine and seleno-D,L-methionine were purchased from Sigma (L'Isle d'Abeau, France). Sodium selenite is a Merck (Nogent sur Marne, France) suprapur reagent and sodium selenate (98%) was obtained from Aldrich (L'Isle d'Abeau, France). The 1000 mg l^{-1} stock solutions are prepared monthly in deionised water (Milliro-MilliQ system from Millipore (Saint Quentin Yvelines, France). Selenocystine was dissolved in 3% hydrochloric acid (Merck suprapur reagent).

Chromatographic ion-pairing reagents were sodium naphthalenesulfonate (purchased from Sigma) and tetraethylammonium bromide from Kodak (Touzart and Matignon, Vitry sur Seine, France). The ion-exchange mobile phases were solutions of nickel sulfate (Merck, analytical-reagent grade) and nickel acetate (Normapur-Prolabo, Vaulx en Velin, France). The nitric acid (65%) was of analytical-reagent grade from Merck.

ETAAS matrix modifiers: the two latter nickel solutions were used as matrix modifiers as well as for the electrothermal atomic absorption spectrometer detector. For the ion-pairing method a palladium nitrate solution obtained from Sigma was used.

2.2. Chromatographic analysis

A Varian (Les Ulis, France) 5020 liquid chromatograph equipped with a 100-μl loop was used. Two columns were tested; a Hamilton PRP-1 reversed-phase (250×4.1 mm I.D., 10 μm particles) (Interchrom, Montlucon, France) whose stationary phase was poly(styrene–divinylbenzene) and an ion-exchanger Hamilton PRP-X100 (250×4.1 mm I.D., 10 μm particles) whose stationary phase consisted of poly(styrene–divinylbenzene)trimethylammonium sites. Precolumns of the corresponding stationary phase were used in the analysis of natural matrices. The retention time of unretained species (t_0) was evaluated for the two columns using an UV detector

(254 nm). The unretained species were NH_4^+, which was not retained on the ion-exchange column, and NO_3^-, which was not retained on the reversed-phase column. The pH was adjusted with nitric acid or ammonia.

2.3. HPLC–ETAAS

The chromatograph was coupled to the ETAAS assembly with a laboratory-made flow-through cell. The automatic sampling device of the ETAAS regularly sampled 20 μl of the eluent. Two spectrometers were successively employed.

— A Varian SpectrAA30-GTA96 assembly: A special atomization program was optimized to obtain higher sensitivity in the shortest time. Background correction was achieved with a deuterium arc. The spectrometer operated with a selenium hollow cathode lamp (Photron, λ=196 nm, intensity, I=8 mA). The furnace program was divided into four steps; drying at 90° (15 s), temperature increase from 90 to 900°C (30 s), ashing 900°C (4 s) and atomization 2400°C (3 s). The overall time period between two consecutive measurements was 94 s.

— A Unicam (Argenteuil, France) 939QZ-FS 90 assembly with both quadline (D2 Unicam) and Zeeman background correction. The selenium hollow cathode lamp was a photron super lamp (λ=196 nm, instrument power supply I=11 mA, boost current: I=15 mA). For all coupled experiments, quadline correction was used. The furnace program was divided into a drying step at 90°C (5 s), an ashing step at 800°C (10 s) and an atomization step at 2200°C (3 s). The overall time period was 95 s.

2.4. Extraction procedure

Yeast enzymic hydrolysis was carried out either in a phosphate citric acid buffer or in a nickel acetate solution. For a 200-mg portion of the dried material, 20 mg of protease (pronase E) purchased from Sigma was added together with 5 ml of solution. The mixture was magnetically stirred overnight and was then centrifuged (20 min, 6000 rpm, approx. 6000 g), filtered (Millipore filters, 0.45 μm) and dissolved in mobile phase before injection. Direct ETAAS measurements of enzyme solutions have shown that no selenium was present in the extractants.

3. Results and discussion

3.1. Ion-pairing method with a cationic reagent (tetraethylammonium bromide) {I}

Initial investigation was carried out using a reversed-phase Hamilton PRP-1 column with an ion-pairing method, the mobile phase consisting of a water–acetonitrile solution (99:1, v/v) [15]. A (TeABr) counter-ion was studied to achieve separation of the four selenocompounds, selenocystine, selenomethionine, selenite and selenate. The quaternary ammonium function of TeABr is likely to form an ion-pair with selenated species, with the retention being governed by the partition between the ion-pair, the stationary and the mobile phases. Fig. 1 reports the variation of capacity factors (k') with an increase of the concentration of the ion-pairing reagent. Selenocystine appears to be eluted in the dead volume ("hold-up" time=9 min) and the absence of evolution of capacity factors with concentration of the reagent shows that apparently no ion-pair formation occurs either with the selenocystine or with the selenomethionine. Concerning retention of selenomethionine, a hydrophobic interaction between the "seleno alkyl chain" (CH_3-Se-$(CH_2)_2$-) of the molecule and the relatively unpolar stationary phase would be responsible for its better retention. With regard to inorganic species, their capacity factors increase with increasing concentration of the ion-

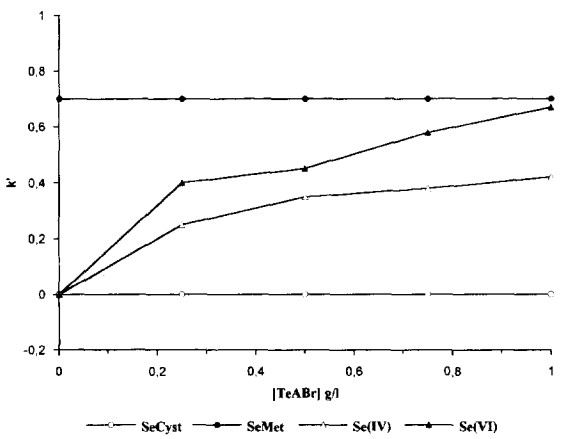

Fig. 1. Capacity factors of selenocystine (SeCyst), selenomethionine (SeMet), Se(IV) and Se(VI) as a function of TeABr concentration.

pairing reagent. These conditions led to the good detection limits reported in Table 2. Nevertheless, the absence of retention of selenocystine is a major disadvantage, when this compound is present together with other unretained species it is impossible to quantify it. The insufficient selectivity between selenite and selenate peaks is a limit to the application of this method to high selenium-containing matrices.

3.2. Ion pairing mechanism with an anionic reagent (sodium heptanesulfonate) {II}

Using the same column and a water–acetonitrile mobile phase, another ion-pairing mechanism was investigated. Alkyl and aryl sulfonate are commonly used in amino acid analysis [17], the ion-pair formation occurs at a suitable pH between the positively charged ammonium part of the amino acid and the sulfonate group. According to the values we previously determined [15], the first pK_a of seleno amino acids is in the pH 2.4 region (Table 1). At this pH, selenite and selenate are negatively charged and thus are eluted in the dead volume of the column ("hold-up" time=9.4 min). We have investigated the influence of the concentration of the ion-paring reagent on the capacity factors of the seleno amino acids and found that selenocystine is slightly retained by these conditions whereas selenomethionine forms the expected ion-pair with heptane sulfonate with k' increasing with increasing concentration of the ion-pairing reagent (Fig. 2).

Optimization of the amount of acetonitrile in the mobile phase is presented in Fig. 3. A water–acetonitrile, (90:10, v/v) solvent system was shown to give more rapid elution of the compounds without affecting the resolution. The optimal conditions of analysis must take into account the use of the ETAAS detector. The influence of the mobile phase

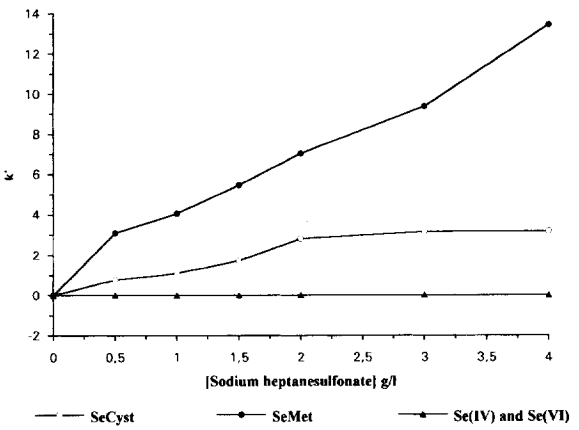

Fig. 2. Capacity factors of selenocystine (SeCyst), selenomethionine (SeMet), Se(IV) and Se(VI) as a function of sodium heptanesulfonate concentration.

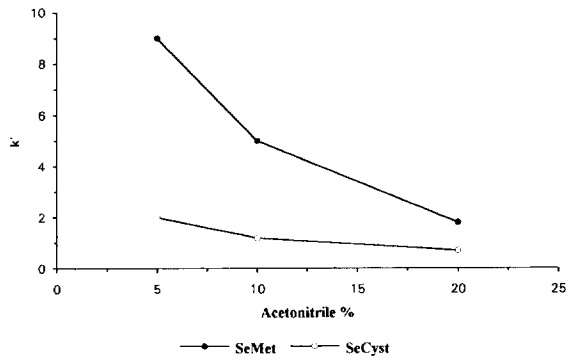

Fig. 3. Capacity factors of selenocystine (SeCyst), selenomethionine (SeMet), Se(IV) and Se(VI) versus percentage of acetonitrile in the mobile phase.

on the sensitivity of the detector has lead to the study of various matrix modifiers usually employed in selenium determinations [18]. Measurements have been carried out for a selenomethionine standard solution (50 $\mu g\ l^{-1}$) under different conditions and the results are summarized in Fig. 4. The best

Table 1
Formulae and pK_a of seleno compounds

Name	Formula	pK_a	Reference
Hydrogen selenite	H_2SeO_3	2.35 and 7.94	[21]
Hydrogen selenate	H_2SeO_4	<0 and 1.7	[21]
Selenocystine	$HOOC(NH_2)CH_2SeSeCH_2CH(NH_2)COOH$	2.4 and 8.9	[15]
Selenomethionine	$CH_3Se(CH_2)_2CH(NH_2)COOH$	2.6 and 8.9	[15]

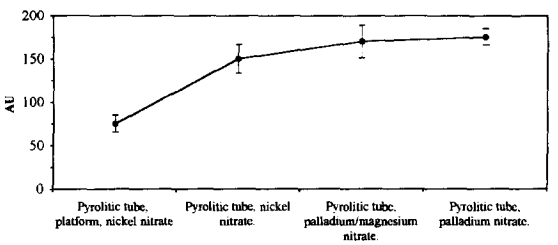

Fig. 4. Influence of the nature of the tube and the matrix modifier on the ETAAS signal of a standard solution of selenomethionine (50 $\mu g\ l^{-1}$) prepared in the chrmatographic mobile phase.

sensitivity was obtained with a palladium matrix modifier added to the chromatographic eluent through the automatic sampling device of the ETAAS assembly; retention times and detection limits are reported in Table 2.

These conditions only gave a partial speciation, as selenite and selenate were eluted together in the dead volume, the interference of the sulfonate-containing mobile phase led to high detection limits that were five-to-ten times higher than those obtained with the TeABr method. However, sufficient selectivity of the amino acid separation allowed for their determination in a selenium-rich material (Table 3).

3.3. Ion-exchange method {III}

An ion-exchange method was then investigated. A Hamilton PRPX-100 column was chosen and the mobile phase used normally consisted of a solution of sodium carbonate, phosphate or sulfate. To ensure sensitive detection with ETAAS, a nickel salt was preferred, as it is a matrix modifier [19]. Nickel acetate and sulfate solutions were tested as mobile phases. The separation mechanism in anion-exchange chromatography is governed by ionisation of species. In our case, negatively charged compounds interact with quaternary ammonium sites bound to the polymeric stationary phase and therefore are more retained. Thus pH is an important parameter to investigate. Fig. 5 demonstrates that with increasing

Table 2
Analytical criteria for the three conditions

	Condition	Selenocystine	Selenomethionine	Selenite	Selenate
Relative detection limit ($\mu g\ l^{-1}$)	I	10	8	12	10
	II	33	47	93	93
	III	8	15	17	12
Absolute detection limit (ng)	I	1.0	0.8	1.2	1.0
	II	3.3	4.7	9.3	9.3
	III	0.8	1.5	1.7	1.2
Retention time (min)	I	9.5	16.5	12	13.5
	II	14.5	29.7	9.4	9.4
	III	6	15	20	29.5
Reproducibility ($n=5$) Relative standard deviation (%)	I	4	4	4	4
	II	7	8	18	18
	III	5	4	4	2

Table 3
Selenium speciation in a selenium-enriched yeast, concentrations are obtained as the mean of five determinations (± confidence limits)

Conditions	Inorganic selenium	Selenocystine	Selenomethionine	Σ (identified compounds)	Total extracted[a]
II (%)[b]	7±1.5	35±9	42±4	84±11	92±11
mg kg^{-1}	76±17	365±91	436±42	878±101	955±10
III (%)	Se(IV): 3±0.3	33±2	44±2	80±3	90±2
mg kg^{-1}	Se(IV): 29.5±2.5 Se(VI) <0.02	343±20	457±21	831±31	935±20

[a] Analysed after mineralization of the extract with HNO_3–H_2O_2.
[b] Expressed as the percentage of total Se found in the yeast.

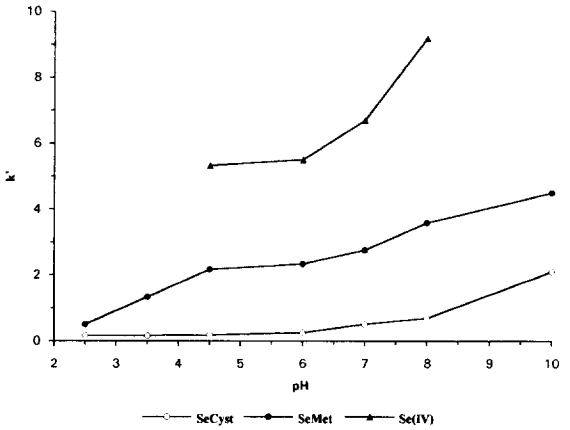

Fig. 5. Capacity factors of selenocystine (SeCyst), selenomethionine (SeMet), Se(IV) and Se(VI) as a function of the pH of the mobile phase.

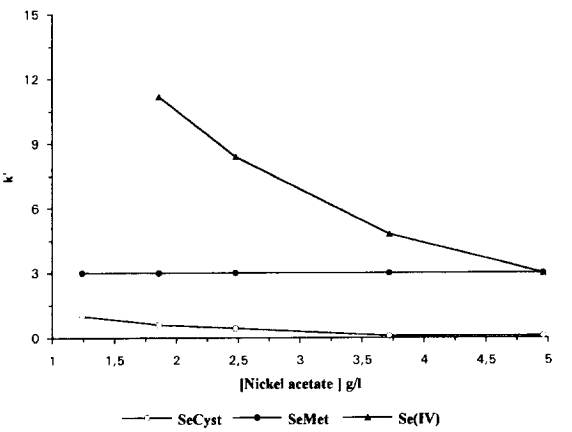

Fig. 6. Capacity factors of selenocystine (SeCyst), selenomethionine (SeMet), Se(IV) and Se(VI) as a function of the concentration of the nickel acetate in the mobile phase.

pH, the retention factors of seleno amino acids increase (t_0=2 min). Selenocystine is only evolving after pH=9 in the retention range of its third pK_a, corresponding to an equilibrium between a zwitterionic species and a negatively charged compound that is more retained on the column. Selenomethionine capacity factor increases both in the first pK_a region and also after the second (Table 1). The better retention of this compound could be explained by interaction of the hydrophobic part of the molecule (alkyl chain) with the polymeric backbone. Retention of Se(IV) is following its global charge. Therefore pH=6.5 was evaluated as the optimum working pH for good separation without a long analysis time.

The concentration of the nickel acetate mobile phase was investigated and led to a satisfactory separation at 2 g l^{-1} (Fig. 6). Selenate has two negative charges over the pH range studied and with a poor developing ion, such as acetate, it is irreversibly retained on the column. Therefore, gradient elution of nickel acetate to nickel sulfate is used to elute this species. The composition of the mobile phase is modified after the elution of selenite, a linear gradient from nickel acetate (2 g l^{-1}) to nickel sulfate (2 g l^{-1}) in 1 min is programmed.

Nickel from the mobile phase provides a matrix modifier (Fig. 7) and sensitivity of the detector has been evaluated with these conditions, the sulfate interfering agent [19] decreases the performance of the detector. Using direct ETAAS measurements, a sensitivity decrease of more than 30% is evaluated compared with the sensitivity found in the nickel acetate solution.

Table 2 summarizes retention times and detection limits obtained under the three conditions. Detection limits are calculated with the IUPAC formula L_D= 3SD/m, SD is the standard deviation of the blank evaluated on the basis of twenty measurements and m is the slope of the calibration curve. Values calculated for the ion-pairing method using TeABr are of the same order of magnitude as those obtained with the ion-exchange method using nickel acetate as the mobile phase. They range from 8 to 12 μg l^{-1} and 8 to 17 μg l^{-1}, respectively. The elution of selenocystine in the dead volume with the ion-pairing method is a major drawback of this method. The second one led to high detection limits with incomplete speciation. Despite its longer analysis time, the third one gave low detection limits and high res-

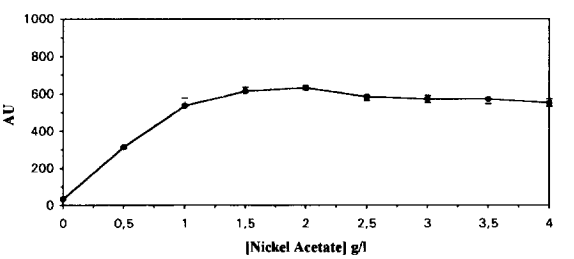

Fig. 7. Influence of the concentration of nickel acetate on the ETAAS signal.

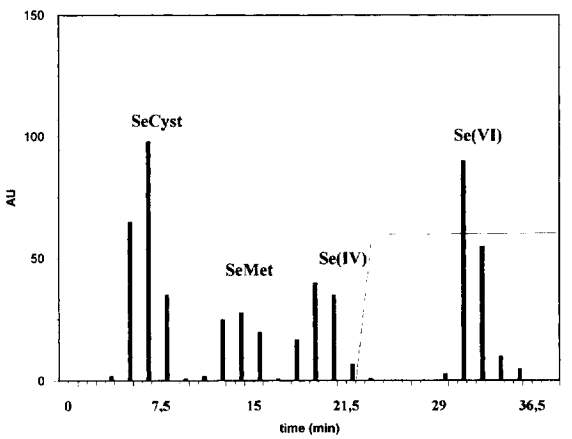

Fig. 8. Chromatogram of a standard solution of SeCyst (250 $\mu g\ l^{-1}$), SeMet (100 $\mu g\ l^{-1}$), Se(IV) (100 $\mu g\ l^{-1}$) and Se(VI) (300 $\mu g\ l^{-1}$).

olution between the four compounds, as shown in Fig. 8, and thus appears to give best results.

3.4. Application

These new chromatographic conditions were applied to the analysis of a natural material that contains significant level of selenium. Selenium enriched yeast is a *Saccharomyces cerevisiae* brought up in a sodium selenite-rich medium. Starting from Se(IV) it synthetizes organic selenium. After pasteurization it is used as a supplementation in human nutrition [20]. Total selenium determination was carried out after mineralization of the material with $H_2O_2-HNO_3$ and a high content (1038 ± 101 mg kg^{-1}) was evaluated. Enzymic extraction was found to give the best recoveries (around 90%) on this material [16]. Hydrolysis is carried out either in a phosphate buffer (pH 7.5) or in a nickel acetate solution. Table 3 summarizes the repartitioning of seleno-compounds in the extract the speciation was achieved in, with both the ion-pairing method with heptane sulfonate reagent and in the ion-exchange method. Results are in good agreement with regard to seleno amino acid analysis. The determination of selenomethionine as the major constituent in selenium-rich yeast has already been mentioned by other authors [9]. The lower value found for the determination of Se(IV) could be explained by the evolution of the natural material with time.

4. Conclusion

The comparison of the three chromatographic modes illustrates their advantages and drawbacks. The ion-pairing method with TeABr ensures low detection limits but no retention of the selenocystine and poor selectivity between the two inorganic species. When heptane sulfonate is employed as the counter-ion, detection limits are strongly affected by sulfonate interferences, selectivity of the amino acids is satisfactory and inorganic species are eluted in the dead volume. Finally, for optimum analysis with regard to both selectivity and detection limits, the ion-exchange method might be preferred. In this case, the nickel-containing mobile phase provides the matrix modifier required by ETAAS detection that ensures low detection limits. These performances are compatible with the selenium speciation in biological matrices such as those present in yeast, plant and animal tissues which will be further studied. Nevertheless, HPLC–ETAAS is a difficult technique, with the major restrictions of a long analysis time enhanced by discontinuous signal measurement that reduces peak resolution. The use of a continuous detector such as ICP-MS would increase performance of the method. Despite these considerations, HPLC–ETAAS could appear as an alternative to HPLC–ICP-MS, whose detection limits are one order of magnitude lower for a higher investment [5].

Acknowledgments

The authors thank Mr. S. Hannedouche for his help in the realization of this work which was carried out during a "maîtrise" training period in the Analytical Chemistry Laboratory.

References

[1] T.C. Stadtman, J. Biol. Chem., 266 (1991) 257.
[2] M.A. Beilstein and P.D. Whanger, J. Nutr., 116 (1986) 1711.

[3] F. Dubois and F. Belleville, Path. Biol., 38 (1988) 1017.
[4] X. Dauchy, M. Potin Gautier, A. Astruc and M. Astruc, Fresenius' J. Anal. Chem., 348 (1994) 792.
[5] G. Kölb, K. Kalcher, K.J. Irgolic and R.G. Magee, Appl. Organomet. Chem., 7 (1993) 443.
[6] M.G. Cobo Fernandez, M.A. Palacios and C. Camara, Anal. Chim. Acta, 283 (1993) 386.
[7] D. Chakraborti, D.C. Hillman, K.J. Irgolic and R.A. Zingaro, J. Chromatogr., 249 (1982) 81.
[8] A. Elaseer and G. Nickless, J. Chromatogr. A, 664 (1994) 77.
[9] Z. Ouyang and J. Wu, J. Biomed. Chromatogr., 2 (1988) 258.
[10] H. Kataoka, Y. Miyanaga and M. Makita, J. Chromatogr. A, 659 (1994) 481.
[11] S. Cavalli and N. Cardellicchio, J. Chromatogr. A, 706 (1995) 429.
[12] S.J. Jiang and R.S. Houk, Spectrochim. Acta, 43B (1988) 405.
[13] G. Matni, R. Azani, M.R. Van Calsteren, M.C. Bissonette and J.S. Blais, Analyst, 120 (1995) 395.
[14] G. Kölb, Marine Chem., 48 (1995) 185.
[15] M. Potin Gautier, C. Boucharat, A. Astruc and M. Astruc, Appl. Organomet. Chem., 7 (1993) 593.
[16] N. Gilon, A. Astruc, M. Astruc and M. Potin Gautier, Appl. Organomet. Chem., 9 (1995) 623.
[17] A. Fialaire, E. Postaire, R. Prognon and D. Peradeau, J. Liq. Chromatogr., 16 (1993) 3003.
[18] J.K. Johannessen, B. Gammelgaard, O. Jons and S.H. Hansen, J. Anal. At. Spectrom., 8 (1993) 999.
[19] K. Matsumoto, Anal. Sci., 9 (1993) 447.
[20] J. Nève, M. Henry, A. Peretz and J.P. Mareshi, Cath. Nutr. Diet., XXII (1987) 145.
[21] R.M. Smith and A.E. Martell, Critical Stability Constants, Vol 4, Plenum Press, London, 1976, pp. 91–93.