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Abstract

Increasing speciation demands in clinical chemistry, toxicology and nutrition have made the determination of the total elements in a sample inadequate; the amount of an element and the chemical forms in which it is present need to be known. Inductively coupled plasma mass spectrometry (ICP-MS) was used after high-performance liquid chromatographic (HPLC) separation, as was electrospray ionization mass spectrometry (ESI-MS). The effect of variation of the number of carbon atoms in perfluorinated carboxylic acids used as ion-pairing agents for the separation of selenium compounds was examined. Trifluoroacetic acid (0.1%), pentafluoropropanoic acid (0.1%) or heptafluorobutanoic acid (0.1%; HFBA) were alternatively used as additives to methanol–water (1:99, v/v) solutions as mobile phases. Reversed-phase HPLC–ICP-MS with 0.1% HFBA in the mobile phase allowed more than 20 selenium compounds to be separated in 70 min in an isocratic elution mode; the separation of natural selenium-enriched sample extracts was examined and explained. The pH of the 0.1% HFBA solution was modified with hydrochloric acid or ammonia and the pH of the sample extracts before injection was modified in order to overcome unwanted double peak formation in the chromatograms of sample extracts. Oxidations of standard γ-glutamyl-Se-methylselenocysteine and Se-methylselenocysteine were carried out using 30% H_2O_2 solution and identifications of selenium-containing oxidation products were made using HPLC–ICP-MS and HPLC–ESI-MS. The principal organic oxidation product in both cases was methaneseleninic acid (MeSeO H).

Keywords: Ion-pairing reagents; Food analysis; Organoselenium compounds; Selenium compounds

1. Introduction

The importance of selenium as a trace element in human diet has long been known. The toxicity, nutritional essentiality, and cancer preventive effects of selenium have been the driving forces in the development of analytical methodology for the determination of selenium. A major clinical development was the finding of Clark et al. [1] that human dietary supplementation with selenium-enriched

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yeast decreased cancer incidence and mortality rates by almost 50%. Increase in selenium intake by consuming a diet with natural selenium levels is problematic due to the low abundance of selenium in common food [2]. Hence knowledge about the selenium content of selenium-enriched supplements or proposed supplements is important.

The cancer chemopreventive effect of selenium has been tentatively attributed to the biological functions of selenoamino acids [3,4]. Since this effect strongly depends on the form of selenium, speciation and identification of these different forms are needed to understand the efficacy of selenium supplementation. Our earlier publications [5–8] and a number of excellent review papers [9–16] give a good coverage of the research in this field.

The methodology for the detection and identification of chromatographically resolved selenium compounds has relied extensively on retention time matching of selenium standards with sample chromatograms using elemental selective detection. Inductively coupled plasma (ICP)-MS [7,8,17–21] or sometimes ICP atomic emission spectrometry (AES) [22] are detection methods of choice due to their ability to monitor transient signals and the former's superior sensitivity. Atomic absorption spectrometry (AAS) and electrothermal AAS (ETAAS) have also been used for detection in on- or off-line modes [19,20,22–24]. Casiot et al. [25] identified Se-adenosyl-selenohomocysteine in a yeast extract using off-line electrospray ionization (ESI)-MS detection. We independently identified this same compound together with selenomethionine in yeast by on-line HPLC–ESI-MS [21]. Identification of γ-glutamyl-Se-methylselenocysteine and possibly γ-glutamyl-Se-selenomethionine in garlic were also made in the same publication. Related sulfur compounds have been previously identified in garlic [26–28].

Ion-exchange chromatography, initially the preferred separation method for speciation of ionic selenium species, is still utilized [18,19,22–24], though ion-pair chromatography, often used for the separation of amino acids [29,30], is increasingly popular for the speciation of selenoamino acids due to its superior performance [6–8,25]. The ion-pairing agent most often used in the separation of peptides and proteins is trifluoroacetic acid (TFA) [31], due in part to its volatility and availability in high purity. The ion-pairing role of TFA and other perfluorinated carboxylic acids for the separation of amino acids has been evaluated [32–34].

Other nonvolatile organic selenium species, which may be present in natural samples, are the selenoxides of selenoamino acids. Wrench has reported the presence of selenomethionine selenoxide in marine phytoplankton [35]. Bottino et al. suggested the presence of Se-methylselenocysteine selenoxide in marine algae, based upon similar retention (ion-exchange) and similar mobility (thin-layer chromatography) to those of the sulfur analogs [36]. Sulfoxide analogs are known to be present in *Allium* species, which along with γ-glutamylcysteine peptides account for more then seventy percent of the sulfur in garlic [37]. The sulfoxides are formed in the garlic by oxidation of the *S*-alk(en)ylcysteines and are precursors of the thiosulfinates, disulfides and trisulfides which are responsible for the flavor and odor of garlic [38,39]. The only report of the presence of selenoxides in higher plants was made by Spåre and Virtanen who tentatively identified Se-methylselenocysteine selenoxide and Se-(β-carboxypropyl)-selenocysteine selenoxide in addition to γ-glutamyl-Se-1-propenylselenocysteine in onion [40]. Selenoxides of selenoamino acids have been synthesized and studied in connection with interest in the antioxidant activity of selenium; thus selenomethionine selenoxide was produced by oxidation of selenomethionine [41–43].

As a part of an ongoing study of the cancer chemopreventive activity of selenium, we have developed an ion-pairing reversed-phase separation method. Over 20 selenium-containing compounds were separated by HPLC with a mobile phase containing 0.1% heptafluorobutanoic acid (HFBA) and were detected by ICP-MS. The oxidation products of two important selenium standards (γ-glutamyl-Se-methylselenocysteine and Se-methylselenocysteine) were identified with HPLC–ICP-MS and HPLC–ESI-MS.

2. Experimental

2.1. Instrumentation

An Elan 5000 inductively coupled plasma mass

spectrometer (Perkin-Elmer Sciex, Thornhill, Canada) was used as the HPLC detector. Samples were introduced using a Meinhard nebulizer with a laboratory-fabricated spray chamber containing an impact bead [8]. The spray chamber had a path length of 8.4 cm and a volume of 14 ml. Instrumental conditions were as follows: radio frequency (RF) forward power: 1100 W; plasma flow-rate: 15.0 l/min; auxiliary flow-rate: 0.8 l/min; nebulizer flow-rate: 0.80–0.95 l/min; resolution: normal; scanning mode: peak hop; dwell time: 500 or 1000 ms; isotope monitored: mass 82.

The chromatographic system consisted of a liquid chromatographic pump (SP8810, Spectra-Physics, San Jose, CA, USA) and a 5-μm Symmetry Shield RP8 (15 cm×3.9 mm) column (Waters, Milford, MA, USA), which has a polar modifier group between the C_8 group and the silica base [44,45]. The column was connected to the nebulizer with polyether ether ketone (PEEK) tubing (30 cm×0.25 mm I.D.). The mobile phase compositions were as follows: water–methanol (99:1, v/v) was used in each case. (a) 0.1% TFA at the resulting pH, (b) 0.1% pentafluoropropanoic acid (PFPA) at the resulting pH and (c) 0.1% HFBA at the resulting pH and in addition at a half pH unit higher and lower (modified with HCl or ammonia). As a flow-rate 1 ml/min was used without flow splitting.

A Bruker–Hewlett-Packard Esquire~LC mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) was used for the molecular mass spectral studies. For the analysis of standard materials, the samples were infused to the ESI source at a flow-rate of 1–2 μl/min. For HPLC–ESI-MS analysis, the 1 ml/min column eluent was split 1/5 with a T flow splitter. The T splitter was connected to the ESI source with PEEK tubing (8 cm×0.25 mm I.D.). Mass calibration and optimization of operating parameters were done daily and generally followed the manufacturer's guidelines. Instrumental conditions were as follows: mode: positive ion, standard scan range, normal scan resolution; ESI source: capillary: −3500 V, capillary exit: 65 V, end plate: −3000 V, nebulizer (N_2) pressure: 20 p.s.i., drying gas (N_2) flow-rate: 12 l/min, drying gas temperature: 350°C; lens pass voltages: skimmer 1: 15 V, skimmer 2: 5 V; ion charge control: off; accumulation time: 0.5 ms; cut off: 45 m/z; scan: 50–800 m/z; averages: 15; no rolling average (1 p.s.i.=6894.76 Pa).

Peak integration and other chromatographic calculations were performed using Peakfit software.

2.2. Chemicals

Barnstead E-pure 18 MΩ water (Boston, MA, USA), nitric acid, hydrochloric acid (purified by sub-boiling), 30% hydrogen peroxide and ammonium hydroxide (Certified ACS Plus, Fisher, Fair Lawn, NJ, USA), TFA, PFPA, HFBA (Aldrich, Milwaukee, WI, USA), and methanol (HPLC grade) were used.

The selenium compounds studied are listed in Table 1. Compounds **1**, **2**, **3**, **6**, **7**, **11**, **18** and protease XIV, were obtained from Sigma (St. Louis, MO, USA). Compounds **4**, **9**, **17**, and **14** were obtained from Professor Howard Ganther (University of Wisconsin, Madison, WI, USA), while **5**, **8**, **10**, **13**, **15**, **16**, **19**, **21** and **22** were synthesized in-house. Plasma selenium standard solution (1000 μg/ml) was obtained from Spex (Spex Industries, Edison, NJ, USA).

Selenium-enriched yeast (1922 μg/g Se dry sample) was obtained from Nutrition 21 (San Diego, CA, USA), Professor Helen Crews (Norfolk and Norwich Hospital, Norwich England), and Professor Richard Zitomer (State University of New York, Albany, NY, USA). Selenium-enriched ramp (*Allium tricoccum*; 252 μg/g Se dry sample) was provided by Professor Philip Whanger (Oregon State University, Corvallis, OR, USA). Selenium-enriched garlic (*Allium sativum*; 68 μg/g Se dry sample) was obtained from Professor Donald Lisk (Cornell University, Ithaca, NY, USA). The samples were freeze-dried and stored in a freezer at −20°C. 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. All solutions were stored in the dark at 0–4°C.

2.3. Sample preparation

2.3.1. Extractions

The enzymatic and hot water extractions followed the procedures reported earlier [7], and are only briefly summarized here. For the hot water extraction, 5 ml of distilled deionized water was added to a 0.2 g sample in a 15-ml centrifuge tube and the tube

Table 1
List of selenium compounds in standard solution

1	Selenic acid — selenate — SeO_4^{-2} (Na_2SeO_4)
2	Selenous acid — selenite — SeO_3^{-2} (Na_2SeO_3)
3	Selenocyanate — $SeCN^-$ (KSeCN)
4	Methaneseleninic acid — $CH_3Se(O)OH$
5	Se-lanthionine — $NH_2CH(COOH)CH_2SeCH_2CH(COOH)NH_2$
6	Trimethyl selenonium — $(CH_3)_3Se^+$ (($CH_3)_3SeI$)
7	Selenocystine — $NH_2CH(COOH)CH_2SeSeCH_2CH(COOH)NH_2$
8	Se-cystathionine — $NH_2CH(COOH)CH_2SeCH_2CH_2CH(COOH)NH_2$
9	Se-methylselenocysteine — $CH_3SeCH_2CH(COOH)NH_2$
10	Se-2-propynylselenocysteine — $HC\equiv CCH_2SeCH_2CH(COOH)NH_2$
11	Selenomethionine — $CH_3SeCH_2CH_2CH(COOH)NH_2$
12	Degradation product of Se-2-methyl-2-propenylselenocysteine
13	γ-Glutamyl-Se-methylselenocysteine — $CH_3SeCH_2CH(COOH)NHC(O)CH_2CH_2CH(COOH)NH_2$
14	Se-allylselenocysteine — $CH_2=CHCH_2SeCH_2CH(COOH)NH_2$
15	*Cis*-Se-1-propenylselenocysteine — $CH_3CH=CHSeCH_2CH(COOH)NH_2$
16	*Trans*-Se-1-propenylselenocysteine — $CH_3CH=CHSeCH_2CH(COOH)NH_2$
17	Se-1-propylselenocysteine — $CH_3CH_2CH_2SeCH_2CH(COOH)NH_2$
18	Selenoethionine — $CH_3CH_2SeCH_2CH_2CH(COOH)NH_2$
19	Selenohomocystine — $NH_2CH(COOH)CH_2CH_2SeSeCH_2CH_2CH(COOH)NH_2$
20	Degradation product of Se-1-methyl-2-propenylselenocysteine
21	Se-2-methyl-2-propenylselenocysteine — $CH_2=C(CH_3)CH_2SeCH_2CH(COOH)NH_2$
22	Se-1-methyl-2-propenylselenocysteine — $CH_2=CHCH(CH_3)SeCH_2CH(COOH)NH_2$
23	Se-adenosyl-selenohomocysteine — $NH_2CH(COOH)CH_2CH_2SeCH_2C_4H_5O_3C_5N_4NH_2$

was placed in a double, boiling water bath for an hour. The mixture was shaken every 15 min. For the enzymatic extraction, 5 ml distilled deionized water was added to 0.2 g sample and 0.02 g 'protease XIV' enzyme in a 15-ml centrifuge tube. Then the mixture was shaken for 24 h at room temperature. After the extraction, the samples were centrifuged and filtered. To acidify samples, 0.3 ml of extract was mixed with 0.06 ml of 0.5 *M* HCl.

2.3.2. Oxidation

For oxidation, excess oxidant (0.1 ml of 30% hydrogen peroxide) was added to 1–2 ml of selenoamino acid solutions (50–200 μg/ml Se). After mixing, the solutions were left for an hour and then analyzed by HPLC–ESI-MS. The analysis was repeated after a total of 4, 8, and 24 h. The first hour was considered to be enough for the completion of the oxidation, while the later measurements monitored further changes in the state of oxidation. The HPLC–ICP-MS analysis was done only after 48 h of oxidation following appropriate dilution with water.

2.4. Experiments

2.4.1. Chromatography of selenium standards

The effect of the increase of the number of carbon atoms in the perfluorinated carboxylic acid ion pairing agents was examined for the separation of selenium standards; 0.1% TFA, 0.1% PFPA or 0.1% HFBA were added to methanol–water (1:99, v/v) solutions and used as mobile phase.

2.4.2. Chromatography of natural sample extracts

The extracts of selenium-enriched garlic and yeast were chromatographed with 0.1% HFBA in 1% methanol as mobile phase. The pH of the 0.1% HFBA solution was also modified using either HCl or ammonia and the pH of the sample extracts before injection was also modified.

2.4.3. Oxidation of selenium standards

Standards **9** and **13** were oxidized with 30% H_2O_2 solution. Results were recorded 1, 4, 8 and 24 h after the H_2O_2 was added to the samples using HPLC–ESI-MS, and after 48 h using HPLC–ICP-MS.

3. Results and discussion

3.1. Separation of selenium standards

Fig. 1a and b show the HPLC–ICP-MS chromatograms of the compounds listed in Table 1. Each of the three ion-pairing agents, TFA, PFPA and HFBA, was used in 0.1% concentration and at the resulting pH, while the standard solutions were prepared at acidic pH ($<$2). The compound identification numbers from Table 1 are indicated next to the peaks. Fig. 1a shows the complete chromatograms and Fig. 1b shows the first 15 min of the same chromatograms. The peak intensities were normalized to the highest peak in each chromatogram individually. The concentrations of the standards were in the 0.2–2 μg/ml range, with concentrations increasing as retention time increased.

In our earlier work, TFA was exclusively used as the ion-pairing agent, first at 0.1% concentration with a Zorbax C_8 column [6,7], then with the Symmetry Shield modified C_8 column at 0.1% [21] or at 0.6–0.7% with ammonia addition to correct for the decrease in pH [8]. The improved separation efficiency as a result of increase in TFA concentration still did not give enough power at the beginning of the chromatogram for the separation of the early eluting peaks in sample extracts or for the separation of the oxidation products of selenoamino acids. Thus, investigation of perfluorinated carboxylic acids of greater chain length seemed appropriate, as earlier reports showed their successful application for the separation of amino acids [32].

The operational requirements of the ICP-MS detection system mandate a limit of a few percent of organic content in the mobile phase, which presents a serious limitation in the possible use of ion-pairing agents, narrowing the choices to TFA, PFPA and HFBA. Although retention times with HFBA increased to more than an hour for later eluting standards, it was preferred over the PFBA, because of higher available purity, and substantial retention time drift noted over several hours for the latter acid.

The increase in the separation power at the beginning of the chromatogram as the chain-length of the ion-pairing acid was increased is clearly seen in Fig. 1b. The separation, provided by the HFBA, gave satisfactory results throughout the chromatogram. Compounds **2** (selenite) and **4** (methaneseleninic acid) also could be resolved by the use of Peakfit chromatographic software and in individual standard injections they were easily distinguishable based on retention times. Compound **3** (selenocyanate) was not routinely included in the standard mixture, because of degradation to elemental selenium due to the acidity of the solution. A single standard injection of aqueous potassium selenocyanate gave a retention time close to those of selenite and methaneseleninic acid, indicating that satisfactory separation of these three compounds could not be achieved with the present ion-pairing method.

Comparison of elution order among the three chromatograms in Fig. 1 is of interest. Compounds **19** (selenohomocystine) and **23** (Se-adenosyl-selenohomocysteine) coeluted with compounds **12** (degradation product) and **15** (cis-Se-propenyl-selenocysteine), respectively, when TFA was used. Compounds **19** and **23** eluted after compounds **16** and **22**, respectively, with PFPA ion pairing while with HFBA, compound **19** coeluted with **20** and **23** did not elute within 75 min. Most other compounds retained their elution order sequence when ion-pairing agents were changed, the only exceptions being compounds **13** (γ-glutamyl-Se-methylselenocysteine) and **14** (Se-allylselenocysteine), which switched elution order between TFA and HFBA. The increase in pH from TFA to HFBA could cause such a switch due to the slightly different pK_a values of compounds **13** and **14**. The large retention time increase of compounds **19** and **23**, compared to the increase in retention time of the other compounds, could be attributed to differences in their ion-pair formation capabilities from the other compounds in the standard mixture. An obvious difference is the presence of two -NH_2 groups, which would be responsible for ion-pairing after protonation. The distance between the two -NH_2 groups may be the major factor in determining the strength of the ion pairing; thus the 'double' ion-pairing was more emphatic in compound **23** than in compound **19**. Other compounds in the standard mixture with two -NH_2 groups were **5** (Se-lanthionine), **7** (selenocystine) and **8** (Se-cystathionine), but retention data suggests that these were less capable of strong double ion-pairing.

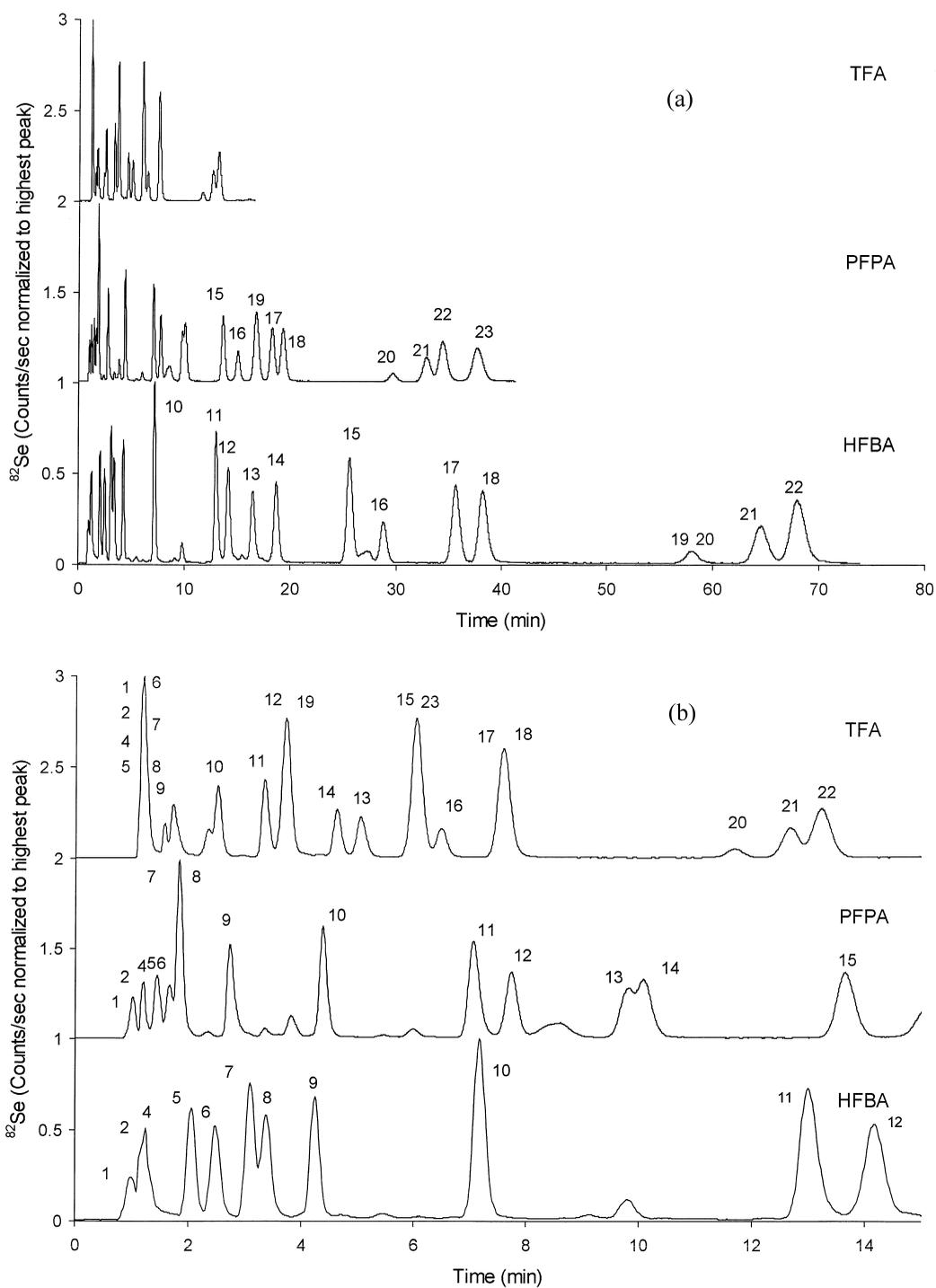Table 2 shows the chromatographic data for

Fig. 1. (a) HPLC–ICP-MS chromatograms of selenium standards using 0.1% TFA, PFPA or HFBA as ion-pairing agents (full time scale). (b) HPLC–ICP-MS chromatograms of selenium standards using 0.1% TFA, PFPA or HFBA as ion-pairing agents (extended time scale, 15 min).

Table 2
Chromatographic data averages and standard deviations from the measurement of the selenium standard mixture using 0.1% HFBA as ion-pairing agent on three different days

| Compound	t_R (min)	RSD (%)	k'	RSD (%)	Asym50	RSD (%)	N	RSD (%)	R_s	RSD (%)
1	0.97	0.2	0.00		1.08	6.2	151	16		
2	1.16	0.7	0.20	2.8	1.04	0.2	722	56	0.6	7.3
4	1.25	0.6	0.29	2.1	1.64	36.8	522	41	0.3	32.4
5	2.05	0.0	1.12	0.5	1.22	25.9	797	10	2.4	11.9
6	2.47	0.4	1.55	0.3	1.14	12.1	899	4	1.1	1.3
7	3.10	0.2	2.21	0.2	1.07	4.3	1254	15	1.6	6.3
8	3.38	0.3	2.50	0.5	1.23	26.2	1779	21	0.7	5.2
9	4.23	0.6	3.37	0.5	1.07	4.4	2826	2	2.2	4.5
10	7.16	0.6	6.41	0.6	1.04	0.4	5205	10	6.9	3.5
11	12.96	0.8	12.41	0.7	1.06	3.3	7487	6	9.9	2.6
12	14.01	1.4	13.49	1.3	1.06	2.5	6576	3	1.4	9.0
13	16.58	0.7	16.16	0.8	1.08	6.0	6674	7	2.9	8.9
14	18.59	1.0	18.24	0.9	1.06	3.5	7868	2	2.1	8.1
15	25.47	1.0	25.35	0.8	1.05	0.8	8994	5	6.1	1.7
16	28.58	1.1	28.57	0.9	1.09	5.1	8965	16	2.3	4.5
17	35.29	1.3	35.51	1.1	1.16	18.1	9360	7	4.2	3.4
18	38.01	0.8	38.32	0.6	1.31	16.9	9522	9	1.5	7.1
20	57.48	1.0	58.47	0.8	1.16	13.5	11 038	19	8.5	4.0
21	64.01	1.0	65.23	0.8	1.04	0.0	9778	9	2.3	4.8
22	67.24	1.3	68.57	1.1	1.18	13.5	10 256	3	1.0	9.4
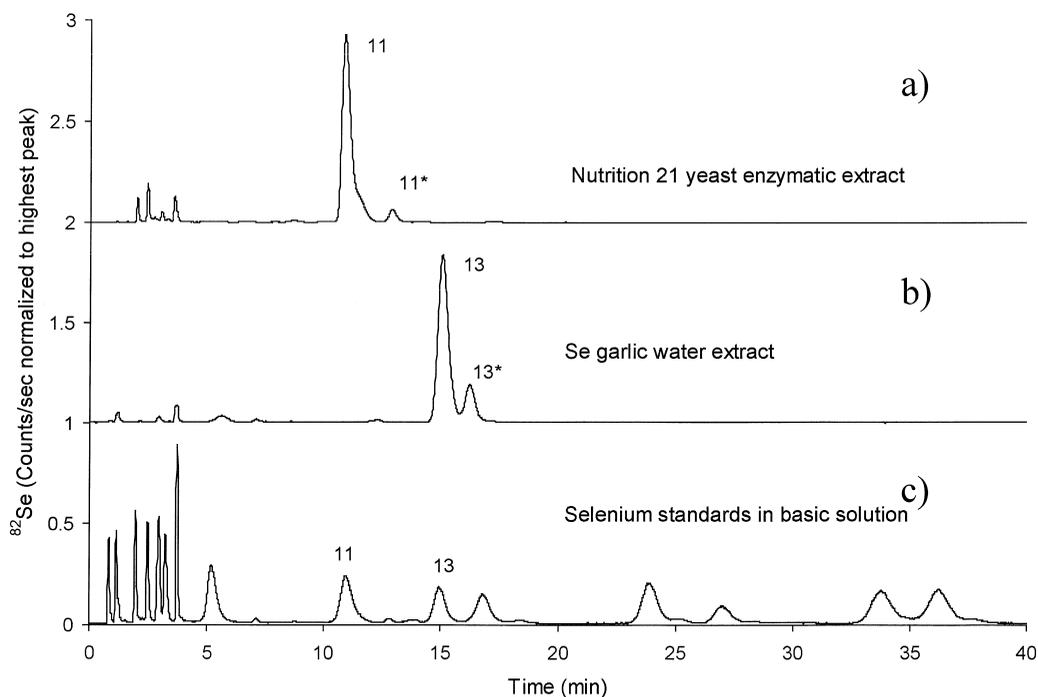
Fig. 2. HPLC–ICP-MS chromatograms of yeast (a) and garlic (b) extracts compared to chromatogram of selenium standards in basic solution (c) using 0.1% HFBA as ion-pairing agent at the naturally occurring pH.

standard chromatograms using 0.1% HFBA. The standard deviations were based on chromatographic data, as calculated by Peakfit software, from three chromatograms recorded on three different days and indicated good day-to-day retention time reproducibility.

3.2. Separation of components in natural sample extracts

Analysis of selenium-enriched natural sample extracts with the mobile phase containing 0.1% HFBA gave unexpected additional peaks as seen in chromatograms (a) and (b) in Fig. 2. Chromatogram (a) shows the enzymatic extract of the Nutrition 21 selenium-enriched yeast, with 11 (selenomethionine) as the principal selenium compound [6]. Chromatogram (b) shows the hot water extract of a selenium-enriched natural garlic sample, with 13 (γ-glutamyl-Se-methylselenocysteine) as the principal selenium compound [21]. In each case there was only a relatively small peak at 13 min (a-11*) and at 16.6 min (b-13*) where the standard compounds elute (Fig. 1, Table 2).

It was initially believed that the separation power of HFBA had revealed the presence of a new selenium compound in yeast, which had been mistakenly identified as selenomethionine using less powerful separation techniques. The 'misplaced' additional peaks (Fig. 2 a-11 and b-13) were transformable to peaks at the expected retention times by dilution or by acidification of the sample before injection. This effect was initially attributed to hydrolysis of the misplaced compounds to give selenomethionine and γ-glutamyl-Se-methylselenocysteine. However when the same effect was observed for compound 9 (Se-methylselenocysteine) in standard solutions when the only difference between two solutions was in pH, it was postulated that different forms of the same compounds rather than new compounds might be responsible for this behavior.

The pH of the 0.1% HFBA solution is 2.55 which is in the pK_a region for carboxyl groups of amino
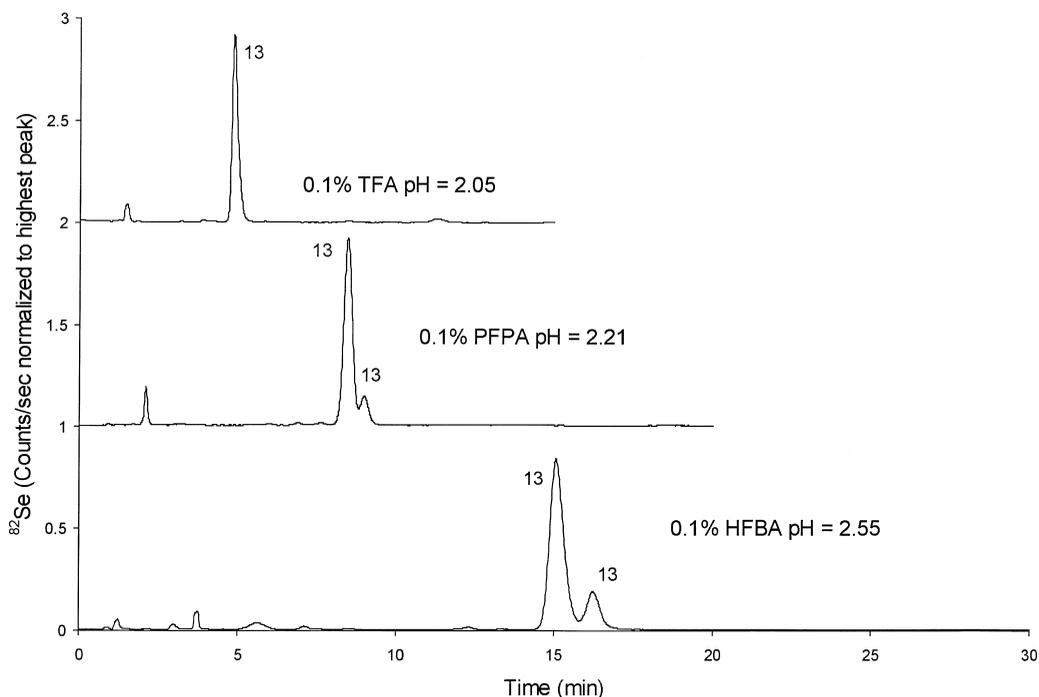

Fig. 3. HPLC–ICP-MS chromatograms of garlic extract using 0.1% TFA, PFPA or HFBA as ion-pairing agents at the naturally occurring pHs.

acids. Thus the conditions in the column with the 0.1% HFBA-containing mobile phase could be considered as giving rise to equilibrium conditions between protonated and unprotonated amino acid carboxylate functions, such that the unprotonated forms predominate. At this pH, depending on the exact value of the pK_a for specific amino acids, similar quantities of carboxyl groups would be protonated or deprotonated, giving rise to badly tailing double peaks (one corresponding to each form) due to the on-column equilibrium process, as is seen in Fig. 2a. Furthermore, a change in pH of the mobile phase or sample could readily modify the equilibrium conditions and consequently the double-peaking effects. Use of a standard C_8 column without the internal modifier group of the Symmetry Shield column did not produce double peaks, indicating a role for the modified stationary phase in the double peak formation.

For the noted 0.1% HFBA conditions, Se-methylselenocysteine (compound **9**) had retention times of 4.2 and 3.7 min, respectively when the solution was acidic (pH<2) or basic (pH=7–10). The additional charge on the molecule present under the basic conditions gives rise to the retention time decrease. The relative difference in retention times between the peaks in the acidic and the basic sample solutions remained the same when the pH of the mobile phase was increased to 3.15, while the absolute retention times decreased by 30–40%. When the pH of the mobile phase was decreased to 2.18, only one peak at 4.2 min was seen. It may be concluded that all amino acid molecules are in the protonated form at pH 2.18.

Fig. 2c shows the chromatogram of the basic (pH=7–10) standard mixture under the naturally occurring pH of the 0.1% HFBA. As is clearly seen, the new retention times of compounds **11** (seleno-methionine) and **13** (γ-glutamyl-Se-methyl-selenocysteine) match the retention times of the large peaks in Fig. 2a and b, in agreement with the argument above.

The change in the pH of the sample solution had the same effect for the natural extract sample as for
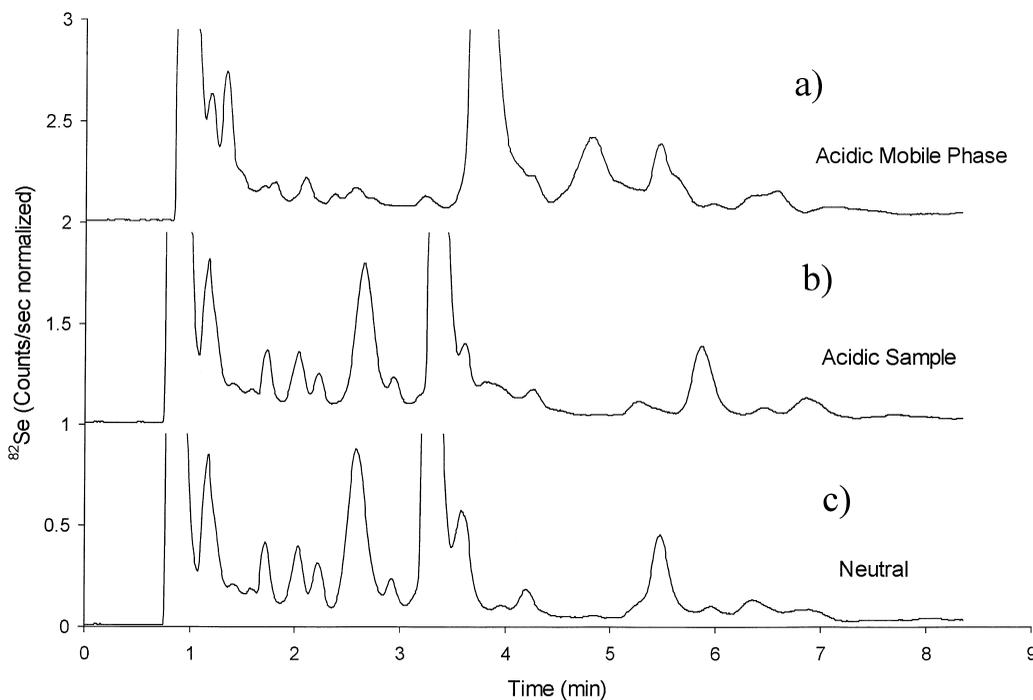

Fig. 4. HPLC–ICP-MS chromatograms of ramp extracts: (a) nonacidified extract injected into acidified 0.1% HFBA containing mobile phase, (b) acidified extract injected into a 0.1% HFBA at naturally occurring pH, (c) nonacidified extract injected into a 0.1% HFBA at naturally occurring pH.

the standard solution. The earlier eluting additional peak (13, Fig. 2b) was transformed into the peak with retention time equal to that of the standard (16.5 min). Fig. 3 shows how the double peak formation disappeared when the pH of the mobile phase was changed, here not by the addition of HCl to the HFBA solution, but by replacement of HFBA with 0.1% PFPA or 0.1% TFA resulting in pH values of 2.21 or 2.05, respectively.

There were three ways to avoid double peak formation. The use of TFA was the least desirable, since the separation efficiency at the beginning of the chromatogram was not satisfactory as demonstrated in Fig. 1b. Fig. 4 compares the chromatograms of enzymatic extract of ramp under three different conditions: (a) nonacidified extract was injected into an acidified 0.1% HFBA containing mobile phase, (b) acidified extract was injected into a 0.1% HFBA containing mobile phase at the naturally occurring pH, (c) nonacidified extract was injected into a 0.1% HFBA containing mobile phase at the naturally occurring pH. As can be seen, acidification of the

sample did not affect the early eluting peaks, but using a mobile phase with lower pH changed the front portion of the chromatogram considerably.

It may be concluded that the best separation was achieved using 0.1% HFBA in 1% methanol solution, and that the extracts of natural samples needed to be acidified in order to avoid the double peak formation due to the equilibrium between protonated and deprotonated forms of the carboxylic group in the selenoamino acid.

3.3. Oxidation

Oxidation studies of selenoamino acids that are principal components in selenium-enriched natural samples are important in order to identify possible metabolic endproducts of these compounds. Also some of the early eluting peaks in the natural extracts could be oxidation products of the principal selenium compounds.

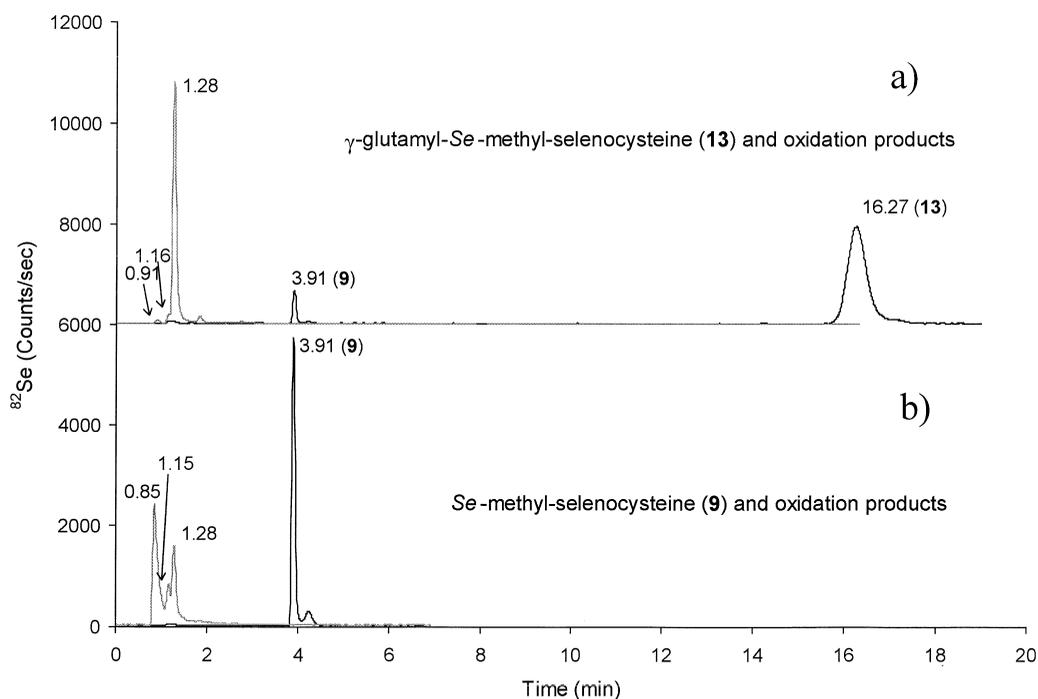In these experiments the results of oxidation of compounds **9** (Se-methylselenocysteine) and **13** (γ-

Fig. 5. HPLC–ICP-MS chromatograms of γ-glutamyl-Se-methylselenocysteine (**13**) (a), Se-methylselenocysteine (**9**) (b) and their oxidation products using 0.1% HFBA as ion-pairing agent.

glutamyl-Se-methylselenocysteine) were interpreted, these compounds being the principal components of selenium enriched garlic with high (1355 μg/g) and moderate (296 μg/g) total selenium content, respectively [7,21].

Fig. 5 shows the HPLC–ICP-MS chromatograms of standards **9** (b) and **13** (a). The retention time of **9** was 3.91 min, while the retention time of **13** was 16.27 min (standard **13** had a slight contamination of **9**). The chromatograms depicted with darker and lighter lines were the original standards and their oxidation products respectively. The retention data of the oxidation products indicated the presence of compounds **1**, **2** and **4** in both cases. Compound **4** (methaneseleninic acid) was the principal oxidation product formed upon oxidizing **13**, while compounds **1** (selenate) and **4** were formed upon oxidizing **9**.

The HPLC–ESI-MS total ion chromatogram (TIC) of the oxidation product of standard **13** (γ-glutamyl-Se-methylselenocysteine) is shown in Fig. 6 indicating six peaks. Only the peak eluting at 1.291 min had a spectrum with the selenium isotope pattern, as predicted by the HPLC–ICP-MS chromatogram (Fig. 5). The mass spectrum in Fig. 6 is the ionic sum of the spectra under the peak at 1.291 min. The $m/z=129$ indicates the M+1 ion, and $m/z=111$ indicates a loss of water. The spectrum of the 1.291 min peak in Fig. 6 was identical to that of the standard **4**. Similar total ion chromatograms and spectra were seen when oxidation products of compound **9** were investigated. These findings support the identification made by HPLC–ICP-MS that compound **4** was the principal organic product obtained upon oxidation of the major components of different garlic samples. Compounds **1** and **2** (selenate and selenite) could not be identified using
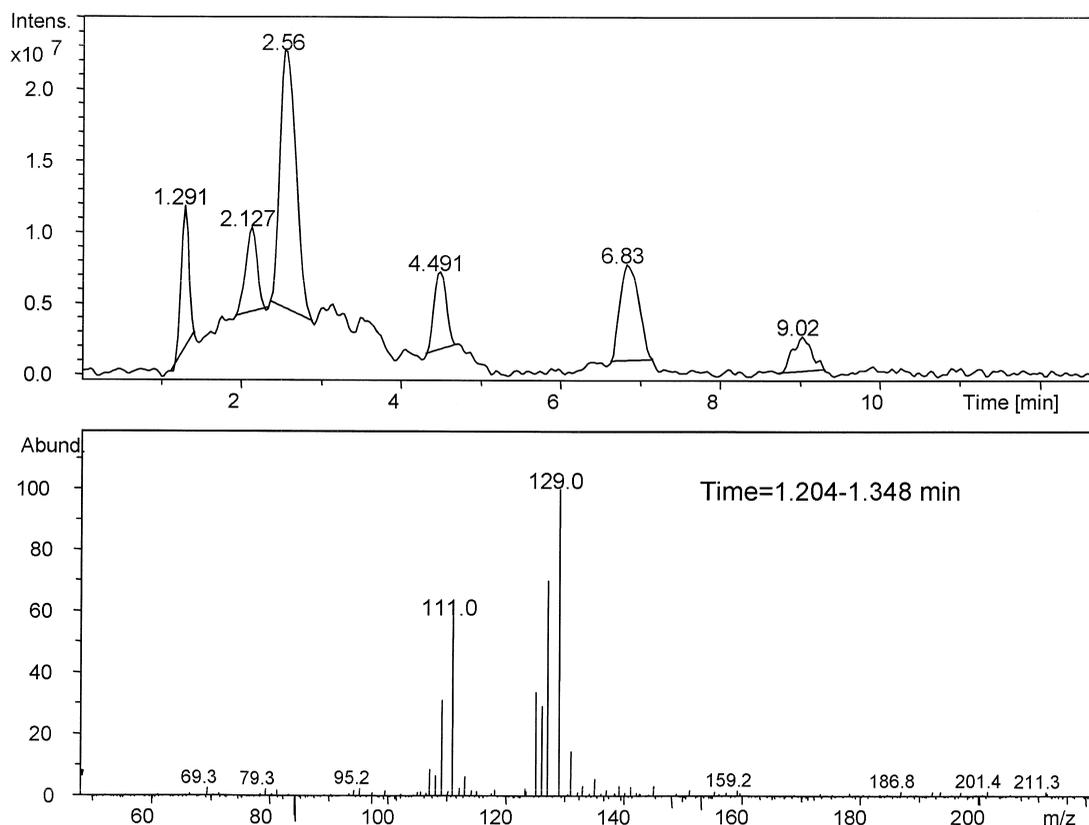

Fig. 6. HPLC–ESI-MS TIC of oxidation product and spectrum of γ-glutamyl-Se-methylselenocysteine (**13**) using 0.1% HFBA as ion-pairing agent.

HPLC–ESI-MS, because they do not give signals under the positive ionization mode in ESI-MS. In this case the lack of such a signal was the supporting factor in identifying them.

4. Conclusion

Three alternative ion-pairing agents were evaluated for the separation of selenium compounds using reversed-phase HPLC with ICP-MS or ESI-MS detection systems, as the result of recognizing the need for improved separation power at the beginning of the chromatogram. Double peak formation, which appeared for HFBA as the ion-pairing agent was explained as the consequence of the relationship between the pH of the sample and the mobile phase and the pK_a value of the carboxyl group in the selenoamino acid. 0.1% HFBA in water–methanol (99:1, v/v) solution at the resulting pH was chosen as the most suitable mobile phase, with the requirement of acidification of the sample before injection.

γ-Glutamyl-Se-methylselenocysteine (**13**) and Se-methylselenocysteine (**9**) were oxidized with 30% hydrogen peroxide. Methaneseleninic acid was identified as the organic oxidation product of **9** and **13**, and subsequently that of different selenium-enriched natural garlic samples.

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