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Selenium-enriched garlic and yeast sample extracts and digests were analyzed using ion-pair high performance liquid chromatography (HPLC) with on-line inductively coupled plasma-mass spectrometric (ICP-MS) and electrospray ionization-mass spectrometric (ESI-MS) detection. The principal selenium compounds in these samples were identified as selenomethionine, and Se-adenosyl-selenohomocysteine in yeast, and γ-glutamyl-Se-methyl-selenocysteine and possibly γ-glutamyl-selenomethionine in garlic. The compounds identified account for 85 and 90% of the total selenium content of the yeast and the garlic samples, respectively. On-line HPLC-ESI-MS selected ion chromatograms (SIC) and mass spectra of selenium compounds extracted from selenium enriched samples are presented. Limits of quantification (LOQ, defined as S/N = 10) for HPLC-ICP-MS were in the range 10–50 ng mL^{-1} Se in the injected extracts. LOQ values for HPLC-ESI-MS were *ca.* 100 times higher than those of HPLC-ICP-MS.

Introduction

The importance of selenium in human nutrition and in cancer chemo-prevention has been demonstrated,[1–3] directing a major interest from the analytical community towards the separation and identification of selenium compounds in various matrices.[4–7] The techniques employed have mainly been capable of identifying the presence of selenium compounds without providing structural information, thus leaving retention time matching of available standards as the major form of identification. Recently heart-cutting and preconcentration were employed to obtain a high enough concentration for off-line electrospray ionization-mass spectrometric (ESI-MS) analysis to identify Se-adenosyl-selenohomocysteine as the major selenium compound in the water extract of a selenium-enriched yeast sample.[8] On-line ESI-MS identification was reported by Momplasir *et al.*[9] Detailed reference lists of different existing methods for selenium compound separation are available from earlier publications.[6,10–12]

The importance of the extraction method employed must also be noted, since the selenium compounds can exist in a free form or are covalently bound in proteins. Thus, by employing simple water extraction, only the free selenium compounds will be detected, thus leading to incomplete differentiation of identified compounds into major and minor categories. As demonstrated in our earlier work, establishing a selenium mass balance is important.[6] To release the covalently bound selenoamino acids, enzymatic or acidic hydrolysis is necessary. This is especially important for yeast in which selenomethionine was found to be the major component after hydrolysis, but only a minor component after simple hot water extraction.[11] The reason for this is that the selenomethionine is incorporated into the proteins in place of methionine, which results in undesirable accumulation of selenium following ingestion of selenium-enriched yeast supplements.[13]

Experimental

Instrumentation

An Elan 5000 inductively coupled plasma mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) was used as the detector for HPLC-ICP-MS. Samples were introduced by a Meinhard nebulizer with an in-house fabricated spray chamber containing an impact bead.[12] The spray chamber had a path length of 8.4 cm and a volume of 14 mL. The ICP-MS parameter settings were as follows: forward power 1003 W; plasma argon flow 15 L min^{-1}; auxiliary argon flow 0.8 L min^{-1}; nebulizer flow 0.860–1.060 L min^{-1} (optimized daily); nickel sampler and skimmer cones; normal resolution; peak hop mode; and dwell time 250 ms. The selenium signal at m/z = 82 was monitored without krypton correction.

The chromatographic system consisted of a liquid chromatographic pump (SP8810, Spectra-Physics, San Jose, CA, USA) and a 5 μm Symmetry Shield RP8 (3.9 mm × 15 cm) column (Waters Corporation, Milford, MA, USA). The column was connected to the nebulizer with PEEK® tubing (30 cm × 0.25 mm id). The mobile phase composition was 99 + 1 (v/v) water–methanol with 0.1% trifluoroacetic acid.[11] The mobile phase flow rate was typically 1.0 mL min^{-1} and the sample injection volume was 20 μL.

A Bruker-Hewlett Packard Esquire-LC Mass Spectrometer (Bruker-Franzen Analytik Gmbh, Bremen, Germany) was used for the molecular mass spectral studies. For HPLC-MS analysis the 1 mL min^{-1} column output was split in a 1 : 5 ratio wtih a T flow splitter. The column was connected to the ESI source with PEEK® tubing (8 cm × 0.25 mm id). Mass calibration and optimizing of the operating parameters were performed daily and generally followed the manufacturer's guidelines.

Chemicals

Barnstead E-pure 18 Mohm water (Boston, MA, USA), hydrochloric acid (purified by sub-boiling), trifluoroacetic acid (Aldrich, Milwaukee, WI, USA) and methanol (HPLC grade) were used.

DL-Selenomethionine, and Protease XIV, were obtained from Sigma (St. Louis, MO, USA). Se-Adenosyl-DL-selenohomocysteine and γ-glutamyl-Se-methyl-L-selenocysteine were synthesized in-house. Selenium-enriched yeast (1922 μg Se per g dry sample) was obtained from Nutrition 21 (San Diego, CA, USA),

Dr Helen Crews (Norfolk and Norwich Hospital, Norwich, UK), and Dr Richard Zitomer (State University of New York, Albany, NY, USA). Selenium-enriched garlic (296 μg Se per g dry sample) was from Dr Clement Ip (Rsowell Park Cancer Institute, Buffalo, NY, USA). Stock solutions of selenoamino acids were prepared in 0.2 M HCl. All solutions were stored in the dark at 0–4 °C.

Sample preparation

The enzymatic digestion and hot water extractions followed the procedures reported earlier,[6] and they are only briefly summarized here. For the hot water extraction, 5 mL distilled deionized water was added to 0.2 g sample in 15 mL centrifuge tube and the tube was placed in a double, boiling water bath for an hour. The mixture was shaken well every 15 min. For the enzymatic digestion, 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, and shaken for 24 h at room temperature. For the preparation of the concentrated extracts, the original sample to water ratio (0.2 g:5 mL) was increased 5-fold. After the extraction, the samples were centrifuged and filtered.

Results and discussion

HPLC-ICP-MS analysis

Figs. 1A and B show the on-line HPLC-ICP-MS chromatograms of selenium-enriched yeast and garlic, respectively. The somewhat poorer separation at the beginning of the chromatogram, as compared to our earlier work, using 0.1% TFA in the mobile phase,[6] is due to the change of the column from a Zorbax SB-C8 (4.6 mm × 15 cm) to a Symmetry Shield RP8 (3.9 mm × 15 cm). The latter has a polar modifier group between the silica base and the C8 group[14] and the advantages of the modified column will be evident in upcoming publications. Since the goal of this work was to identify the later eluting compounds, the low separation efficiency at the beginning of the chromatogram did not represent a problem and was in fact desirable to achieve faster separation.

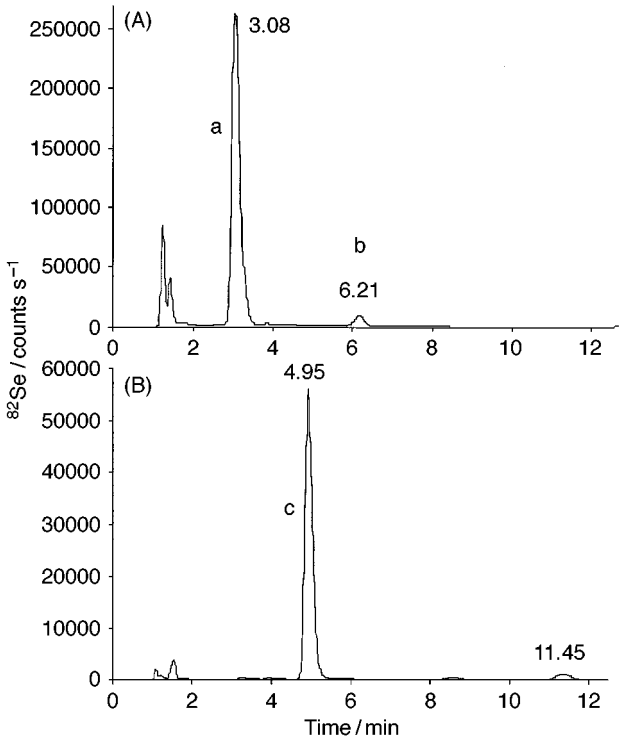

Fig. 1 (A) HPLC-ICP-MS chromatogram of the enzyme digest of selenium-enriched yeast (Nutrition 21) containing 1922 μg g^{-1} total selenium. Identified peaks are: a, selenomethionine and b, Se-adenosyl-selenohomocysteine. (B) HPLC-ICP-MS chromatogram of the water extract of selenium-enriched garlic containing 296 μg g^{-1} total selenium. The identified peak is c, γ-glutamyl-Se-methyl-selenocysteine.

The yeast analyzed was that used in the now definitive anticarcinogen studies,[3] which is also commercially available in the form of Selenomax (Nutrition 21) selenium supplement. The total selenium contents of the samples were 1922 μg g^{-1} in the yeast and 296 μg g^{-1} in the garlic.[6,11] The identities of the peaks eluting at 3.08 and 6.21 min in Fig. 1A and at 4.95 min (Fig. 1B) were first established by spiking the samples with the appropriate standards. The peaks were identified as selenomethionien, Se-adenosyl-selenohomocysteine and γ-glutamyl-Se-methyl-selenocysteine, respectively. The chromatograms shown in Figs. 1A and B were recorded using the 'regular' 0.2 g:5 mL sample extracts. The peak at 11.45 min (Fig. 1B) did not match the retention time of any of our standards.

Comparison of the chromatogram of the yeast's enzymatic digest (Fig. 1A) with its water extract (not shown) showed the absence of the major selenomethionine peak in the water extract, showing that the selenomethionine is covalently bound in proteins. In the water extract Se-adenosyl-selenohomocysteine appeared as the major selenium compound as shown by Casiot *et al.*[8] The then unidentified Se-adenosyl-selenohomocysteine peak was also present in our chromatogram as presented earlier.[11] The extraction efficiency and column recovery values for the enzymatic digests of yeast were in the range 80–90%, while the hot water extraction efficiency was only approximately 10%.[6,11] The two identified compounds account for 82% of the total selenium compounds eluting from the column after the injection of the enzymatic digest.

Comparison of the chromatogram of the garlic's enzymatic digest (not shown) with its water extract (Fig. 1B) shows no major difference in the peak height of γ-glutamyl-Se-methyl-selenocysteine. This indicated that the γ-glutamyl-Se-methyl-selenocysteine is not incorporated into proteins. This also supports the suggestion that potentially dangerous accumulation of selenium in body proteins does not occur upon selenium-enriched garlic ingestion, which finding, with the support of animal studies, could be important for choosing which form of selenium supplement would be best.[13] The efficiency of the hot water extraction was in the range 80–90% with similar column recovery values, with γ-glutamyl-Se-methyl-selenocysteine accounting for 85% of the total selenium compounds eluting from the column.

HPLC-ESI-MS analysis

All HPLC-ESI-MS analyses were carried out on-line instead of by the heart-cutting method employed by Casiot *et al.*,[8] thus avoiding any possible decomposition of the sample during the preconcentration procedure. Fig. 2 shows the overlaid selected ion chromatograms (SIC) of selenomethionine (3.41 min), γ-glutamyl-Se-methyl-selenocysteine (5.07 min) and Se-adenosyl-selenohomocysteine (6.39 min) at selenium concentrations of 50, 20 and 10 μg mL^{-1} The SIC of the three standards were recorded in one injection. The whole mass range (*m/z* 50–500) was recorded and the SIC's were calculated by the computer after the run was completed. Such an approach gives superior S/N ratios compared to total ion chromatograms (TIC). The selenomethionine has molecular ion M + 1 at 198 with a loss of 17, which is attributed to ammonia, producing a fragment ion at mass 181. The M + 1 values for γ-glutamyl-Se-methyl-selenocysteine and Se-adenosyl-selenohomocysteine are 313 and 433, respectively. The selenium isotopic pattern is clearly seen in each case.

Fig. 3 shows the SIC of *m/z* = 313 (5.11 min) and *m/z* = 327 (10.2 min) from 'regular' (0.2 g:5 mL) and 'concentrated' (1 g:5 mL) hot water extracts of garlic, respectively. The two SICs were superimposed in one figure. It is necessary to note here that the use of 'concentrated' sample extracts resulted in a

decrease of the retention times as is shown by comparing those in Fig. 1B (11.45 min) and Fig. 3 (10.12 min) for the same compound. The reason for the shift in retention time was the overloading of the column. The same effect was also observed when the injection volume of the 'regular' extract was increased from 20 to 100 μL. The mass spectrum of the 5.11 min peak supports the identification of this peak as γ-glutamyl-Se-methyl-selenocysteine. The peak at 10.12 min was tentatively identified as γ-glutamyl-Se-selenomethionine based on its molecular weight and the region where it elutes, but this identification needs further proof since a standard to confirm its retention time is not yet available.

Fig. 4 shows the SIC of *m/z* = 198 (3.14 min) and *m/z* = 433 (6.31 min) from the 'regular' enzymatic digest of selenium-enriched yeast. A similar spectrum can be obtained for the peak eluting at 6.3 min from the hot water extract of the sample. The mass spectra of the peaks at 3.14 and 6.31 min support the identification of these peaks as selenomethionine and Se-adenosyl-selenohomocysteine, respectively, confirming the off-line identification of Se-adenosyl-selenohomocystiene in the

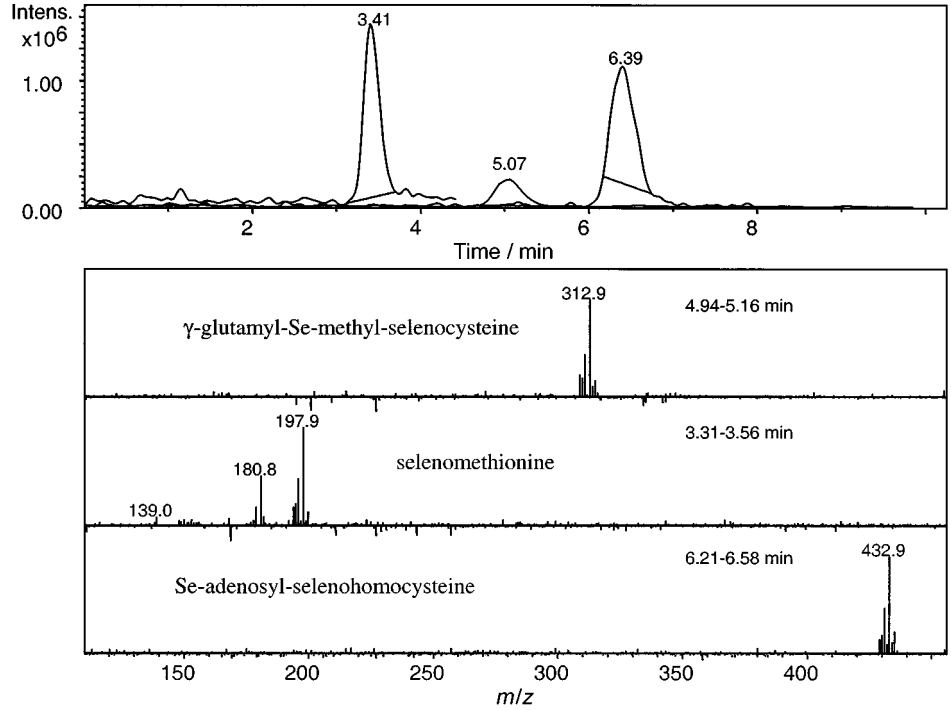

Fig. 2 HPLC-ESI-MS SIC of DL-selenomethionine (3.41 min, *m/z = 198)*, γ-glutamyl-Se-methyl-selenocysteine (5.07 min, *m/z* = 313) and Se-adenosyl-selenocysteine (6.39 min, *m/z* = 433) with concentrations of 50, 20 and 10 μg ml^{-1} selenium, respectively, recorded in 3 separate injections and overlaid in one chromatogram.

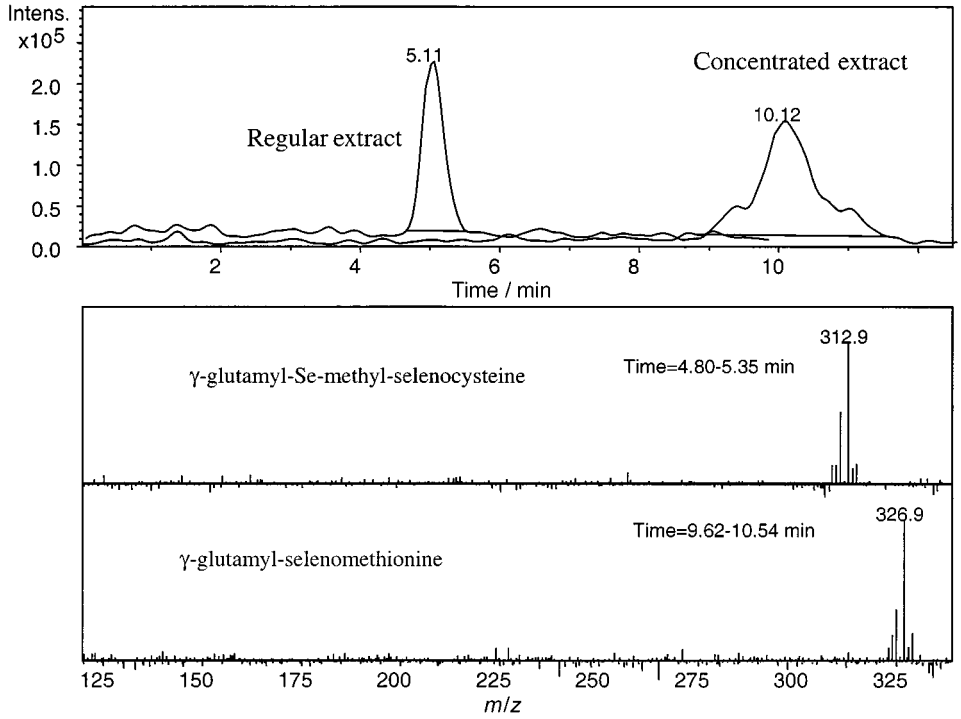

Fig. 3 HPLC-ESI-MS SIC chromatograms of *m/z* = 313 (5.11 min) and 327 (10.12 min) with their spectra from the water extract of selenium-enriched garlic containing 296 μg g^{-1} total selenium.

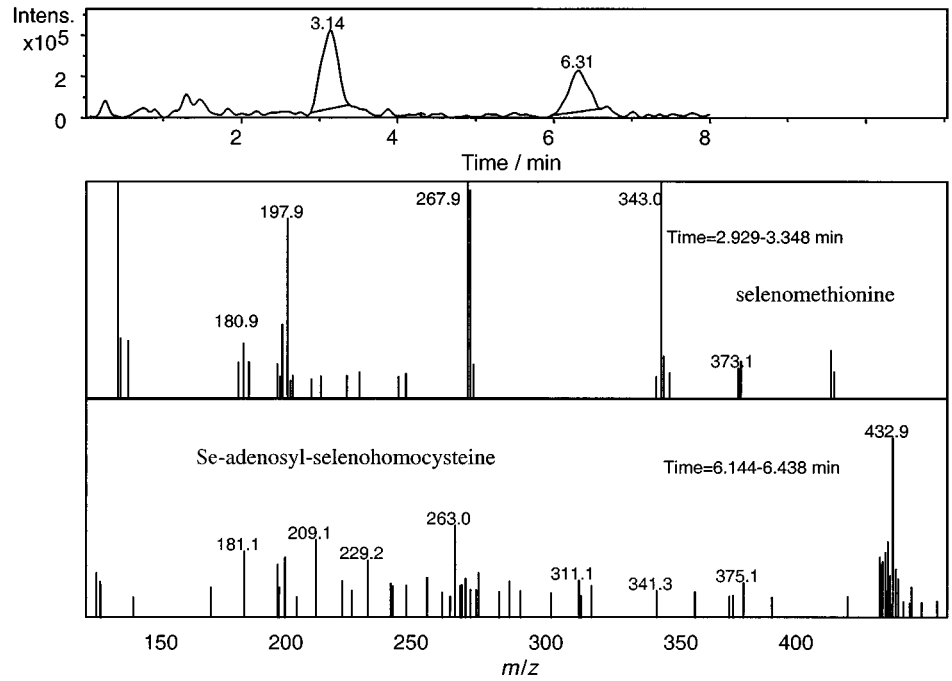

Fig. 4 HPLC-ESI-MS SIC chromatograms of *m/z* = 198 (3.14 min) and 433 (6.31 min) with their spectra from the enzyme digest of selenium-enriched yeast (Nutrition 21) containing 1922 μg g^{-1} total selenium.

water extract.[8] Peaks observed, in addition to the selenium-containing ion (*m/z* = 198), in the mass spectrum of selenomethionine in Fig. 4, were from the compounds co-eluting with selenomethionine. The *m/z* = 268 ion, for example, corresponds to adenosine.

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