

Determination of selenoamino acids using two-dimensional ion-pair reversed phase chromatography with on-line detection by inductively coupled plasma mass spectrometry

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Abstract

Analytical methods for the speciation of nine selenium species (selenite, selenate, selenourea, trimethylselenonium ion, selenocystamine, selenocystine, selenocysteine, selenomethionine and selenoethionine) that are commonly encountered in biological and environmental samples were developed. Good separation was achieved by either a mixed ion-pair reversed phase chromatography (LiChrosorb RP 18, 2.5 mM 1-butanefulfonate–8 mM tetramethylammonium hydroxide–4 mM malonic acid–0.05% methanol, pH 4.5) or a conventional ion-pair reversed phase chromatography (Inertsil ODS, 10 mM tetraethylammonium hydroxide–4.5 mM malonic acid, pH 6.8) with on-line ICP-MS detection. Using a 20- μ l sample loop, low detection limits around 1 ng ml⁻¹ expressed as Se were achieved for the examined selenium species. The methods were used for the determination of selenoamino acids in a selenium nutritional supplement. The developed methods were found to be rather robust. No alteration of the separation was observed when the protease enzymatic extracts were analyzed without dilution. Both water extracts and enzymatic extracts were chromatographed first with the mixed ion-pair reversed phase chromatographic system, then the major chromatographic peaks were collected and analyzed by the second ion-pair reversed phase chromatographic system for a further verification of their identity. Selenomethionine was found to be the major selenium species in the supplement. A major unknown species, probably Se-adenosylhomocysteine, could be determined in the extracts. A biological reference material, Dolt-2, was also examined for the selenoamino acids. Selenocystine and selenomethionine could be detected in its enzymatic extract, suggesting that Dolt-2 may be used as a reference material for the identification of selenoamino acids in biological and environmental samples. As selenoethionine does not occur naturally in the investigated samples, it is added as an internal standard in this study.

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1. Introduction

The effectiveness of the supplementation in the prevention of chronic selenium deficiency symptoms such as those in Keshan disease in China was well recognized [1]. Recently, a sufficient selenium supplement to protect against cancer was also reported [2]. As a public health concern, the dietary selenium supplementation has become a subject of increasing interest. However, at present, which chemical form of selenium can achieve best nutritional effect still remains an open question. It was generally believed that organic selenium compounds such as selenoamino acids are better absorbed by the human body [3]. Selenomethionine can be metabolized in the human body into selenocysteine [4], an essential component of glutathione peroxidase, which inhibits the oxidative role of peroxides and hydroperoxides, thereby protecting immunocompetent cells and slowing down ageing process. On the other hand, a certain percentage of selenomethionine is known being incorporated nonspecifically into various proteins in place of methionine, which could introduce certain health risks when supplementing infants [5].

To date, a variety of selenium-enriched materials, such as garlic [6], yeast [7], and lactic acid bacteria [5] have been commercialized or proposed as a supplement. Selenomethionine and selenocysteine have been reported to be the major Se species in Se-enriched yeast and in lactic acid bacteria, respectively. Se-methylselenocysteine, Se-homocysteine and Se-cystathionine were also detected in some Se-enriched plants [8]. Since the potential benefits of a dietary supplementation in selenium depend to a great extent on the chemical forms of selenium and are limited by its relatively narrow nutritional window above which toxic symptoms may occur, it is essential to identify and quantify the selenium species present in the supplements.

To determine the selenium species in biological and environmental samples, methods based on the hyphenation of chromatography with either ICP-MS or electrospray mass spectrometry (ES-MS) have been widely reported [9–14]. With the use of ES-MS, the structural characterization and quantification of selenium compounds become possible,

which is extremely useful for the identification of unknown selenium compounds present in natural samples. Their wide application, however, is limited due to the critical requirements for HPLC mobile phase components, the lower sensitivity compared with ICP-MS, as well as the relatively severe matrix effect requiring special pre-treatment and preconcentration of the analytes. On the other hand, the hyphenation of HPLC with ICP-MS provides a powerful tool for the determination of structurally known selenium compounds, especially for the compounds that have standards commercially available or the standards can be synthesized readily. Because of the high sensitivity offered with ICP-MS, the less matrix effect, and the relatively wide compatibility with HPLC mobile phase composition, HPLC–ICP-MS technique remains to be the main stream method for elemental speciation analysis.

To separate Se species, a various of HPLC separation modes, such as ion-exchange, reversed-phase, ion-pair reversed-phase and vesicle mediate chromatography have been examined in the literature [15]. Among them, ion-pair reversed phase chromatography has been found to be increasingly used in recent years for Se speciation, especially, the use of perfluoroaliphatic acid as ion pairing reagent has showed an impressive success in the speciation analysis of selenium [16–18].

The aim of the current investigation is to evaluate the feasibility of ion-pair reversed phase chromatography combined with ICP-MS as a robust and reliable analytical method for the routine speciation analysis of low molecular mass selenium compounds such as selenoamino acids in biological and environmental samples. In this study the selenium species in a nutritional supplement (Selenoprecise, Pharma Nord, UK) was investigated. This work was initiated due to a request on Se speciation in the Se supplement because this Se supplement was selected as a source of selenium for an on-going research project on the study of Se metabolism in rats. Considering the primary selenoamino acids, such as selenomethionine, selenocysteine and selenocystine, might be the main forms of selenium in the supplement, two chromatographic separation systems were employed in this work, involving a

mixed ion-pair and a conventional ion-pair reversed phase chromatography. Full separation of nine selenium species, including four selenoamino acids (selenomethionine, selenocystine, selenocysteine and selenoethionine), was achieved with our chromatographic separation systems. The selenium speciation was carried out in the water-soluble fraction and in the enzymatic extracts, and selenomethionine was found to be the major selenium species. With an attempt to provide a reference material for selenoamino acids speciation, the selenium species in the enzymatic extract of Dolt-2 was also examined.

2. Experimental

2.1. Chemical and reagents

All commercial chemicals were of analytical-reagent grade and were used without further purification. Sodium selenate [Se(VI)] and sodium selenite pentahydrate [Se(IV)] were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Seleno-DL-ethionine (SeEt), seleno-DL-methionine (SeMet), seleno-DL-cystine (SeCys), selenocystamine dichloride (SeCM), and selenourea (SeUr) were obtained from Sigma. Trimethylselenonium iodide (TMSe^+) was obtained from Tri Chemical Laboratory (Yamanashi, Japan). Selenocysteine was prepared in our laboratory. Sodium 1-butan-2-sulfonate (98%) was obtained from Aldrich. Tetramethylammonium hydroxide (10% in water), tetraethylammonium hydroxide (10% in water) and malonic acid were purchased from Nacalai Tesque Inc. HNO_3 (70%), NH_4OH (29%) and methanol (99.9%) were obtained from Kanto Chemical, Co. (Tokyo, Japan). Protease (XIV) was obtained from Sigma. A selenium supplement used in this study is Selenoprecise, a product of Pharma Nord, UK.

Stock solutions of selenium standards were prepared with Milli-Q water (18.3 M Ω cm) (Milli-Q SP ICP-MS, Millipore, Tokyo, Japan) according to the procedures described previously [9]. The stock solutions were stored in a refrigerator at -20°C before use. Work solutions in ng/ml range were prepared daily by appropriate

dilution of the stock solutions. The mobile phase used in this study was prepared by dissolving an appropriate amount of sodium 1-butan-2-sulfonate, malonic acid and tetramethylammonium hydroxide or tetraethylammonium hydroxide to 1 l Milli-Q water to get the required concentrations. The pH of the mobile phases was adjusted by a dropwise addition of dilute nitric acid or 20% NH_4OH . All mobile phases were filtered through 0.45 μm nitrocellulose membrane filters (Millipore, USA) and degassed before use.

2.2. Instrumentation

The ICP-MS instrument used was HP 4500 (Yokogawa Analytical Systems, Tokyo, Japan). The sample introduction system included a Scott-type spray chamber fitted with a concentric nebulizer. The chromatographic system consisted of a Perkin Elmer model series 410 B10 solvent delivery unit (Perkin Elmer, Norwalk, USA), a syringe-loading injector (model 9725i, Rheodyne six-port injection valve) with a 20- μl loop and a LiChrosorb RP 18 or an Inertsil ODS reversed phase column (GL Sciences, Tokyo, Japan, 250×4.6 mm i.d., 5 μm particle size). The chromatographic system was interfaced with the ICP-MS instrument using a 120-mm PEEK (polyether ether ketone) capillary tubing (0.25 mm i.d.) to connect the column outlet to the inlet hole of the nebulizer. The column was conditioned by passing at least 100 ml of the used mobile phase through the column before injection of the Se standards and samples. The chromatographic results were processed using ChromatOSOFTWARE (Yokogawa Analytical Systems, Tokyo, Japan). Quantifications were performed in the peak area mode. The operating conditions for ICP-MS are as follows: rf forward power, 1200 W; plasma Ar flow rate, 16.0 l min^{-1} ; auxiliary Ar flow rate, 1.0 l min^{-1} ; nebulizer Ar flow rate, 1.02 l min^{-1} ; integration time, 0.5 s; data acquisition mode, time resolved analysis; and isotope monitored, ^{77}Se and ^{82}Se .

2.3. Preparation of selenocysteine

Selenocysteine (Sec) was prepared by reduction of selenocystine (SeCys) according to the method

of Baker and Tappel [19] with a slight modification. To 1.35 mg selenocystine was added 1.65 ml of a mixture that contained 200 μl of β -mercaptoethanol, 423 μl of 2 mol l^{-1} ammonium hydroxide, and 3.45 ml of 1 mg ml^{-1} sodium borohydride. The mixture was heated for 5 min at 45 °C in a water bath and then dried under nitrogen with a Rotavapor at 45 °C. After drying, the volume of liquid in the tube was brought to 1 ml with 0.01 mol l^{-1} HCl-1 mmol l^{-1} dithioerythritol to give an 8 mmol l^{-1} solution of selenocysteine. This solution serves as the stock solution. Working solution of selenocysteine was prepared prior to use by appropriate dilution of the stock solution with Milli-Q water.

2.4. Sample preparation

Se supplement (Selenoprecise, Pharma Nord, UK) was weighed, then homogenized with a mortar and pestle. Aliquots (~ 0.1 g) of powder for Se supplement and Dolt-2 reference material (dogfish liver, National Research Council of Canada) were digested with 3 ml HNO_3 and 0.5 ml 30% H_2O_2 using a microwave digestion procedure described previously [20]. Subsequently the digestate was made up to 100 ml in a volumetric flask with Milli-Q water. The total selenium was determined by a microwave-induced plasma mass spectrometry (MIP-MS, Hitachi P 6000, Japan). The operation conditions of MIP-MS were set as in Ref. [21].

Both water and enzymatic extraction methods were used for the extraction of selenium compounds. In the water extraction, supplement powder (0.1 g) or Dolt-2 reference material (0.5 g) was added to 10 ml of Milli-Q water in a 15 ml polyethylene centrifuge tube, and shaken at room temperature for 24 h in a shaking water bath. In enzymatic extraction, protease (20 mg) and supplement powder (0.1 g) or Dolt-2 (0.5 g) were added to 10 ml of a 5 mM phosphate buffer (pH 7.5) in a 15 ml polyethylene centrifuge tube, and shaken at 37 °C for 24 h in a shaking water bath. The solutions were then centrifuged for 20 min at 3000 rpm. The supernatant was removed and filtered through a 0.45- μm membrane filter. The obtained extracts were injected into HPLC–ICP-

MS system for speciation analysis. The enzyme solution was checked by HPLC–ICP-MS, no detectable selenium was found in the extractants. The total selenium concentration in the extracts was determined using MIP-MS. For Se supplement, the determination was performed after tenfold dilution for water extracts and 100-fold dilution for enzymatic extracts, respectively. As an internal standard, indium with a concentration of 50 ng ml^{-1} was added into the calibration standard solutions and the samples prior to the analysis for total selenium concentration.

3. Results and discussion

3.1. Chromatographic separation of selenium compounds

Many analytical methods for the speciation of selenium have been available in the literature [22,23]. However, a close look at the published methods revealed that the lack of ruggedness in the separation of selenium species in real matrix was common. Also, the studied selenium species was limited to a few selenium compounds, in most cases, the most important selenoamino acid, the so-called 21st amino acid, selenocysteine was not considered. In this work, two chromatographic separation systems were developed for the separation of nine selenium species, four selenoamino acids (selenocysteine, selenocystine, selenomethionine, and selenoethionine) and other five selenium species (selenite, selenate, trimethylselenonium ion, selenocystamine and selenourea) commonly encountered in biological and environmental samples. One system (LC-1) was the previously developed mixed ion-pair reversed phase chromatography [9], in which the nine selenium species were separated in a single run. However, the retention times between selenomethionine and selenocysteine were rather close, resulting in a difficulty for the quantification if both of them exist in the sample (see Table 1). The other ion-pair reversed phased chromatographic system (LC-2), which is initially developed for the separation of arsenic compounds [24], employed tetraethylammonium ion (TEA) as a pairing ion for the

Table 1
Retention times of nine selenium standards

Se species	Formula	Retention time (s) ^a	
		LC-1	LC-2
Se(IV)	H ₂ SeO ₃	181	207
Se(VI)	H ₂ SeO ₄	164	268
SeCys	(HOOCCH(NH ₂)CH ₂ Se ⁻) ₂	206	176
SeUr	Se=C(NH ₂) ₂	262	223
TMSe ⁺	(CH ₃) ₃ Se ⁺	305	179
Sec	HSeCH ₂ (NH ₂)CHCOOH	410	436
SeMet	CH ₃ SeCH ₂ CH ₂ CH(NH ₂)COOH	421	395
SeCM	H ₂ NCH ₂ CH ₂ Se–SeCH ₂ CH ₂ NH ₂	775	– ^b
SeEt	CH ₃ CH ₂ SeCH ₂ CH ₂ CH(NH ₂)COOH	1010	982
Cl ^{-c}		181	206

^a Chromatographic conditions: LC-1, column = LiChrosorb RP 18 (4.6 × 250 mm, 5 μm particle size), mobile phase = 2.5 mM 1-butanefulfonic acid sodium salt–8 mM tetramethylammonium hydroxide–4 mM malonic acid–0.05% methanol, pH 4.5, 1.0 ml min⁻¹. LC-2, column = Inertsil ODS (4.6 × 250 mm, 5 μm particle size), mobile phase = 10 mM tetraethylammonium hydroxide–4.5 mM malonic acid, pH 6.8, 1.0 ml min⁻¹. For all the investigated selenium compound, the stability of the retention time was evaluated by the relative standard deviation of three successive injections (< 1%).

^b Selenosystemine (SeCM) was not eluted in LC-2 system.

^c The polyatomic ions, such as ⁴⁰Ar³⁷Cl⁺ and ¹²C³⁵Cl⁺, may cause interference for ICP-MS detection at *m/z* of 77 and 82.

separation anionic species. In this system, the pH of the mobile phase was set at 6.8, which is close to the permitted pH limit of the ODS reversed phase column, in order to keep the deprotonation of inorganic selenite and selenate, also to deprotonize the carboxylic acid group of the selenoamino acids. The formation of ion pairs between tetraethylammonium ion and selenium species (selenite, selenate and the negatively charged carboxylate group of the selenoamino acids) was evidenced by the concentration effect of tetraethylammonium ion on the retention of selenium species shown in Fig. 1. With a decrease of tetraethylammonium ion concentration in the mobile phase, the retention times of selenium species also decreased, showing a typical characteristic of an ion-pair reversed phase chromatography. After a series of optimization, it was found that this system was particularly suited for the separation of seleno-

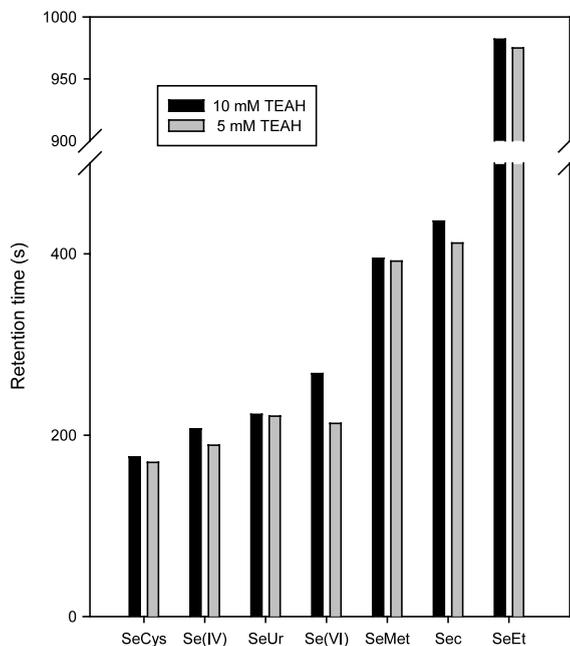


Fig. 1. The effect of tetraethylammonium ion concentration on the retention of selenium species. Chromatographic conditions: Inertsil ODS column; *x* mM tetraethylammonium hydroxide–4.5 mM malonic acid, pH 6.8 (1.0 ml min⁻¹).

amino acids and inorganic selenite and selenate. The retention times of selenium species and the chromatographic conditions were summarized in Table 1. Kotrebai et al. have demonstrated a successful application of ion-pair reversed phase chromatography containing perfluoroaliphatic acid agents to the separation of more than ten organic selenium compounds with high resolution [16–18]. The results obtained in the present work further showed a great potential to separate both inorganic and organic selenium compounds with ion-pair reversed phase chromatography. With a 20-μl sample loop, low detection limits ca. 1 ng ml⁻¹ were achieved for the examined selenium species. These two chromatographic separation systems were employed in this work for the identification and determination of selenium species in the selenium supplement.

3.2. Selenium compounds in Se supplement

In an on-going research project, a selenium supplement (Selenoprecise, Pharma Nord, UK)

was administered to rats to study the metabolism of selenium species, a request on the speciation of selenium in this supplement, therefore, was raised in order to get information on the selenium species in the supplement. Se-yeast was the source of selenium in this supplement as indicated by the producer. At first, the total selenium concentration in the supplement was determined using MIP-MS after a microwave digestion with HNO_3 and H_2O_2 . The selection of MIP-MS for the determination of total Se is due to its characteristic of the lack of spectra interferences such as $^{40}\text{Ar}^{40}\text{Ar}$ and $^{38}\text{Ar}^{40}\text{Ar}$ at m/z 80 and 78 [25]. The obtained result ($502 \pm 45 \text{ mg kg}^{-1}$ Se) was in a good agreement with the formulation value (512 mg kg^{-1} Se). The determination of total Se content was also validated by the analysis of a biological reference material Dolt-2 (Dogfish liver, National Research Council of Canada): certified value, 6.06 mg kg^{-1} ; we found, $6.01 \pm 0.08 \text{ mg kg}^{-1}$. When a water extraction was carried out for the selenium supplement, it was found that $18 \pm 1\%$ ($n = 3$) of the total selenium was water-soluble. This observation is in consistent with other reports on Se-yeast or Se-yeast based supplements [26,27]. Yeast is known for its high protein content, which is approximately 40% dry mass [28]. Because of the similarity of selenium to sulfur, selenoamino acids can also be metabolized into proteins. It was reported that about 80% of Se incorporated in yeast was found associated with high molecular mass compounds (cell walls, mitochondria, microsomes, proteins and nucleic acids). Therefore, subsequently, a proteolytic enzymatic extraction was employed aiming to extract the selenoamino acids associated with proteins because protease is known to be able to break the peptic bonds of proteins present in the sample. The obtained extraction yield (%) was 77 ± 5 ($n = 3$) of the total selenium in the supplement. This extraction yield is lower than that obtained by Gilon et al. [7] in Se-yeast, but in good agreement with our previous work on other selenium supplements [9,27]. As discussed before [27], the incomplete extraction may be attributed to the interference on the enzyme activity resulting from the additives added in the supplement.

The selenium species present in the water extracts and enzymatic extracts were first investigated with LC-1 chromatographic system with ICP-MS detection (Fig. 2). Except a filtration with a $0.45 \mu\text{m}$ membrane, there is no need for any sample pre-treatment; no dilution and no adjustment of the pH value. Therefore, low-concentrated Se species can be seen in the chromatograms. Although the protein load in the sample was high, the separation was matrix tolerant; good resolution and constant retention times of selenium species were remained. However, in order to avoid the accumulation of proteins containing in the samples, which may hinder the separation at a later stage, the column should be cleaned regularly using water and acetonitrile with a gradient elution when the decrease of separation efficiency is observed. As can be seen in Fig. 2(A and B),

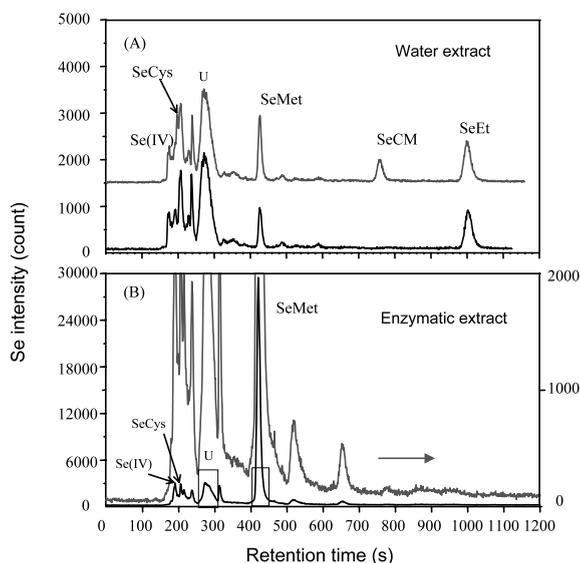


Fig. 2. Representative chromatograms of water extracts (A) and enzymatic extracts (B) of the examined selenium supplement with LC-1 chromatographic system. All the extracts were directly injected for analysis without any dilution. In the water extracts, selenoethionine (100 ng ml^{-1}) was added as internal standard. The upper chromatogram in (A) was the water extracts spiked with selenomethionine, selenocystine and selenocystamine (30 ng ml^{-1} for each) for species identification. In (B) the black line shows the chromatogram as a total. The grey line is a zoomed plot of the chromatogram to show smaller peaks more clearly. The shadowed parts show the fractions collected for a further analysis with LC-2 chromatographic system.

similar chromatographic profiles were obtained in the water and enzymatic extracts. At least 12 selenium species could be seen in the chromatograms with good reproducibility, thanks to the high sensitivity of ICP-MS detection. By comparison with the retention times of authentic selenium standards and the spiking experiment, inorganic Se(IV) and selenoamino acids (selenocystine and selenomethionine) could be identified. The selenoethionine was not present in the original sample but added in the water extracts as a chromatographic internal standard. A major unknown selenium species (U) was found in both water extracts and enzymatic extracts. Owing to its relative broad peak shape and similar retention time with selenourea, it was considered that this peak may consist of more than one species. Also, due to the similar retention time of selenomethionine and selenocystine in LC-1 chromatographic system, the purity of the peak of selenomethionine in the enzymatic extracts was an issue of concern. Therefore, an aliquot of 100 μl of the enzymatic extracts was chromatographed with LC-1 chromatographic system, and the fractions corresponding to the major unknown peak (U) and selenomethionine peak were collected (the shadowed parts in Fig. 2B showed how the fraction was collected). The collected fractions were injected into the LC-2 chromatographic system to further check their identity. The obtained chromatograms were shown in Fig. 3. It was found that the unknown species had a retention time of 244 s in LC-2 system, not matching with the retention time of selenourea (223 s). A trace peak with a retention time of 492 s was also seen in the chromatogram. This trace peak was most probably resulted from the neighbor unknown peak of the major unknown species U in LC-1 system. The identity of the major unknown species (U) could not be identified in this work because its retention time did not match the retention times of selenium standards available in our laboratory. However, based on its chromatographic behaviors, it is most probably the same species, Se-adenosylhomocysteine, identified in Se-yeast by Casiot et al. [26] using pneumatically-assisted electrospray tandem mass spectrometry. The identification of selenomethionine in LC-1 system was further confirmed

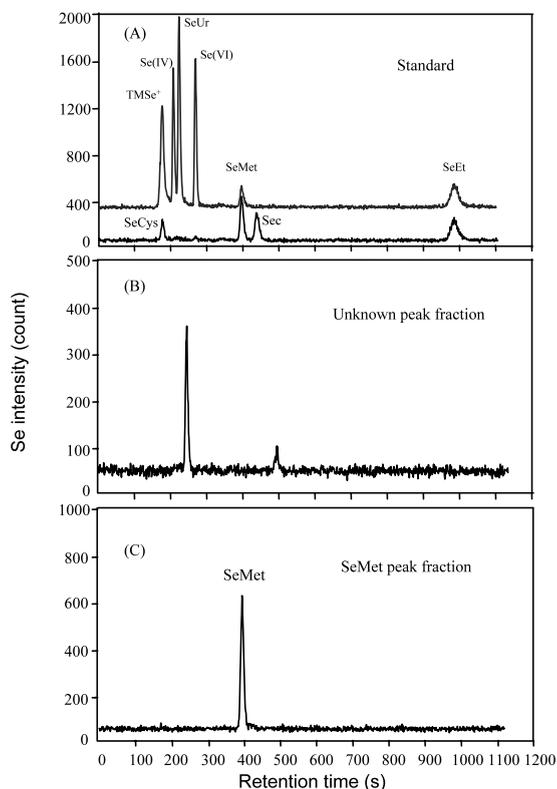


Fig. 3. Chromatograms obtained with LC-2 chromatographic system for: (A) the lower chromatogram, the standards of four selenoamino acids (SeCys, 16 ng ml^{-1} ; SeMet, 30 ng ml^{-1} ; Sec, 25 ng ml^{-1} ; and SeEt, 25 ng ml^{-1}); the separation of other Se compounds was shown in the upper chromatogram (TMSe⁺, Selenite, SeUr, Selenate, 50 ng ml^{-1} ; SeMet, 20 ng ml^{-1} ; and SeEt, 25 ng ml^{-1}); (B) the fraction of the major unknown peak U in LC-1 system; (C) the fraction of SeMet in LC-1 system.

because the fraction only produced one peak in LC-2 system, and the retention time was exactly the same as that of selenomethionine standard. No detectable selenocystine could be seen in the chromatogram. Since the complexity of various selenium species in the Se-yeast based supplement, it is essential to have different chromatographic systems for the identification of detected selenium species in order to avoid the potential misidentification.

Quantitative calculations indicated that the accounted percentage of total Se in the water extracts for the detected selenium species were as follows: Se(IV), 1.8%; Selenocystine, 2.9%; selenomethionine, 3.2%; the major unknown U, 16.5%

and the other unknowns, 9.4%. These results showed that the on-column recovery for the water extracts was only 34% of the total Se present in the extracts, indicating most of the selenium compounds in the water extracts may remain to be protein-associated, which could not be eluted in the chromatographic column. When a protease was used in the extraction process, the big Se-proteins were broken down and Se was released as smaller molecular mass compounds, such as selenoamino acids and small peptides that could be eluted in our chromatographic system. The on-column recovery of selenium, therefore, was increased up to 93% in the case of enzymatic extracts. Selenomethionine was found to be the predominant species (78% of the total Se) in the enzymatic extracts. Selenite (2%), selenocystine (1.9%), the major unknown species U (10%) and the other unknowns (7.8%) accounted for the remaining selenium in the enzymatic extracts. The chloride concentration in the extracts was determined using an ICP-AES to be less than $2 \mu\text{g ml}^{-1}$, therefore, the interferences of $^{40}\text{Ar}^{37}\text{Cl}$ and $^{12}\text{C}^{35}\text{Cl}_2$ on the ICP-MS detection at m/z 77 and 82 were negligible.

3.3. Selenium compounds in Dolt-2 reference material

The interest in the occurrence, speciation and cycling of selenium in marine systems is increasing considerably in recent years [10,29], the knowledge of Se species in the marine ecosystem, however, is still limited. It was believed that selenium in animal tissues was associated with proteins and present as selenomethionine and possibly selenocysteine. Jakubowski et al. [10] also reported the presence of selenocystamine in herring gull eggs. To explore the potential application to the determination of selenoamino acids in marine ecosystem, and the possibility of providing a reference material for the speciation of selenoamino acids, the selenium compounds in a biological reference material, NRCC Dolt-2 (dogfish liver) (National Research Council of Canada) were examined using LC-1 system. This reference material was certified for the total Se concentration at 6.06 mg kg^{-1} . The extraction of Se species was performed using

water extraction and protease digestion. The water extractable selenium is only 3% of the total Se. This is not a surprise because most of selenium present in the marine animals was associated with insoluble proteins [30]. The extraction yield (%) could be improved up to 15.7 ± 2 with a protease digestion. The extract was directly injected into the HPLC-ICP-MS system without any dilution. A typical chromatogram of the enzymatic extract of Dolt-2 is shown in Fig. 4. The preliminary results indicated that both SeCys and SeMet existed in the extract. Two major unknown compounds with a retention time of 235 s and 377 s, respectively, were also observed in the chromatogram. Although it was believed that selenomethionine and selenocysteine were the basic forms of selenium associated with proteins in animal tissues, no detectable selenocysteine could be observed in the enzymatic extract of Dolt-2. The presence of selenocystine, however, may suggest that the oxidation of selenocysteine occurred during the extraction process. Further work is needed to improve the extraction to avoid the species transformation. The total Se in the extract, determined with N_2 -MIP-MS using isotope ^{80}Se , which is free from the Ar associated spectra interference, is $46.2 \pm 3.1 \text{ ng ml}^{-1}$. The sum (ng ml^{-1}) of the Se species, SeCys (7.7 ± 0.4), SeMet (6.8 ± 1.8), and the unknown (29.2 ± 1.9), is 43.7, giving an on-column recovery of 94.6%. This good mass balance demonstrated the applicability of the developed analytical method to the quantitative determination of

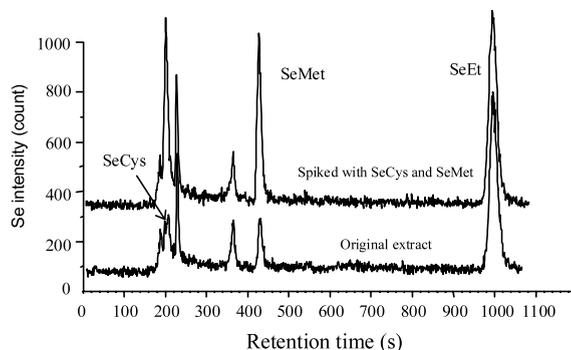


Fig. 4. Chromatograms of the enzymatic extracts of Dolt-2 obtained with LC-1 chromatographic system. SeEt was spiked in the extracts as an internal standard.

selenoamino acids in marine animal tissue samples.

4. Conclusion

Selenoamino acids attract much interest, because these selenium compounds take part in the biological selenium cycle and are incorporated into proteins. In this work, the selenium species, particularly, selenoamino acids in a selenium supplement were determined using two ion-pair reversed phase chromatographic systems with on-line ICP-MS detection. Through the investigation of both water extracts and enzymatic extracts of the supplement, it was found that there was a trace of free selenoamino acids (SeMet and SeCys) in the supplement. However, most of the selenium was associated with proteins in a form of selenomethione, which accounted for ca. 60% of the total selenium in the supplement. Compared with the conventional methods of selenoamino acid determination, such as using amino acid analyser and selective detection with different derivatization approaches, several advantages of the developed HPLC-ICP-MS methods are notable. Firstly, the cross-check of selenium species with two different chromatographic systems strengthen the correct identification of selenium species with HPLC-ICP-MS technique. Secondly, the developed chromatographic systems were demonstrated to be highly matrix tolerant and suited for the separation of mixtures of inorganic and organic selenium compounds consisting of neutral, anionic and cationic species. Finally, the sulfur-analogues of selenium compounds present in the samples do not interfere with the identification of selenium compounds because of the use of ICP-MS as element-specific detector. In addition, preliminary results indicated that both SeCys and SeMet could be detected in the enzymatic extract of Dolt-2. With a further effort to improve the extraction efficiency, this reference material may be employed for the validation of analytical method for selenoamino acids speciation analysis.

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