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Speciation of selenium compounds with ion-pair reversed-phase liquid chromatography using inductively coupled plasma mass spectrometry as element-specific detection

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Abstract

For selenium speciation analysis, the hyphenation of chromatographic separation with element-specific detection has proved a useful technique. A powerful separation system, which is capable of resolving several biologically and environmentally important selenium compounds in a single column, is greatly needed. However, that has been difficult to achieve. In this paper eight selenium compounds, namely, selenite [Se(IV)], selenate [Se(VI)], selenocystine (SeCys), selenourea (SeUr), selenomethionine (SeMet), selenoethionine (SeEt), selenocystamine (SeCM) and trimethylselenonium ion ($TMSe^+$), were separated by using mixed ion-pair reagents containing 2.5 m*M* sodium 1-butanesulfonate and 8 m*M* tetramethylammonium hydroxide as a mobile phase. The separation of these anionic, cationic and neutral organic selenium compounds on a LiChrosorb RP18 reversed-phase column took only 18 min at a flow-rate of 1.0 ml/min with isocratic elution, and baseline separation among the six organic Se compounds was achieved. Inductively coupled plasma mass spectrometry (ICP-MS) was employed as element-specific detection. A comparison of ICP-MS signal intensity obtained with a Barbington-type nebulizer and with an ultrasonic nebulizer (USN) was made. Different signal enhancement factors were observed for the various selenium compounds when a USN was used. The speciation technique was successfully applied to the study on chemical forms of selenium in a selenium nutritional supplement. Selenomethionine was found to be the predominant constituent of selenium in the supplement.

Keywords: Inductively coupled plasma mass spectrometry; Detection, LC; Speciation; Selenium compounds

1. Introduction

In environmental and biological systems, selenium can exist either in inorganic forms or as organic species with direct Se–C bonds. Selenium is primarily present as selenite [Se(IV)] and selenate [Se(VI)] ions in soil and water. A number of organic selenium compounds occur in plants and microorganisms as well as in their associated environments because of the biomethylation of inorganic selenium species by microorganisms and plants [1]. In mammalian tissues, selenoamino acids are the predominant forms of selenium. Selenocystamine (SeCM), recently, was identified in human milk [2] and in the extracts of

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sea-gull eggs [3]. The trimethylselenonium ($TMSe^+$) ion has been identified in human urine, and is thought to indicate excessive intake of selenium [4]. Evidence for selenourea (SeUr) in human urine was also reported [5]. It has been recognized that the nutritional bioavailability, toxicity and cancer chemopreventive activity of selenium are species-dependent [6]. The speciation of selenium therefore became the subject of increasing interest in recent years, and much effort has been devoted to the development of analytical methods for selective determination of naturally occurring selenium compounds in environmental and biological materials.

For elemental speciation analysis, the hyphenation of high-performance liquid chromatography (HPLC) with element-specific detection, such as hydride generation-atomic absorption spectrometry (HG-AAS) [7], graphite furnace atomic absorption spectrometry (GF-AAS) [8], inductively coupled plasma atomic emission spectrometry (ICP-AES) [9], atomic fluorescence spectrometry (AFS) [10], and inductively coupled plasma mass spectrometry (ICP-MS) [3,6,11], has been an ideal choice. This is due to the separation power provided by HPLC leading to the separation of element species of interest, which is a precondition for elemental speciation, thus the separated species could be determined by the coupled element-specific detectors. Among the commonly used element-specific detection methods, ICP-MS has been increasingly employed in recent years. The combination of HPLC–ICP-MS provides a powerful and sensitive technique for on-line elemental speciation analysis. Because of the high sensitivity of this technique, the analysis can usually be carried out without sample pre-concentration steps which may influence the composition of the species in the samples. In addition to the high sensitivity, the sample uptake rate of ICP-MS is in the same range of the eluent flow-rate of the most HPLC systems, therefore, the connection between HPLC and ICP-MS can be achieved easily without any special interface.

In terms of selenium speciation, various HPLC separation modes, such as ion-exchange chromatography, reversed-phase chromatography, and ion-pair reversed-phase chromatography as well as size-exclusion chromatography, have been used. The number of separated selenium compounds has increased from two inorganic species, Se(IV) and Se(VI), to several organic compounds in a single chromatographic run. On an anion-exchange column six selenium compounds, namely, Se(IV), Se(VI), SeCys, SeMet, methylselenocysteine and allylselenocysteine, were separated with an aqueous solution of ammonium citrate [12], four compounds, Se(IV), Se(VI), SeCys and SeMet, were separated with an aqueous solution of salicylate under alkaline conditions [13] and $TMSe^+$, SeCys, SeMet and Se(IV) were separated with tartaric acid as a mobile phase [11]. On a silica-based cation-exchange column six selenium compounds, namely, Se(IV), Se(VI), SeCys, SeMet, SeEt and $TMSe^+$, were separated with 20 m*M* pyridine as a mobile phase [14]. Recently, SeCys, SeMet, SeEt, SeUr, Se(IV) and Se(VI), were separated by vesicle-mediated reversed-phase chromatography [15]. The application of ion-pair reversed-phase chromatography with appropriate counter-ions for the speciation of selenium compounds has increased considerably in recent years due to its capacity of simultaneous separation of anionic, cationic ions and neutral molecules. For instance, the pentanesulfonate anion which served as an ion pairing agent for the separation of $TMSe^+$, SeCys and SeMet [16] was reported. Cationic reagent tetraethylammonium bromide or anionic reagent sodium heptanesulfonate was used separately as an ion pairing reagent for the separation of Se(IV), Se(VI), SeCys and SeMet on a reversed-phase column [8]. SeMet, SeCys and SeEt were separated on a C_{18} column by using trifluoroacetic acid as an ion-pair reagent [10]. Normally, the anionic or cationic ion-pair reagent was used separately for the speciation of selenium compounds by using ion-pair reversed-phase chromatography. In the previous work [17], a mixed ion-pair reversed-phase chromatographic technique was demonstrated to be useful for the speciation of selenium compounds using flame atomic absorption spectrometry (FAAS) as an element-specific detector. Sodium 1-butanesulfonate and tetramethylammonium hydroxide were added simultaneously into an aqueous solution. Under suitable pH conditions, the ion-pair formation occurs between the positively charged ammonium part of the selenoamino acids ($TMSe^+$ is a cation irrespective of pH) and the sulfonate group. The added second ion-pair reagent tetramethylam-

monium hydroxide was used to control the capacity factor *k* of selenium compounds selectively, thus to improve the separation. By using this separation approach, the simultaneous separation of seven selenium compounds, namely, Se(IV), Se(VI), SeCys, SeMet, SeEt, $TMSe^+$ and SeCM, could be realized in a single chromatographic run. However, since FAAS was used as an element-specific detection method, the obtained detection limits were found to be ca. 1 μg Se/ml for the investigated Se compounds. The obtained detection limits were not low sufficient for the determination of Se compounds in most environmental and biological samples.

In the present work, the speciation of eight environmentally and biologically important organic selenium compounds, namely, Se(IV), Se(VI), SeCys, SeMet, SeEt, SeUr, SeCM and $TMSe^+$, was carried out by using the developed mixed ion-pair reversed-phase chromatography. These selenium compounds were separated in a single chromatographic run on a silica-based reversed-phase column. The separation system was coupled with ICP-MS, and low detection limits (ca. 2 μg Se/l) for the investigated Se compounds were achieved with a Barbington-type nebulizer. The sensitivity difference between a Barbington-type nebulizer and an ultrasonic nebulizer (USN) was compared. In terms of the enhancement of sensitivity, different signal enhancement factors were observed for the various selenium compounds when using a USN. The applicability of the mixed ion-pair reversed-phase chromatographic separation technique to the speciation of selenium compounds in real world samples was demonstrated by the analysis of a selenium nutritional supplement.

2. Experimental

2.1. Chemicals and reagents

All commercial chemicals were of analytical grade and were used without further purification. Sodium selenate [Se(VI)] was purchased from Nacalai Tesque (Kyoto, Japan), seleno-D,L-ethionine (SeEt), seleno-D,L-methionine (SeMet), seleno-D,L-cystine (SeCys), selenocystamine dichloride (SeCM), selenourea (SeUr) and Protease XIV (5.6 units/mg solid) from Sigma. Trimethylselenonium iodide ($TMSe^+$) was obtained from Tri Chemical Lab. (Yamanashi, Japan). Sodium 1-butanesulfonate (98%) was obtained from Aldrich. Tetramethylammonium hydroxide (10% in water) was purchased from Merck. Malonic acid and ammonium phosphate were obtained from Kanto (Tokyo, Japan).

Stock solutions were prepared with Milli-Q water (18.3 MΩ cm) from anhydrous sodium selenate (1196.4 mg to 500 ml, 1000 mg Se/l), from trimethylselenonium iodide (63.6 mg to 20 ml, 1000 mg Se/l), from selenomethionine (24.8 mg to 20 ml, 500 mg Se/l), from selenoethionine (26.6 mg to 20 ml, 500 mg Se/l), from selenocystine (21.2 mg to 20 ml 0.2 m*M* HCl, 500 mg Se/l), from selenourea (15.6 mg to 100 ml, 100 mg Se/l) and from selenocystamine dichloride (20.2 mg to 20 ml, 500 mg Se/l). A Spex Plasma Standard Solution of selenite [Se(IV)] was also used as a stock solution. The stock solutions were stored in a refrigerator at −20°C before use. No degradation of the compounds was observed over 3 months of storage [18]. Work solutions in the ng/ml range were prepared daily by appropriate dilution of the stock solutions. Ammonium phosphate solution used for the enzymatic extraction was prepared by dissolving 1.15 g $NH_4H_2PO_4$ in 100 ml Milli-Q water, and the pH was adjusted to 7.5 by the addition of 20% NH_4OH.

2.2. Mobile phases

The mobile phases used in this study were prepared by dissolving an appropriate amount of sodium 1-butanesulfonate, malonic acid and tetramethylammonium hydroxide (TMAH) in 1 l Milli-Q water to get the required concentrations. The pH of the mobile phases was adjusted by 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.

2.3. Instrumentation

The chromatographic system consisted of a JASCO Tri-Rotar-V HPLC pump (Japan Spectroscopic Cooperation, Japan), a syringe-loading injector (Model 7125, Rheodyne six-port injection valve)

with a 100-μl loop and a LiChrosorb RP18 reversed-phase (Seibersdorf, Austria, 250×4.6 mm I.D., particle size 5 μm). The chromatographic system was interfaced with ICP-MS instrument using a 300 mm PEEK (polyether ether ketone) capillary tubing (0.25 mm I.D.) to connect the column outlet to the inlet hole of the Barbington-type nebulizer of the ICP-MS instrument or to a USN (Cetac U-5000 AT^+). The outlet of the USN was connected with an additional Ar supply and then with the torch of the ICP-MS instrument. The ICP-MS instrument used was a Model HP 4500 (Yokogawa Analytical Systems, Tokyo, Japan). The column was conditioned by passing 100 ml of the used mobile phase through the column before injection of the selenium standard solution and samples. Chromatographic results were processed using Chromatosoftware (Yokogawa Analytical Systems). Quantifications were performed in the peak area mode. The operating conditions for HPLC–ICP-MS are summarized in Table 1. The ICP-MS measurement conditions, given in Table 1, were optimized daily using a standard built-in software procedure for injection of a 10 μg/l solution containing Li, Y, Ce and Tl. Three selenium isotopes, ^{77}Se, ^{78}Se and ^{82}Se were monitored. Since the ion intensity at m/z 82 presents a better signal-to-noise ratio than the other Se isotopes, it was used for quantification.

Total selenium concentrations in the extracts of selenium supplement and in the extraction residues were also determined with ICP-MS. Instrumental parameters of ICP-MS were the same as used for HPLC–ICP-MS measurements.

2.4. Extraction of selenium compounds

An enzymatic extraction method was used for the extraction of selenium compounds from a selenium nutritional supplement. The extraction procedure has been described in detail elsewhere [11]. Briefly, protease (20 mg) and supplement powder (0.1 g) were added to 5 ml of a 100 m*M* ammonium phosphate solution (pH 7.5) in a 15-ml polyethylene centrifuge tube, and shaken at 37°C for 24 h in a shaking water-bath (Iuchi WCS-150, Osaka, Japan). The solution was then centrifuged in a centrifuge

Table 1
Operating conditions for HPLC and ICP-MS instruments

HPLC:	
Column	LiChrosorb RP18 (250×4.6 mm I.D., 5 μm particle size)
Mobile phase	2.5 m*M* sodium 1-butanesulfonate–8 m*M* tetramethylammonium hydroxide–4 m*M* malonic acid–0.05% methanol, pH 4.5
Flow-rate	1.0 ml/min
Injection volume	100 μl
Column temperature	Ambient
ICP-MS:	
Forward RF power	1300 W
Plasma Ar flow	15.0 l/min
Auxiliary Ar flow	1.0 l/min
Nebulizer Ar flow	1.18 l/min
Data acquisition mode	Time resolved analysis
Integration time	100 ms
Isotopes monitored	^{77}Se, ^{78}Se and ^{82}Se
Total analysis time	18 min
USN	
Nebulizer gas	0.8 l/min
Heater temperature	140°C
Condenser temperature	2°C
Membrane desolvator:	
Heater temperature	160°C
Sweep gas flow	2.6 l/min

(Iuchi Pasolina, Osaka, Japan) for 25 min at 1500 *g*. The supernatant was removed and filtered through a 0.45-μm nitrocellulose membrane filter (Millipore). The filtrates were diluted appropriately (ca. 20-fold dilution) with a mobile phase before being chromatographed. The enzyme solution was checked by HPLC–ICP-MS, and no detectable selenium was present in the extractants [14].

2.5. Determination of total selenium concentration

The total selenium concentration in the investigated selenium nutritional supplement was determined by using both GF-AAS and ICP-MS in the previous work [14]. The GF-AAS and ICP-MS results were in good agreement with the formulation value 400 μg/g. In this work, to evaluate the extraction efficiency and establish the mass balance, the total selenium concentration in extracts obtained from enzymatic extraction, was determined with ICP-MS. The total selenium concentration in the residues was determined also with ICP-MS after microwave digestion (3 ml HNO_3+0.5 ml 30% H_2O_2) of the residues. An MLS 1200 MEGA microwave digestion system from Milestone (Italy) was used for the mineralisation of the extraction residues. The following microwave digestion program was used: 250 W, 2 min; 0 W, 30 s; 250 W, 10 min; 0 W, 30 s; 450 W, 5 min; 0 W, 30 s; 600 W, 5 min; 500 W, 7 min [19].

3. Results and discussion

3.1. Selenium compounds in aqueous solutions

Selenium compounds can be present in aqueous solutions as cations, anions or zwitterions under different pH values due to their different dissociation constants. Selenic acid (pK_2 1.7) and selenous acid (pK_1 2.46, pK_2 7.31) can be present in aqueous solutions as anions with one or two negative charges. At pH values less than 4.0 a portion of selenous acid may remain undissociated. SeCM and SeUr may carry two positive charges at low pH, and be present as neutral molecules at high pH, the dissociation constants for SeCM and SeUr are, at present, not available. $TMSe^+$ is a cation irrespective of pH. SeMet (pK_1 2.19, pK_2 9.05) and SeEt will exist in an aqueous solution of low pH as cations (carboxyl group not deprotonated and uncharged, amino group protonated and positively charged), at intermediate pH as a zwitterionic (carboxyl group deprotonated and negatively charged, amino group protonated and positively charged), and at high pH as anion (carboxyl group deprotonated and negatively charged, amino group not protonated and uncharged). The dissociation constants for SeEt are not known, at present, but can be expected to be almost the same as the constants for SeMet, because methionine (pK_1 2.23, pK_2 9.08) and ethionine (pK_1 unknown, pK_2 9.02), the corresponding thioammino acids, have very similar constants. Based on the p*K* values, five SeCys (pK_1 1.68, pK_2 2.15, pK_3 8.07, pK_4 8.94) species may be present in aqueous solutions. SeCys carries two positive charges at very low pH (localized to the two protonated amino groups), one positive charge at low pH, and becomes zwitterionic at intermediate pH. It carries one negative charge at higher pH, and converts to divalent anions at high pH. Since cationic, anionic selenium species and zwitterionic species may exist simultaneously in the solution, their separation is still a great challenge for analytical scientists.

3.2. Chromatographic separation of selenium compounds

The retention behavior of seven environmentally and biologically important Se compounds, namely, Se(IV), Se(VI), SeCys, SeMet, SeEt, $TMSe^+$ and SeCM, on a LiChrosorb RP18 reversed-phase column using sodium 1-butanesulfonate and TMAH as mixed ion-pair reagents, has been studied in the previous work [17]. Several chromatographic parameters, such as the pH of the mobile phase, the concentrations of ion-pair reagents, and the content of methanol in the mobile phase, were optimized. A mobile phase containing 10 m*M* sodium 1-butanesulfonate–4 m*M* TMAH–4 m*M* malonic acid–0.05% methanol at pH 4.5 was found to be suitable for the separation of above-mentioned seven Se compounds.

The presence of SeUr in human urine has been suggested [5], therefore, in the present work, the separation power of the mixed ion-pair chromatog-

raphy was extended to include this organic Se compound. ICP-MS was employed as an element-specific detector to achieve the low detection limits needed for the speciation of Se compounds in real samples. Due to the great separation capability of the mixed ion-pair reversed-phase chromatographic separation technique, the separation of eight Se compounds in a single chromatographic run was achieved readily by adjusting the concentration ratio between butanesulfonate and TMAH, and it was found that good separation for these eight Se compounds could be achieved on a LiChrosorb RP18 reversed-phase column with a mobile phase of 2.5 m*M* sodium 1-butanesulfonate–8 m*M* TMAH–4 m*M* malonic acid–0.05% methanol at pH 4.5 with a flow-rate of 1.0 ml/min (Fig. 1). It can be seen that the separation of eight environmentally and biologically important selenium compounds could be completed within 18 min. Baseline separation is achieved for all the six organic selenium compounds. To our best knowledge, this is the first report on the separation of these eight selenium compounds on a single chromatographic run with an isocratic elution. The separation can be reduced to 15 min by adding 2% methanol to the mobile phase. This is because the introduction of organic modifier into the mobile phase reduces the hydrophobic interaction between the C_{18} stationary phase and the ion-pair reagent to which analyte ions are paired [20]. However, the use of a higher concentration of organic solvent is not recommended for ICP-MS detection, because the sample cone of the ICP-MS interface may become clogged due to the deposition of carbon residue resulting from incomplete combustion of organic compounds [21,22]. Therefore, 0.05% methanol was added to the mobile phase as a compromised condition. If a large number of samples need to be analyzed, to improve the sample throughput, 2% methanol can be added into the mobile phase, while a small amount of O_2 should be added to the nebulizer gas flow to avoid the deposition of carbon residue on the ICP-MS sample cone.

Table 2 summarizes the separation stability and efficiency of the developed ion-pair chromatography. The separation stability was evaluated in three different days using the same mixed standard solution (50 ng Se/ml each). Relative standard deviation (RSD) of the peak retention times is always less than 1% for the eight selenium compounds (Table 2). The separation efficiency (N) was calculated by using the following equation [23]:

$$N = 5.56(t_r/W_{1/2})^2$$

where $W_{1/2}$ is the bandwidth at half-height in a unit

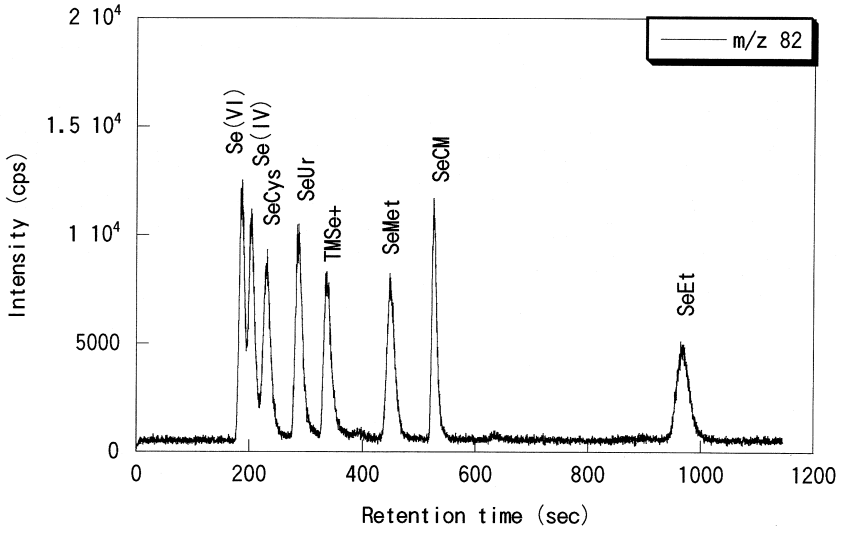

Fig. 1. HPLC–ICP-MS chromatogram obtained from a mixed selenium standard solution (50 μg/l each). Chromatographic conditions: column, LiChrosorb RP18; mobile phase, 2.5 m*M* sodium 1-butanesulfonate–8 m*M* tetramethylammonium hydroxide–4 m*M* malonic acid–0.05% methanol, pH 4.5; flow-rate, 1.0 ml/min; column temperature, ambient. ICP-MS detection at *m*/*z* 82 was used.

Table 2
Stability and separation efficiency of the chromatographic separation for selenium compounds[a]

Compound	Retention time (mean, s)	RSD (%)	N^b
Se(VI)	186	0.7	1500
Se(IV)	203	0.7	1200
SeCys	231	0.6	1100
SeUr	287	0.3	1900
$TMSe^+$	336	0.7	2700
SeMet	450	0.4	3500
SeCM	526	0.4	13 800
SeEt	968	0.4	6800

[a] Estimated on three different days ($n=3$).
[b] $N=5.56(t_r/W_{1/2})^2$.

of time. This equation was used because all the peaks in the chromatogram are symmetric peaks. It can be seen that high separation efficiency was obtained even for the last eluted Se compound, SeEt, in our chromatographic separation system.

3.3. Comparison of the pneumatic nebulizer and the USN

The developed mixed ion-pair reversed-phase HPLC system was coupled either directly to a Barbington-type nebulizer or to a USN. Using the Barbington-type nebulizer, the detection limits for the investigated selenium compounds were found to be ca. 2 ng Se/ml (3σ, according to IUPAC). In order to obtain further improvement in detection limits, a USN was coupled between the HPLC and ICP-MS instruments. It is well known that, in general, using a USN the signal intensity can be enhanced up to a factor of 10 due to the better aerosol efficiency. For a USN, the formed aerosol is dried via a heating/condensation process to reduce the water introduced into the plasma. This drying process is dependent on the temperatures and on the dwell time of the aerosol in the desolvatization area, which can be controlled by the nebulizer gas flow-rate.

The chromatograms obtained with on-line ICP-MS detection by using a Barbington-type nebulizer and a USN under the optimized conditions are shown in Fig. 2. The peak areas (10 ng Se/ml for each selenium compound) of the six organic selenium compounds were almost identical when a Barbington-type nebulizer was used [considering the diffu-

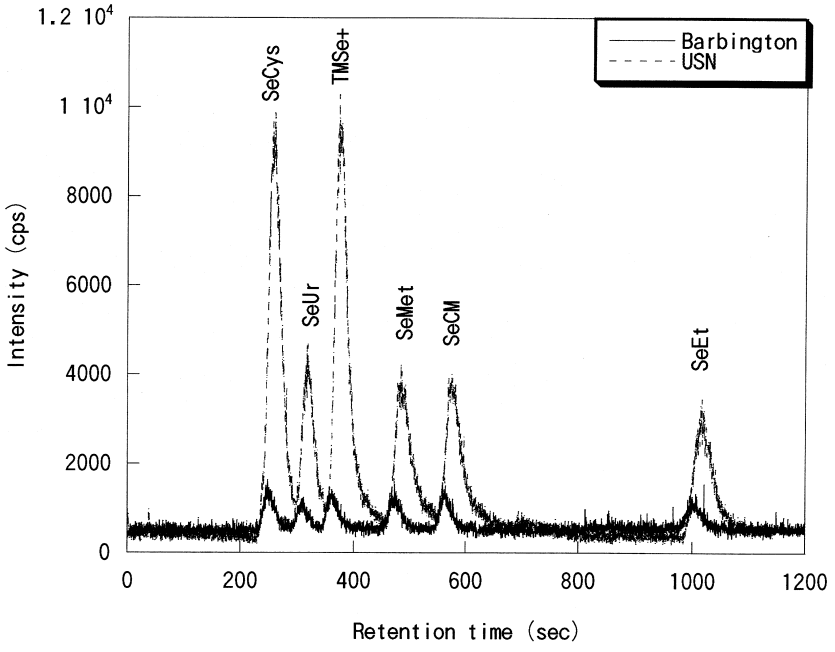

Fig. 2. HPLC–ICP-MS chromatograms obtained from a mixed selenium standard solution (10 μg/l each) using a Barbington-type nebulizer and a USN. Experimental conditions see Table 1.

sion of separated Se(IV) and Se(VI) when a USN was used, these two Se compounds were not included in this investigation]. However, the signal intensity enhancement of the ICP-MS with a USN was found to be species dependent. Compared with the signals obtained with a Barbington-type nebulizer, the signals obtained with a USN increased about 11.2-times for SeCys, 6.60-times for SeUr, 13.5-times for $TMSe^+$, 5.13-times for SeMet, 5.16-times for SeCM and 5.84-times for SeEt, respectively. Similar species discrimination phenomena were also reported by Li et al. [24]. They compared a USN and a Meinhard nebulizer with Se(IV), Se(VI), $TMSe^+$, and SeMet as target compounds, and found that the signals increased about 7-times for Se(IV) and 24–31-times for $TMSe^+$, SeMet and Se(VI) when a USN was used. The observed species discrimination may be attributed to the different properties, such as chemical reactivity and vaporization temperature of the different selenium compounds during the desolvation process.

Using a USN can improve the detection power of ICP-MS, however, this approach was found to be useless in our analytical system due to the problems of rapid decrease of sensitivity resulting from the used mobile phase, which contained 2.5 m*M* sodium. Due to the high introduction efficiency of the USN and the used mobile phase containing sodium, the clogging of sample cone of the ICP-MS interface may occur during the analysis. In fact, the decrease of sensitivity was observed after 1 h of operation when a USN was used. However, with a Barbington-type nebulizer, the used mobile phase was found to be suitable for ICP-MS detection. No significant sensitivity decrease was observed even with a long operation time (>6 h).

3.4. Determination of selenium compounds in selenium nutritional supplement

A selenium nutritional supplement (Selen ACE-Kapseln, Germany) was studied for selenium speciation using the developed ion-pair reversed-phase chromatography with ICP-MS. According to the supplier's specifications, selenium-enriched yeast is the source of selenium present in this supplement. The total selenium concentration in this supplement has been determined after microwave digestion of the supplement with HNO_3–H_2O_2, using both ICP-MS and GF-AAS [14], and the results obtained (426 ± 4 μg/g) were in good agreement with the formulation value (400 μg/g). Since the extraction efficiency with water was found to be ca. 15% in the previous work [14], an enzymatic extraction method was employed in this work. The total selenium concentrations in the extracts and in the residues were determined with ICP-MS. The results indicated that the extraction yield (%) obtained in this work was 74.1 ± 3.2 ($n=3$). This extraction yield is lower than that obtained in selenium enriched-yeast (~90%) reported by Gilon et al. [25]. The incomplete extraction may be attributed to the interference resulting from additives in the supplement. The original extracts were diluted (20-fold) with the used mobile phase, and injected into the chromatographic system. Fig. 3 shows the HPLC–ICP-MS chromatograms resulting from the extract and the extract spiked with SeCys, SeUr and SeEt (50 ng/ml each) standards. In the supplement extract, SeCys and SeMet were detected, while two unknown peaks (U1 and U2) were also observed. Since three isotopes, ^{77}Se, ^{78}Se and ^{82}Se were monitored simultaneously in this study, the obtained unknown peaks were checked for their isotope signature, and the results showed that these unknown peaks were not due to polyatomic interferences. The unknown peak U2 was suspected to be SeUr according to its retention time, however, the spiking result (dotted line in Fig. 3) excluded this possibility. SeEt was not detected in the original extract, thus, the spiked SeEt standard was used as an internal standard in this work. Table 3 summarizes the speciation results obtained with ion-pair reversed-phase HPLC–ICP-MS. It was found that SeMet was the major constituent in the supplement extract (73.6%), and it made up ca. 55% of the total Se in the supplement. This result is in good agreement with the previous work performed with cation-exchange HPLC–ICP-MS [14], which revealed that ca. 69% of selenium in the enzymatic extract was SeMet. SeMet has also been reported in the literature as the major selenium compound in selenium-enriched yeast [8,16].

In conclusion, we have successfully developed an ion-pair reversed-phase chromatographic separation system for the separation of eight environmentally and biologically important Se compounds using

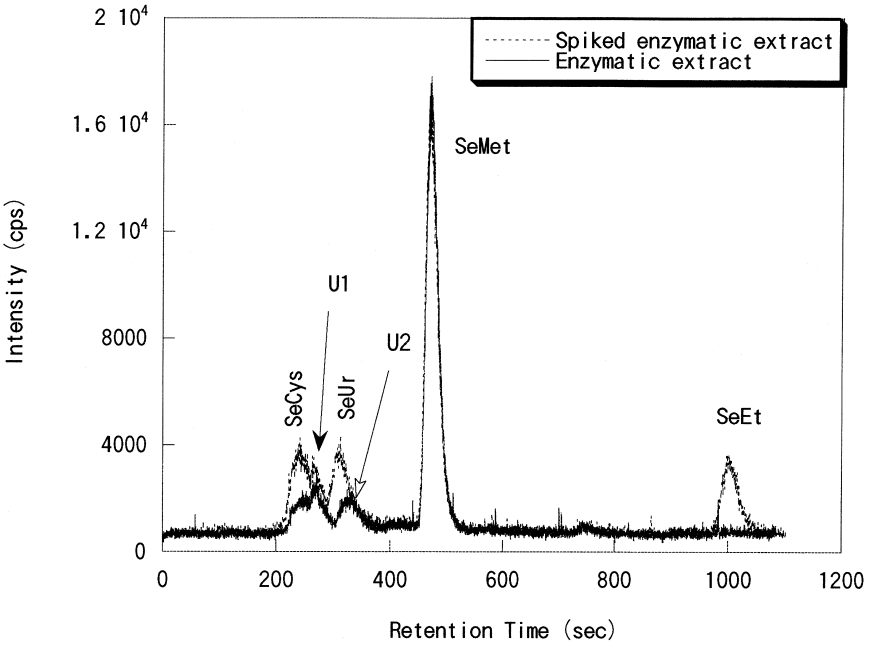

Fig. 3. HPLC–ICP-MS chromatograms obtained from the enzymatic extracts of a selenium supplement. The dotted line shows the chromatogram of enzymatic extract spiked with selenium standard compounds (50 μg/l each). Chromatographic conditions see Fig. 1.

mixed ion-pair reagents, and combined this separation system with ICP-MS. The use of cationic and anionic ion-pair reagents simultaneously in the mobile phase was found to be a very useful approach for the separation of selenium compounds. Different signal enhancement factors were observed for the various selenium compounds when a USN was used. The analysis of a selenium nutritional supplement demonstrated a successful application of the developed mixed ion-pair reversed-phase HPLC–ICP-MS method to the speciation of selenium in complicated real-world matrix. The application feasibility to other environmental and biological samples, such as human urine and serum, will be investigated in a future work.

Table 3
Selenium speciation results in a selenium supplement, concentrations are obtained as the mean of three determinations

	SeCys	SeMet	Unknowns	Sum	Total extracted
μg/g	23.8±0.6	226±11	38.2±3.2	288±5	307±11
(%)[a]	7.8	73.6	12.4	93.8	74.1±3.2[b]

[a] Expressed as the percentage of total Se found in the supplement extract.

[b] Calculated according to the total Se concentration in supplement (400 μg/g).

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