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Selenium speciation studies in Se-enriched chives (*Allium schoenoprasum*) by HPLC-ICP–MS

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Abstract

In this study, three liquid chromatographic techniques were employed to better understand selenium species distribution in Chives (*Allium schoenoprasum*) separately grown in three different supplementation media Se(IV), Se(VI), and SeMet. The highest selenium accumulation up to 700 μg Se g^{-1} was observed in the case of the Se(VI)-enriched samples on the basis of total selenium measurements. Size-exclusion chromatography (SEC-HPLC) was performed for investigation of selenium containing proteins in chives. For speciation of selenium containing amino acids, reversed-phase ion-pairing chromatography (RP-IP-HPLC) and for enantiomeric separations, a crown ether column was used. In all three cases online detection with inductively coupled plasma mass spectrometry was performed for selenium specific detection. Two extractions (perchloric acid–ethanol and enzymatic) were carried out on chive samples. Speciation analysis on the chives grown in three different media revealed that selenium distribution among different forms of amino acids in the sample strongly depends on the type of enrichment employed. Enrichment with Se(VI) leads to accumulation of selenium in inorganic forms, while in case of Se(IV) and SeMet-enriched samples, methyl-selenocysteine and selenocystine were found to be present. Not surprisingly, chiral speciation revealed the presence of the L-enantiomeric forms of selenoamino acids in the sample. The major enantiomer found in the perchloric acid–ethanol extracts was L-MeSeCys, while in the enzymatic extracts L-SeMet was also detected.

Keywords: Chives; Selenium enrichment; RP-IP-HPLC-ICP–MS; SEC-HPLC-ICP–MS; Chiral speciation

1. Introduction

Selenium (Se) is a key trace element required in small amounts in humans and animals for the function of a number of selenium-dependent enzymes, such as glutathione peroxidase (GPX) and thioredoxin reductase, however this element can also be toxic in larger doses (Arthur & Beckett, 1990; Holben & Smith, 1999; Rotruck, Ganther, Swanson, Hafeman, & Hoekstra, 1973). Both the beneficial and the toxic effects of selenium are based on concentration ingested and on its chemical forms (Fairweather-Tait, 1997; Vadhanavikit, Ip, & Ganther, 1993). Selenium content of food varies widely among different world regions, depending on the selenium content of the soil. Selenium deficiency may play a significant role in the increasing number of cancer patients in several countries (Garland et al., 1995; Maihara et al., 2004; Tiran, Tiran, Petek, Rossipal, & Wawschinek, 1992; van den Brandt et al., 1993). Many commercial dietary supplements are available for selenium supplementation purposes. Information on total selenium content of a proposed Se-supplement is insufficient to assure the viability or safety of the product. The bioavailability of selenium from these dietary supplements depends on factors such as selenium species distribution (Combs, 1986; Fairweather-Tait, 1997) and the consumer's health, age and genetic disposition (de Jong et al., 2001; Thomson, 2004). Analytical speciation studies helps to elucidate the

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different forms of selenium present in a sample. Most common selenium supplements are selenized yeast of which the primary selenium species often is selenomethionine (SeMet) (Kotrebai, Birringer, Tyson, Block, & Uden, 1999). Other food based species that have extensively been studied for selenium speciation include selenium-enriched garlic, onion, ramp, cabbage, sprout, and broccoli (Hamilton, 1975; Kotrebai et al., 1999, Kotrebai, Birringer, Tyson, Block, & Uden, 2000; Lintschinger, Fuchs, Moser, Kuehnelt, & Goessler, 2000; Roberge, Borgerding, & Finley, 2003; Shah, Kannamkumarath, Wuilloud, Wuilloud, & Caruso, 2004; Whanger, Ip, Polan, Uden, & Welbaum, 2000). Major Se-species reported in these selenized vegetables are Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenocysteine, which are known to be more effective inhibitors of tumor formation than other seleno species like SeMet that causes an undesirable accumulation of selenium in tissues (Ip et al., 2000).

Bioactive selenium containing molecules present in food, like amino acids possess chiral centers leading to the occurrence of D- and L-enantiomers. In living organisms the L-form is the more predominant enantiomer (Day, Kannamkumarath, Yanes, Montes-Bayon, & Caruso, 2002; de Leon, Sutton, Caruso, & Uden, 2000; Montes-Bayon, B'Heimer, de Leon, & Caruso, 2001; Sutton et al., 2000), although it should be noted that L-form could be more toxic than the D-enantiomer. Because of the diverse properties of enantiomers, identification of particular enantiomeric form in which selenoamino acid is present also needs to be verified. Separation of amino acid enantiomers can be performed basically by three different chromatographic ways: derivatization with a chiral reagent (Day et al., 2002; Montes-Bayon et al., 2001), addition of a chiral reagent to the mobile phase (Nakagawa, Mizunuma, Shibukawa, & Uno, 1981; Nakagawa, Shibukawa, & Uno, 1982) or the use of a chiral stationary phase column (de Leon et al., 2000; Peters, Davis, & Jones, 2004; Sutton et al., 2000). The derivatization step could cause contamination and depending on the reagent used, the derivatization could take several hours. When using chiral stationary phases sample preparation does not differ from that applied in non-enantiomeric separations. Several studies have shown the applicability of chiral stationary phases (Blanco-Gónzalez, Fernández Sánchez, & Sanz-Medel, 1998; de Leon et al., 2000; Pérez Méndez, Blanco González, & Sanz Medel, 2000; Sutton et al., 2000). The most commonly used chiral stationary phases are the crown ethers, which are synthetic macrocyclic polyethers that can form selective complexes with cations. The chiral crown ether column resolves compounds with a primary amino group near the chiral center by forming a complex between the crown ether and the ammonium ion moiety from the sample. The D-form amino acids always elutes faster than the L-form when using a Daicel Crownpak CR(+).

Several Allium vegetables have already been investigated from Se-accumulation (total Se content) and also from species distribution point of view. Se-enriched chives (*Allium schoenoprasum*) have not been extensively studied from these two aspects not mentioning the investigation of the enantiomer distribution among Se-containing amino acids or their derivatives. This vegetable is widely consumed and since some populations do not consume adequate amounts of Se, a natural supplement of selenium, which can be included in every day diet is essential.

The goal of this study was to determine the effect of various kinds of selenium fortifications – accomplished with three Se-species on the selenium species and their enantiomeric distribution in chives. For investigating the possible presence of selenium in proteins, size-exclusion chromatography (SEC-HPLC) coupled to ICP–MS was applied. For speciation of selenium containing amino acids, reversed-phase ion-pairing high performance liquid chromatography (RP-IP-HPLC) and for the separation of the enantiomers of three selenoamino acids a crown ether column was coupled online to ICP–MS detection.

2. Materials and methods

2.1. Instrumentation

An Agilent 7500ce ICP–MS (Agilent Technologies, Tokyo, Japan) was used for total selenium determination. The ICP–MS is equipped with an octopole reaction cell and can be operated with or without the collision/reaction gas. Cell conditions were optimized using H_2 as cell gas with a flow rate between 0 and 5 mL min^{-1} monitoring ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se isotopes. A conventional micromist nebulizer was used for the sample introduction under standard plasma conditions.

For chromatographic separations an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, a temperature column compartment and a diode array detector was utilized. The coupling between the corresponding column outlet and the sample introduction system of ICP–MS was achieved through a 300 mm long 0.25 mm i.d. PEEK tubing. Chromatographic columns used were a Superdex Peptide 10/300 GL (Tricorn, Amersham Biosciences) for size-exclusion chromatography, a C8 Alltima (Alltech, Deerfield, IL, USA) (150 mm × 4.6 mm × 5 μm particle size) equipped with a guard column with the same stationary phase material for RP-HPLC studies and a Daicel Crownpak CR(+) (Chiral Technologies, Exton, PA, USA) column (150 mm × 4.0 mm × 5 mm particle size) for enantiomeric separation of selenoamino acids. The chiral column was fitted with a thermostated jacket (Chiral Technologies) and water was circulated through the jacket applying a Model RTE-110 recirculating water bath (Neslab Instrument, Inc., Newington, NH, USA). A guard column (10 × 4.0 mm) with the same stationary phase material was also used in this case. The instrumental operating conditions are given in Table 1.

Table 1
Instrumental operating conditions

ICP–MS parameters	
Forward power	1500 W
Plasma gas flow rate	15.0 L min^{-1}
Carrier gas flow rate	1.20 L min^{-1}
Dwell time	0.1 s per isotope
Isotopes monitored	^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se
RP-IP-HPLC parameters	
Column	Alltima C8 (150 mm × 4.6 mm × 5 μm)
Mobile phase	0.1% (v/v) heptafluorobutyric acid, 5% (v/v) methanol, pH 2.5, isocratic elution
Flow rate	0.9 mL min^{-1}
Injection volume	50 μL
SEC-HPLC parameters	
Column	Superdex Peptide HR 10/300 (4.1 mm × 250 mm × 10 μm)
Mobile phase	30 mM Tris buffer, pH 7.5
Flow rate	0.6 mL min^{-1}
Injection volume	100 μL
Chiral-HPLC parameters	
Column	Daicel Crownpak CR(+) (150 mm × 4.0 mm × 5 mm)
Mobile phase	0.1 M perchloric acid, pH 1.0, isocratic elution
Flow rate	0.5 mL min^{-1}
Column temperature	22 °C
Injection volume	20 μL

2.2. Reagents

All reagents were of analytical reagent grade. Doubly deionised water (18 MΩ cm) obtained with a NanoPure treatment system (Barnstead, Boston, MA, USA) was used throughout the experiments. HPLC-grade methanol, perchloric acid (Fisher Scientific, Pittsburgh, PA, USA), and acetone (Tedia Co., Fairfield, OH, USA) were used throughout. Nitric acid (Suprapure, 68%) obtained from Pharmaco (Brookfield, CT, USA) and hydrogen peroxide (30%) from Fisher Scientific (Fair Lawn, NJ, USA) was used for total selenium determination.

Individual stock solutions of Se-(methyl) selenocysteine hydrochloride (MeSeCys; 95%; Sigma–Aldrich, Milwaukee, WI, USA), seleno-DL-methionine (SeMet; 99% +, Acros Organics, Fair Lawn, NJ, USA), seleno-L-cystine ($SeCys_2$; 98%, Sigma), Se(VI) (Na_2SeO_4; 98%; Sigma), and Se(IV) (Na_2SeO_3; 98%; ICN Biomedicals, Aurora, OH, USA) at concentration 100 mg Se L^{-1} were obtained by dissolving the appropriate amount of the corresponding compound in doubly deionised water. Concentrated HCl was obtained from J. T. Baker Inc. (Phillipsburg NJ, USA). Heptafluorobutyric acid (HFBA), sodium dodecyl sulfate (SDS), phenylmethylsulfonil fluoride (PMSF), protein standards: cytochrome *c* (125 kDa); vitamin B_{12} (1.35 kDa); and cytidine (0.246 kDa), and enzymes proteinase K (from *Tritiachium album*), protease XIV (from *Streptomyces griseus*) were purchased from Sigma (Sigma–Aldrich Co., St-Louis, MO, USA). $CaCl_2$ was obtained from Fisher (Fisher Scientific, Fair Lawn, NJ, USA). Chromatographic standard and other working solutions were prepared daily as required.

2.3. Cultivation and preparation of Se-enriched chives

The chive type selected was *A. schoenoprasum*. The seeds were purchased from a local food market. Seeds were soaked in water for a day before planting. Approximately 0.5 g of chive seeds were planted in a pot which was watered daily with deionized water. After 4 weeks when chives had attained optimal growth, selenium supplementation was started. Plants were watered with the solution containing Se(IV), Se(VI), and SeMet, at the concentration of 10 μg mL^{-1} every other day for two weeks. Each set of chives was selenized altogether with 200 mL of the corresponding Se enrichment solution. The total cultivation period (including the time of soaking, growing, and the enrichment) was 6 weeks. Treatments were carried out with eight replicates.

After harvesting, chives were washed first with tap water then deionized water to exclude contamination from the surface. Samples were then separated into the roots and the leaves. These two parts were cut into small pieces to increase the surface of the samples so that the heat transfer during freeze-drying could be more sufficient. After minimizing the moisture, which was 90% of the sample weight, samples were ground in a household coffee-grinder (Bomann CB425, Kempen, Germany) to a powder consistency for complete homogenization of the sample.

2.4. Sample preparation for total selenium

Complete digestion of the chive samples was performed with a microwave digestion system (Discover, CEM Corp., Matthews, NC) for total selenium determination. The mineralization program involved two steps. In the first step temperature was ramped to 125 °C and held there for 1 min and then in the second step increased to 150 °C and again held for 1 min. In both the steps, power was held constant at 150 W.

Approximately 0.05 g samples were weighed into microwave vials. One millilitre of concentrated HNO_3 (65%) was added and samples which were left overnight. H_2O_2 (30%, 200 μL) solution was added to the solution after 24 h. The resulting solutions were digested with the microwave system using the above program. After digestion, the final volume was made to 25 mL. A digestion blank was also applied. Both internal calibration and standard addition methods were used for the quantification of total selenium present in the sample. Analyses were performed in triplicate for each sample.

2.5. Extraction of selenium for chromatographic speciation studies

Size-exclusion chromatography was used for investigating possible presence of selenoproteins in selenium-enriched

chive samples. Extraction of selenoprotein was carried out according to Mounicou, Meija, and Caruso (2004) with slight modifications in the procedure.

Proteins were extracted with 10 mL of 30 mM Tris–HCl (pH 7.5) buffer containing 1% SDS and protease inhibitor (2 mM PMSF). Tris–HCl buffer allows recovery of the water soluble proteins from the sample while SDS is known to be a good anionic detergent for solubilizing water insoluble proteins by forming ion-pairs and PMSF minimizes protease activity (Mounicou et al., 2004). Approximately 0.30 g sample was weighed and dissolved in the above solution. The extraction was carried out at room temperature with magnetic stirring for 24 h. The solution was centrifuged (25 min, 3214*g*) and after this the supernatant was collected from the residue. Proteins were precipitated by adding 80% acetone to the supernatant. The sample was placed at −4 °C for 12 h to precipitate the proteins. After this treatment, the mixture was centrifuged (25 min, 3214*g*) and the proteins were finally collected. The supernatant was discarded again and proteins were re-solubilized in 1.5 mL Tris–HCl buffer (pH 7.5) containing 1% SDS. 100 μL of this solution was injected into the SEC-HPLC-ICP–MS system.

Two extraction methods were employed to study the selenium species distribution in chives. The extraction of non-protein bound selenium was based on a paper by Wrobel et al. Approximately 0.10 g of the samples were weighed in a glass vial and 2.4 mL of 0.4 M perchloric acid–ethanol (8:2) was added to it. Extract was then centrifuged (Sorwall RC-5B Centrifuge, Du Pont Instrument, CT, USA) at 3214*g* for 10 min. After centrifugation, samples were filtered through a 0.45 μm PVDF filter. This solution was diluted as required to 3 mL and 50 μL of the resulting solution was introduced into the RP-IP-HPLC-ICP–MS system.

In order to release the selenoamino acids presumably bound to high molecular weight compounds (presumably proteins), enzymatic hydrolysis was performed (Wrobel, Kannamkumarath, Wrobel, & Caruso, 2003). Proteinase K (0.03 g)was dissolved in 5 mL of Tris buffer (pH 7.5) containing 1 mM $CaCl_2$. The resulting solution was added to 0.10 g of chive sample and constantly stirred for 15 h at 50 °C. After this, another proteolytic enzyme, protease XIV was added to the above and stirred continuously at 50 °C for another 15 h. The final mixture was then centrifuged and filtered through a 0.45 μm PVDF filter.

In both cases, about 0.5 mL of each of the above extracts was centrifuged through 5 kDa molecular weight cutoff filters (Ultrafree-0.5, Millipore) to remove high molecular weight species from the extracts before the analysis.

3. Results and discussion

3.1. Total selenium determination

The selenium uptake from different enrichment solutions was determined by measuring the total selenium content of the chive samples. The results derived from total selenium determinations are presented in Table 2. All concentration values presented in this paper are calculated for dry weight of the sample. Both leaves and roots were analyzed for total selenium content. The method of standard additions (at μg L^{-1} Se levels) was applied for the quantification of the selenium present in the samples. The total selenium concentration was determined by ICP–MS. The ^{78}Se, ^{77}Se, ^{82}Se isotopes were monitored. The total selenium concentration of control samples was approximately 1 μg g^{-1} (1.07 ± 0.03 μg Se g^{-1} in the leaves and 0.94 ± 0.47 μg Se g^{-1} in the roots).

Chives fortified with Se(VI) showed to have accumulated the highest amount of selenium, up to 600–700 μg Se g^{-1}. The translocation of Se from root to leaf depends on the species used for supplementation (Terry, Zayed, de Souza, & Tarun, 2000). A study by Zayed, Lytle, and Terry (1998) clearly demonstrated that selenate is transported much more easily than selenite or any organic species, like SeMet. Another study (Arvy, 1993) showed that within 3 h, 50% of selenate taken up by bean plant roots moved to the leaves, whereas in the case of selenite, most of the Se remained in the root. Zayed et al. (1998) also stated that the reason why selenite is poorly translocated to leaves may be explained by the fact that it is rapidly converted to organic forms of Se such as SeMet, which are more likely to be retained in the roots. It can be clearly seen from the table that a higher level of selenium was present in roots than in leaves in case of all three enrichments. This might be attributed to the fact that chives were harvested only after 2 weeks of supplementation with the Se. As people consume the green tops of chives and as not adequate amount of roots were avail-

Table 2
Total selenium content in dried samples and the extraction efficiencies in the chive extracts

Sample	Se form used for enrichment	Se in dried samples ± SD (μg g^{-1})	% of total Se in perchloric acid-EtOH extract	% of total Se in enzymatic extract
Leaves	Se(IV)	222 ± 5	24	69
	Se(VI)	613 ± 5	32	77
	SeMet	265 ± 7	27	74
Roots	Se(IV)	494 ± 6	–	–
	Se(VI)	722 ± 6	–	–
	SeMet	422 ± 8	–	–

able, only chives leaves were further investigated for selenium species distribution.

3.2. Chromatographic speciation studies

To study the possible forms of absorbed selenium in chives, speciation studies were carried out with different HPLC-ICP–MS systems. Chives were extracted with various extraction media to obtain information about the availability and chemical forms of selenium present in the sample.

3.2.1. SEC-ICP–MS

It was of interest to study whether chives incorporate selenium into high molecular weight biomolecules such as proteins like other Allium vegetables. A systematic approach leading to specific extraction and precipitation of proteins was followed as described above. The calibration of the SEC column was accomplished with a standard mixture of cytochrome *c* (125 kDa), vitamin B_{12} (1.35 kDa), and cytidine (0.246 kDa) with online UV-detection at $\lambda = 280$ nm. The chromatographic conditions are given in Table 1. The re-solubilized protein extracts as obtained in the previous section was introduced into the SEC-ICP–MS system. The chromatograms obtained for the chive samples enriched with three different selenium supplement procedures are shown in Fig. 1. It is clear from the figure that in all three cases, the elution profiles of selenium in both high molecular weight (HMW; about 6.5 kDa) and low molecular weight (LMW; molecular weight between 1.3 and 0.1 kDa) fractions is similar. Differences could only be observed in the intensities of the peaks detected. Since the protein specific extraction procedure was applied, it is assumed that the peak 1 (Fig. 1) corresponding to molecular weight of about 6.5 kDa in size exclusion chromatograms represent selenium containing proteins. In the LMW region (peak 2 and 3), two major peaks are also observed. These later eluting peaks might correspond to the selenium containing peptides, selenoamino acids, and inorganic selenium. It is interesting to note (Fig. 1(b)) that supplementation with Se(VI) leads to more pronounced incorporation of selenium in low molecular weight species when compared to Se(IV) and SeMet.

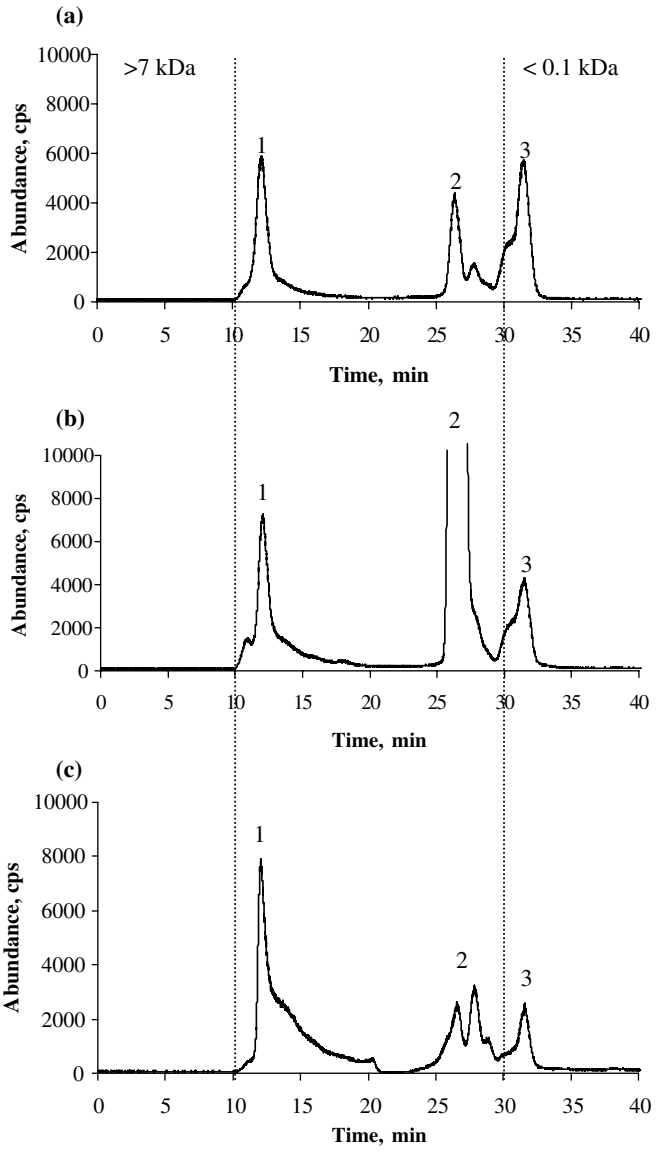

Fig. 1. SEC-ICP–MS chromatograms of chive samples grown in the presence of: (a) Se(IV); (b) Se(VI); and (c) SeMet.

3.2.2. RP-IP-HPLC-ICP–MS

To extract the low molecular weight free selenium containing compounds from plant matrix, two kinds of extraction methods were employed. First procedure involved treatment of the sample with perchloric acid–ethanol mixture for extraction of free selenium compounds. Perchloric acid was used for the elimination of proteins from the matrix and ethanol was added to decrease the solubility of polysaccharides. Another extraction method was aimed at the extraction of selenium species bound to proteins. For that purpose, proteolytic digestion of the plant sample was performed. Extraction efficiencies of the two extraction procedure applied are depicted in Table 2. With the perchloric acid–ethanol extraction, approximately 30% of the total selenium content could be extracted while the proteolytic enzymes increased the recovery of selenium from the samples up to 70% of total amount. Fig. 2(a) shows the selenium species distribution in perchloric acid–ethanol extract expressed in the percentage of total selenium content of the extract. A notable amount of inorganic selenium was found in the Se(VI)-enriched chive sample. However, in the other two cases where supplementation with Se(IV) and SeMet was done, the metabolism of the selenium species in the leaves leads to the formation of more organic forms. As already mentioned, extraction efficiencies increased with the enzymatic hydrolysis since the protein bound selenium becomes available for detection (Kotrebai et al., 2000). As depicted in Fig. 2(b) – after enzymatic hydrolysis of the samples – $SeCys_2$ and MeSeCys became accessible for identification, which suggests that a part of

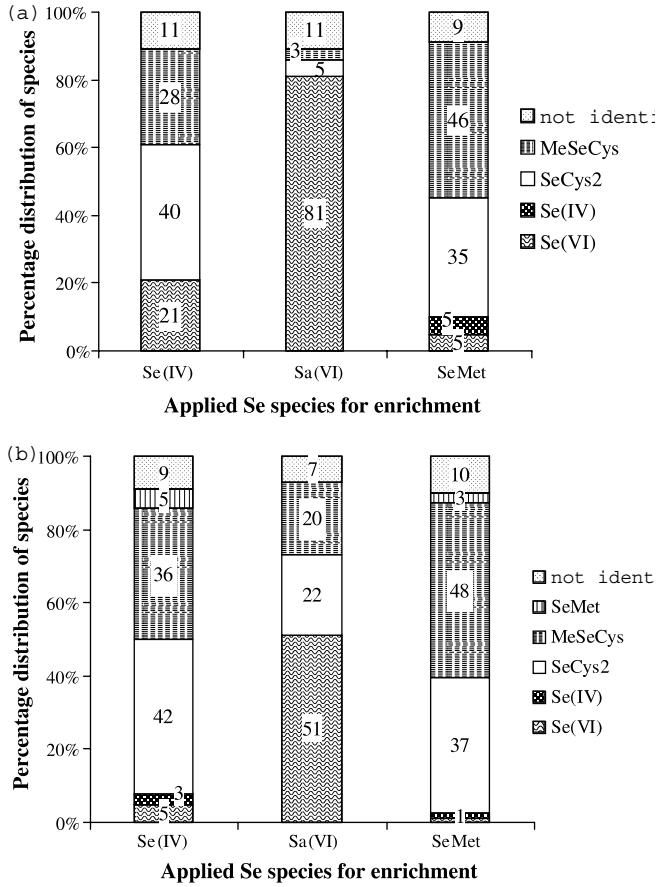

Fig. 2. Selenium species distribution expressed in the percentage of total selenium content of: (a) perchloric acid–ethanol extract; and (b) enzymatic extract.

these species are also bound to proteins. Samples enriched with Se(IV) showed to contain approximately 49%, 38%, 5%, and 8% of $SeCys_2$, MeSeCys, SeMet, and inorganic selenium, respectively. Se(VI) fortification produced 24% of $SeCys_2$ and 22% MeSeCys and more than 50% Se(VI) was also detected. In the case of SeMet-enriched chives the highest amount of organic Se compounds was observed. MeSeCys, $SeCys_2$, and SeMet constitute 52%, 44%, and 3%, respectively, of the total selenium found in the extract. These speciation distribution results agree well with the studies reported earlier on Allium vegetables; the major forms of Se reported in these kinds of plants are MeSeCys, $SeCys_2$, and only enzymatic extraction reveals a slight amount of SeMet (de Leon et al., 2000; Kotrebai et al., 2000; Shah et al., 2004; Wrobel et al., 2004). The insufficient column recovery, the unknown compound and the low extraction efficiencies account for the difference between the total selenium content and the sum of the identified peaks.

The separation of selenium compounds was performed by ion-pairing RP-HPLC using heptafluorobutyric acid as ion-pairing reagents. Fig. 3(a) shows a typical chromatogram of a mixture of selenium standards with the following retention times: Se(VI) ($t_{ret} = 2.83 \pm 0.01$ min), Se(IV) ($t_{ret} = 4.01 \pm 0.04$ min), $SeCys_2$ ($t_{ret} = 4.98 \pm 0.03$ min), MeSeCys ($t_{ret} = 7.27 \pm 0.04$ min), and SeMet ($t_{ret} = 16.9 \pm 0.11$ min). The extracts were appropriately diluted before being injected onto the column. To avoid the excess loading of the column with the sample matrix and to improve the chromatographic separation, a final filtration step was used for all extracts with 5 kDa molecular weight cutoff filters before introducing them into the chromatographic system. To confirm that this additional cleaning step does not cause any loss in the selenium content, samples were measured before and after the filtration for total Se content and found no difference in signal intensities. Fig. 3(b) indicates the perchloric acid–ethanol extract of Se(IV)-enriched chive sample. In this case, the major compound is identified as $SeCys_2$ while MeSeCys was also present but in smaller amount. Fig. 3(c) shows the chromatogram gained after the enzymatic extraction of Se(IV)-enriched chive sample. Fig. 3(d) is identical to Fig. 3(c) but the *y*-scale is expanded as the high amount of inorganic species would not allow showing the smaller peaks. Additional peaks were observed in this case which can be attributed to the proteolytic digestion, which results in release of numerous protein bound selenium compounds. Proteolytic digestion also increased the amount of $SeCys_2$ and MeSeCys released from the samples. In both the extracts analyzed, it can be observed that retention time of the species changed as shown in Fig. 3(a). This might be due to the change in pH and plant matrix present (Sloth & Julshamn, 2003). So in order to confirm the identity of the various selenium species standard addition was performed.

In all three types of enrichments, the chromatographic separations of both extracts revealed the presence of an unknown peak, eluting directly after MeSeCys. Identification of this peak could not be performed due to lack of commercially available selenium standards. It may be presumed that this peak is from γ-glutamyl-Se-methyl-selenocysteine, identified previously from Allium vegetables (Kotrebai et al., 1999, 2000).

3.3. Chiral speciation

The enantiomeric distribution of selenoamino acids in chive samples was also investigated. In this study, a Daicel Crownpak CR(+) crown ether column was used for separation of the enantiomers. As the chiral separation of amino acids on this type of column is strongly dependent on the temperature, the column was fitted with a thermostated jacket providing a constant temperature control during the separations. Chromatographic conditions were adopted from a previous study by Sutton et al. (2000).

A summary of the predictions and results for the enantiomeric separations at 22 °C for three selenoamino acids studied in the present study are depicted in Table 3. The number of peaks expected is 2_n where n is the number of chiral centers. The enantiomers observed also depend on whether one chiral center is the mirror image of the other. With a mirror image, one enantiomer from each of the chi-

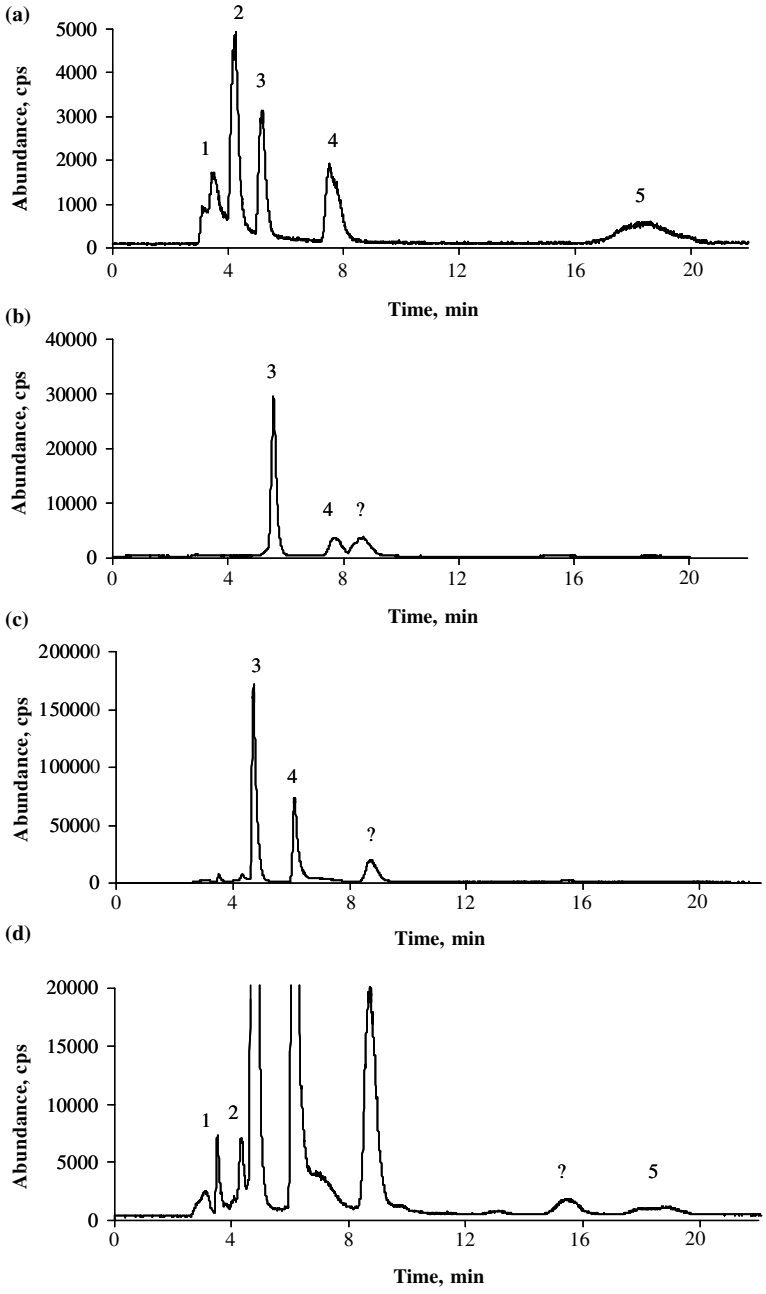

Fig. 3. RP-IP-HPLC-ICP–MS chromatogram of: (a) a mixture of selenium species containing 100 ng Se mL^{-1} separately. Peak: 1 = Se(VI); 2 = Se(IV); 3 = $SeCys_2$; 4 = MeSeCys; 5 = SeMet; (b) perchloric acid–ethanol extract of Se(IV)-enriched chive sample; (c) enzymatic extract of Se(IV)-enriched chive sample; and (d) magnified profile of chromatogram (c).

Table 3
Summary of the chiral properties and experimental results for three selenoamino acids under investigation

Compound	Number of chiral centers	Mirror images	Number of peaks	
			Expected	Obtained
$SeCys_2$	2	Yes	3	3
MeSeCys	1	No	2	2
SeMet	1	No	2	2

ral centers is equivalent to the other, giving the meso form, i.e., when such a molecule has 2 chiral centers, only 3 of the 4 enantiomers expected are seen in a mixture of D- and L-isomers (de Leon et al., 2000), this is the case when analyzing selenocystine.

In the case of chiral speciation the goal was only to identify the isomers rather than quantification. For this purpose standard addition was applied.

Fig. 4(a) represents the separation of a mixture of D,L-selenocystine, D,L-selenomethionine, and D,L-methyl-selenocysteine standard solution containing 250 ng Se mL^{-1} of each. As depicted in Table 3, seven peaks should be obtained when analyzing the racemic mixture of these three amino acids. The first peak in the chromatogram corresponds to inorganic selenium originating from the

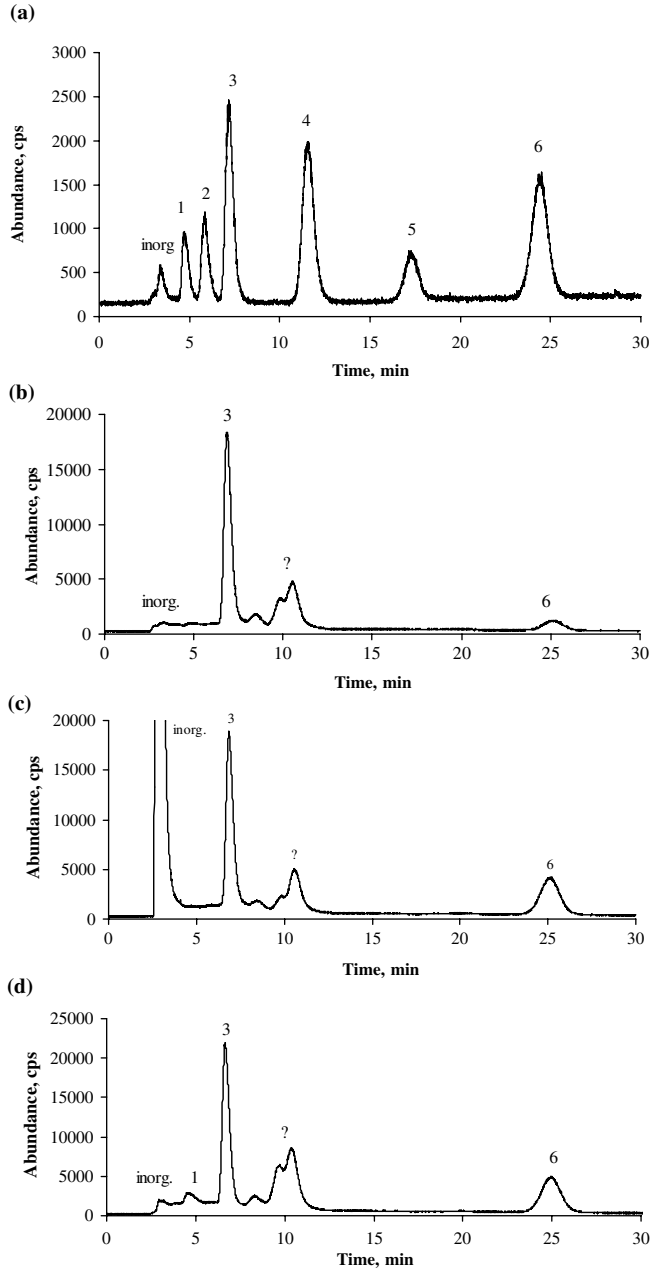

Fig. 4. Chiral separation of: (a) a mixture of D,L-selenocystine, D,L-selenomethionine, and D,L-methyl-selenocysteine standard solution containing 250 ng Se mL^{-1} separately. Peak: 1 = D-MeSeCys; 2 = D-$SeCys_2$; 3 = L-MeSeCys; 4 = meso-$SeCys_2$ and D-SeMet; 5 = L-$SeCys_2$; 6 = L-SeMet; (b) Se(IV)-enriched chive; (c) Se(VI)-enriched chive; and (d) SeMet- enriched chive.

contamination in the standards. The elution order of the various enantiomeric forms of the selenospecies is as follows: 1: D-MeSeCys ($t_{ret} = 4.45 \pm 0.02$ min); 2: D-$SeCys_2$ ($t_{ret} = 5.50 \pm 0.04$ min); 3: L-MeSeCys ($t_{ret} = 6.76 \pm 0.04$ -min); 4: meso-$SeCys_2$ and D-SeMet ($t_{ret} = 10.77 \pm 0.08$ -min); 5: $SeCys_2$ ($t_{ret} = 16.28 \pm 0.05$ min); 6: L-SeMet ($t_{ret} = 23.16 \pm 0.04$ min). The meso form of $SeCys_2$ could not be resolved from the D-SeMet in the present separation conditions. Both extracts were analyzed for the chiral separation of selenoamino acids present in the sample. Samples were appropriately diluted before injection onto the column.

The species found in Se-enriched chives when using chiral speciation were in agreement with the results derived from the RP-IP-HPLC speciation analysis. L-MeSeCys and L-SeMet and an unknown at $t_{ret} = 10.33$ are the three species found in the extracts irrespective of the enrichment types. The most predominant species is all three cases is L-MeSeCys. The unknown peak at $t_{ret} = 10.33$ may be corresponding to the enantiomeric form of unknown peak in the reversed-phase chromatogram. In all cases the first peak in the chromatograms corresponds to the inorganic selenium which has no retention on the crown ether column. When analyzing Se(VI)-enriched chive extract (Fig. 4(c)) a very high amount of inorganic selenium was detected. This agrees with the fact that a large amount of inorganic selenium is found when enriched with Se(VI). It is interesting to note that peak intensity of SeMet is much higher in all three enrichment cases when analyzed in crown ether column as compared to that obtained from reversed-phase column. This can be attributed to the fact that better column recovery of SeMet is obtained from crown ether column.

4. Conclusions

Three liquid chromatographic methods were employed for investigating selenium distribution in chives enriched with Se(IV), Se(VI), and SeMet. Total selenium measurements indicated that supplementation with Se(VI) resulted in the highest total selenium level in chive samples as compared to other two cases. However, the speciation studies revealed that approximately 30% of the total selenium was not metabolized in the sample and resulted in accumulation of selenium as inorganic seleniun. Therefore, this kind of fortification of chives could not likely be used for dietary purposes. In chives samples fortified with SeMet and Se(IV), high amounts of $SeCys_2$ and MeSeCys were found, especially in the case of SeMet enrichment. An unknown peak is seen in all three cases, which may be attributed to γ-glutamyl-Se-methyl-selenocysteine. Chiral speciation revealed the major enantiomers as L-MeSeCys and L-SeMet in all cases. The comprehensive analysis on the chives grown in three different media reveal that selenium distribution in different enantiomeric forms of amino acids in the sample, and strongly depends on the type of enrichment applied.

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References

Arthur, J. R., Nicol, F., & Beckett, G. J. (1990). Hepatic iodothyronine 5′-deiodinase. *Biochemical Journal, 272*, 537–540.

Arvy, M. P. (1993). Selenate and selenite uptake and translocation in bean plants (*Phaseolus vulgaris*). *Journal of Experimental Botany, 44*, 1083–1087.

Blanco-Gónzalez, E., Fernández Sánchez, M. L., & Sanz-Medel, A. (1998). Speciation of D,L-selenomethionine enantiomers on a β-cyclodextrin column with fluorimetric and on-line hydride generation inductively coupled plasma mass spectrometric detection. *Journal of Analytical Atomic Spectrometry, 13*, 893–898.

Combs, G. F., & Combs, S. B. (1986). *The role of selenium in nutrition*. New York: Academic Press.

Day, J. A., Kannamkumarath, S. S., Yanes, E. G., Montes-Bayon, M., & Caruso, J. A. (2002). Chiral speciation of Marfey's derivatized DL-selenomethionine using capillary electrophoresis with UV and ICP-MS detection. *Journal of Analytical Atomic Spectrometry, 17*, 27–31.

de Jong, N., Gibson, R. S., Thomson, C. D., Ferguson, E. F., McKenzie, J. E., Green, T. J., et al. (2001). Selenium and zinc status are suboptimal in a sample of older New Zealand women in a comunity-based study. *Journal of Nutrition, 131*, 2677–2684.

de Leon, C. A. P., Sutton, K. L., Caruso, J. A., & Uden, P. C. (2000). Chiral speciation of selenoamino acids and selenium enriched samples using HPLC coupled to ICP-MS. *Journal of Analytical Atomic Spectrometry, 15*, 1103–1107.

Fairweather-Tait, S. J. (1997). Bioavailability of selenium. *European Journal of Clinical Nutrition, 51*, S20–S23.

Garland, M., Morris, J. S., Stampfer, M. J., Colditz, G. A., Spate, V. L., Baskett, C. K., Rosner, B., Speizer, F. E., Willett, W. C., & Hunter, D. J. (1995). Prospective study of toenail selenium levels and cancer among women. *Journal of the National Cancer Institute, 87*, 497–505.

Hamilton, J. W. (1975). Chemical examination of seleniferous cabbage *Brassica oleracea* capitata. *Journal of Agricultural and Food Chemistry, 23*, 1150–1152.

Holben, D. H., & Smith, A. M. (1999). The diverse role of selenium within selenoproteins: a review. *Journal of the American Dietetic Association, 99*, 836–843.

Ip, C., Birringer, M., Block, E., Kotrebai, M., Tyson, J. F., Uden, P. C., et al. (2000). Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. *Journal of Agricultural and Food Chemistry, 48*, 2062–2070.

Kotrebai, M., Birringer, M., Tyson, J. F., Block, E., & Uden, P. C. (1999). Identification of the principal selenium compounds in selenium-enriched natural sample extracts by ion-pair liquid chromatography with inductively coupled plasma- and electrospray ionization-mass spectrometric detection. *Analytical Communications, 36*, 249–252.

Kotrebai, M., Birringer, M., Tyson, J. F., Block, E., & Uden, P. C. (2000). Selenium speciation in enriched and natural samples by HPLC- ICP-MS and HPLC-ESI-MS with perfluorinated carboxylic acid ion-pairing agents. *Analyst, 125*, 71–78.

Lintschinger, J., Fuchs, N., Moser, J., Kuehnelt, D., & Goessler, W. (2000). Selenium-enriched sprouts. A raw material for fortified cereal-based diets. *Journal of Agricultural and Food Chemistry, 48*, 5362–5368.

Maihara, V. A., Gonzaga, I. B., Silva, V. L., Favaro, D. I. T., Vasconcellos, M. B. A., & Cozzolino, S. M. F. (2004). Daily dietary selenium intake of selected Brazilian population groups. *Journal of Radioanalytical and Nuclear Chemistry, 259*, 465–468.

Montes-Bayon, M., B'Heimer, C., de Leon, C. A. P., & Caruso, J. (2001). Resolution of seleno-amino acid optical isomers using chiral derivatization and inductively coupled plasma mass spectrometric (ICP-MS) detection. *Journal of Analytical Atomic Spectrometry, 16*, 945–950.

Mounicou, S., Meija, J., & Caruso, J. (2004). Preliminary studies on selenium-containing proteins in *Brassica juncea* by size exclusion chromatography and fast protein liquid chromatography coupled to ICP-MS. *Analyst, 129*, 116–123.

Nakagawa, T., Mizunuma, H., Shibukawa, A., & Uno, T. (1981). Liquid-chromatography with crown ether-containing mobile phases.1. Capacity factors of amino-compounds on a hydrophobic stationary phase. *Journal of Chromatography, 211*, 1–13.

Nakagawa, T., Shibukawa, A., & Uno, T. (1982). Liquid-chromatography with crown ether-containing mobile phases.2. Retention behavior of beta-lactam antibiotics in reversed- phase high-performance liquid-chromatography. *Journal of Chromatography, 239*, 695–706.

Pérez Méndez, S., Blanco González, E., & Sanz Medel, A. (2000). Chiral speciation and determination of selenomethionine enantiomers in selenized yeast by HPLC-ICP-MS using a teicoplanin-based chiral stationary phase. *Journal of Analytical Atomic Spectrometry, 15*, 1109–1114.

Peters, H. L., Davis, A. C., & Jones, B. T. (2004). Enantiomeric separations of amino acids with inductively coupled plasma cabon emission detection. *Microchemical Journal, 76*, 85–89.

Roberge, M. T., Borgerding, A. J., & Finley, J. W. (2003). Speciation of selenium compounds from high selenium broccoli is affected by the extracting solution. *Journal of Agricultural and Food Chemistry, 51*, 4191–4197.

Rotruck, J. T., Ganther, H. E., Swanson, A. B., Hafeman, D. G., & Hoekstra, W. G. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science, 179*, 588–590.

Shah, M., Kannamkumarath, S. S., Wuilloud, J. C. A., Wuilloud, R. G., & Caruso, J. A. (2004). Identification and characterization of selenium species in enriched green onion (*Allium fistulosum*) by HPLC-ICP-MS and ESI-ITMS. *Journal of Analytical Atomic Spectrometry, 19*, 381–386.

Sloth, J. J., Larsen, E. H., & Julshamn, K. (2003). Determination of organoarsenic species in marine samples using gradient elution cation exchange HPLC-ICP-MS. *Journal of Analytical Atomic Spectrometry, 18*, 452–459.

Sutton, K. L., de Leon, C. A. P., Ackley, K. L., Sutton, R. M. C., Stalcup, A. M., & Caruso, J. (2000). Development of chiral HPLC for selenoamino acids with ICP-MS detection: application to selenium nutritional supplements. *The Analyst, 125*, 281–286.

Terry, N., Zayed, A. M., de Souza, M. P., & Tarun, A. S. (2000). Selenium in higher plants. *Annual review of plant physiology and plant molecular biology, 51*, 401–432.

Thomson, C. D. (2004). Selenium and iodione intakes and status in New Zealand and Australia. *British Journal of Nutrition, 91*, 661–672.

Tiran, B., Tiran, A., Petek, W., Rossipal, E., & Wawschinek, O. (1992). Selenium status of healthy-children and adults in Styria (Austria) - investigation of a possible undersupply in the Styrian population. *Trace Elements in Medicine, 9*, 75–79.

Vadhanavikit, S., Ip, C., & Ganther, H. E. (1993). Metabolites of sodium selenite and methylated selenium- compounds administered at cancer chemoprevention levels in the rat. *Xenobiotica, 23*, 731–745.

van den Brandt, P. A., Goldbohm, R. A., van't Veer, P., Bode, P., Dorant, E., Hermus, R. J., & Sturmans, F. (1993). A prospective cohort study of toenail selenium levels and risk of gastrointestinal cancer. *Journal of the National Cancer Institute, 85*, 224–229.

Whanger, P. D., Ip, C., Polan, C. E., Uden, P. C., & Welbaum, G. (2000). Tumorgenesis, metabolism, speciation, bioavailability, and tissue deposition of selenium in selenium-enriched ramps (*Allium tricoccum*). *Journal of Agricultural and Food Chemistry, 48*, 5723–5730.

Wrobel, K., Kannamkumarath, S. S., Wrobel, K., & Caruso, J. A. (2003). Hydrolysis of proteins with methanesulfonic acid for improved HPLC-ICP-MS determination of seleno-methionine in yeast and nuts. *Analytical and Bioanalytical Chemistry, 375*, 133–138.

Wrobel, K., Wrobel, K., Kannamkumarath, S. S., Caruso, J. A., Wysocka, I. A., Bulska, E., Swiatek, J., & Wierbicka, M. (2004). HPLC-ICP-MS speciation of selenium in enriched onion leaves a potential dietary source of Se-methylselenocysteine. *Food Chemistry, 86*, 617–623.

Zayed, A., Lytle, C., & Terry, M. N. (1998). Accumulation and volatilization of different chemical species of selenium by plants. *Planta, 206*, 284–292.