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HPLC–ICP-MS speciation of selenium in enriched onion leaves – a potential dietary source of Se-methylselenocysteine

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Abstract

In this work chromatographic separation was coupled with mass spectrometry detection to study the speciation of selenium in selenium enriched onion leaves. The plants of onion (*Allium cepa* L.) were grown hydroponically for one week in standard medium containing inorganic selenium. The leaves and bulbs were separated from roots, the two types of material (leaves and bulbs) were dried and homogenized. The sodium hydroxide (0.1 mol l^{-1}) extracts were analyzed by size exclusion chromatography (with spectrophotometric and mass spectrometric detection) showing the incorporation of selenium to the high molecular weight fraction, which was more pronounced in the leaves relative to the bulbs. This incorporation was better with Se(IV) enrichment (33% in leaves and 26% in bulbs) than with Se(VI) (3% and 5%, respectively). After extraction of the low molecular weight compounds (chloroform–methanol–water or 0.4 M perchloric acid–ethanol) and elimination of inorganic selenium (Dowex 1X8), selenium speciation was carried out by ion-pairing high performance chromatography [5 mmol l^{-1} citric acid, 5 mmol l^{-1} hexanesulfonic acid, pH 4.5: methanol (95:5)]. The primary organic selenium species found in leaves extracts was Se-methylselenocysteine, which has been reported as one of the most active chemopreventive selenium species as found in cell and animal experiments. The percentage contribution of selenium in this species was 4.0% and 1.9%, respectively, of the total selenium in leaves enriched with Se(IV) and Se(VI). It may be possible to use selenium enriched leaves as a dietary source of Se-methylselenocysteine. The important advantage of this source is the simple and fast enrichment process.
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Keywords: Analytical speciation; Selenium enriched onion leaves; Se-methylselenocysteine

1. Introduction

The anticarcinogenic potential of different dietary products rich in selenium has been observed in various epidemiological and laboratory studies (Clark et al., 1996; Ip, 1998; Ip & Lisk, 1994a; Ip & Lisk, 1994b; Irion, 1999). To elucidate which selenium forms are responsible for the suggested cancer-preventive effects, analytical speciation studies were carried out in selenium-enriched yeasts and in different vegetables from Allium family (Ip et al., 2000; Kotrebai, Bird, Tyson, Block, & Uden, 1999a; Kotrebai, Birringer, Tyson, Block, & Uden, 1999b; Kotrebai, Birringer, Tyson, Block, & Uden, 2000; Whanger, Ip, Polan, Uden, & Welbaum, 2000).

In parallel, the biological activity of enriched products was tested on rats with both chemically and virally induced tumors (Combs & Gray, 1998; Ip et al., 2000; Whanger et al., 2000). It was observed that selenium enriched yeast were considerably less active in tumor prevention relative to enriched garlic. Moreover, the ingestion of yeast caused an undesirable accumulation

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of selenium in tissues, more pronounced than that observed after consuming Se-enriched garlic (*Allium sativum*), onion (*Allium cepa*) or ramps (*Allium tricocum*) (Ip & Lisk, 1994a; Whanger et al., 2000).

The primary species found in yeast enzymatic digests was Se-methionine (Bird, Uden, Tyson, Block, & Denoyer, 1997; B'Hymer & Caruso, 2000; Kotrebai et al., 1999b) and it has been assumed that this species may be a key link in understanding the proposed cancer-preventive properties of yeast.

On the other hand, the protective effect of vegetables from Allium family against stomach, colorectal and mammary cancers has been attributed to the presence of organosulfur compounds and mainly allyl derivatives that inhibit carcinogenesis in the forestomach, esophagus, colon, mammary glands, and lungs of experimental animals (Bianchini & Vainio, 2001). Enhanced anticarcinogenic activity was observed for Se-enriched garlic (Ip, Lisk, & Stoewsand, 1992), in which few selenium analogs of organosulfur compounds were identified (Ip et al., 2000).

Depending on the type of vegetable, on the total selenium level after enrichment and on the analytical procedure applied, Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine were the two primary species reported (Ip et al., 2000; Kotrebai et al., 2000; Whanger et al., 2000). The speciation results obtained in Se-enriched garlic indicated that the samples containing <300 μg g^{-1} Se produced the γ-glutamyl-Se-methylselenocysteine as the main species and that the contribution of Se-methylselenocysteine increased with the increasing total selenium content (Ip et al., 2000). The authors assumed that the former species serves as a carrier of Se-methylselenocysteine, which is further metabolized to methylselenol by the action of β-lyase enzyme. It was also suggested that the endogenous production of monomethylated selenium could be a critical factor in selenium chemoprevention (Ip et al., 2000).

In more recent studies, different Se-alk(en)ylselenocysteines and their γ-glutamyl derivatives were synthesized and their biological activity was tested (Block et al., 2001). The highest chemopreventive activity was observed for Se-methylselenocysteine and Se-2-propenyl-L-selenocysteine, in agreement with the results reported for the respective organosulfur compounds (Fukushima, Takada, Hori, & Wanibuchi, 1997). Studies carried out with other selenium enriched vegetables (broccoli, onion, ramp) revealed the presence of essentially these same selenium species, although selenium uptake was lower and the biological effects less pronounced (Finley, Davis, & Feng, 2000; Ip & Lisk, 1994b; Irion, 1999; Kotrebai et al., 1999b).

In the case of vegetables from Allium family, the research interests have been selectively focused on bulbs and there have been no data reported on selenium speciation in the leaves. However, in various geographical regions, people consume the green tops of regular onion, so called "green onions" or "chives". In the present work, the incorporation of selenium to onion leaves was studied and speciation analysis in the leaves extracts was carried out by high performance liquid chromatography (HPLC) coupled with mass spectrometric (MS) detection. The finding that Se-methylselenocysteine is the principal organoselenium species in leaves extracts suggests the consideration of dried and powdered green shoots as an alternative for Se supplementation with the additional advantage of leaves as an easy and fast process of growing/enrichment.

2. Materials and methods

2.1. Instrumentation

High performance liquid chromatography (HPLC) used an Agilent Technologies (Palo Alto, CA, USA) series 1100 instrument equipped with an autosampler, a diode-array detector and Chemstation. The chromatographic columns were C8 Alltima (150 × 4.6 mm, 5 μm particle) and Superdex Peptide HR 10/30 (Pharmacia Biotech).

An Agilent 7500s inductively coupled plasma mass spectrometer (ICP-MS) connected to a concentric nebulizer and Scott-type double-pass spray chamber was used for selenium-specific detection in effluent after chromatographic separation. The column effluent was introduced on-line to ICP-MS. An Elan 9000 inductively coupled plasma mass spectrometer (Perkin Elmer Sciex, Canada) equipped with Meinhard nebulizer and Scott-type double-pass spray chamber made from Ryton was used for the determination of total selenium. The instrumental operation conditions are given in Table 1.

A model RC5C centrifuge (Sorvall Instruments, DuPont) and rotavap RE 111 (Buchi Laboratoriums Technik AG, Schwitzerland) were also used.

Anton Paar Multiwave Sample Preparation System (Austria) was used for acid digestion of the sample. The mineralization program is given in Table 2.

2.2. Reagents

Analytical reagent grade chemicals and HPLC-grade methanol, ethanol and chloroform (Fisher Scientific, Pittsburgh, PA, USA) were used. Double deionized water (18.2 MΩ cm), NanoPure treatment system (Barnstead, Boston, MA, USA) was used throughout.

L-selenomethionine, selenocystine, sodium selenite and sodium selenate were purchased from Aldrich (Milwaukee, WI, USA), Se-methylselenocysteine was from Sigma (St. Louis, MO, USA). The stock solutions containing 1 mg ml^{-1} selenium compound were pre-

Table 1
Instrumental operating conditions for HPLC–ICP-MS

SEC parameters		
Column	Superdex peptide HR 10/30	
Mobile phase	CAPS 10 mmol l^{-1}, pH 10.0	
Flow rate	0.6 ml min^{-1}	
Volume injected	100 μl	
UV detection (λ)	280 nm	
Ion-pairing HPLC parameters		
Column	Alltima C8, 150 × 4.6 mm, 5 μm	
Mobile phase	(5 mmol l^{-1} citric acid, 5 mmol l^{-1} hexanesulfonic acid, pH 4.5): methanol (95:5)	
Flow rate	0.9 ml min^{-1}	
Volume injected	50 μl	
ICP-MS parameters	Agilent 7500 s	Elan 9000
Forward power	1300 W	1000 W
Nebulizer gas flow	1.07 l min^{-1}	0.92 l min^{-1}
Dwell time	0.1 s per isotope	0.1 s per isotope
Isotopes monitored	^{77}Se, ^{82}Se	^{77}Se, ^{78}Se

Table 2
Mineralization program used for the microwave digestion of onion

Step	Time (min)	Power (W)
1	5	100
2	5	600
3	10	1000
4	15	cooling

pared in 10 mmol l^{-1} hydrochloric acid (Sigma) and were stored frozen. Working solutions were prepared daily by appropriate dilution.

For the determination of total selenium the working solutions were prepared daily by appropriate dilution of standard solution of selenium 1 mg ml^{-1} (Se(IV), Merck, Germany). Nitric acid 65% (Suprapure) was from Merck (Germany), perhydrol 30% from POCH (Poland) and deionized water from Milli-Q system (Millipore, USA, 18 MΩ).

The chromatographic mobile phases (Table 1) were prepared from Sigma reagents. Solutions of the following Sigma reagents were used: sodium hydroxide, perchloric acid, acetic acid, myoglobin, lysozyme, substance *P* and $(Gly)_6$.

2.3. Plant growth and samples

The experiment was conducted on onion (*Allium cepa* var. Blonska). The onion bulbs were prepared for the experiments according to the method described by Wierzbicka (Wierzbicka, 1987) in order to stimulate the growth of plants. The bulbs were hydroponically grown in black containers of 320 ml at room temperature, in aerated 1/8 Knopp nutrient solution (Wierzbicka & Antosiewicz, 1988). When the root reached a length of 2–3 cm, the experimental onions were transferred to 5 mg l^{-1} selenium in the form of selenite or selenate, to add to nutrient solution. Pools of the onion were left in the nutrient solution as controls.

The plants were incubated in the selenium-containing medium for 8 days. During the incubation with selenium, the length of the roots and leaves of each plant was measured every 24 h using a millimeter ruler (Wierzbicka, 1999). In treatment combination 12 plants were cultivated. In total, 36 plants were grown. After incubation, onion plants were rinsed with tap water and distilled water and were separated into roots, bulbs and leaves.

The onion parts were dried in the oven during 48 h at a temperature of 50–60 °C. After drying all parts of onion were homogenized.

2.4. Total selenium determination

For the determinations of total selenium by ICP-MS the microwave digestion was employed (see Table 2). Approximately 0.3 g of dried onion (leaves or bulbs) was digested by using 3 ml HNO_3 (65%) and 1 ml H_2O_2 (30%). The end-solutions were diluted with distilled water up to 25 ml. The standard solution and reagent blanks were digested in the same way. The total selenium concentration was directly determined by ICP-MS. Two isotopes ^{77}Se and ^{78}Se were used for the measurements (Table 1).

2.5. Extraction procedures

About 0.1 g of dried and powdered leaves or bulbs was weighted precisely in the plastic tubes and the extraction was carried out with 3 ml of sodium hydroxide (0.1 mol l^{-1}). The samples were centrifuged (10 min, 5000 rpm) and the supernatant (100 μl) was introduced to the SEC-ICP-MS system (Table 1).

The method of Lancaster and Kelly (1983) was used for the extraction of low molecular weight compounds from leaves. A volume of 10 ml of the mixture methanol:chloroform:water (12:5:3) was used for 0.1 g of the dried sample, the extraction was repeated three times, the liquid phases were combined and evaporated (rotavapor). For dissolution, 10 ml of chloroform and 10 ml of water were added. After separation of phases, the aqueous phase was collected and evaporated. The residue was dissolved in water (1 ml), the anionic species were eliminated by ion exchange on Dowex 1X8 (acetate form) and the final solution was analyzed by ion-pairing HPLC–ICP-MS (Table 1).

Alternatively, the extraction of low molecular weight species was carried out with 1.2 ml of 0.4 mol l^{-1} perchloric acid:ethanol (8:2). The sample was then centrifuged (10 min, 5000 rpm), 10 times diluted with deionized water and introduced to the ion-pairing HPLC–ICP-MS system (Table 1).

2.6. Calibration of size exclusion column

The conditions of chromatographic separation are given in Table 1. Calibration of the SEC column was accomplished with a standard mixture of myoglobin (17 kDa), lysozyme (14.4 kDa), substance *P* (1.35 kDa) and $(Gly)_6$ (0.36 kDa), showing in this range of a good linear response for the $\log_{10}$ of molecular weight vs. retention time ($r^2 = 0.9914$).

2.7. Ion-pairing HPLC separation

The column type, the composition of mobile phase and the elution conditions are listed in Table 1 (Kannamkumarath, Wrobel, Wrobel, Vonderheide, & Caruso, 2002). The assignment of chromatographic peaks in the vegetable extracts was accomplished by matching the retention times with those of commercial selenium standards and by spiking experiments. To do so, the sample was mixed with the standard solution (containing four species at the concentrations corresponding to 200 μg l^{-1} of selenium) in relation 4:1. The concentrations of Se-methylselenocysteine, Se-methionine and Se-cystine/Se-cysteine were estimated based on the results of spiking experiments.

3. Results and discussion

The plants were grown hydroponically using the standard medium that contained 5 mg l^{-1} selenium in the form of selenite (Se(IV)) or selenate (Se(VI)). During the experiment (8 days) a millimeter scale was used to measure the length of the roots and leaves of each onion. In the presence of the each form of selenium, the growth of leaves was inhibited about 50% with respect to the control plants. Root length measurements proved that selenium present in the growing medium caused inhibition of the root growth, the more pronounced effect was observed in the presence of Se(IV) (roots about 25% shorter than in the presence of Se(VI)) (Fig. 1).

After one week, the roots and bulbs were separated from leaves and both were dried and powdered. For the analysis of total selenium, the samples were digested and the quantification was carried out by ICP-MS (Tables 1 and 2). As can be seen in Table 3, higher selenium levels were found in leaves (601 ± 7 and 154 ± 6 mg kg^{-1} in the samples enriched with Se(VI) and Se(IV)) as compared to bulbs (51.3 ± 1.8 and 15.6 ± 0.7 mg kg^{-1}), which is in agreement with data reported elsewhere (Whanger et al., 2000). In the first approach to selenium speciation, the dried samples were treated with sodium hydroxide for protein solubilization. The obtained extracts were analyzed by size exclusion chromatography (SEC) with a tandem spectrophotometric (UV) and ICP-MS detection. Calibration of the SEC column is described in the previous section. The chromatograms obtained for leaves samples are presented in Fig. 2(a) and (b). As can be observed in Fig. 2(a), different UV (280 nm) elution profiles were obtained for the samples grown in the presence of two selenium forms. A higher contribution of high molecular weight compounds (apparent MW > 10 kDa) was observed in the sample enriched with Se(IV) as compared to the sample grown with Se(VI). In the case of Se(VI), the elution profile was more complex in the region of apparent MW < 10 kDa. This possibly could be due to the activation of lyzosomal enzymes caused by the excess of Se(VI), resulting in protein degradation (oxidative shock). In the case of ICP-MS chromatogram (Fig. 2(b)), two broad selenium peaks can be observed in the elution region of compounds with apparent molecular weight higher and lower than 10 kDa. The retention time of the first peak matched that observed with UV detection (280 nm), which indicates that a fraction of selenium was bound to proteins (apparent MW > 10

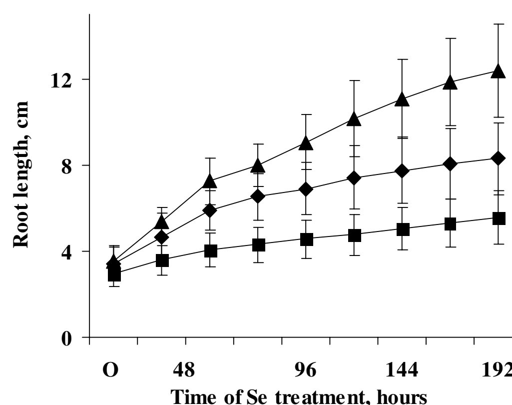

Fig. 1. Root growth of Allium cepa during 8 days of treatment with Se(IV) and Se(VI) (average ± SD). (▲) control; (◆) nutrient with Se(VI); (■) nutrient with Se(IV).

Table 3
Total selenium content in dried samples and SEC-ICP-MS results obtained in sodium hydroxide extracts

Sample	Se form used for enrichment	Se in dried samples ± SD ($\mu g\ g^{-1}$)	Se in alkaline extract ± SD ($mg\ l^{-1}$)	% of total Se in HMW fraction
Leaves	Se(IV)	154 ± 6	1.30 ± 0.12	33
	Se(VI)	601 ± 7	13.0 ± 0.2	3
Bulb	Se(IV)	15.6 ± 0.7	0.31 ± 0.05	26
	Se(VI)	51.3 ± 1.8	2.83 ± 0.31	5

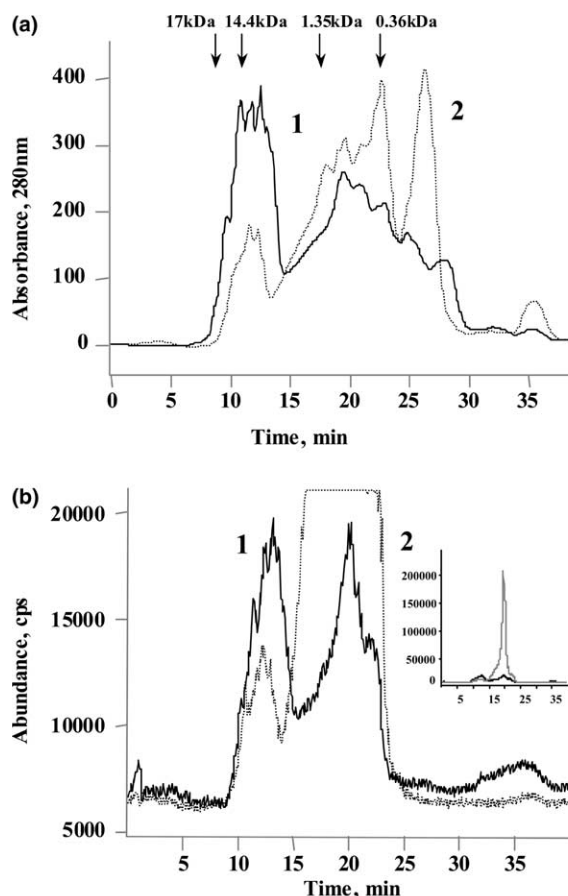

Fig. 2. Typical size exclusion chromatograms of sodium hydroxide extracts of dried onion leaves grown in the presence of Se(IV) (1) and in the presence of Se(VI) (2). (In the upper part the elution of molecular weight standards is marked.) (a) Spectrophotometric detection (280 nm). (b) ICP-MS detection for ^{82}Se (the insert shows the full-scale chromatogram).

kDa). Moreover, higher presence of selenium in HMW compounds was observed in the Se(IV) enriched sample. The second selenium peak corresponded to the elution of low molecular weight compounds, including two forms of inorganic selenium (Se(IV) and Se(VI)). Similar selenium elution profiles were obtained in the analysis of bulb samples, however the percentage contribution of the element in the low and high molecular weight fractions was different. The incorporation of inorganic selenium to HMW compounds (MW > 10 kDa) was estimated based on the peak area measurements. The obtained results are presented in Table 3, where better incorporation can be observed for the samples enriched with Se(IV) (33% in leaves and 26% in bulbs) than in those containing Se(VI) (3% and 5%, respectively).

The two common procedures used for the speciation of organic selenium forms in vegetables from Allium family have been hot water extraction and enzymatic digestion (Ip et al., 2000; Uden et al., 1998; Whanger et al., 2000). The results presented above indicated that only a fraction of total selenium in leaves was bound to proteins. Our interest was focused on the identification of low molecular weight selenium compounds that could be responsible for the suggested cancer-preventive properties of onion shoots (Block et al., 2001; Ip et al., 2000). Thus, in the preliminary experiments hot water extractions were carried out. However, due to the high content of polysaccharides, a high viscosity extract hindered its direct introduction to the chromatographic system. To avoid this effect and to assure better selectivity of extraction, a procedure proposed for the analysis of S-alk(en)yl-L-cysteine sulphoxides in onion was adopted (Lancaster & Kelly, 1983). The anionic species were removed from the extract by ion exchange on Dowex 1X8. The separation of selenium species was carried out by ion-pairing HPLC with hexanesulfonate (Kannamkumarath et al., 2002). In Fig. 3(a), typical chromatogram of mixed selenium standards is presented with the following order of elution: Se(IV), $t_{ret} = 72.0 \pm 0.2$ s; Se-cystine, $t_{ret} = 113.1 \pm 0.5$ s; Se-methylselenocysteine, $t_{ret} = 150.4 \pm 0.8$ s; Se-methionine, $t_{ret} = 229.6 \pm 0.6$ s. The ICP-MS chromatogram of shoots grown in the presence of Se(IV) is shown in Fig. 3(b). The assignment of chromatographic peaks was accomplished by matching the retention times with those of commercial selenium standards and by spiking experiments. The primary species identified in extract was Se-methylselenocysteine, in addition Se-methionine and Se-cystine were found at considerably lower levels. The small elution peak, not resolved from the later species corresponded to Se-cysteine. Because of the easy conversion between Se-cystine and Se-cysteine during sample handling, in further text these are referred to as a couple Se-cystine/Se-cysteine. The elution of at least four unidentified selenium species can be observed on the chromatogram with the retention times 90.4 s, 98.4 s, 170.7 s, 262.0 s, respectively. With the selenium standards available it was concluded that none of these peaks corresponded to Se(VI) (incomplete separation

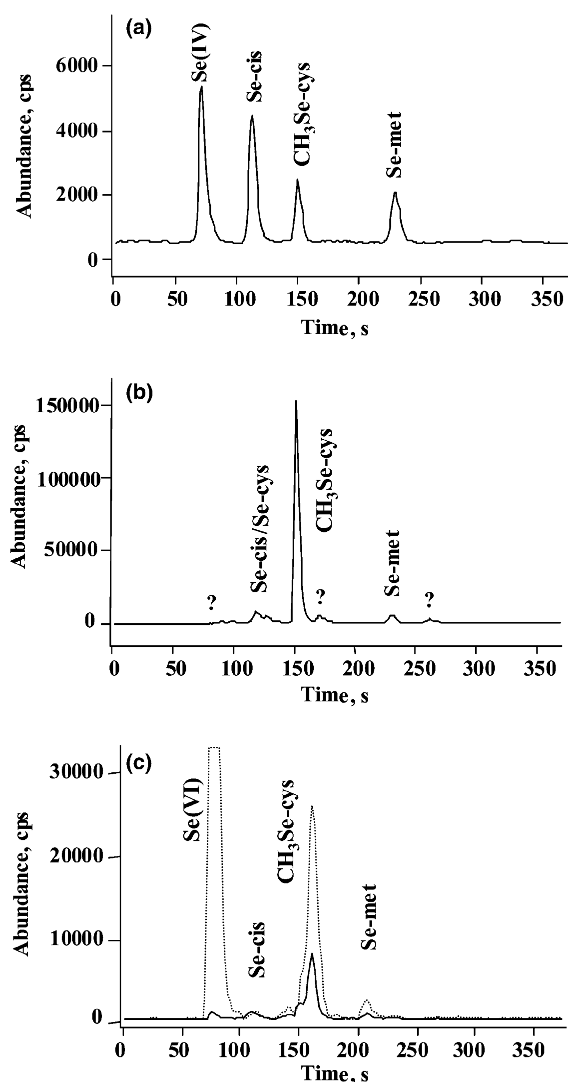

Fig. 3. ICP-MS chromatograms of selenium (^{82}Se) species. (a) Mixed standard solution: Se(IV), 50 μg l^{-1}; Se-cystine, 100 μg l^{-1}; Se-methylselenocysteine, 100 μg l^{-1}; Se-methionine, 50 μg l^{-1}. (b) Methanol:chloroform:water extract of leaves grown in the presence of Se(IV). (c) Perchloric acid:ethanol extract of Se-enriched leaves: (–) grown with Se(IV) and (- - -) with Se(IV).

from Se(IV)), trimethylselenium ($t_{ret} = 266.7$ s) or Se-methylselenomethionine ($t_{ret} = 323.8$ s). According to the reports on selenium speciation in onion bulbs, the unknown peaks with higher retention times could be attributed to the presence of Se-containing γ-glutamyl peptides (Kotrebai et al., 1999a, 1999b, 2000; Uden et al., 1998).

Currently, experiments are being carried out for the identification of these species, after elimination of inorganic selenium and Se-methylselenocysteine from the extract. To confirm the presence of Se-methylselenocysteine, Se-cystine or Se-cysteine and Se-methionine in leaves, the second extraction procedure was applied (see procedures). Perchloric acid was used for the elimination of proteins and ethanol was added to minimize solubility of polysaccharides. Under these extraction and chromatographic conditions, the elution of Se(IV) was not observed, in agreement with previous reports (Wrobel, Wrobel, & Caruso, 2002). The extracts obtained from leaves grown in the presence of Se(IV) and Se(VI) were introduced to the ion-paring HPLC–ICP-MS system and typical chromatograms are presented in Fig. 3(c). The elution peaks with retention times 82.2 s, 111.0 s, 140.6 s and 208.7 were identified by standard addition (described in Materials and methods) as Se(VI), Se-cystine, Se-methylselenocysteine and Se-methionine, respectively. The retention times changed with respect to those observed in Fig. 3(a) because of different pH of the two extracts analyzed, in both cases the identity of species was confirmed by standard addition. The presence of Se-methylselenocysteine and Se-methionine was also observed in the hot water extract from onion bulbs (Cai, Block, Uden, Zhang, Quimby, & Sullivan, 1995; Uden et al., 1998). Where the enzymatic hydrolysis was carried out (Kotrebai et al., 2000), the main species found in bulbs was γ-glutamyl-Se-methylselenocysteine. On the other hand, it was suggested that the relative contribution of Se-methylselenocysteine and γ-glutamyl selenopeptides in Se-enriched garlic depends on the total amount of the element in the enriched vegetable from Allium family. The results obtained in this work indicate that selenium incorporates into the leaves producing essentially these same primary organic species as observed in bulbs.

The percentage of selenium found in the form of Se-methylselenocysteine, Se-methionine and Se-cystine/Se-cysteine with respect to total selenium in the dried samples was estimated based on the results of spiking experiments. For the leaves enriched with Se(IV) and Se(VI), respectively, 4.0% and 1.9% of total selenium was in the form of Se-methylselenocysteine, 0.3% and 0.2% in the form of Se-methionine, 0.5% and 0.1% in the form of a couple Se-cystine/Se-cysteine. These results clearly indicate the more effective incorporation of Se(IV) to organic species in leaves. It should be mentioned, however, that the utter content of each species was higher in leaves extract enriched with Se(VI) than in that enriched with Se(IV).

4. Conclusions

The chromatographic separation was coupled with ICP-MS detection to study the speciation of selenium in onion leaves grown in the presence of Se(VI) or Se(IV). In SEC experiments, more effective incorporation of element into high molecular weight compounds (apparent MW > 10 kDa) was observed in leaves with respect to bulbs. The ion-pairing HPLC–ICP-MS results allowed for identification of Se-methylselenocysteine as the primary species in the leaves extracts. Better incorporation of selenium to organic species was observed for

the plants grown in the presence of Se(IV). In this case, about 4.0% of total selenium in leaves was present as Se-methylselenocysteine. Considerably lower levels of Se-methionine, of a couple Se-cysteine/Se-cystine and few unidentified species were also found. It may be possible that onion leaves could be a valuable dietary source of Se-methylselenocysteine, the species associated with cancer-preventive properties. Further studies are in progress for identification of unknown selenium species and for determining the most appropriate enrichment procedure.

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