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As part of an ongoing study to identify selenium compounds with cancer chemopreventive activity, selenium-enriched yeast was analyzed by HPLC–ICP-MS. More than twenty selenium-containing species were found in hot water and enzymatic hydrolysis extracts of the yeast. Trifluoroacetic acid was used as an ion-pairing agent in a water–methanol mobile phase with reversed-phase chromatography on an octylsilane stationary phase. The presence of selenocystine, selenomethionine and methylselenocysteine was confirmed by comparative retention of standards. The column efficiency was 8500 theoretical plates and the mobile phase was compatible with standard ICP-MS operating conditions.

Keywords: *Selenoamino acids; selenium; speciation; high-performance liquid chromatography; inductively coupled plasma mass spectrometry; ion pair; selenium-enriched yeast*

Selenium is an essential element which, if consumed in excess, is toxic. Selenium has also been associated with cancer chemoprevention. The nutritional bioavailability,[1] toxicity[2,3] and cancer chemopreventive activity[1,4] of selenium have been found to be species-dependent. Thus, speciation of selenium in many matrices such as soils, plants and animals has been and continues to be an area of research interest.

The speciation of selenium in yeast is of interest from both a nutritional and a cancer preventive perspective. Selenium-enriched yeast is used commonly as a source of selenium in nutritional supplements. Recently, it was reported that the consumption of yeast-derived selenium nutritional supplements is associated with statistically significant reductions in total cancer mortality and total cancer incidence.[5]

Dialysis,[2,6] gas chromatography,[7] ion-exchange chromatography[7–10] and ion pair chromatography[11,12] have been used to study the distribution of selenium in selenium-enriched yeast. Dialysis results indicated only a general distribution of selenium, such as 95% of the selenium in yeast being water insoluble and present in a bound form,[2] or that more than 50% of the selenium is probably protein bound or in other large organic species.[6] Gas chromatographic and liquid chromatographic results have provided more specific selenium distribution information. Gas chromatography of the reaction products of a selenium-enriched yeast with cyanogen bromide indicated that 30% of the selenium was selenomethionine.[7] Analysis of collected eluent fractions after ion exchange chromatography indicated that more than 60% of the selenium in a selenium yeast hydrolysate co-chromatographed with selenomethionine.[10] Using a different hydrolysis and ion exchange chromatographic method, 30% of the selenium in a selenium yeast hydrolysate was found to be in the form of four selenoamino acids: selenomethionine, selenocystine, selenocysteine, and Se-methylselenocysteine.[9]

More recently, the coupling of ion exchange or ion pair high-performance liquid chromatography with element-specific detectors for selenium yeast speciation has been employed,[8,11,12] eliminating the need for eluent fraction collection. Results from ion pair HPLC with electrothermal atomic absorption spectrometry (ETAAS) on an enzymatic yeast hydrolysate indicated the selenium to be in three forms: inorganic selenium, selenocystine, and selenomethionine.[11] Ion exchange HPLC–ETAAS separated the inorganic selenium into Se^{IV} and Se^{VI}, as well as separating selenocystine and selenomethionine.[8] Ion pair HPLC inductively coupled plasma mass spectrometry (ICP-MS) of an enzymatic yeast hydrolysate once again indicated the selenium to be in three forms: inorganic selenium, selenocystine and selenomethionine.[12]

Clearly, progress has been made in speciating selenium in yeast; however, the importance of definitively identifying and quantifying all selenium compounds in yeast necessitates further research. In this paper, as part of an ongoing study of the cancer chemopreventive activity of selenium, a new ion pair HPLC–ICP-MS method for the speciation of selenium in a selenium-enriched yeast, shown to be associated with cancer prevention,[5] is reported. The HPLC conditions are compatible with standard ICP-MS operating conditions and result in the separation of more than twenty selenium species in yeast extracts.

EXPERIMENTAL

Instrumentation

An Elan 5000 inductively coupled plasma mass spectrometer (Perkin-Elmer SCIEX, Norwalk, CT, USA) was used for total selenium determination and HPLC–ICP-MS. Samples were introduced using a cross-flow nebulizer and double-pass spray chamber. The chromatographic system consisted of a HP1090 liquid chromatograph (Hewlett-Packard, Wilmington, DE,

USA) and a Zorbax SB-C_8 column (4.6 mm × 15 cm) preceded by a Zorbax SB-C_8 guard column (4.0 mm × 12.5 mm) (MAC-MOD Analytical, Chadds Ford, PA, USA). A piece of 30 cm long PEEK tubing (0.25 mm id) was used to transfer the column eluent to the nebulizer. Typical HPLC–ICP-MS conditions are shown in Table 1.

Data were processed using Perkin-Elmer ELAN, Microsoft Excel, Extend and Lightstone Labs software. Chromatograms shown were smoothed in ELAN graphics signal display using a four-point moving average.

Reagents and Samples

Barnstead E-pure 18 MΩ water (Boston, MA, USA), nitric acid and hydrochloric acid (Certified ACS Plus, Fisher, Fair Lawn, New Jersey, USA), nitric acid purified by sub-boiling, trifluoracetic acid (Aldrich, Milwaukee, WI, USA) and methanol (HPLC grade, Fisher, Fair Lawn, New Jersey, USA) were used for sample or mobile phase preparation.

Sodium selenate, DL-selenomethionine, DL-selenoethionine, DL-selenocystine and protease XIV were obtained from Sigma (St. Louis, MO, USA). Methylselenocysteine, propylselenocysteine, and allylselenocysteine were obtained from Dr. Howard Ganther (University of Wisconsin, Madison, WI, USA). Plasma selenium standard solution (1000 ppm) was obtained from Spex (Spex, Industries Inc., Edison, New Jersey, USA). Selenium-enriched yeast was obtained from Nutrition 21 (San Diego, CA, 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.

Sample Preparation

Two methods of extraction were used. In the first method, yeast (0.3 g) was added to 6 ml of H_2O in a 15 ml polypropylene centrifuge tube, and heated and shaken in a hot water bath at 85–90 °C for 1 h. In the second method, a variation of that reported by Gilon *et al.*,[8] yeast (0.3 g) and protease (30 mg) were added to 6 ml of H_2O in a 15 ml polypropylene centrifuge tube and shaken in the dark for 24 h using a wrist-action shaker (Burrell, Pittsburgh, PA, USA). Protease XIV is a non-specific protease, which breaks peptide bonds of any protein present in the yeast. The solutions were then centrifuged for 30 min using a Beckman GPR centrifuge (Fullerton, CA, USA) at 3000*g*. The supernatant was removed and filtered through a 0.45 μm polypropylene filter (Arbor Technologies, Ann Arbor, MI, USA). Three millilitres of each of the filtered solutions were passed through 10 000 Da molecular weight cutoff filters (Micron Separations Inc., Westboro, MA, USA) by centrifuging overnight. Solutions (0.45 ml) were then spiked with 50 μl of a 10 ppm selenoethionine solution before being chromatographed.

Table 1 Typical conditions for HPLC–ICP-MS of selenium species

Chromatographic conditions—	
Stationary phase	Zorbax SB-C8 (4.1 mm × 15 cm)
Mobile phase	98 + 2 water–methanol, 0.1% (v/v) TFA
Flow rate	1.0 ml min^{-1}
Injection volume	10 μl
ICP-MS conditions—	
rf Forward power	1003 W
Gas flow rates:	
Plasma	15.0 l min^{-1}
Auxiliary	0.800 l min^{-1}
Nebulizer	0.925 l min^{-1}
Resolution	Normal
Scanning mode	Peak hop
Dwell time	500 ms
Isotope monitored	^{82}Se (with krypton correction)

Total Selenium Determination and Recovery

Total selenium in the extracts was determined by direct nebulization ICP-MS of the undigested extracts. Total selenium in the yeast was determined by ICP-MS after digestion in a closed-vessel, microwave digestion system (Q Max 4000, Questron Corporation, Mercerville, NJ, USA). The digestion and analysis procedures were the same as reported for selenium-enriched garlic.[13] Recovery of the selenium injected on the column was evaluated using column switching on the HP 1090 liquid chromatograph set up in a flow injection mode. To determine the column recovery of injected selenium, the average of the flow injection peak areas was compared with the total area of the chromatogram.

Selection of Chromatographic Conditions

The challenge to separate by HPLC a complex mixture of selenium species potentially present in selenium-enriched yeast requires efficient chromatography. The known presence of ionic species, such as selenoamino acids, requires derivatization prior to reversed-phase HPLC, or limits the chromatographic options to ion exchange or ion pair chromatography. The use of ICP-MS as a detector for HPLC further constrains chromatographic choices. The percentages of dissolved solids and organic solvents must be minimal under normal ICP-MS sample introduction and operating conditions. Thus, based on high column efficiency and compatibility with ICP-MS, ion pair chromatography was selected as the separation method. Trifluoroacetic acid (TFA), a commonly used ion pairing agent for reversed-phase HPLC of underivatized peptides and proteins,[14,15] was selected as the ion pairing agent. Methanol was used as a mobile phase additive to reduce retention times of later eluting peaks, and also to increase the sensitivity of the selenium signal.

RESULTS AND DISCUSSION

The 95% confidence interval for total selenium in the yeast was determined to be 1922 ± 113 ppm ($n = 6$). Fig. 1 shows a typical chromatogram of six selenoamino acid standards. The peaks are well resolved with the exception of selenoethionine and propylselenocysteine, which have some baseline overlap.

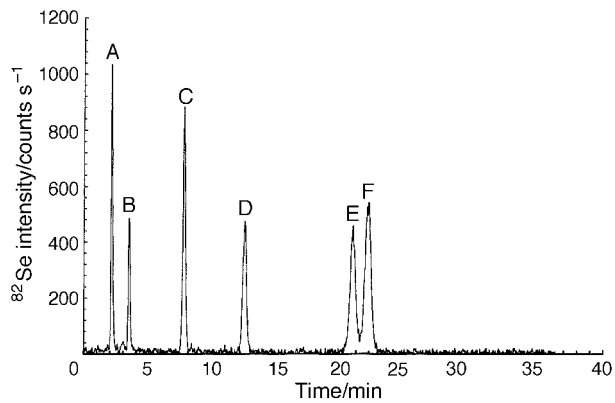

Fig. 1 Chromatogram of selenoamino acid standards; approximately 300 ppb Se each. Peaks: A, selenocystine; B, methylselenocysteine; C, selenomethionine; D, allylselenocysteine; E, selenoethionine; F, propylselenocysteine.

Despite the post-column band broadening from the interfacing and sample introduction to the ICP-MS, an efficiency of 8500 theoretical plates (calculated using the selenomethionine peak) were obtained for a 15 cm column.

In comparison with recently published retention times for ion pair HPLC–ICP-MS,[12] the retention times of the selenoamino acids are increased significantly. This can be explained by the mobile phase composition. A 1% (v/v) solution of TFA ($pK_a = 0.25$) has a pH of approximately 2. At this pH, the carboxylic acid groups and the amine groups of the amino acids are protonated, giving the amino acids a net positive charge. The use of an anionic pairing agent results in an increased retention of positively charged species in comparison with that of zwitterionic species at a higher pH.

Figs. 2 and 3 [(*a*) and (*b*)] show typical chromatograms of the extracts of 1922 ppm selenium yeast. In both extracts more than 20 selenium-containing peaks were detected with good

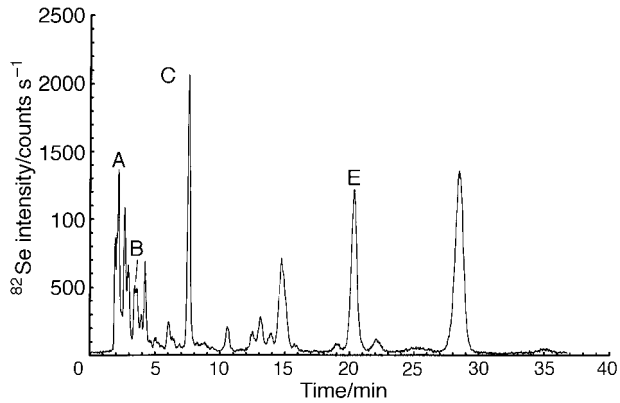

Fig. 2 Chromatogram of hot water extract of 1922 ppm selenium yeast. See Fig. 1 for peak notation.

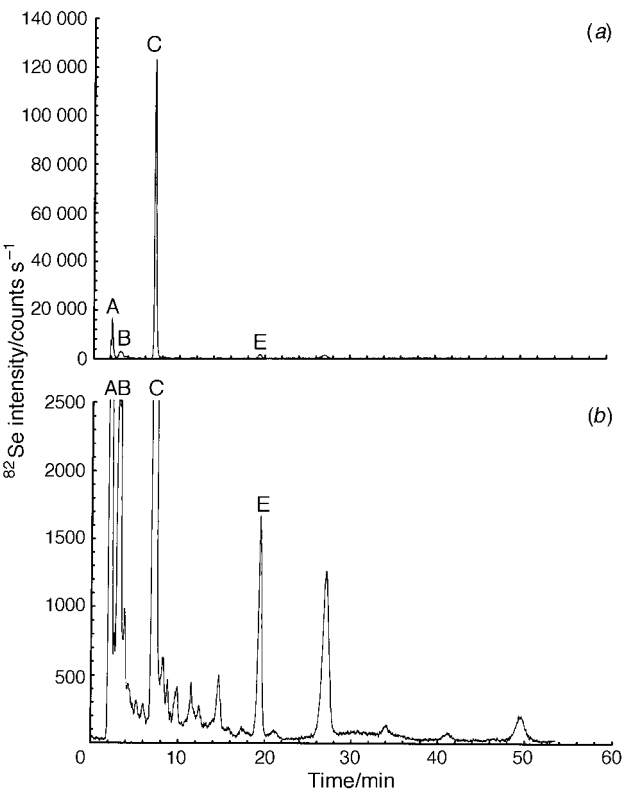

Fig. 3 (*a*) Chromatogram of enzymatic hydrolysis water extract of 1922 ppm yeast. (*b*) Same as Fig. 3(*a*), but with *y* axis scale changed. See Fig. 1 for peak notation.

reproducibility. As expected, inorganic selenium, as selenate and selenite, is not retained and eluted in the dead volume (1.8 ml). In Fig. 2, 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. The selenoethionine was used as a chromatographic internal standard to adjust for changes in retention, and will be used in the future for quantification purposes.

Semi-quantitative calculations indicated that 10% of the selenium was extracted by the hot water method, while 90% of the selenium was extracted by the enzymatic hydrolysis. The enzymatic hydrolysis extraction efficiency was comparable to the 92% obtained by Gilon *et al*., who performed the experiment at room temperature[8] and at 37 °C.[11] This incomplete recovery may be due to either an insufficient amount of enzyme or insufficient extraction time. Optimization of the enzymatic hydrolysis procedure is in progress.

In terms of recovery for the column, all of the selenium was recovered from the injection of selenoamino acid standards, while 80% of the selenium in the hot water extract was recovered, and over 90% of the selenium in the enzymatic hydrolysis extract was recovered. One possible explanation for less than 100% recovery for the extracts is that strongly hydrophobic species would be expected not to elute under the chromatographic conditions used.

Measurement of retention times indicated the presence of inorganic selenium, selenocysteine, methylselenocysteine and selenomethionine in the hot water extract. Selenomethionine appears as the major peak in the enzymatic hydrolysate, which also contains inorganic selenium, selenocystine and methylselenocysteine concentrations higher than those in the water extract. Confirmations of the identity of these peaks and identification of the remaining peaks are currently being made.

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