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Selenium Species in Aqueous Extracts of Alfalfa Sprouts by Two-Dimensional Liquid Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry and Electrospray Mass Spectrometry Detection

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The complementary use of two different liquid chromatographic mechanisms coupled to inductively coupled plasma mass spectrometry (ICP-MS) for selenium (Se) specific detection has permitted the screening of the most abundant Se-containing fractions in selenized alfalfa sprouts (*Medicago sativa*). Aqueous extracts of the sprouts were fractionated first by size exclusion chromatography (SEC) using a Superdex Peptide column and a mobile phase containing an ammonium acetate buffer (pH 7). Further purification of the individual SEC Se-containing fractions was carried out using two different chromatographic systems: a Shodex Ashaipack column, with a mixed mechanism of size exclusion and ion exchange, and a conventional reversed phase C_8 using ion-pairing reagents. In both cases, the columns were coupled to an inductively coupled plasma mass spectrometer equipped with an octapole reaction system for Se specific detection. This system allowed the on-line monitoring of the most abundant Se isotopes (^{78}Se, ^{80}Se) by reducing the possible polytomic interferences affecting these ions by adding hydrogen (2 mL min^{-1}) to the octapole reaction cell. The results obtained by both separation mechanisms were highly comparable, revealing the presence of Se-methionine and Se-methyl selenocysteine. Both compounds were then confirmed by analyzing the corresponding fractions by electrospray quadrupole-time-of-flight (ESI-Q-TOF) mass spectrometry. Finally, an additional Se-containing species showing Se isotope distribution was detected at a molecular ion *m*/*z* 239 in the ESI-Q-TOF. The collision-induced dissociation of the *m*/*z* 239 and 237 ions (corresponding to ^{80}Se and ^{78}Se isotopes, respectively) revealed the possible presence as well of a derivative of the Se-2-propenyl selenocysteine.

KEYWORDS: Selenium; alfalfa sprouts; HPLC-ICP-MS; ESI-Q-TOF

INTRODUCTION

The recognition of selenium in the prevention and treatment of various diseases, including cancer, has resulted in an increasing popularity of selenium supplementations (*1*, *2*). Consumption of Se-enriched food and feed supplements has grown tremendously in the last years as a result of the numerous health benefits reported in the literature. Several industrial companies are now involved in the production and marketing of such nutritional Se supplements. Considering the ability of some plants to accumulate and transform selenium into bioactive compounds, it follows that the use of Se-enriched or selenized plant species can have important implications in human nutrition and health (*2*). However, the bioavailability but also the toxicity of selenium are closely correlated to the chemical form ingested; therefore, selenium species characterization in food and food supplements has become necessary. In this regard, organic selenium species [selenomethionine (SeMet), selenocysteine, and Se-methyl selenocysteine (SeMC), etc.] have shown more bioavailability than inorganic species for humans (*3*, *4*). However, Whanger et al. (*5*) have shown that Se-Met tends to accumulate in tissues; thus, it is desirable to find effective Se sources that exhibit high anticarcinogenic activity and low Se tissue accumulation. In this regard, SeMC is more bioactive but not so easily accumulated in tissues. This is of great nutritional interest since the primary forms of Se found in selenium enriched vegetables from the *Allium* family (garlic and onions) and from the *Brassicaceae* family (broccoli and Indian mustard) are SeMC and derivatives such as γ-glutamyl-Se-methylselenocysteine (*5*−*7*).

Several studies on the speciation of Se plants have been reported in the literature regarding garlic (*8*, *9*), onion (*6*), Indian mustard (*7*), and fungi such as mushrooms (*10*). However, the

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literature concerning sprouts is rather limited. Sprouts are one of the most complete and nutritionally beneficial of all foods providing minerals, vitamins, and enzymes. Their nutritional value was discovered by the Chinese thousands of years ago. Recently, numerous scientific studies suggest the importance of alfalfa sprouts (*Medicago sativa*) for a healthy diet since they contain about 3.78% carbohydrate, 3.99% protein, and 0.69% total lipid (fat), with the rest consisting of minerals and vitamins (*11*). Edible alfalfa sprouts are a good source of vitamins A, B_1, B_2, B_6, and C and folic acid as well as many mineral elements, e.g., Fe, P, Zn, Mg, and Ca (*12*). Additionally, sprouts are known to be selenium accumulators (*13*) and it is known that this plant species is able to accumulate selenium from different inorganic sources (e.g., selenite) and/or organic ones (e.g., SeMet and Se-methylselenocysteine).

Inductively coupled plasma mass spectrometry (ICP-MS) is an attractive detector for selenium speciation due to its sensitivity for Se detection and the specificity when coupled to different separation methods [e.g., high-performance liquid chromatography (HPLC), gas chromatography (GC), etc.] (*14*−*16*). Different types of liquid chromatographic separations including size exclusion and ion pair reversed phase chromatography have been successfully applied for separating selenium species in different biological samples (*17*−*19*). The first screening of the Se species is classically performed by HPLC-ICP-MS and comparing the retention times observed in samples and those for commercially available or chemically synthesized standards. However, for many organo- and bioselenium compounds, no standards are available. Hence, the use of complementary molecular characterization techniques, such as electrospray mass spectrometry (ESI-MS), is more and more in demand (*20*−*22*).

In the present work, the distribution of selenium in alfalfa sprout is studied by means of a two-dimensional (2D) HPLC separation followed by ICP-MS and then by electrospray quadrupole-time-of-flight (ESI-Q-TOF) detection. The sprout sample was extracted in water and then fractionated by preparative size exclusion chromatography (SEC). The chromatographic purity of the resulting fractions was verified by reversed phase chromatography (using an ion-pairing reagent) and also by a combined mechanism of ion exchange/size exclusion with ICP-MS detection. Then, the observed major selenium compounds were characterized by ESI-MS.

MATERIALS AND METHODS

Instrumentation. Chromatographic separations were carried out with a liquid chromatograph pump (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) equipped with a sample injection valve Rheodyne, model 7125 (Cotati, CA). The ICP-MS detector was an Agilent 7500c (Agilent Technologies, Tokyo, Japan). The ICP-MS was equipped with an octopole reaction cell and can be operated with or without the collision/reaction gas. Conventional Meinhard and PFA micronebulizer (Agilent microflow nebulizer, 100 μL min^{-1}) were used as nebulization devices for the SEC/ion exchange and the C_8 column, respectively. Collision cell conditions were optimized using H_2 as a collision gas, and a final flow of 2 mL min^{-1} was used for monitoring ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se isotopes.

For SEC, a Superdex Peptide HR10/30 (10 mm × 300 mm × 13 mm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used. The other columns were a C_8 (250 mm × 2.0 mm i.d., 5 μm) (Alltima, Alltech Associates, Deerfield, IL) and a Shodex Asahipak GS-220 HQ (300 mm × 7.6 mm i.d.) with an exclusion size >3000 Da (Showa Deko, Tokyo, Japan), respectively. Both HPLC columns were connected to the ICP-MS nebulizer with PEEK tubing (30 cm × 0.25 mm i.d.). Chromatographic conditions are summarized in **Table 1**.

A Heraeus refrigerated ultracentrifuge (Kendro Instruments, Hanau, Germany) was used for the separation of the supernatant after extraction of Se species from the solid plant. The eluates were afterward concentrated by freeze drying (LYOLAB 3000, Heto-Holten A/S, Allerod, Denmark).

Table 1. Chromatographic Conditions and ICP-MS Parameters

ICP-MS parameters	
forward power	1500 W
plasma gas flow rate	15.0 L min^{-1}
carrier gas flow rate	1.13 L min^{-1}
dwell time	0.1 s per isotope
isotopes monitored	^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se
collision gas	2.5 mL H_2 min^{-1}
quadrupole bias	−13 V
octopole bias	−15 V
size exclusion chromatographic parameters	
column	Superdex Peptide HR 10/30
separation range	100–7000 Da
mobile phase	30 mM ammonium–acetate buffer, pH 7.4
flow rate	0.4 mL min^{-1}
injection volume	100 μL
Shodex Asahipack chromatographic parameters	
column	Shodex Asahipack GS-220 HQ (300 × 7.6 mm)
separation range	>3000 Da
mobile phase	10 mM ammonium–acetate buffer, pH 6.5
flow rate	0.6 mL min^{-1}
injection volume	50 μL
RPIP chromatographic parameters	
column	Alltima C_8 (250 mm × 4.6 mm, 5 μm)
mobile phase	0.2% (v/v) heptafluorobutyric acid (HFBA), 10% (v/v) methanol
flow rate	0.2 mL min^{-1}
injection volume	20 μL

The ESI-Q-TOF instrument used for this study was a QStar XL model (Applied Biosystems) equipped with the ion spray source working in positive mode and using N_2 as the nebulization gas. The scanned range went from *m*/*z* 50−1000, and the instrument was daily calibrated using a standard solution of renine (peptide of *m*/*z* 1757.9 Da).

Reagents. Optimization of the ICP-MS instrumental conditions was performed using standard calibration solution containing Li, Co, Y, and Tl in a concentration of 10 μg L^{-1} in 2% HNO_3 solution. All reagents were of analytical reagent grade, and the presence of selenium was not detected in the working range of these experiments. HPLC grade methanol (Merck, Darmstadt, Germany) was used. All of the solutions were prepared in 18 MΩ cm distilled deionized water (Millipore, Bedford, MA). SeMet and SeMC were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Working solutions were prepared daily by appropriate dilution. For the determination of total selenium, working solutions were prepared daily by appropriate dilution of 1 mg mL^{-1} Se(IV) standard solution (Sigma). Nitric acid (Suprapure) (68%) from Sigma and 30% hydrogen peroxide from Merck were used for sample digestion. Hepta-fluorobutyric acid (HFBA) was purchased from Sigma, and ammonium acetate solid salt was from Riedel-de-Haën (Honeywell Chemicals, Seelze, Germany) for the preparation of the chromatographic mobile phases (**Table 1**). Standards for calibration of the SEC column were as follows: MT (Sigma), MW = 6500 Da; GS-SG (Fluka-Sigma), MW = 612 Da; GSH (Fluka-Sigma), M = 307 Da; and Gly (Sigma), M = 75 Da.

Germination of *M. sativa* Seeds. The germination of alfalfa (*M. sativa*) seeds was performed with a solution containing 10 μg mL^{-1} of SeMet (analytical grade). *M. sativa* is a member of Leguminosae family. The seeds were bought in the market (Okoszerviz Ltd., Budapest, Hungary) for the purpose of home germination. For sprouting, the seeds were placed into a synthetic resin container. The base of the container was filled with absorbent cotton. Germination was accomplished at ambient room temperature and under the prevailing light and dark conditions during day and night, respectively. The total germination period was 168 h. During the cultivation, the sprouts were watered once each day with the SeMet solution. After they were harvested, all sprouts were washed carefully with deionized water to exclude

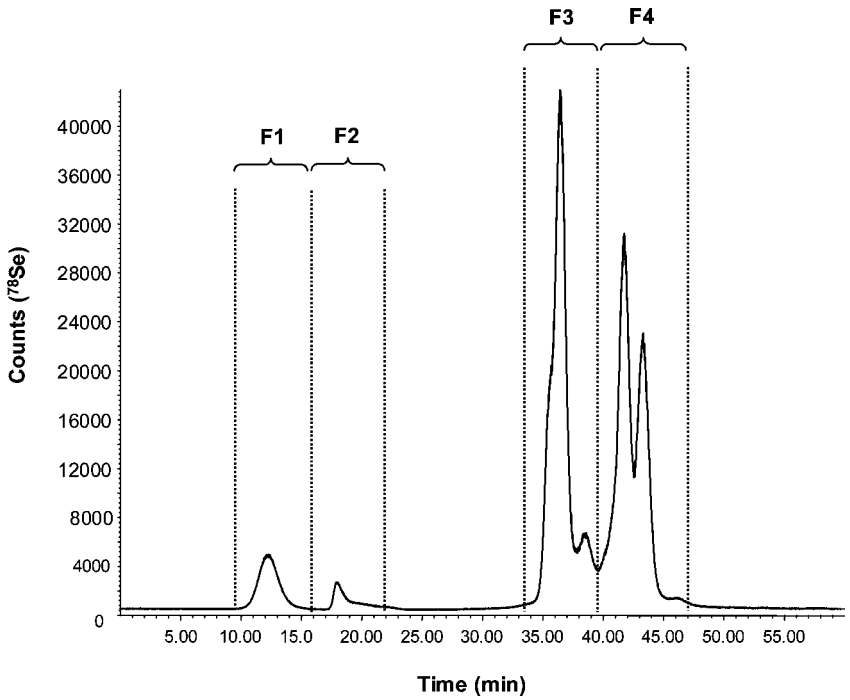

Figure 1. Chromatogram corresponding to the aqueous extract of the selenized sprouts obtained by SEC with ICP-MS detection; fractions labeled as F-1, F-2, F-3, and F-4 were collected and preconcentrated.

contamination of the surface of the sprouts of the standard solution. Afterward, the sprouts were put into plastic tubes and stored at −18 °C in a deep freezer. The entire sample was freeze-dried. After that, the dry samples were ground into a consistent powder.

Determination of Total Selenium Concentrations. For determinations of total selenium by ICP-MS, an Agilent 7500c was employed. Approximately 0.01 g of sprout powder was digested using 10 mL of HNO_3 (50%) and 1 mL of H_2O_2 (30%). To complete digestion, samples were covered with a watch glass and heated at reflux on a hot plate for ∼18 h. The final solutions were diluted with deionized water to 50 mL. Three replicates of each sample were prepared and analyzed. The reagent blank was digested in the same way. The method of external calibration was applied for the quantification of the selenium present in the samples. The total selenium concentration was determined by ICP-MS monitoring ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se isotopes.

Extraction of the Selenium-Containing Compounds. Five milliliters of deionized water (20 °C) was added to 0.25 g of selenized afalfa sprout powder in a 15 mL polypropylene tube. The tube was placed in an ultrasonic bath for 30 min at room temperature. After ultrasonication, the sample was centrifuged at 12000*g* for 15 min at 4 °C. The supernatant was filtered through a 0.22 μm PVDF membrane filter (Millex-GV, Millipore, Tokyo, Japan), and then, the extract was stored in a refrigerator.

RESULTS AND DISCUSSION

Fractionation and Purification of Se-Containing Species in Alfalfa Sprouts by 2D LC-ICP-MS. *SEC-ICP-MS.* Initial quantification of total Se present in the sprouts was obtained after microwave digestion and turned out to be 770 ± 19 μg/g (dry weight) while the concentration of the water-soluble Se was 559 ± 31 μg/g. Therefore, about 72% of the total Se present on the sprouts was solubilized using this aqueous extraction procedure. These water extracts were used to perform fractionation studies of the high and low molecular weight Se-containing species present in this sample first by using SEC on-line coupled to ICP-MS detection. Because few high molecular weight species (e.g., proteins) are expected in plant tissues (*23*), a Superdex Peptide column (with the fractionation range M_r = 100−10000) has been used for this study. The column was calibrated with a standard protein mixture of lactoalbumin, MW = 14200 rabbit liver MT, MW = 6500 Da; GS-SG, MW = 612 Da; GSH, MW = 307 Da; and Gly, MW = 75 Da. These standards were chosen to cover the separation range of the column and serve as molecular mass markers.

Figure 1 shows the HPLC-ICP-MS chromatogram corresponding to the Se species present in the aqueous extract of the alfalfa sprouts (^{78}Se is shown in the trace). As can be observed, the highest Se signals are encountered between 35 and 45 min, which, according to the previous calibration of the column, could correspond to species in the 600−100 Da MW range. This can probably be ascribed to the presence of small Se-amino acids or Se-peptides in the aqueous extracts. Additionally, some minor Se signals are observed within the first 20 min of the chromatogram that perhaps could be ascribed to some higher molecular mass species (e.g., Se-containing proteins). Therefore, four different fractions (F-1, F-2, F-3, and F-4, as shown in **Figure 1**) were collected, freeze-dried, and stored at −20 °C until further purification by a second dimension liquid chromatographic separation.

Shodex Ashaipack ICP-MS. The purity of the fractions labeled in **Figure 1** as F-2, F-3, and F-4 was subsequently studied by means of a novel chromatographic column with a combined mechanism of ion exchange and size exclusion. This column has been successfully used for the separation of Se metabolites in mushrooms and other plant tissues; therefore, it could be an interesting possibility in this study (*24*, *25*). Chromatographic conditions are also summarized in **Table 1**. **Figure 2** shows the elution profile observed for the F-3 and F-4 fractions (from SEC) in the Shodex column by monitoring the ^{78}Se channel (F-3 in **Figure 2A** and F-4 in **Figure 2B**). Additionally, the chromatogram corresponding to the separation of the standards containing Se-methionine and SeMC in this column is superimposed in **Figure 2B**. In the case of **Figure 2A**, most of the Se present in the fraction seems to elute in the peak at about 12 min, slightly after the void volume (which is approximately 10 min). No standards matched the retention time of this species;

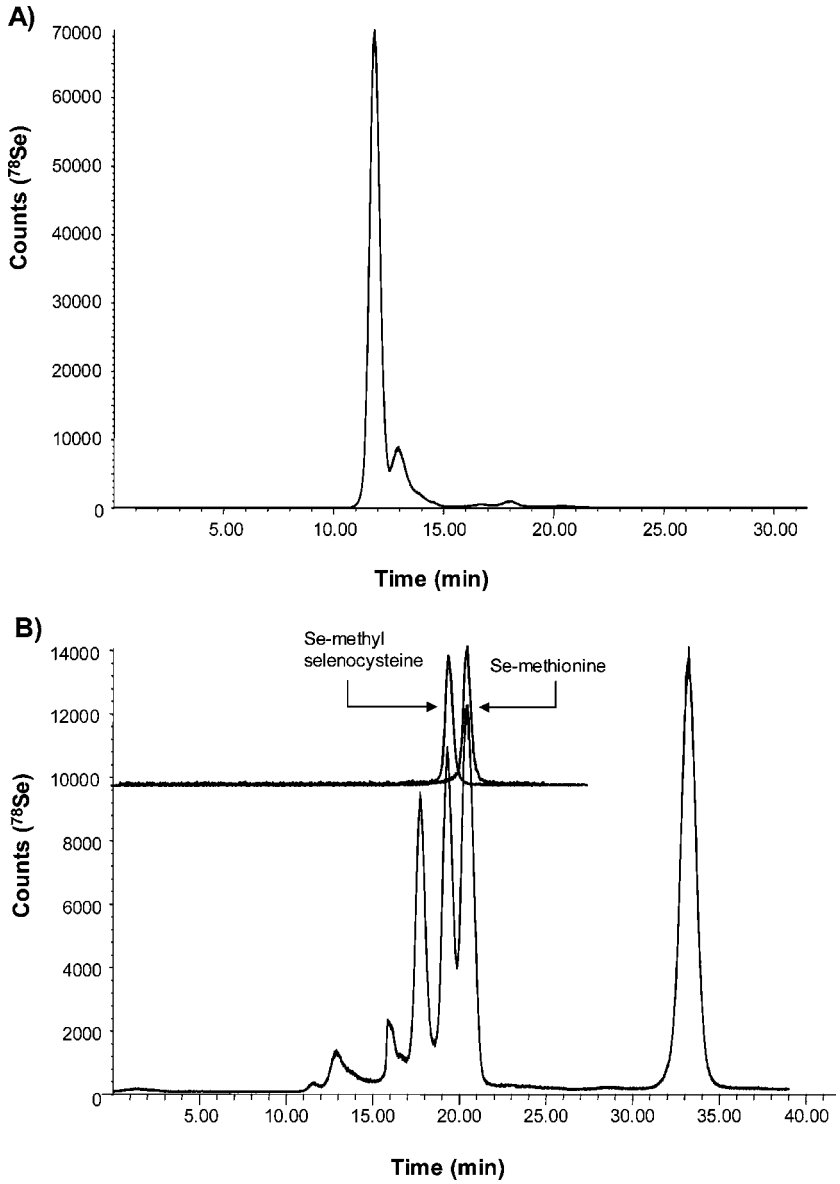

Figure 2. Chromatograms of the fractions collected by SEC using a Shodex Asahipak column and ICP-MS detection: (**A**) fraction 3 and (**B**) fraction 4. Chromatographic conditions are summarized in **Table 1**.

therefore, its purity had to be further tested by reversed phase chromatography and afterward by ESI-Q-TOF.

The species found in the sample extract shown in **Figure 2B** (corresponding to F-4 in **Figure 1**) matched the retention time of the injected standards of SeMC and Se-methionine, respectively. As previously reported, two of the most abundant Se species encountered in exposed plants are these two Se-amino acids (*3*, *4*). This fraction was also analyzed by reversed phase ion-pairing chromatography in order to compare the obtained results. Additionally, the presence of other Se-containing species eluting at 18 and 33 min, respectively, which did not fit to any of the available standards, is also noteworthy.

Finally, no Se-containing species were found in a similar procedure either in F-1 or in F-2 probably because of their low concentration (see **Figure 1**) and the fact that they could be partially lost or degraded in the collection/preconcentration procedures.

Reversed Phase Ion Pairing (RPIP)-ICP-MS. The fractions obtained from SEC were analyzed also by reversed phase (C_8) using HFBA (0.2%) as an ion-pairing reagent. In this case, the column was a narrow-bore system (2.0 mm i.d.) and a flow of 0.2 mL min^{-1} was used. This type of chromatography in connection with ICP-MS detection has been successful in previous speciation studies in plant tissues (*5*). Therefore, fractions F-3 and F-4 were subjected to RPIP-HPLC-MS and the obtained results are shown in **Figure 3 A**,**B**.

Figure 3A (corresponding to F-3 in **Figure 1**) shows at least three unknown selenium peaks eluting from the column within the first 10 min and a predominant Se-containing species at about 15 min. Neither SeMC nor Se-methionine could be identified in this fraction (either by comparing retention times or by sample spiking), and so, the Se-containing fractions eluting from the C_8 column at 6−8 and 14−17 min were independently collected in two fractions to then be lyophilized and stored at −20 °C for ESI-Q-TOF analysis.

Figure 3B shows the chromatographic profile of F-4 fraction obtained from SEC. Similarly to the observed chromatographic profile of this fraction in the Shodex column, SeMC and Se-methionine were positively identified by matching retention times of standards and also by spiking the sample. This species could be part of the metabolic pathway, more likely when the Se enrichment of the plant takes place with Se-met as the Se

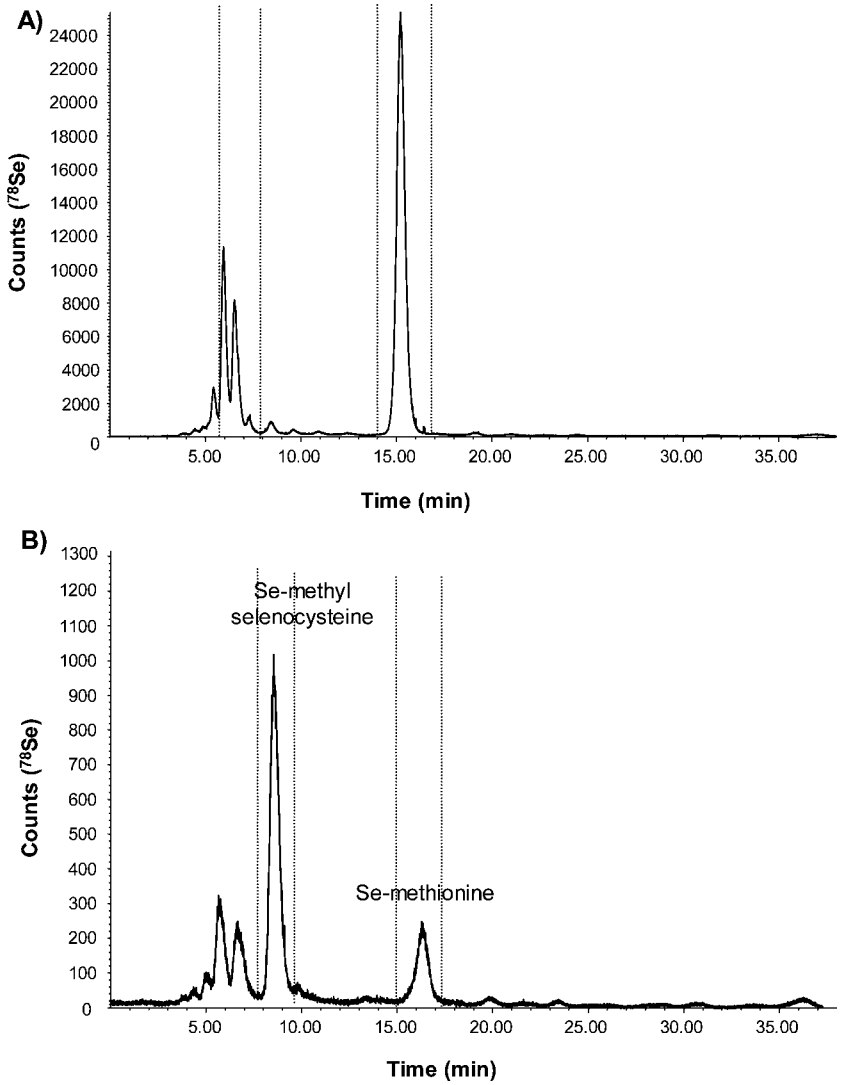

Figure 3. Chromatograms of the fractions collected by SEC using RPIP chromatography (C_8 column) and ICP-MS detection: (**A**) fraction 3 and (**B**) fraction 4. Chromatographic conditions are summarized in **Table 1**.

source. Similar results have been observed for selenium-enriched onions and mustard (*5*, *7*). Also, the two Se species were collected for further confirmation by ESI-Q-TOF analysis. Additional peaks corresponding to unknown selenium-containing species were also observed as in the case of the Shodex column at shorter retention times.

ESI-Q-TOF of the Collected Fractions. To unequivocally identify the small Se metabolites present in the sprout extracts, all of the collected Se-containing species (after SEC + RPIP-HPLC) were processed by ESI-Q-TOF. Collections from five to ten individual chromatograms were necessary in order to achieve enough concentration for final detection by ESI-Q-TOF. **Figure 4** shows the mass spectra obtained for the fraction eluting from 14 to 17 min in the RP-HPLC chromatogram of **Figure 3A**. The clearly observed isotope pattern of Se is showed by **Figure 4A** while the corresponding MS/MS of the *m*/*z* 239 and 237 is given in **Figure 4B**,**C**, respectively. As can be seen in **Figure 4A**, a good Se isotope pattern can be observed in the range of *m*/*z* 230 to 243 where the most abundant ion occurs at *m*/*z* 239 (corresponding to the ^{80}Se isotope). Similarly, the corresponding potassium conjugate ($[M + K]^+$) of the same ion at *m*/*z* 277 was observed containing the Se pattern (data not shown).

When collision-induced detection (CID) was taken on the *m*/*z* 239 and 237, respectively (^{80}Se and ^{78}Se isotopes), some fragments with two mass units difference were found (see **Figure 4B**,**C**). This could be ascribed to Se-containing fragments of the molecular ion at *m*/*z* 239 and 237, respectively. Those fragments of **Figure 4B**,**C**, correspond to the successive loss of H_2O, CO, CO_2H, and $(CH)NH_2CO_2H$ and are common to both spectra just two mass units apart. Additionally, the fragment corresponding to the molecule CH_3SeCH_2 can be observed at *m*/*z* 107 and 109, respectively. Considering all this information, these spectra could correspond to the oxidation product of Se-2-methyl-2-propenylselenocysteine $[CH_2{=}C(CH_3)CH_2{-}Se{-}CH_2{-}CH(NH_2)CO_2H]$ or to the carboxymethylated conjugate (due to the reconstitution of the fraction in 50% MeOH). This species has been found initially as the S analogues in plant tissues and further on in Se-enriched onions from the *Allium* family (*26*) and could be also produced in alfalfa sprouts (*M. sativa*, *Leguminosae* family). Synthesis and in vitro studies of the propenyl derivatives have revealed that these species exhibited higher chemopreventive effects than SeMC, which could be of crucial interest in the investigation of Se nutritional supplements (*26*). Unfortunately, we were not able to identify in this way other Se species in the fraction collected from 6 to 8 min (**Figure 3A**).

Similarly, the fractions collected in the RP-HPLC chromatogram showed in **Figure 3B**, in which SeMC and Se-methionine were identified by matching the retention time with the standards

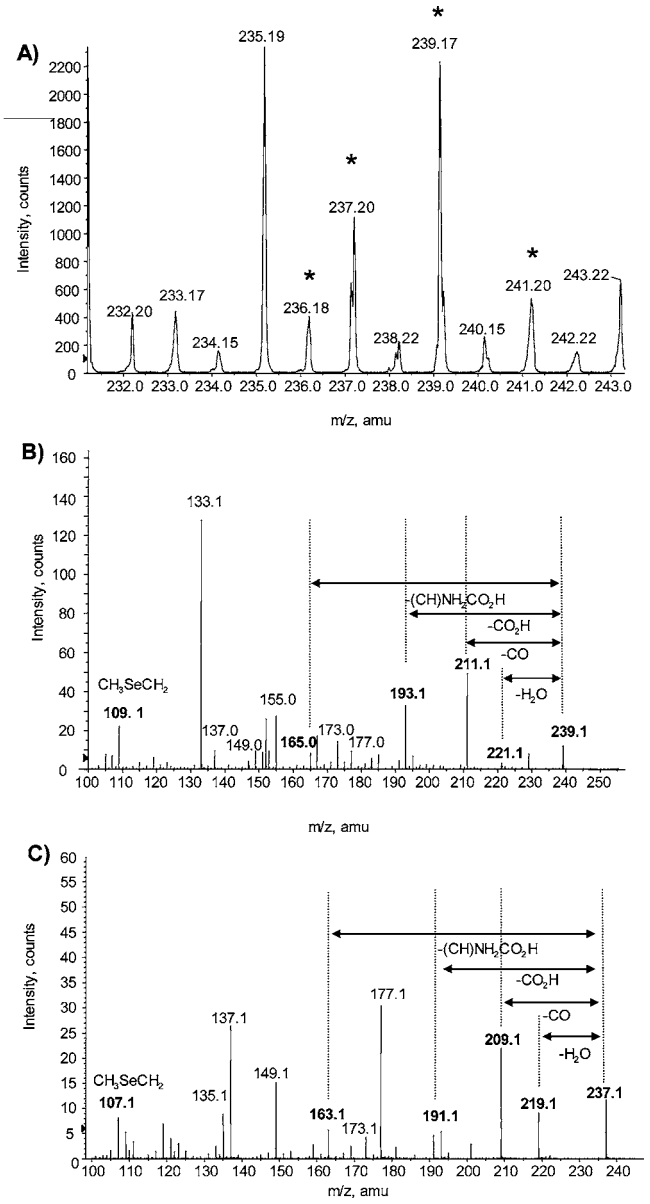

Figure 4. Mass spectra of the species isolated from **Figure 3A** eluting at 15 min: (**A**) magnified mass spectra to observed Se isotope pattern, (**B**) MS/MS on the *m*/*z* 239, and (**C**) MS/MS on the *m*/*z* 237.

(see also **Figure 2B**), were analyzed by ESI-Q-TOF. It is noteworthy that, in this case, the mass spectra showed higher noise and not so clear Se-isotope pattern (data not shown). However, the instrumental capabilities of the ESI-Q-TOF permitted to selectively isolate the Se-containing ions at *m*/*z* 198 and *m*/*z* 196 (corresponding to ^{80}Se and ^{78}Se in the molecular ion of Se-methionine) to perform CID on each one. **Figure 5** (**A**,**B**, respectively) shows the fragmentation pattern of both ions with the successive loss of H_2O, CO, CO_2H, and $(CH)NH_2CO_2H$ two units apart confirming again Se-methionine as the main Se species present in this fraction. In a similar way, the presence of SeMC was confirmed by CID (ions *m*/*z* 184 and 182, data not shown).

Conclusions. The use of 2D LC has been applied to the isolation of Se species in alfalfa sprouts. The initial fractionation by SEC followed by ion exchange or reversed phase chromatography, respectively, has been successful to isolate some important Se species present in the aqueous extracts. Most Se species occur in the low molecular mass range, even when the extraction takes place in water. Therefore, no water-soluble Se-

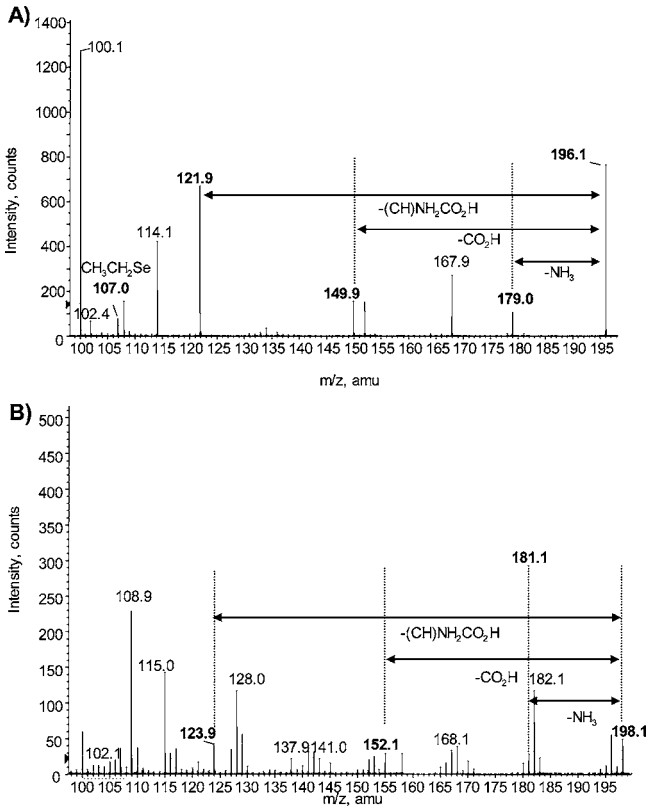

Figure 5. MS/MS spectra of the species isolated from **Figure 3B** that matched the Se–methionine retention time with a molecular ion at *m*/*z* 198: (**A**) MS/MS on the *m*/*z* 198 and (**B**) MS/MS on the *m*/*z* 196.

containing proteins seem to be formed in the sprouts grown in the presence of Se-methionine. On the other hand, similar results have been encountered by using both separation methods (reversed phase and ion exchange) and have revealed the presence of SeMC and Se-methionine in the extracts together with other unknown Se-containing species. When using the ESI-Q-TOF, the possible presence of Se-2-methyl-2-propenylselenocysteine in the extract has been studied by doing CID on the main corresponding Se ions (^{80}Se and ^{78}Se). For confirmation, however, further experiments synthesizing the standard of this compound and studying its chromatographic and MS behavior have to be conducted. Finally, the presence of Se-methionine and SeMC in the sprout aqueous extracts has also been concluded by ESI-Q-TOF experiments.

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