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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · APRIL 2008

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Selenium Speciation and Bioavailability in Biofortified Products Using Species-Unspecific Isotope Dilution and Reverse Phase Ion Pairing−Inductively Coupled Plasma−Mass Spectrometry

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In some regions of the world, where the bioavailability of selenium (Se) in soil is low and/or declining (e.g., due to use of high-sulfur fertilizers), there is increased risk of adverse affects on animals and human health. In recent years, increased research attention has focused on understanding the relationships between Se contents in foods and supplements and their nutritional benefits for animal and humans. The objective of this study was to use a species-unspecific isotope dilution and reverse phase ion pairing−inductively coupled plasma−mass spectrometry techniques for the identification and quantification of Se species in biofortified grains (i.e., wheat and triticale), flour, and wheat biscuits. The information on Se species was used to gain an understanding of the bioavailability of Se in biofortified and process-fortified wheat biscuits used in a clinical trail. The major Se species identified in biofortified and process-fortified samples were selenomethionine (76–85%) and selenomethionine selenoxide (51–60%), respectively. Total plasma Se concentrations in the biofortified Se exposure group were found to increase throughout the 6 month trial period (mean = 122 μg L^{-1} at 0 months to 194 μg L^{-1} at 6 months). In contrast, the trial group exposed to process-fortified Se biscuits showed little increase in mean total Se plasma concentrations until 4 months of exposure (mean = 122 μg L^{-1} at 0 months to 140 μg L^{-1} at 4 months) that remained constant until the end of the trial period (mean = 140 μg L^{-1} at 4 months to 138 μg L^{-1} at 6 months). The difference in total Se plasma concentrations may be due to the presence and bioavailability of different Se species in biofortified and process-fortified biscuits. An understanding of Se speciation in foods enables better understanding of pathways and their potential benefits for animals and humans.

KEYWORDS: Selenium; speciation; biofortification; bioavailability; HPLC-ICP-MS

INTRODUCTION

Selenium is an essential micronutrient required in animals and humans for successful growth, reproduction, and survival (e.g., antioxidant defense and thyroid hormone catalysis) (*1–3*). Selenium deficiency can result in an increased risk of thyroid and immune dysfunction, infections, cancer and various inflammatory conditions (*1–3*). There is increasing evidence to suggest that diets supplemented with Se can reduce incidences of certain types of cancers, such as prostate and breast cancer (*2, 4, 5*). This anticarcinogenic property of Se has been suggested to be associated with its production or conversion to certain metabolites such as methylselenol (CH_3SeH) (*3, 6*).

Human dietary requirements for Se are in a relatively narrow range, with food containing <0.1 mg kg^{-1} possibly leading to deficiency, whereas concentrations above 1 mg kg^{-1} can cause toxic effects (*7*). In some regions of the world where the bioavailability of Se in soil is low, Se-deficient diseases in humans have been observed, such as Keshan and Kaschin-Beck diseases (*8*). Where the bioavailability of soil Se is low, the associated food chains may also be Se deficient (*3, 9*). A declining trend in the Se content of soils has been noted in some regions of the world that may be associated with the burning of fossil fuels and the release of sulfur (a Se antagonist), acid rain, soil acidification and the use of high-sulfur fertilizers (*9, 10*). Therefore, in recent years attention has been focused on increasing the Se content of foods for improved animal and human nutritional benefits (*9, 11*). However, the different pathways by which Se delivers its nutritional benefits are still to be elucidated. This is in part due to a lack of information on the identification and concentrations of Se species present in foods.

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10.1021/jf073030v CCC: $40.75
Published on Web 02/07/2008

Globally, wheat is one of the most important dietary sources of Se (*2*). In Australia, it is estimated that nearly half of the nutritional requirements for Se are provided by wheat (*2*). A study of the Se status in Australia has shown a 20% decline in total plasma Se concentrations of South Australians sampled from 1977 to 2002 (*12*). This decline may be due to changes in dietary composition and/or a decline in the mean Se concentrations of South Australian wheat (*Triticum aestivum*) (*2*). The use of biofortification strategies such as supplementation of livestock and fertilization of food crops has been shown to increase the uptake and accumulation of Se in food crops (*4, 12*). The fertilization of food crops with selenate in Finland since 1984 has increased Se in food chains, with consequent increases in human Se status (*4*). Given the importance of adequate Se concentrations for animal and human health, the biofortification of Se in foods may be an effective strategy to improve nutritional requirements. However, an understanding of Se species present in foods is essential in understanding their metabolic pathways and potential health benefits.

The recent advancements in inductively coupled plasma−mass spectrometry (ICP-MS) with the development of collision-reaction cell technology have enabled the routine use of stable isotope dilution procedures to accurately determine Se in environmental samples (*13, 14*). Isotopic dilution procedures (i.e., species specific and species unspecific) can further be combined with high-performance liquid chromatography−ICP-MS (HPLC-ICP-MS) techniques for the identification and quantification of known (i.e., through retention time comparisons with synthesized standards) and unidentified Se species in biological tissues (*13, 15*). The further offline confirmation and/or identification of Se species can occur through the use of complementary mass spectrometry techniques such as tandem mass spectrometry (*16*).

The objective of this study was the identification and quantification of Se species present in biofortified grains (i.e., wheat and triticale), flour, and wheat biscuits using species-unspecific isotope dilution and reverse phase-ion pairing−inductively coupled plasma mass spectrometry. This information on Se species will be used to improve our understanding of the bioavailability of Se in biofortified and process fortified wheat biscuits used in a clinical trial undertaken by the University of Adelaide (South Australia), Commonwealth Scientific and Industrial Research Organisation (CSIRO)-Division of Human Nutrition, and the National Centre of Excellence in Functional Foods (NCEFF) in 2005.

MATERIALS AND METHODS

Selenium Standards. Selenomethionine (SeM), selenocystine (SeCys), sodium selenate (Se(VI)), and sodium selenite (Se(IV)) were purchased from Sigma (Australia). Individual stock solutions (1000 mg Se L^{-1}) were acidified using hydrochloric acid (0.01 M) and refrigerated at 4 °C until analysis. Selenomethionine selenoxide (SeOMet) was prepared through the addition of excess hydrogen peroxide (0.1 mL of 30% H_2O_2) to 2 mL of SeM (100 mg of Se L^{-1}) (*17*). The enriched ^{76}Se (99.8% metal) was purchased from Cambridge Isotope Laboratories (Andover, MA) and dissolved in a minimum amount of ultrapure nitric acid (HNO_3) (Sigma).

Grain, Flour, and Wheat Biscuit Samples. Wheat (cultivar Whylah) and triticale (cultivar Abacus) grains were biofortified with Se in October 2003 by applying sodium selenate as a foliar spray to plants. The recovery of Se in grain of cereal crops biofortified in this manner was approximately 7% (*18*). The commercial products, wafer grain biscuits (~3 μg of Se g^{-1}) and wholemeal bread flour (~1 μg of Se g^{-1}) with biofortified Se (Bio-Fort), were purchased from a local business in South Australia in September 2006. These products were made with wheat that was biofortified with Se by the application of sodium selenate to the soil around planting time.

Sample Preparation. Samples of grains, flour, and wafer biscuits were freeze-dried for approximately 24 h (Labconco) to a constant mass and ground to an homogeneous powder using a ZM 100 ultra centrifugal mill (ring sieve = 0.5 mm) (Retsch, Germany). Approximately 0.5 g of sample was added to 50 mL polypropylene vials and 10 mL of acetone (HiPerSolv, BDH) added. Samples were shaken for 1 h on an end over end mixer and supernatants removed after centrifugation at 1200*g* for 15 min. This acetone extraction procedure was repeated twice, and supernatants were removed after centrifugation at 1200*g* for 15 min. After acetone extraction, the residue pellet was dried at room temperature (~25 °C) for approximately 12 h.

Total Selenium. Total Se concentrations were determined following a closed vessel microwave digestion procedure using concentrated HNO_3 (Suprapur, Sigma). Approximately 0.5 g of sample was weighed into a Teflon digestion vessel and 10 mL concentrated HNO_3 added. The samples were digested using a closed vessel microwave oven (Milestone) and a time program consisting of two stages: 5 min at 300 W and 40 min at 500 W. After digestion, vessels were allowed to cool at room temperature (~25 °C) for approximately 30 min and diluted to 50 mL with ultrapure deionized water (Millipore). Digests were stored in a cool room (~0–5 °C) until total Se concentrations were determined by ICP-MS (Agilent 7500ce). Total Se concentrations were determined using *m*/*z* 78 in collision cell mode with hydrogen gas (flow rate = 4 mL min^{-1}). The possible interference on *m*/*z* 78 from the formation of Se hydrides (i.e., $^{77}SeH^+$) was corrected using equations previously published by Hinojosa Reyes et al. (*13*). The recovery of Se from reference material NIST 8436 (1.19 ± 0.05 μg g^{-1}) was in close agreement with the reference value (1.23 ± 0.09 μg g^{-1}).

Enzymatic Hydrolysis. Approximately 0.2 g of acetone extracted sample was digested using 0.02 g of Protease XIV (Sigma) in a 15 mL glass incubation tube with 5 mL of ultrapure deionized water (Millipore) (*19*). The samples were shaken in an incubation chamber at 37 °C for 24 h. After incubation, samples were centrifuged for 20 min at 1200*g*, filtered <0.2 μm (Sartorius), and diluted 1:10 (v/v) with ultrapure deionized water (Millipore, Australia) prior to total Se and speciation analysis. Total Se and speciation analysis occurred on all samples within 4 h of enzymatic digestion.

Selenium Speciation. Selenium speciation occurred using a reverse phase−ion pairing chromatography procedure coupled with ICP-MS (*17, 19*). The enzymatic digested samples (1:10 (v/v) dilution with ultra pure deionized water) were injected (100 μL) onto an Alltech C_8 (Alltech Associates) (250 × 4.6 mm, 5 μm) column and eluted with an isocratic mobile phase consisting of 10% methanol (v/v) (Malachroft) and 0.1% (v/v) heptafluorobutanoic acid (HFBA) (Sigma) at a flow rate of 1 mL min^{-1} and a column temperature of 25 °C. The identification of Se species occurred through retention time comparisons with available standards (i.e., SeM, SeCys, Se (VI), and Se (IV)).

The quantification of Se species occurred using a postcolumn species-unspecific isotope dilution procedure. This isotopic dilution procedure was selected in this study because it allows for the quantification of both identified (i.e., through retention time comparisions with synthesized standards) and unidentified Se species directly in the sample matrix. The isotopes selected in this study for isotope dilution were ^{76}Se (9.37%) and ^{78}Se (23.77%) due to their relatively high abundance and previous research that has demonstrated the successful removal of argide-based interferences on these isotopes using collision-reaction cell ICP-MS (*13, 20*). The most abundant isotope of Se at *m*/*z* 80 was not selected in this study due to the possibility for further correction on this isotope due to the presence of bromide that may occur in biological samples (i.e., $^{79}Br^1H$) (*13*).

The ^{76}Se spike (Cambridge Isotope laboratories, MA) (5 μg L^{-1}) was continually mixed at a flow rate of 0.1 min min^{-1} with the eluent of the HPLC column through a T piece. The mixed mobile phase spike (1.1 mL min^{-1}) was coupled directly to the nebulizer of the ICP-MS (Agilent 7500ce), and intensities were measured at *m*/*z* 75, 76, 77, 78, 82, and 83. The accurate quantification of Se species by species-unspecific HPLC-ICP-MS requires an understanding and if necessary

the correction for potential errors in $^{78/76}$Se isotope ratio determinations, such as detector dead time errors and mass bias discrimination.

The error introduced into Se isotope measurements due to detector dead time losses was determined using a procedure proposed by Vanhaecke et al. (*21*). The main isotopes of Se (76, 77, 78, 80, and 82) were monitored with dead time correction disabled in standard solutions ranging from 50 to 1000 μg Se L^{-1}. A detector dead time of 39 ns was calculated and applied to all measured intensities in further experiments.

Mass bias discrimination is known to cause deviations in measured Se isotopic ratios from those expected during ICP-MS analysis (*13, 22*). The mass bias factor (K) for elements can be determined by measuring the isotope ratio of samples with known isotopic composition that can then be used to correct individual isotope ratios in unknown samples (*13, 22*). The mass bias effect on measured $^{78/76}$Se ratios is known to be complicated further through the formation of hydrides (i.e., ^{77}SeH$^+$) during collision-reaction cell ICP-MS (*13, 14, 23*). These hydrides are believed to be derived from the sample matrix (e.g., water and methanol) or through impurities in cell gases (*13, 14*). The measured intensities for ^{78}Se were corrected for ^{77}Se^{1}H$^+$ using the intensity equation (*13*)

$$\mathrm{Se} = {}^{78}I - f\mathrm{Se}({}^{77}I) \quad (1)$$

where fSe is the ratio ^{82}Se^{1}H$^+$/^{82}Se$^+$ based on the measured ^{83}I/^{82}I ratios in samples; ^{77}I, ^{78}I, ^{82}I, and ^{83}I are the measured intensities at m/z ^{77}Se, ^{78}Se, ^{82}Se, and ^{83}Kr, respectively.

The possible interference from the formation of ^{75}As^{1}H$^+$ on ^{76}Se intensities was determined daily through the analysis of 0.5–100 μg of As L^{-1} in 10% methanol/0.1% HFBA. The error introduced into ^{76}Se intensities through the formation of ^{75}AsH$^+$ was found to be ~0.85% (fAs = 0.82–0.87%). The measured intensities at ^{76}Se were corrected for the possible presence of ^{75}AsH$^+$ using the intensity equation

$$\mathrm{Se} = {}^{76}I - f\mathrm{As}({}^{75}I) \quad (2)$$

where fAs is an arsenic factor; ^{75}I and ^{76}I are the measured intensities at m/z ^{75}As and ^{76}Se, respectively.

In this study, using hydrogen as a reaction gas at 4 mL min^{-1} and samples introduced in a mobile phase of 10% methanol/0.1% HFBA (10–100 μg of Se L^{-1}) the measured ^{82}Se^{1}H$^+$/^{82}Se$^+$ (fSe) ratios were ~2.2% (fSe range = 0.021−0.024). This error in Se isotope measurements due to hydride formation is consistent with the error of ~3% reported by Hinojosa Reyes et al. (*13*). However, it is significantly lower than that previously reported by Sloth and Larsen (*14*) and Boulyga and Sabine Becker (*23*) of ~9%.

The mass bias in $^{78/76}$Se isotope ratios was corrected using the exponential model (*13*). The mass bias error can be determined by a plot showing the relative error Ln ($R_{\mathrm{exptl}}/R_{\mathrm{theor}}$) in experimental isotope ratios with respect to ^{78}Se versus the mass difference between the measured isotopes, ΔM. The mass bias factor (K) derived from the slope of the regression lines (10–100 μg of Se^{1-} natural abundant Se standard) was ~−4% per mass unit. This mass bias factor is similar to that previously reported for Se using collision-reaction cell ICP-MS (*13, 14*). The measured $^{78/76}$Se ratios for 10–100 μg of Se L^{-1} standards (Inorganic Ventures, Astral Scientific, Pty, Ltd., Australia) without mass bias correction were 92 ± 0.8% accurate, whereas after mass bias correction $^{78/76}$Se ratios were found to be 100 ± 0.4%. The mass bias correction factor was determined every four samples during HPLC-ICP-MS analysis through the direct nebulization post column of a 10 μg of Se L^{-1} natural abundant standard (Inorganic Ventures, Astral Scientific, Pty, Ltd., Australia).

The intensity chromatograms of samples following HPLC-ICP-MS analysis were corrected for potential hydride interferences using eqs 1 and 2, corrected for mass bias, and converted into mass flow chromatograms (ng min^{-1}) using a previously reported isotope dilution equation (*24*). The total amount of Se in each chromatographic peak was determined by area integration. The accuracy of the isotopic dilution−HPLC-ICPMS procedure to determine Se species was assessed through the spiked recovery of a 10 μg L^{-1} SeM standard. The recovery of the SeM spike was in close agreement for all samples analyzed (98 ± 0.1%).

Table 1. Estimated Daily Average Se Intakes of Trial Participants from Wafer Biscuits

	μg of Se per day		
trial group	0−2 months	2−4 months	4−6 months
control	1	1	2
biofortified	86	172	258
process-fortified	105	205	315

Tandem Mass Spectrometry. A direct infusion tandem mass spectrometry procedure was used to confirm the structure of known standards and attempt the identification of an unidentified Se peak in process-fortified wheat biscuit samples. The unidentified Se peak was collected after multiple injections (n = 8, 100 μL) onto an Alltech C8 column (250 × 4.6 mm, 5 μm) (Alltech Associates) column using a 10% (v/v) methanol/0.1% (v/v) HFBA mobile phase at a flow rate of 1 mL min^{-1} (column temperature = 25 °C). The collected fractions (~2 mL) containing the unknown Se peak were evaporated to dryness using a stream of ultrapure nitrogen (BOC Gases) for approximately 2 h.

The dried unknown Se peak and SeM standard were suspended in 30% (v/v) methanol (ChromAR HPLC, Mallinckrodt) acidified with hydrochloric acid (Trace Pur, Merck) and directly infused into the mass spectrometer of a Thermo TSQ Quantum instrument (Thermo, Australia) using a syringe pump. The instrument was operated in positive ion electrospray ionization acquisition mode and scanned in the m/z range of 50–500. Selected ions were further analyzed for structural information using MS/MS by increasing the collision energy and scanning in the m/z range of 50–500.

Selenium Bioavailability: Clinical Trial Using Se Biofortified Biscuits. This Se bioavailability trial was part of a collaborative research project undertaken through the University of Adelaide, CSIRO, and the NCEFF (human ethical approval granted by the University of Adelaide and CSIRO) to examine potential human health benefits from biofortified Se products (*25*).

The biscuits used in this clinical trial (a low-Se control containing around 80 μg kg^{-1}; biofortified Se, and "process fortified" Se) were prepared by soaking whole grain wheat in water for 12 h, then heating (220 °C) to expand the grain, and compressing into a "puffed wheat" biscuit. A "positive control" was included in the trial labeled "process-fortified" to compare the bioavailability of Se through biofortification processes. The process-fortified samples were prepared through the addition of L-SeM (Eburon Organics, Lubbock, TX) to the soaking water and heated as above.

Plasma Se Concentrations. Total plasma Se concentrations were recorded for 75 men aged between 40 and 70 years living in or near Adelaide, South Australia, with initial average Se plasma blood concentrations of ~122 μg L^{-1}. Trial participants were required to consume as part of their diets one low-Se control, biofortified or process-fortified wafer biscuit per day for the first two months, then two daily for months 3−4, and then three daily for the final 2 months (3 groups of 25 individuals) as part of their regular diets. Peripheral blood samples were collected every 2 months and total plasma Se concentrations determined by ICP-MS (Agilent 7500ce) following digestion with nitric and hydrochloric acids (*25*). The estimated daily average intake of Se by trial participants from wafer biscuits is presented in **Table 1**.

RESULTS AND DISCUSSION

Selenium Extraction Using Enzymatic Hydrolysis. Total Se concentrations in samples ranged from 1.6 ± 0.1 μg g^{-1} in commercially available wheat flour to 8.6 ± 0.2 μg g^{-1} in triticale grains (**Table 2**). Enzymatic hydrolysis using Protease XIV (Sigma) extracted between 87 and 93% of the total Se present in samples (**Table 2**). This high Se extraction efficiency is consistent with previous findings using enzymatic hydrolysis to extract Se from wheat (80–94%) (*26, 27*) and other plant species (e.g., Indian mustard, Brazil nuts, barley, and rye) (70–95%) (*19, 27, 28*). The high extraction efficiency using

Table 2. Total Se, Protease-Extracted Se, and Se Species Identified in Biofortified and Process-Fortified Samples

sample[a]	total Se (μg g^{-1})	protease extracted[c] (%)	SeM[d] (%)	SeOMet[d] (%)
biofortified wheat	8.3 ± 0.3	90 ± 4	87 ± 3	
biofortified flour[b]	1.6 ± 0.1	92 ± 3	82 ± 6	
biofortified wheat biscuit[b]	4.4 ± 0.2	87 ± 4	83 ± 3	
biofortified triticale	8.6 ± 0.2	93 ± 2	83 ± 4	
biofortified wheat wafer[e]	8.3 ± 0.2	93 ± 3	88 ± 5	
process-fortified wafer[e]	8.5 ± 0.2	91 ± 3	5 ± 1	55 ± 4

[a] $n = 4$. [b] Commercial products. [c] Percentage of total Se determined using HNO_3. [d] Percentage of total Se extracted using protease. [e] Wafer biscuits used in clinical trial.

protease suggests that Se may be present in these samples as water-soluble species (e.g., "free" selenoamino acids) and/or associated with Se-containing proteins that can release "free" selenoamino acids (e.g., SeM) following the cleavage of peptide bonds.

Selenium Species Unspecific Isotope Dilution. The recovery of Se ("mass balance") during HPLC-ICP-MS was found be 95–102% (99 ± 3%) for all samples analyzed. Typical HPLC-ICP-MS chromatograms for the Se species identified in biofortified and process-fortified grain and biscuit samples are shown in **Figure 1**. The major Se species identified in biofortified grain, flour, and biscuit samples was the selenoamino acid, SeM (76–85%) (**Figure 1A**; **Table 2**). Selenomethionine has previously been identified as the major Se species present in nonaccumulator plants and Se-yeast products (*17, 26–30*). In process-fortified samples, SeM was identified as a minor constituent (5 ± 1%) with a large unidentified Se peak present at 4.8 min (**Figure 1B**; **Table 2**). This unidentified Se species was found to contain between 51 and 60% of the total Se extracted from process-fortified samples (**Figure 1B**; **Table 2**).

Selenium in nonaccumulator plants is believed to be metabolized nonspecifically along sulfur pathways (*30, 31*). In this pathway, inorganic Se (e.g., Se (VI) and Se (IV)) can be reduced to selenide (Se^{2-}) either by nonenzymatic reactions and glutathione reductase or enzymatic reactions via adenylyl sulfate reductase and sulfite reductase and further converted to selenocysteine using *O*-acetylserine and the action of cysteine synthase (*30*). Selenocysteine is thought to be metabolized to SeM through the intermediate species, selenocystathionine and selenohomocysteine (*30, 32*). The selenoamino acids, SeM and selenocysteine, can then be nonspecifically incorporated into proteins in place of methionine and cysteine, respectively (*30, 32*).

Identification of Unknown Se Species. Initial attempts to identify the unidentified Se peak at 4.8 min in process-fortified biscuits samples using tandem mass spectrometry proved to be unsuccessful in this study. The inability to recognize a natural Se isotopic region may be due to matrix-induced suppression effects associated with electrospray tandem mass spectrometry.

It has been reported in the literature that SeM can be readily converted to SeOMet in the presence of oxidizing agents, such as peroxynitrite, cyanogen bromide and hydrogen peroxide (*33–35*). The presence of SeOMet in natural samples remains unclear. It is uncertain if SeOMet in samples occurs due to natural pathways or is an artifact of sample preparation procedures (*22, 29*). In this study, to determine if the unknown peak at 4.8 min can be attributed to SeOMet a standard was prepared using H_2O_2 and used in species-unspecific isotope dilution HPLC-ICP-MS.

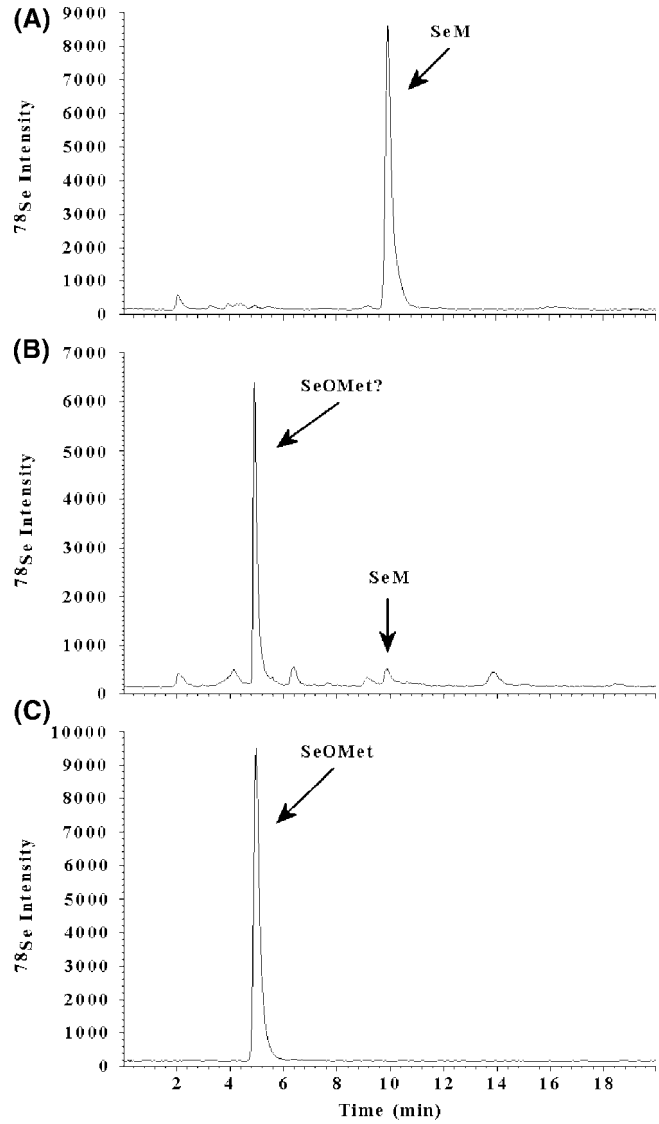

Figure 1. Typical HPLC-ICP-MS chromatograms of Se species identified in (**A**) biofortified wheat biscuit, (**B**) process-fortified wheat biscuit, and (**C**) SeOMet standard.

A SeM standard was initially analyzed by tandem mass spectrometry to determine the accuracy of the direct infusion procedure to identify molecular and fragment ions containing Se. The direct infusion of SeM (10 mg L^{-1}) ($^{+}NH_3CH(COOH)$-$CH_2CH_2SeCH_3$) produced one major natural Se isotopic region at *m/z* 198 (**Figure 2**) and five fragment ions from collision-induced dissociation (CID) at *m/z* 181, 152, 135, 109, and 102 (**Figure 3**). The ion at *m/z* 198 corresponds to the molecular ion ($[M + 1]^{+}$ ^{80}Se), *m/z* 181 corresponds to the loss of OH, *m/z* 152 corresponds to the loss of CO and H_2O, *m/z* 135 corresponds to the loss of CO, NH_3, and H_2O, *m/z* 109 is the ion $CH_3SeCH_2^{+}$, and *m/z* 102 is the ion fragment $NH_2CH(COOH)CH_2CH_2^{+}$. These molecular and fragmentation patterns are similar to those previously reported for SeM by Dumont et al. (*32*) and Goenaga Infante et al. (*29*).

The direct infusion of the SeOMet standard ($^{+}NH_3CH(COOH)$-$CH_2CH_2SeOCH_3$) produced one major natural Se isotopic region at *m/z* 196 ($[M+1]^{+}$ ^{80}Se) (**Figure 4**). A lower abundant natural Se isotopic region was identified at *m/z* 214 ($[M + 1]^{+}$ ^{80}Se). The CID of the *m/z* 196 produced fragment ions at *m/z* 182, 150, and 102. The presence of major Se ion regions at *m/z* 214 and 196 is consistent with previous findings for SeOMet (*34*). The molecular ion at *m/z* 196 can be attributed to SeOMet,

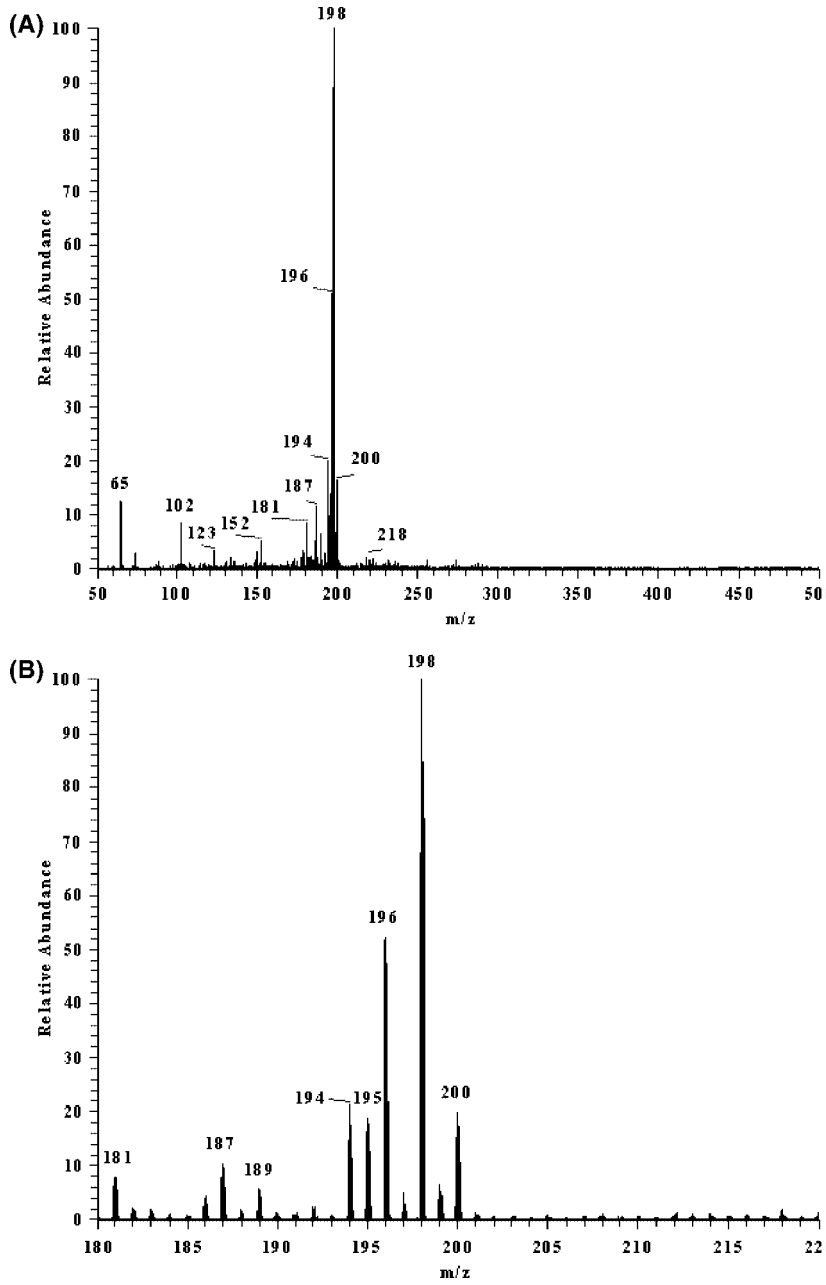

Figure 2. Tandem mass spectrometry of SeM standard: (**A**) molecular ions in *m*/*z* 50–500; (**B**) major natural isotopic region at *m*/*z* 198 $[M + 1]^{+}$ ^{80}Se.

whereas the presence of a natural Se isotopic region at *m*/*z* 214 can be attributed to the hydrated form of this species (i.e., Se-dihydroxy selenomethionine). The final products of SeM oxidation, Se(IV) and methylseleninic acid (CH_3SeOOH), were not identified or could not be identified in this study using positive ion acquisition mode. The presence of ions at *m*/*z* 230 (**Figure 4**) that do not display a natural Se isotopic pattern in this study may be due to a combination of ions generated from the SeM standard, oxidation products, and matrix constituents.

Selenomethionine selenoxide was identified through retention time comparison with the tandem mass spectrometry confirmed standard as the major peak in process-fortified wheat biscuits using HPLC-ICP-MS (**Figure 1B**,**C**). The earlier elution of SeOMet (or its hydrated species) relative to SeM in reverse phase-ion pairing-ICP-MS can be attributed to its enhanced polarity due to the addition of oxygen and water to the Se atom, producing a greater affinity for the acidic mobile phase (*7*). Selenomethionine selenoxide has previously been identified in natural products, such as yeast extracts (*22, 29, 36*). The presence of SeOMet in processed fortified biscuits in this study is probably due to the biscuit production procedure. In process-fortified biscuits, SeM was added to the soaking water as the "free" selenoamino acid that can potentially be exposed to oxidation in further production steps (i.e., 220 °C), whereas in biofortified samples, SeM may be present in Se-containing proteins, which protects it from oxidation during biscuit production.

Selenium Bioavailability: Biofortified Biscuit Trial. Total plasma Se concentrations were found to increase in relation to the control after 6 months of exposure through their diets to biofortified and process-fortified Se biscuits (**Table 3**). Mean Se plasma concentrations were higher in the trial group exposed to biofortified Se biscuits (**Table 3**). Total plasma Se concentrations in the biofortified Se exposure group were found to increase throughout the 6 month trial period (mean = 122 μg L^{-1} at 0 months to 194 μg L^{-1} at 6 months). An increase in

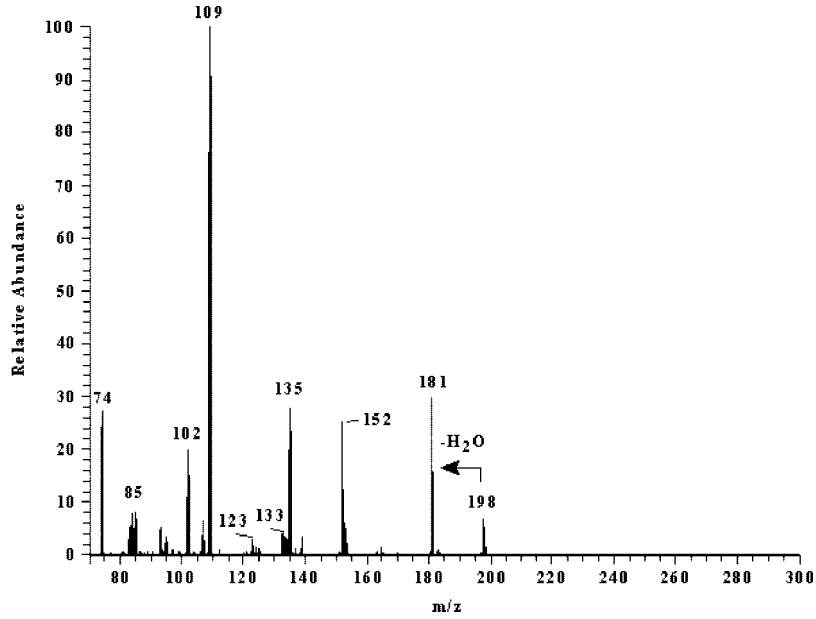

Figure 3. Collision-induced dissociation of SeM standard at *m*/*z* 198.

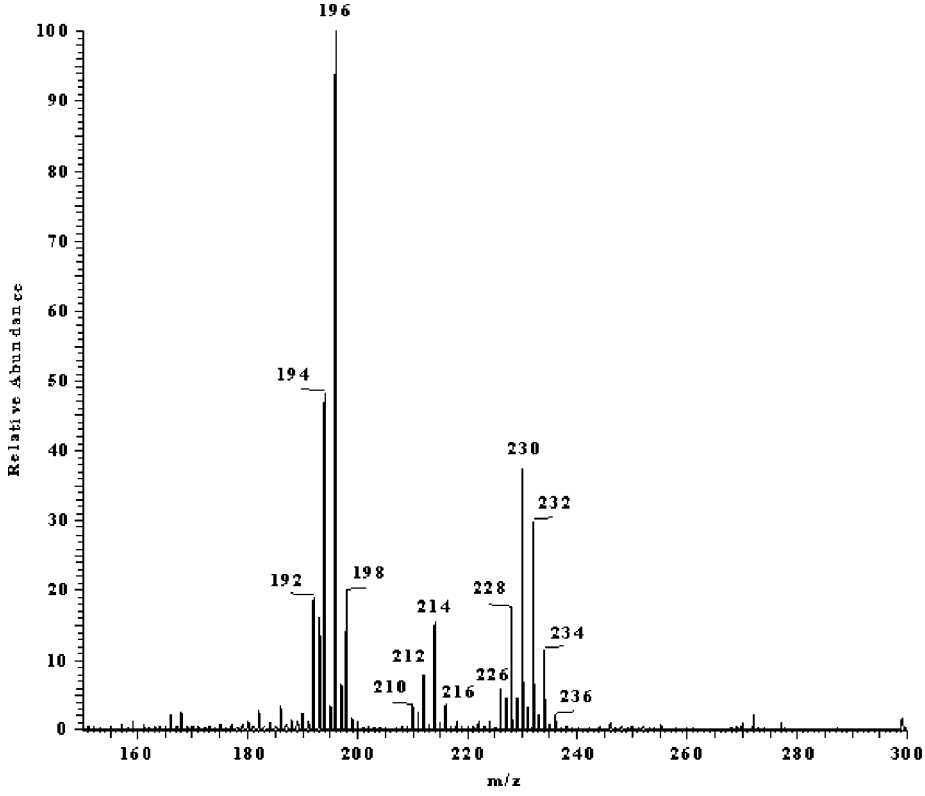

Figure 4. Tandem mass spectrometry of SeOMet in *m*/*z* 50–500.

Table 3. Total Plasma Se Concentrations (Mean ± SD) in Men Exposed to Biofortified or Process-Fortified Se Wheat Wafer Biscuits

		μg L^{-1a}			
trial group	no.	0 months	2 months	4 months	6 months
control	22	121 ± 9a	117 ± 11a	122 ± 12a	116 ± 14a
biofortified[b]	19	122 ± 17a	144 ± 11b	170 ± 16c	194 ± 19d
process-fortified[c]	21	122 ± 13a	126 ± 6a	140 ± 13b	138 ± 15b

[a] Different letters indicate significant differences based on one-way analysis of variance. [b] $p < 0.01$ [c] $p < 0.05$.

total plasma Se concentrations was observed to occur within 2 months of exposure (mean = 122 μg L^{-1} at 0 months to 144 μg L^{-1} at 2 months) (**Table 3**). In contrast, the trial group exposed to process-fortified Se biscuits showed little increase in mean total Se plasma concentrations until 4 months of exposure (mean = 122 μg L^{-1} at 0 months to 140 μg L^{-1} at 4 months) and remained constant until the end of the trial period (mean = 140 μg L^{-1} at 4 months to 138 μg L^{-1} at 6 months) (**Table 3**).

The high bioavailability of Se in biofortified and process-fortified wheat biscuits is consistent with previous findings for Se in wheat (*37–39*). The higher bioavailability of Se in biofortified biscuits, in relation to process-fortified biscuits, may be due to differences in Se speciation. In this study, SeM was the main Se species identified in biofortified samples, whereas

SeOMet was the main constituent present in process-fortified samples (**Table 3**). Supplementation studies have shown organic Se (i.e., SeM) in wheat and Se-yeast is more bioavailable and retained longer than inorganic Se species (*31, 39*). This difference is suggested to occur due to the nonspecific incorporation of SeM from digested wheat and Se-yeast into tissue proteins such as skeletal muscle, erythrocytes, and plasma albumin (*31*). The lower bioavailability of SeOMet in this study may be due to its change in chemical structure through oxidation that prevents it following SeM pathways. These results are consistent with findings by Fox et al. (*40*) and Sloth et al. (*20*), who have examined the absorption and retention of Se following exposure to Se-yeast. Sloth et al. (*20*) found the absorption and retention of Se-yeast in 12 male subjects fed ^{77}Se-labeled SelenoPrecise yeast (Pharma Nord, Vejle, Denmark) to be 90 and 75%, respectively. Fox et al. (*40*), using the same methodology but a different Se-yeast, found a lower absorption and retention of 54 and 59%, respectively. The differences in these two studies may be due to the strain of the yeast and/or the processes used to prepare the Se-yeast.

The analysis of total Se in foods provides insufficient information to determine the fate and behavior of this element in animal and human systems. An understanding of Se species present in food is required to determine metabolic pathways and potential animal and human health effects.

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Received for review October 15, 2007. Revised manuscript received December 12, 2007. Accepted December 20, 2007.

JF073030V