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High-performance liquid chromatography of selenoamino acids and organo selenium compounds
Speciation by inductively coupled plasma mass spectrometry

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Abstract

As part of an ongoing study to identify selenium compounds with cancer chemopreventive activity, extracts of selenium-enriched samples were analyzed by HPLC–inductively coupled plasma (ICP)-MS. Ion-exchange, ion pair and derivatization methods for reversed-phase HPLC were considered and advantages and disadvantages for each compared. Anion exchange allows separation of selenite and selenate, but otherwise provides poor separation. Pre-column derivatization and reversed-phase chromatography provides separation of compounds with terminal amine functionalities, but many other species elute in the void volume. The ion pair method gave optimal separation and was compatible with standard ICP-MS operating conditions. © 1997 Elsevier Science B.V.

Keywords: Selenoamino acids; Selenium; Organoselenium compounds

1. Introduction

Trace element analytical techniques have been widely and effectively applied to gain biologically and clinically significant data for total element determinations in biomedical studies. Increasingly significant, however, is the understanding that the nutritional, toxicological and biochemical impact of any element on a biological system depends on the different elemental forms present, their relative quantities and chemical interactions. Speciation studies are central to broad elemental understanding; qualitative separation schemes need to be coordinated with selective detection and correlated total trace element measurements.

Selenium has attracted extensive analytical study as an essential element which is nevertheless toxic in excess of a rather narrow range of adequacy [1]. The nutritional bioavailability [2], toxicity [3,4] and cancer chemopreventive properties [2,5] of the element are chemical species dependent. Selenium-enriched yeast is often used as a selenium source in nutritional supplements and it has been reported recently that their consumption is associated with statistically significant reductions in cancer mortality and total cancer incidence [5]. Selenium deficiency will cause skeletal and cardiac muscle dysfunction [6,7]. It is known to protect cell membranes from oxidative damage [8,9], but selenite and selenate

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have been suspected to have mutagenic effects [10,11].

Chromatographic separation and speciation of organic and inorganic selenium compounds has followed parallel paths for volatile and non-volatile species. GC–electron-capture detection (ECD) is applicable to trace element determination as piazaselenole derivatives [12]. Volatile compounds such as dimethylselenide, dimethyldiselenide and methaneselenol have been determined by GC [13]. A particularly powerful analytical approach to the determination of low levels of volatile selenium and analogous sulfur compounds has utilized headspace-GC with capillary separation and element selective detection by atomic plasma emission spectroscopy (AED) [14]. Even in such selenium-rich plants as garlic, there is a preponderance of analogous sulfur species present; element selective detection is valuable in differentiating species by element content. Thus 'selenium breath' following ingestion of garlic has been successfully monitored [15]. GC of selenoamino acids has been accomplished by derivatization to volatile species using ethylchloroformate followed by element selective GC–AED [16].

Liquid chromatography is preferred for separation of non-volatile species, ion-exchange, ion pair and reversed-phase partition with derivatization having been utilized for separation of inorganic selenium species and selenoamino acids [17–26]. Selenite and selenate are typically separated on a strong anion-exchange column [17,18]. Trimethylselenonium cation ($TMSe^{+}$) has been included in such separations, eluting in the dead volume, low ng detection being reported by hydride generation or electrothermal atomic absorption spectrometric detection of Se [18,19]. Potin-Gautier et al. [20] separated selenocystine and selenomethionine by ion pair chromatography using electrothermal atomic absorption spectrometric detection, but selenite and selenate were poorly resolved and interfered with amino acid determination.

In a number of selenium speciation investigations, an inductively coupled plasma mass spectrometry (ICP-MS) system has been directly coupled to a HPLC system. There are various capabilities of plasma emission and mass spectral detection which make them valuable tools for elemental speciation in a wide variety of samples, notably those with complex matrices. Interferences from unresolved peaks, which may be present at much greater levels than the targeted analyte, sometimes make it impossible to quantify or even to identify the eluate if a universal detector is used. Element-selective atomic plasma detection can reduce or even eliminate such interferences. Quijano et al. [27] have developed an HPLC–ICP-MS method for the separation determination of selenocystine, selenomethionine, selenite and selenate in aqueous samples, using a mixed-phase ODS-amino column and a pH 6.0 phosphate buffer. This column enabled the amino acids to be eluted outside the dead volume, in contrast to the behavior sometimes seen for strong anion-exchange columns [19]; the noted elution order did suggest an anion-exchange mechanism. Crews et al. [28] investigated selenium speciation in in vitro gastrointestinal extracts of cooked cod by anion-exchange chromatography–ICP-MS using a salicylate buffer at pH 8.5, selenocystine, selenomethionine, selenite and selenate, again being determined. Olivas et al. [29] used a polymer-based reversed-phase column with a pH 4.5 methanol–water mobile phase containing 10^{-4} *M* sodium pentylsulfonate to separate selenocystine, selenomethionine and trimethylselenonium ion. HPLC–ICP-MS detection limits obtained were less than 1 μg/l for each species, but selenocystine could not be resolved from inorganic selenium under their conditions.

Clearly progress has been made in speciating selenium by HPLC, however, the importance of definitively identifying and quantifying all selenium compounds in samples necessitates further research. In this paper, as part of an ongoing study of the cancer chemopreventive activity of selenium, extracts of selenium-enriched samples are analyzed by HPLC–ICP-MS. The known presence of ionic species in the extracts, such as selenoamino acids, requires derivatization prior to reversed-phase HPLC, or limits the chromatographic options to ion-exchange or ion pair chromatography. Results from all three chromatographic approaches are reported.

2. Experimental

2.1. Reagents and samples

Barnstead E-pure 18 MΩ water (Boston, MA, USA), nitric acid and hydrochloric acid (Certified

ACS Plus, Fisher, Fair Lawn, NJ, USA), nitric acid purified by sub-boiling, trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, USA), 2,4-dinitrofluorobenzene, sodium bicarbonate, ethanol and ACS-grade ammonium citrate, (Sigma, St. Louis, MO, USA) and methanol (HPLC grade, Fisher) were used for sample or mobile-phase preparation. Sodium selenate, DL-selenomethionine, DL-selenoethionine and DL-selenocystine, were obtained from Sigma. Se-Methyl-DL-selenocysteine, Se-*n*-propyl-DL-selenocysteine, and Se-allyl-DL-selenocysteine were obtained from Dr. Howard Ganther (University of Wisconsin, Madison, WI, USA). *cis/trans*-Se-1-Propenyl-DL-selenocysteine was synthesized in-house. Plasma selenium standard solution (1000 ppm) was obtained from Spex (Spex Industries, Edison, NJ, USA). Selenium-enriched yeast (1922 ppm) was obtained from Nutrition 21 (San Diego, CA, USA). Freeze-dried selenium-enriched garlic samples (1355 and 235 ppm) were obtained from Dr. Clement Ip (Roswell Park Cancer Institute, Buffalo, NY, USA).

Stock solutions of selenoamino acids were prepared in 0.2 *M* HCl. A stock solution of selenate was prepared in 2% (v/v) HNO_3, while the plasma selenium standard was used as a stock solution of selenite. Working solutions were diluted with mobile phase and stored in the dark between 0 and 4°C.

2.2. Sample preparation

2.2.1. ion-exchange HPLC

Finely ground 1355-ppm Se-garlic (0.12 g) was accurately weighed into 15-ml centrifuge tubes. Four ml of 10% methanol in 0.2 *M* HCl were added to the tube as extraction solvent. The mixture was sonicated for 1 h with an E/MC Model 450 ultrasonic cleaner and left in a refrigerator overnight. The mixture was then centrifuged for 30 min by a Beckman GPR centrifuge (Fullerton, CA, USA) with a relative centrifugal force of 3000 *g*. The solution pH was then adjusted to approximately 7.0 by the addition of ammonia. After a second centrifugation, the extract was filtered through a 0.2-μm IC filter (Gelman Science), and then diluted 2.5-fold.

2.2.2. Reversed-phase HPLC

The 1355-ppm Se-garlic sample extract was prepared as for the ion-exchange experiment through the first centrifugation step. Then 10 μl of the extract were subjected to the following derivatization procedure for the amino acid standards. Selenoamino acid standards were converted to N-2,4-dinitrophenyl (DNP) derivatives with 2,4-dinitrofluorobenzene (FDNB) using a method modified from that developed by Goeger and Ganther [30] for the determination of methionine by HPLC with UV detection. A 10-μl aliquot of amino acid standard was spiked into a 1-ml amber glass vial containing 178 μl of deionized distilled water, 375 μl of 100% ethanol, 375 ml of 1% sodium bicarbonate and 160 μl of FDNB (80 m*M*) in ethanol. After 90 min reaction time, the derivatives were acidified with HCl and then chromatographed.

2.2.3. Ion pair HPLC

The 1355-ppm Se-garlic sample extract was prepared as for the ion-exchange experiment. An alternative extraction method was used for the 235-ppm Se-garlic and 1922-ppm Se-yeast. Yeast (0.3 g) and garlic (0.2 g) were added to 6 and 4 ml of water, respectively, in 15-ml polypropylene centrifuge tubes. The tubes were then heated and shaken in a hot water bath at 85–90°C for 1 h. The solutions were then centrifuged for 30 min using a Beckman GPR centrifuge at 3000 *g*. Supernatant was removed and filtered through a 0.45-μm polypropylene filter (Arbor Technologies, Ann Arbor, MI, USA). Three ml of each of the filtered solutions were passed through 10 000 molecular mass cutoff filters (Micron Separations, Westboro, MA, USA) by centrifuging overnight. Solutions of the 235-ppm Se-garlic and 1922-ppm Se-yeast (0.45 ml) were then spiked with 50 μl of a 10-ppm selenoethionine solution before being chromatographed.

2.3. Instrumentation

2.3.1. Ion-exchange HPLC

The separation system consisted of a Waters (Milford, MA) M-6000 high pressure pump and a Hamilton PRP-X-100 polyether ether ketone (PEEK) anion-exchange column (15 cm×4.6 mm). A Perkin-Elmer Elan 5000 (Perkin-Elmer Sciex, Norwalk, CT, USA) inductively coupled plasma mass spectrometer was used as the detector with the following parameter settings: forward power, 1000 W; plasma Ar flow, 15 l/min; auxiliary Ar flow, 0.8 l/min; nebulizer

flow, 0.75–0.95 l/min; nickel sampler and skimmer cones; normal resolution; peak hop mode; dwell time, 100 ms; and ^{82}Se isotope monitored with krypton correction. A piece of 30 cm×0.58 mm I.D. tubing connected the LC eluent to the cross-flow nebulizer inlet of the ICP-MS. The optimized mobile-phase composition was 5 m*M* ammonium citrate buffer at pH 4.8 with 2% methanol as the organic modifier. The mobile-phase flow-rate was 1.0 ml/min and the sample injection volume was 30 μl.

2.3.2. Reversed-phase HPLC

The separation system consisted of a Perkin-Elmer series 200 LC pump and a Perkin-Elmer Brownlee C_{18} PTC column (22 cm×2.1 mm). A Perkin-Elmer Elan 6000 inductively coupled plasma mass spectrometer was used as the detector with the following parameter settings: forward power, 1000 W; plasma Ar flow, 15 l/min; auxiliary Ar flow, 1.2 l/min; nebulizer flow, 0.84 l/min; nickel sampler and skimmer cones; pulse detection mode; integration time 1 s and ^{82}Se isotope monitored with krypton correction. High purity oxygen (20 ml/min) was introduced to the plasma via a glass T-connector inserted between the Scott double pass spray chamber outlet and the torch adapter. A piece of 22 cm×0.005 in. I.D. PEEK tubing connected the LC eluent to the cross-flow nebulizer inlet of the ICP-MS (1 in.=2.54 cm). The optimized mobile-phase composition, based on a method developed by Goeger and Ganther [30], was a mixture of methanol–water (62:38) with 0.024% TFA. The mobile-phase flow-rate was 0.2 ml/min and the injection volume was 10 μl.

2.3.3. Ion pair HPLC

The separation system consisted of an HP1090 liquid chromatograph (Hewlett-Packard, Wilmington, DE, USA) and a Zorbax SB-C_8 column (15 cm×4.6 mm) (MAC-MOD Analytical, Chadds Ford, PA, USA). For the chromatography of the standards and water extracts, the column was preceded by a Zorbax SB-C_8 guard column (12.5×4.0 mm). UV detection was carried out using an HP1090 diode array detector. A Perkin-Elmer Elan 5000 inductively coupled plasma mass spectrometer was used as the detector with the following parameter settings: forward power, 1003 W; plasma Ar flow, 15 l/min; auxiliary Ar flow, 0.8 l/min; nebulizer flow, 0.925 l/min; platinum sampler and nickel skimmer cones; normal resolution, and peak hop mode. The ^{82}Se isotope with krypton correction was monitored using a 500-ms dwell time, or mass 82 was monitored with a dwell time of 1 s in order to eliminate negative signal spikes resulting from the use of the krypton correction algorithm. A piece of 30 cm×0.25 mm I.D. PEEK tubing connected the LC eluent to the cross-flow nebulizer inlet of the ICP-MS. The optimized mobile-phase composition was water–methanol (98:2) with 0.1% TFA. The mobile-phase flow-rate was typically 1.0 ml/min and the sample injection volume was 10 μl.

3. Results and discussion

3.1. Ion-exchange chromatography

Ion-exchange is a common chromatographic mode for the separation of amino acids. Martin et al. successfully applied a classical anion-exchange method for amino acid separation to selenoamino acids [31]. Our initial attempts at high-performance anion-exchange chromatography produced less than optimal results, the similarity in pK_a values of the selenoamino acids making an isocratic separation difficult.

As can be seen in Fig. 1 [32], baseline resolution was not obtained for any of the six selenium standards used, and peak shape degraded excessively with increased retention. The ion-exchange method developed did allow, however, the separation of selenite and selenate which was not accomplished with the RP-HPLC or the ion pair HPLC. Selenocystine (t_r 1.9 min) is virtually unretained according to the column void volume (1.87 ml) calculated from column dimensions. Ionic interaction alone would predict the anionic selenite to elute after the zwitterionic selenoamino acids. A secondary retention mechanism of hydrophobic interaction of the polystyrenedivinylbenzene column packing and the selenomethionine and Se-allyl-DL-selenocysteine is proposed to explain the retention of these amino acids.

Fig. 2 is a typical chromatogram of an extract of 1355-ppm Se-garlic [32]. Se-Methyl-DL-seleno-

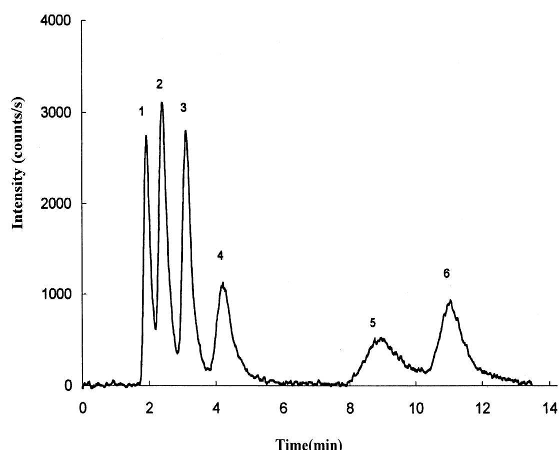

Fig. 1. Anion-exchange chromatogram of selenium standards; 250 ppb Se each. Peaks: 1, selenocystine; 2, methyl-selenocysteine; 3, selenite; 4, selenomethionine; 5, Se-allyl-DL-selenocysteine; 6, selenate.

cysteine, selenomethionine, selenate, selenite and possibly selenocystine were detected in the extract, with Se-methyl-DL-selenocysteine being the predominant form. For these separations, the concentration of ammonium citrate (5 m*M*) was kept as low as possible to avoid matrix effects in the plasma, while not compromising separation. Methanol was added to the mobile phase to increase the sensitivity of the selenium signal, as selenium sensitivity for ICP-MS has been shown to be enhanced by addition of organic modifiers to the sample matrix or addition of methane or nitrogen gas to the plasma [33].

3.2. Reversed-phase chromatography

Precolumn derivatization of amino acids is usually carried out to facilitate ultraviolet or fluorescence detection. Derivatization of the amine functional group with a nonpolar moiety also allows the use of reversed-phase chromatography which is inherently more efficient than ion-exchange chromatography. In this investigation, precolumn derivatization was done not for detection purposes, but solely to exploit the higher efficiency of reversed-phase columns. Comparison of Figs. 1 and 3 illustrates the improved peak shapes of the standards, especially of the later eluting species. In Fig. 3, elution order of the derivatized species is consistent with hydrophobic interaction being the main mechanism of retention. The first peak, eluting in the unretained volume, probably corresponds to inorganic selenium species present in the synthesized Se-*n*-propyl-DL-selenocysteine and Se-methyl-DL-selenocysteine samples, or to non-derivatized selenoamino acids. Column efficiency for the selenomethionine peak was 5400 plates. Fig. 4 shows a chromatogram of selenium species in the extract of 1355-ppm Se-garlic. Peaks 3, 4, and 6 were identified as Se-methyl-DL-selenocysteine, selenomethionine and Se-*n*-propyl-DL-selenocysteine by comparative retention of standards.

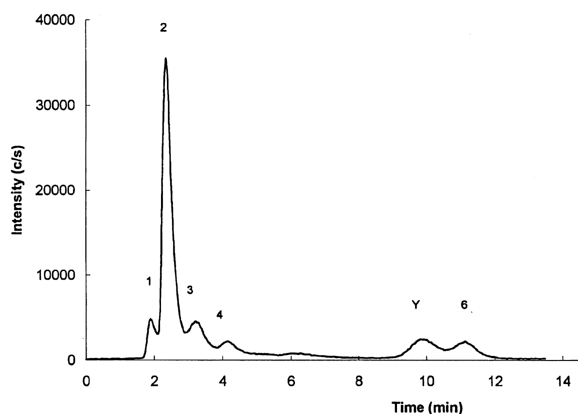

Fig. 2. Anion-exchange chromatogram of 1355-ppm Se-garlic. Peaks: 1, selenocystine; 2, Se-methyl-DL-selenocysteine; 3, selenite; 4, selenomethionine; Y, unknown; 6, selenate.

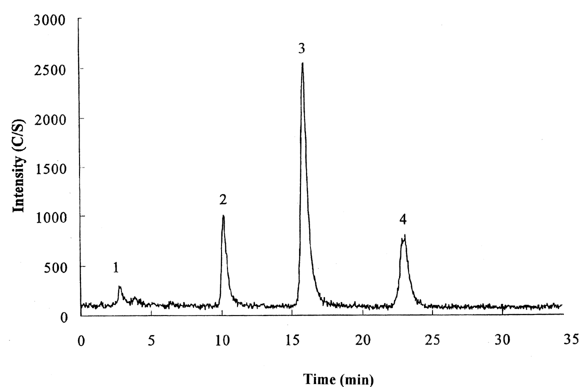

Fig. 3. Reversed-phase chromatogram of DNB derivatized selenium standards; 70–170 ppb Se each. Peaks: 1, unknown; 2, Se-methyl-DL-selenocysteine; 3, selenomethionine; 4, Se-*n*-propyl-DL-selenocysteine.

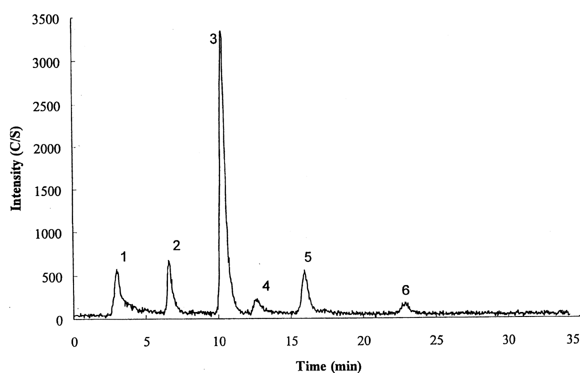

Fig. 4. Reversed-phase chromatogram of 1355-ppm Se-garlic. Peaks: 1, unknown; 2, unknown; 3, Se-methyl-DL-selenocysteine; 4, unknown; 5, selenomethionine; 6, Se-*n*-propyl-DL-selenocysteine.

The chromatographic gains of the derivatized RP-HPLC are offset somewhat by the modifications required to the standard operating conditions of the ICP-MS. A small bore column (2.1 mm I.D.) was selected specifically so that optimal mobile-phase flow-rate was less than 250 μl/min. An inductively coupled plasma is destabilized or extinguished at mobile-phase flow-rates of 1 ml/min with greater than 5–10% organic modifier, depending on the organic modifier used and the power of the plasma. Larger amounts of organics can be used if they are removed by a desolvator before reaching the plasma. At the flow-rates used here, the plasma was stable with 65% methanol. Oxygen gas at 20 ml/min had to be added to the plasma, however, to prevent carbon deposition on and resulting clogging of the sampler and skimmer cones.

3.3. Ion pair chromatography

Ion pair HPLC–ICP-MS offers the advantage of the high column efficiency of reversed-phase chromatography without the need to alter the standard sample introduction and operating conditions of the ICP-MS. TFA, a commonly used ion pairing agent for reversed-phase HPLC of peptides and proteins, was selected as the ion pairing agent for the amino acid separation. The C_8 stationary phase was selected over CN and C_{18} stationary phases. The C_8 and C_{18} provided equivalent separation of the early eluting species, but the C_{18} resulted in excessive retention of the later eluting species. The CN stationary phase resulted in inadequate retention and separation. Methanol was used to reduce retention times of the later eluting species, as well as to increase the sensitivity of the selenium signal.

Fig. 5 is a typical chromatogram of seven selenoamino acid standards. The peaks illustrate good symmetry, with asymmetry factors ranging from 1.03 to 1.09 for peaks 3–8, and 1.15 and 1.20 for peaks 2 and 1, respectively. The peaks are well resolved with the exception of selenoethionine and Se-*n*-propyl-DL-selenocysteine which show some baseline overlap. Column efficiency calculated for the selenomethionine peak was 7200 plates, and for any of the last four eluting peaks was greater than 10 000 plates.

Figs. 6–8 are typical chromatograms of water extracts of selenium-enriched garlic and yeast. Se-Methyl-DL-selenocysteine, selenomethionine, inorganic selenium and possibly selenocystine were identified in the 1355-ppm Se-garlic extract by comparative retention times of standards. In the 235-ppm Se-garlic extract (Fig. 7), inorganic selenium, Se-methyl-DL-selenocysteine, and selenomethionine were identified by comparative retention times of standards. In the yeast extract (Fig. 8), inorganic selenium, selenocystine, Se-methyl-DL-selenocysteine, and selenomethionine were identified by comparative retention of standards. The selenoethionine was used as a chromatographic internal standard in the 235-ppm garlic and 1922-ppm yeast samples to adjust for changes in retention, and will be used in the future for quantification purposes.

None of the selenium species were detectable by UV at the concentrations of standards (300 ppb). Comparison of the UV chromatogram (Fig. 6a) with the MS chromatogram (Fig. 6b) of the garlic extract demonstrates that the selenium species detected by the MS are coeluting with some non-selenium-containing species. The element-selective detection of the ICP-MS limits the separation problem to those species containing selenium. Comparison of the UV and MS chromatograms also demonstrates the loss of column efficiency due to the interfacing to the ICP-MS. Dead volume in the connection of the LC eluent to the nebulizer, and passage of the sample through the nebulizer and spray chamber into the plasma,

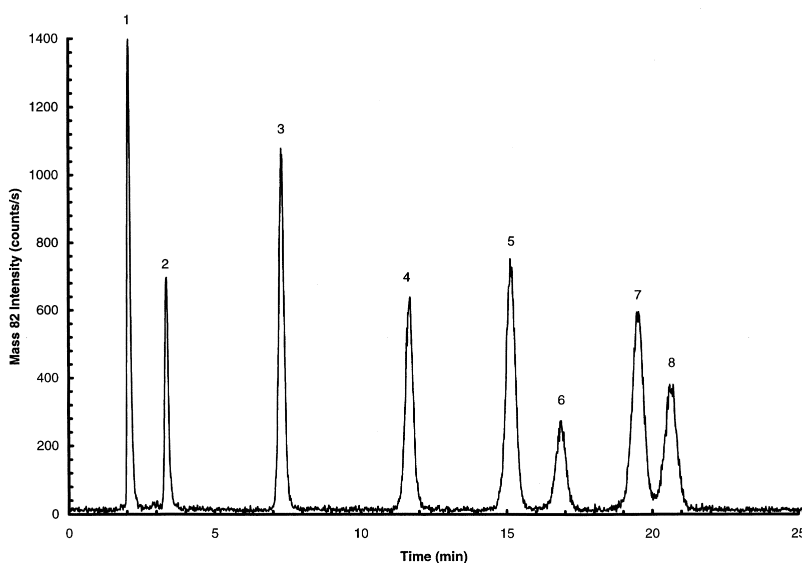

Fig. 5. Ion pair chromatogram of selenoamino acid standards; approximately 300 ppb Se each. Mobile-phase flow-rate, 1.0 ml/min. Peaks: 1, selenocystine; 2, Se-methyl-DL-selenocysteine; 3, selenomethionine; 4, Se-allyl-DL-selenocysteine; 5, *cis*-Se-1-propenyl-DL-selenocysteine; 6, *trans*-Se-1-propenyl-DL-selenocysteine; 7, selenoethionine; 8, Se-propyl-DL-selenocysteine.

result in extra-column band-broadening and a decrease of column efficiency from over 20 000 to 12 000 plates. The theoretical plates were calculated for the non-selenium peak eluting at 8.9 min in the UV chromatogram and the selenium peak eluting at 9.35 min in the MS chromatogram, respectively.

Use of TFA as the ion pair agent gave several advantages over alkane sulfonates when used for the speciation of selenium compounds (29). Firstly, the low pH (pH 2) of the TFA-containing mobile phase results in protonation of the carboxylic acid and amine groups on the amino acids, giving the amino acids a net positive charge. The anionic pairing agent is thus more effective at retaining the positively charged species than the zwitterionic species present at the higher pH values used with the alkane sulfonates. Secondly, the short carbon chain of the TFA compared with the longer carbon chains of the alkane sulfonates, allows for a hydrophobic interaction to be a secondary mode of retention. Pathy reported that the ion pair retention mechanism for TFA is based on non-specific surface adsorption, rather than dynamic ion pair formation [34]. Inchauspe et al. reported the separation of aminoglycoside antibiotics using TFA based on a concerted mechanism involving the stationary phase, the pairing ion and amino and methyl groups [35]. Applying such a proposed mechanism to the retention of selenoamino acids, the resolution of the *cis*/*trans* isomers of Se-1-propenyl-DL-selenocysteine can be explained. The stereochemistry of the *trans* isomer results in interaction of the methyl group with the stationary phase resulting in increased retention over the *cis* isomer.

Thirdly, it has been shown that some of the selenium-containing species in selenium-enriched samples also contain sulfur [15,16]. Detection of these species by ICP-MS by monitoring the ^{34}S isotope and a selenium isotope would be impossible with an alkane sulfonate pairing agent. Detection is theoretically possible with a non-sulfur-containing pairing agent such as TFA, which would allow identification of the sulfur- and selenium-containing species.

3.4. Method comparison for sample extracts

All three chromatographic approaches, ion exchange, reversed phase, and ion pair indicated the presence of selenomethionine, Se-methyl-DL-selenocysteine, and inorganic selenium and the possible presence of selenocystine in the extracts of the 1355-

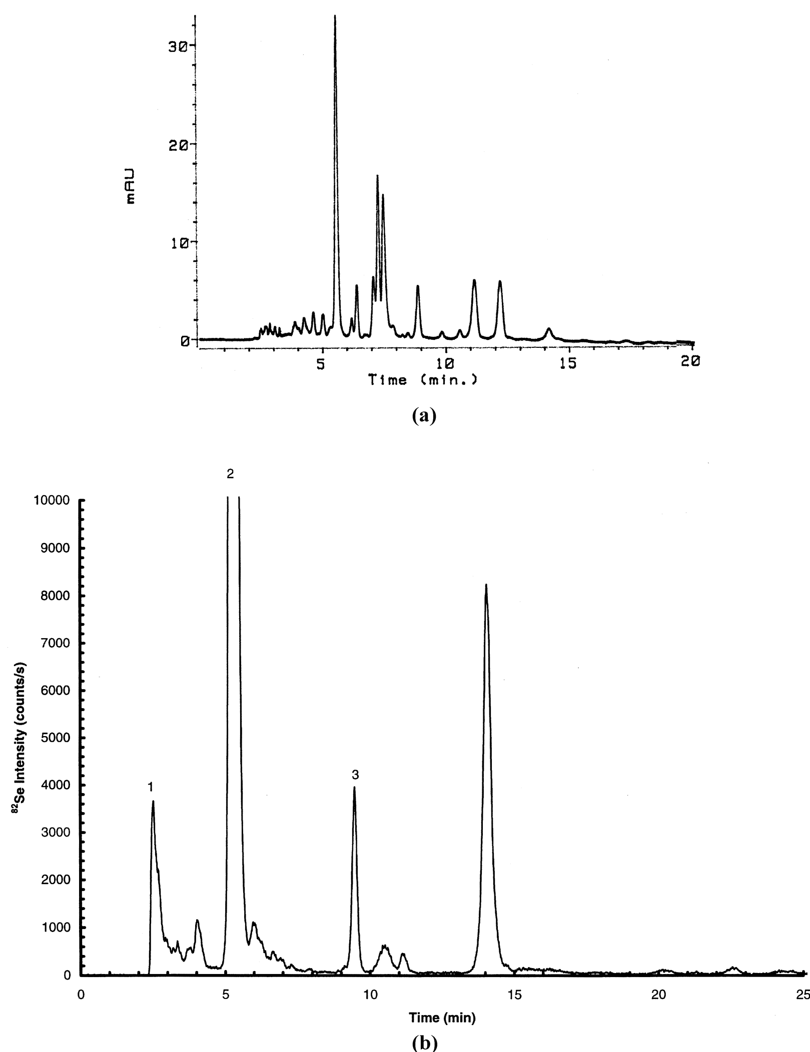

Fig. 6. (a) Ion pair chromatogram of 1355-ppm Se-garlic extract diluted 1:1 with water. Mobile-phase flow-rate, 0.6 ml/min until 6 min, then 1.0 ml/min. UV absorbance monitored at 254 nm. (b) Selenium 82 chromatogram for same sample injection shown in (a). Peaks: 1, inorganic selenium; 2, Se-methyl-DL-selenocysteine; 3, selenomethionine. Maximum intensity of Se-methyl-DL-selenocysteine peak was 110 000 counts/s.

ppm Se-garlic. Likewise, the predominant species in each of the three chromatograms was Se-methyl-DL-selenocysteine. Se-Propyl-DL-selenocysteine was identified by comparative retention time of a standard in the reversed-phase mode and retention times indicate it is possibly present in the ion pair mode, but confirmation is needed.

The three 1355-ppm Se-garlic extract chromatograms also contain unidentified peaks. These unidentified peaks are selenium species, as the only other element with a stable isotope at mass 82 is krypton and a software correction algorithm for the krypton isobaric interference was used. It is also highly unlikely that any of the polyatomic isobaric interferences at mass 82 would be the source of these peaks. Only one unidentified peak was present in the ion-exchange chromatogram, compared with three in the reversed-phase chromatogram and more than 10 in the ion pair chromatogram. The difference in the number of unidentified peaks in the extracts can be

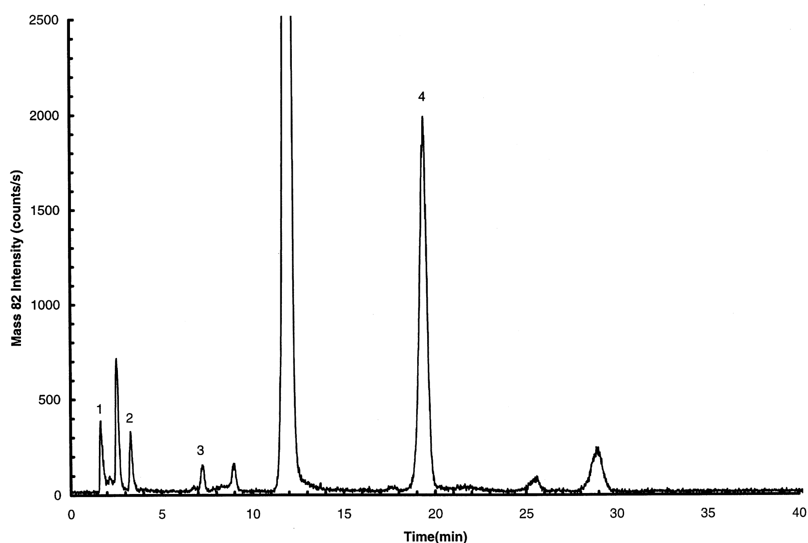

Fig. 7. Ion pair chromatogram of 235-ppm Se-garlic. Mobile-phase flow-rate, 1.0 ml/min. Peaks: 1, inorganic selenium; 2, Se-methyl-DL-selenocysteine; 3, selenomethionine; 4, selenoethionine. Maximum intensity of peak at 12 min was 15 000 counts/s.

explained. Lack of resolution in the ion-exchange chromatography minimized the number of unknown peaks. Except for the unretained peak, the selenium peaks in the reversed-phase chromatogram are limited to selenium species with derivatized amine groups, or strongly hydrophobic species. Underivatized hydrophilic or ionic species would elute in the unretained volume. UV detection coupled with ICP-MS detection could be used to confirm this. The garlic extract was also diluted 100-fold during the derivatization reaction, thus less abundant species in the extract may have been diluted to levels below the

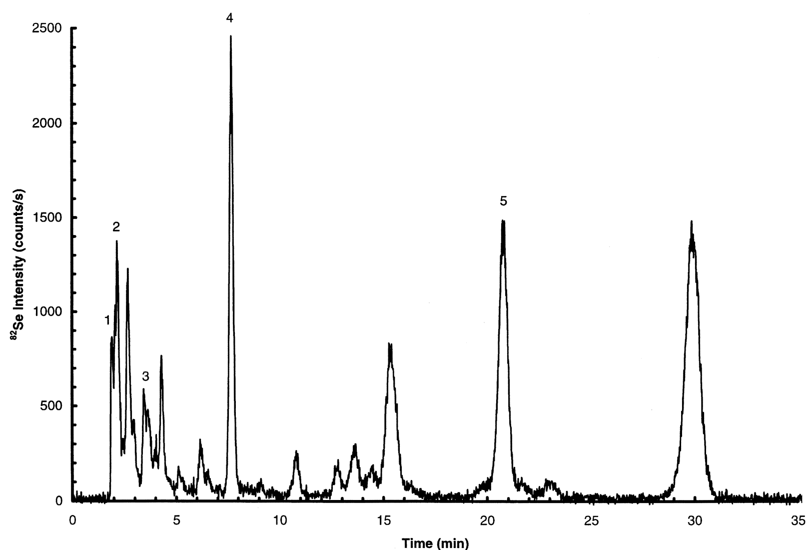

Fig. 8. Ion pair chromatogram of 1922-ppm Se-yeast. Mobile-phase flow-rate, 1.0 ml/min. Peaks: 1, inorganic selenium; 2, selenocystine; 3, Se-methyl-DL-selenocysteine; 4, selenomethionine; 5, selenoethionine.

detection limit. The ion pair chromatography of the garlic extract resulted in the greatest number of selenium species because of the high column efficiency, mixed-mode retention mechanism allowing separation of polar and amino acid species, and minimal dilution of the extract.

The ion pair chromatogram of the 235-ppm Se-garlic indicates a different speciation profile than the 1355-ppm Se-garlic. The major differences in the profiles were found not to be due to differences in the extraction method (hot water versus methanol–HCl). Hot water extractions were substituted for the methanol/HCl extractions to be consistent with ongoing collaborative animal studies. Comparative retention times of standards indicate the presence of inorganic selenium, Se-methyl-DL-selenocysteine, and selenomethionine. The predominant species seen at 12 min (Fig. 7) is unknown. A peak at the same retention volume was present in the 1355-ppm Se-garlic extract (Fig. 6b). Although close to the retention time of Se-allyl-DL-selenocysteine, spiking of Se-allyl-DL-selenocysteine standard into the extract resulted in a doublet peak.

The chromatographic profile of the selenium-enriched yeast differs significantly from the garlic profiles. Recent reports in the literature of HPLC-speciated selenium-enriched yeast found all of the selenium to be in three forms: inorganic selenium, selenomethionine, and selenocystine [29,36]. Those three forms and Se-methyl-DL-selenocysteine are identified as being present, but more than 20 selenium peaks are present in the chromatogram. Selenomethionine is the predominant identified selenoamino acid present, while the most abundant species (23% of total peak area) is an unknown peak at 29.8 min. Selenate and selenite are seen as front shoulders on the selenocystine peak. At pH 2, selenate has a negative charge and elutes first, followed by selenite in the form of selenous acid eluting as a tailing peak.

Each of the three chromatographic modes investigated for the speciation of selenium in selenium-enriched samples have advantages and disadvantages. Anion-exchange chromatography allows for the separation of selenite and selenate, but otherwise provides poor separation. Pre-column derivatization and reversed-phase chromatography improves the separation and allows the identification of selenium compounds with terminal amine functionalities. Many other selenium species are simply eluted in the void volume, however, and special ICP-MS operating conditions are required for the organically rich mobile phase used in reversed-phase chromatography. The ion pair method provided the best separation and was compatible with standard ICP-MS operating conditions. The chromatographic mode which gives the best detection limit for the selenium species has not yet been determined. Detection limits In HPLC–ICP-MS are affected greatly by the chromatographic conditions and are species dependent. Injection volume, column dispersion, and column recovery of a compound all affect the detection limit. Nebulizer efficiency is affected by mobile-phase composition and flow-rate, while ionization efficiency is affected by the mobile phase and may differ for each selenium compound. Detection limit determinations are currently in progress.

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