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Initial Studies of Selenium Speciation in *Brassica juncea* by LC with ICPMS and ES-MS Detection: an Approach for Phytoremediation Studies

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Various *Brassica* species accumulate Se into the thousands of ppm. This suggests some of them as candidates for Se phytoremediation. *Brassica juncea* (Indian mustard) was used to accumulate selenium by growing with sodium selenite as the selenium source under hydroponic conditions resulting in Se accumulation of up to hundreds of ppm in various parts of the plant. To date, few selenium speciation studies have been done in plants, with most studies reporting total selenium concentration in various parts of the plant. Se species extraction was evaluated by several digestion/extraction procedures, including the use of HCl, Tris-HCl buffer, and enzymatic hydrolysis (using proteinase K and protease XIV). The best extraction was obtained with proteinase K (extracting ∼75% of the total Se present in the plant). Some of the species produced by the plant, such as selenomethionine, can be identified at ppb levels by RP-HPLC−ICPMS, since standards are readily available. Others needed to be further characterized by ES-MS. Enzymatic hydrolysis releases mostly Se-methionine from *juncea* leaves, although other Se-containing species can also be observed by HPLC−ICPMS. In this initial study, the possible identification (by ES-MS) of a small chromatographic peak containing a Se−S bridged seleno amino acid with a structure similar to cystine is suggested.

Selenium is a natural constituent in the environment, occurring at elevated levels in some geographic regions. Anthropogenic activities, such as irrigation, tend to mobilize Se increasing the background levels and consequently the exposure to living systems. Exposure is known to produce toxic and teratogenic effects in grazing animals that consume plants containing Se.[1] Many plants are also harmed by root exposure to Se, presumably by replacing sulfur. Due to the continuous presence of selenium in the environment, a number of plants have adopted resistance mechanisms that allow them to survive—or even thrive in its presence. One such resistance strategy is to accumulate Se, differentiate it from S, and use it to build nonessential seleno amino acids.[2,3] Plants that do this are known as hyperaccumulators, as their tissue concentrations of Se exceed the levels found in the environment. *Brassica juncea*, a species of plant in the mustard family (commonly called Indian, wild, or wild brown mustard), has been shown[4] to accumulate Se at moderate levels. Hyperaccumulation of Se and other metals by plants as a potential means of soil and water remediation is an active area of investigation and interest. Remediation could be accomplished by harvesting and disposing of Se-rich plant tissues, or the plant itself may further metabolize Se to gaseous species that are then volatilized to the atmosphere.

Selenium in contaminated environments exists in several chemical forms. Particular species and their relative concentrations can have a direct impact on the rate of Se uptake, accumulation, and volatilization. Some forms of selenium are more readily available to plants than others. Therefore, developing analytical techniques that allow the identification and quantification at ultratrace levels of the various Se species in plant tissues may increase our understanding of the Se chemistry in the phytoremediation process. The uses of techniques that combine a separation of seleno compounds (e.g., HPLC, GC, or CE) with a sensitive and selective Se detector are attractive approaches.[5,6] Some of the studies to date involve GC separation with atomic emission detection (GC-AED)[1−7] or HPLC with UV absorption detection.[8] However, these methods are sometimes limited to certain species (such as derivatizable species in the case of GC) or they may show an important lack of selectivity (UV detection). Therefore, inductively coupled plasma mass spectrometry (ICPMS) is an attractive detector for selenium speciation due to its sensitivity for Se detection and the selectivity when coupled to different separation methods (e.g. HPLC, GC, CE, etc.).[9,10]

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(1) Banuelos, G. S.; Ajwa, H. A.; Wu, L.; Guo, X.; Akohoue, S.; Zambrzuski, S. *Ecotoxicol. Environ. Saf.* **1997**, *36*, 282−287.
(2) Zayed, A. M.; Lytle, C. M.; Terry, N. *Planta* **1998**, *206*, 284−292.
(3) Zayed, A. M.; Terry, N. *J. Plant Physiol.* **1992**, *140*, 646−652.
(4) Rosenfeld, I.; Beath, O. A. *Selenium, Geobotany, Biochemistry, Toxicity and Nutrition*; Academic Press: New York, 1964.
(5) Lobinski, R.; Szpunar, J. *Anal. Chim. Acta* **1999**, *400*, 321−332.
(6) Uden, P. C.; Bird, S. M.; Kotrebai, M.; Nolibos, P.; Tyson, J. F.; Block, E.; Denoyer, E., *Fresenius J. Anal. Chem.* **1998**, *362*, 447−456.
(7) Cai, X. J.; Block, E.; Uden, P. C.; Zhang, X.; Quimby, B. D.; Sullivan, J. J. *J. Agric. Food Chem.* **1995**, *43*, 1754−1757.
(8) Souza, M. P. d.; Lytle, C. M.; Mulholland, M. M.; Otte, M.; L.Terry, N. *Plant Physiol.* **2000**, *122*, 1281−1288.
(9) Gayon, J. M. M.; Thomas, C.; Feldmann I.; Jakubowski, N. *J. Anal. At. Spectrom.* **2000**, *15*, 1093−1102.

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Different types of liquid chromatographic separations including size exclusion,[11] ion exchange,[12] or ion pairing reversed-phase chromatography[13] have been successfully applied for separating selenium species in different biological and environmental samples. The coupling of HPLC to ICPMS is straightforward, and retention time comparisons can be used to identify the species present in the samples with available Se standards. However, for many organo- and bio-selenium compounds, no standards are available. Hence, other identification techniques such as electrospray mass spectrometry (ES-MS) are necessary.[14]

The ultimate goal of the study is to identify and quantify the various selenium species produced by *B. juncea*. In view of the number of possible Se species at relatively low concentrations (ppb) and the lack of retention time standards, this report is only the beginning. Ultimately, comprehensive identification of the various species will help to elucidate the Se metabolic pathway in *B. juncea* and may further the phytoremediation technique by better understanding the uptake mechanisms.

EXPERIMENTAL SECTION

Instrumentation. The chromatographic system was an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with the following: a binary HPLC pump, an autosampler, a vacuum degasser system, a thermostated column compartment, and a diode array detector. The ICPMS was also an Agilent 7500s (Agilent Technologies, Tokyo, Japan). Both systems are connected through a remote cable that allows the simultaneous start of the chromatographic run on both instruments. The system is marketed by Agilent as a "metal speciation" option with the ICPMS.

Two chromatographic columns were used to perform the separations: a Luna C_8 (Phenomenex, Torrance, CA) (250 × 4.6 mm id with a 5-μm particle size) used for ICPMS and a Luna C_8 (Phenomenex) (250 × 2.0 mm id with a 5-μm particle size) used for ES-MS. The chromatographic run was isocratic (for ICPMS) at 1.2 mL min^{-1} with a sample injection volume of 50 μL; in the case of using ES-MS as the detection system, a 0.2 mL min^{-1} chromatographic flow and a sample injection volume of 15 μL were used. The ES-MS instrument used to perform the studies was a Q-TOF from Micromass (Platform, Micromass, Manchester, U.K.). The instrument was operated in the positive ion mode. The applied voltage to the capillary was 3000 V and the cone voltage was 45 V with N_2 as nebulizing gas. The spectra were scanned from 50 to 1000 m/z. Conditions are summarized in Table 1.

Reagents. Commercial chemicals were of analytical reagent grade and were used without further purification. Diluted Hoagland, a nutrient solution used for hydroponic culturing of plants, contained 20 mL of 1 M potassium dihydrogen phosphate, 120 mL of 1 M potassium nitrate, 80 mL of 1 M calcium nitrate, 40 mL of 1 M magnesium sulfate, and 20 mL 0.08 M EDTA in 20 L of solution. All the reagents were from Fisher (Fisher Scientific Co., Fair Lawn, NJ).

Table 1. Instrumental Operating Conditions for the HPLC, ES-MS, and ICPMS

ICPMS Parameters	
forward power	1300 W
external flow	15.0 l min^{-1}
internal flow	1.0 l min^{-1}
carrier gas flow	0.6 l min^{-1}
makeup gas flow	0.5 l min^{-1}
selected isotopes	^{78}Se, ^{82}Se
dwell time	0.1 s per isotope
shield torch	on
HPLC Parameters	
column	C_8 (250 × 4.6/2.0[a] mm)
mobile phase	10% MeOH/H_2O−5% MeOH[a]
pH	1% TFA−0.1% HFBA[a]
flow	1/0.24[a] mL min^{-1}
Injected volume	50/15[a] μL
ES-MS Parameters	
capillary voltage	3000 V
cone voltage	45 V
nebulizing gas	N_2
mass range	50−1000

[a] Values used on the HPLC-ES-Q-TOF.

For HPLC−ICPMS studies, the mobile phase was 1% trifluoroacetic acid (Sigma Chemical Co., St. Louis, MO) and 10% methanol (Fisher Scientific Co.) in 18 MΩ cm^{-1} distilled, deionized water (Sybron Barnstead, Boston, MA). The mobile phase for HPLC−ES-MS was prepared by using 0.1% heptafluorobutyric acid (Sigma Chemical Co.) and 5% methanol (Fisher Scientific).

For extraction procedures, which are described later, hydrochloric acid, nitric acid (Fisher Scientific), Tris, proteinase K, and protease XIV (Sigma) were used to prepare the plant tissue samples. The seleno amino acid standards, selenomethionine, selenocystine, and selenoethionine were obtained from Sigma. Dithiothreitol was obtained from Aldrich (Milwaukee, WI).

Plant Growth and Sample Preparation. *B. juncea* seeds were obtained from the USDA-ARSs in Pullman, WA, and Ames, IA. They were germinated in water-moisturized paper towels for a period of 7 days. After germination, individual seedlings were placed in an amber plastic bottle containing Hoagland's nutrient. Plants were kept in a controlled environment room at a temperature of 25 °C, supplemental full-spectrum lighting, and 12-h days. A total of eight plants were grown, but the present study shows the results obtained for four of them. After 1 week, the hydroponic solution was spiked with aqueous sodium selenite to produce a solution concentration of 3.7 μg mL^{-1} Se. The concentration of Se in the solution was monitored after the first, second, and fifth day of exposure.

Plants were harvested, washed with distilled, deionized water, and separated into roots, stems, leaves, and shoots. The different plant tissues were weighed, frozen in liquid nitrogen to break the cell walls, ground with pestle and mortar, and stored at 7 °C in the refrigerator in high-density polyethylene (HDPE) bottles until digestion or extraction. For the extraction of selenium species from the sample matrix, four different extraction procedures were investigated. These included HCl (1 M), Tris-HCl (pH 8), and enzymatic digestions with protease XIV and proteinase K.

For HCl and Tris-HCl extractions, 10 mL of either acid or buffer system was added to 0.5 g of plant tissue in a 15-mL centrifuge

(10) Kotrebai, M.; Tyson, J. F.; Block, E.; Uden, P. C. *J. Chromatogr., A* **2000**, *866*, 51−63.
(11) Casiot, C.; Szpunar, J.; Lobinski R.; Potin-Gautier, M. *J. Anal. At. Spectrom.* **1999**, *14*, 645−650.
(12) LaFuente, J. M. G.; Dlaska, M.; Sanchez, M. L. F.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **1998**, *13*, 423−429.
(13) B'Hymer, C.; Caruso, J. A., *J. Anal. At. Spectrom.* **2000**, *15*, 1531−1539.
(14) McSheehy, S.; Pohl, P.; Szpunar, J.; Potin-Gautier, M.; Lobinski, R. *J. Anal. At. Spectrom.* **2001**, *16*, 68−73.

Table 2. Total Se Extracted under the Different Extraction Conditions Used[a]

extraction reagent	HCl, 1 M	Tris-HCl, pH 8	proteinase K	protease XIV
Se on the extracts (μg g^{-1})	109 ± 24	94 ± 17	147 ± 5	141 ± 4
% of Se extracted[b]	54	46.5	73	70

[a] $n = 3$. [b] Based on the HNO_3 acid digestion/microwaves.

tube. In both cases, the mixture was shaken for 20 h at 35 °C and then centrifuged for 30 min. The aqueous solution (supernatant) was decanted, filtered through a 0.45-μm membrane filter, and diluted appropriately for analysis. For total Se determination by ICPMS, the samples were diluted in nitric acid (2%). For selenium speciation, the samples were diluted in the corresponding chromatographic mobile phase.

In the case of enzymatic extractions, 0.5 g of fresh or refrigerated plant tissue and 0.02 g of enzyme (protease XIV or proteinase K) were placed in a 15-mL centrifuge tube and 10 mL of distilled water was added. The samples were shaken in darkness for 20 h at 37 °C and then centrifuged at 3000*g*. The solutions were finally passed through 5000-Da molecular weight cutoff filters to retain excess of enzyme. Finally, to perform a total quantification of the Se present in *Brassica* leaves, 0.5 g of tissue from three different plants was digested with 50% HNO_3 using a microwave oven and the total Se content determined (after adequate dilution in Milli Q water) by external calibration using ICPMS.

RESULTS AND DISCUSSION

Selenium Accumulation. The plants evaluated in this study were exposed to Na_2SeO_3 (3.7 μg mL^{-1}), and the total selenium content in the nutrient solution was measured after the first, second, and fifth day of exposure. For total selenium determination, ^{77}Se and ^{82}Se were monitored using ^{89}Y and ^{69}Ga as internal standards. The determination of the Se content in the nutrient solution allowed for the calculation of theoretical selenium uptake by the plant. For this study, the nutrient solution corresponding to four different plants, was adequately diluted and independently analyzed for Se. Results were comparable between plants, and the Se content remaining in the solution after 5 days of exposure was 0.32 ± 0.013 μg mL^{-1}, expressed as the average (and standard deviation) of the four different nutrient solutions. Therefore, taking into account the initial Se content spiked in the solution, it is possible to calculate that 91.4% of the Se was taken up by the plant by the fifth day (either accumulated or volatilized).

This Se may be present as inorganic forms (selenite or selenate) or as seleno amino acids (these could be free in the cytoplasm or forming part of the protein structures). Therefore, after the cell walls were broken using liquid nitrogen and the leaf tissues were ground (as detailed above), four different extraction procedures were evaluated to selectively extract such Se compounds: (i) the use of aqueous solutions containing HCl (1 M) and Tris-HCl (pH 8), respectively. This extraction is suggested to liberate the free or weakly bound inorganic Se (selenite or selenate) and seleno amino acids; (ii) the use of enzymes (protease XIV and proteinase K) to cleave peptide bonds in proteins. The results for the four extractions are summarized in Table 2, which shows the total Se extracted (in μg mL^{-1}; as average obtained for a single extraction of each one of the four plants). As can be

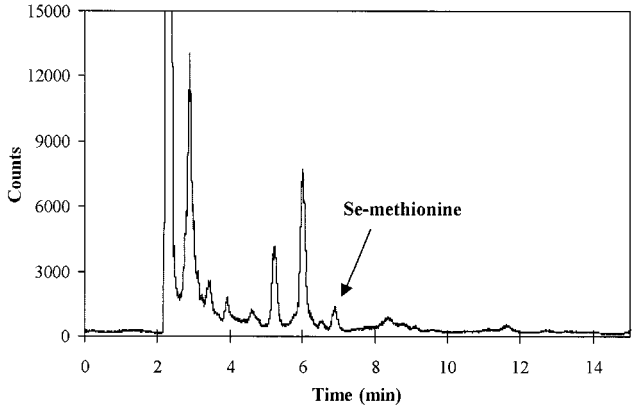

Figure 1. Chromatogram obtained by HPLC−ICPMS (^{82}Se) for the extraction of *B. juncea* leaves with 1 M HCl. Chromatographic conditions detailed in Table 1.

observed in the table, higher Se concentration levels are found when enzymatic hydrolysis is performed instead of extraction with aqueous HCl or Tris-HCl solutions. However, to quantify the yields of all these extraction procedures, a microwave digestion of the tissue (leaves) was carried out using 50% HNO_3 to obtain the total Se present. Table 2 shows that the use of HCl and Tris-HCl as extractants only liberate ~50% of the total Se present in the leaves. This percentage is slightly higher with HCl rather than with Tris-HCl. When proteinase K or protease XIV is used, higher extraction efficiency, ~70% of the Se present in the sample, can be achieved with both enzymes providing similar results. The higher extraction efficiency is in agreement with other authors where related enzymes were used to extract a variety of samples, such as yeast.[11] In these extractions, both free or weakly bound Se compounds plus some protein-incorporated seleno amino acids can be extracted. It was expected that the Se-tolerant *B. juncea* would efficiently accumulate selenium without significant incorporation into proteins.[1] However, it should be considered that the formation of nonfunctional proteins or polypeptides might be a possible detoxification mechanism by the plant.

Selenium Speciation by RP-HPLC−ICPMS. Once the Se species were extracted from the plant leaves and the total Se concentration determined in the extracts, the speciation of the different Se-containing compounds was undertaken. For this purpose, a reversed-phase chromatographic procedure using 10% MeOH and 1% TFA as a perfluorinated carboxylic acid ion pairing reagent was employed as mobile phase in a C_8 column. Under these chromatographic conditions it is possible to resolve several Se species including selenomethionine, selenocystine, and selenoethionine (Se-cystine, Se-methionine, and Se-ethionine, respectively) within 25 min. Figure 1 shows the chromatogram obtained for the HCl extraction of *Brassica* leaves using the chromatographic conditions detailed in Table 1 by HPLC−ICPMS (^{82}Se).

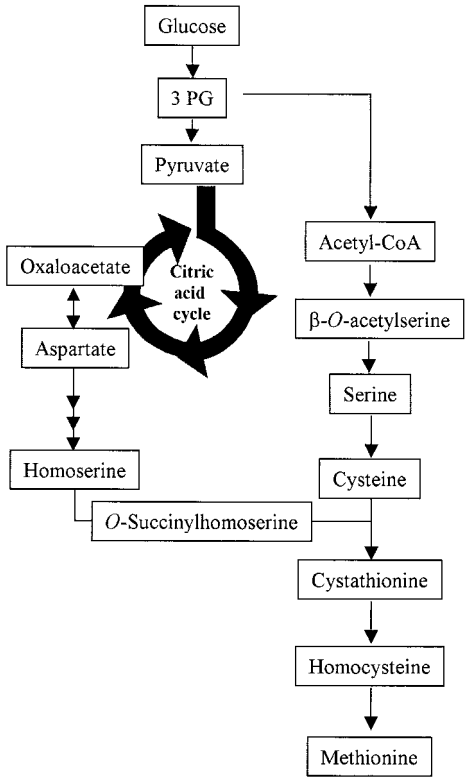

Figure 2. Sufur methabolic pathway in plants (obtained from ref 17).

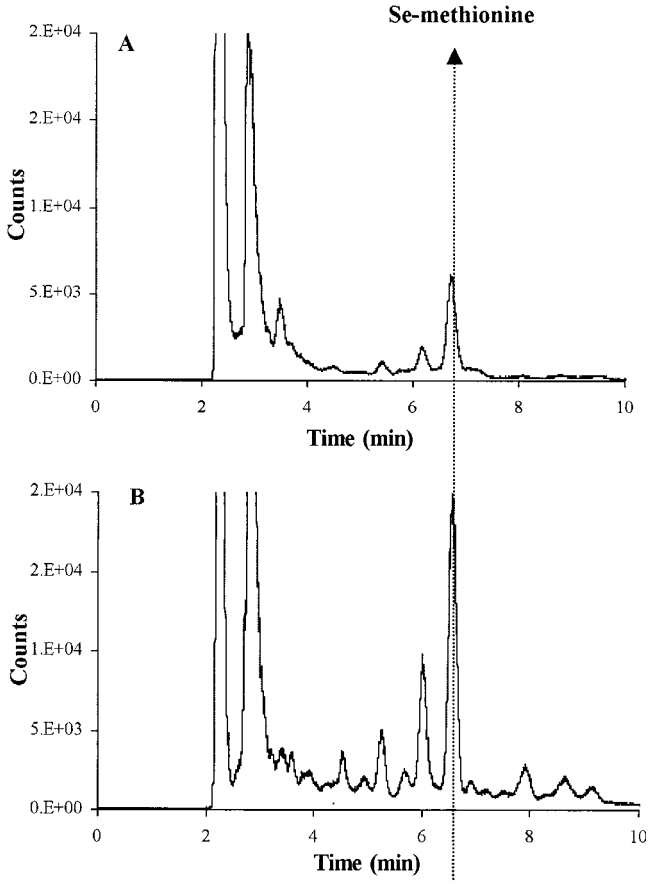

Figure 3. Comparison of different enzymatic extraction procedures in *B. juncea* leaf tissue (^{82}Se): (A) using protease XIV; (B) Using proteinase K.

An important Se signal elutes at the void volume of the column (~2 min). This may represent the inorganic forms of Se or Se-cystine, which cannot be resolved from the inorganic forms under these conditions. At this low retention time, it also was possible to observe a signal corresponding to sulfur (^{34}S). This is the only peak that contains sulfur and is likely to be associated with the cystine residues present in the extracted fraction.

The other major species at 3 and 6 min, respectively, could not be characterized by matching retention times with any known seleno amino acids available in the laboratory. The small signal observed at ~7 min corresponded to the retention time of Se-methionine (~2 ng mL^{-1}). These results indicate that some seleno amino acids were present in the cytoplasm or interstitial fluid that were not incorporated into the plant proteins.[15] The substitution of Se into methionine may be symptomatic of partial physiological toxicity. This can be explained by the pathway for synthesis of methionine in plants (see Figure 2, adopted from ref 16).[16] Methionine is synthesized through formation of cysteine. *Brassica* species may incorporate some Se into cysteine (forming Se-cysteine), which suggests the formation of S−Se bridges between adjacent polypeptide chains. Previous studies using similar extraction conditions have been done using GC-AED as a separation/detection system, and in that case, no Se-amino acids other than Se-methionine were consistently detected in *Brassica* leaves.[1] However, the use of ICPMS as the detector for HPLC permitted observation of a number of Se-containing species (see Figure 1) previously undetected or not suitable for GC derivatization, which do not match retention times of any of the available Se compounds in our laboratory. A similar chromatographic profile was obtained with the Tris-HCl extract.

When the enzymatic (protease XIV or proteinase K) extracts of the *Brassica* leaves were speciated, the chromatogram obtained was similar to that of the HCl extract in terms of the species found. Figure 3 shows the results obtained for the two enzymes compared on the same intensity scale. As can be seen in the figure, the two major Se-containing species are those eluting at 2 and 3 min, respectively, the same as observed with the HCl extract. However, using enzymatic hydrolysis Se-methionine is cleaved from the protein structure, and therefore, a higher signal can be observed at 7 min. The ratio between the peaks eluting at 6 and 7 min, respectively, is reversed in intensity relative to that found from the acid extract.

When enzymatic extractions are compared, proteinase K provides a higher signal for Se-methionine and for the unknown peak eluting at 6 min than protease XIV. In both cases, several minor Se species elute under these chromatographic conditions, and therefore, the use of complementary identification techniques is required for further species characterization

ES -MS for Species Identification. To further identify other species present in the *Brassica* leaf extracts, modification of the chromatographic conditions was required, since TFA is incompatible with an electrospray source. Therefore, the separation was carried out using heptafluorobutyric acid (HFBA) (0.1%) in 5% methanol instead of TFA as perfluorinated ion pairing reagent.

(15) Brown, T. A.; Shrift, A. *Biol. Rev. Cambridge Philos. Soc.* **1982**, *57*, 59−84.
(16) Mathews, C. K.; Holde, K. E. V. *Biochemistry*; Addison Wesley Longman, Inc.: San Francisco, CA, 1995.

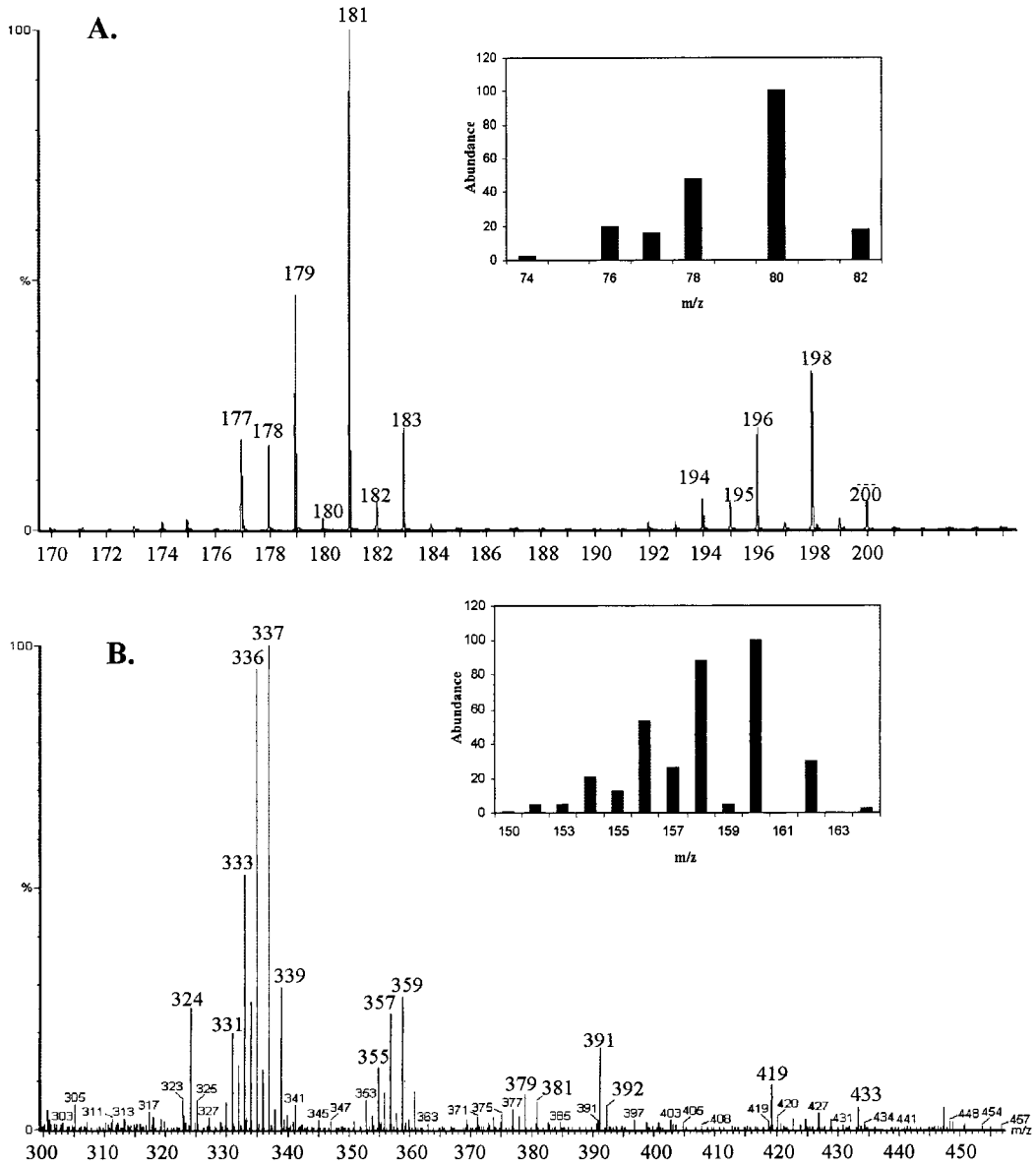

Figure 4. Mass spectra obtained by ES-MS: (A) Se-methionine (Se isotope pattern superimposed); (B) Se-cystine (pattern containing two Se atoms superimposed).

HFBA is appropriate for the ES source, although it produces modified retention times. The conventional C_8 column was replaced with a small-bore C_8 column (2.0-mm i.d.) and the flow decreased to 0.2 mL min^{-1} to facilitate coupling with the ES-MS. These chromatographic conditions were also evaluated using ICPMS as detector.

Unfortunately, HPLC−ES-MS often has lower sensitivity than ICPMS, especially in the presence of the complex matrix from the extractions.[17] Figure 4 shows the mass spectra obtained for standards containing Se-methionine (10 μg mL^{-1} Se) and Se-cystine (5 μg mL^{-1}), respectively. The operating conditions are given in Table 1. A clear selenium isotope pattern (with one or two Se atoms, respectively, as seen in the insets) may be observed for both mass spectra shown in Figure 4, with its major isotope appearing at m/z 198 for Se-met and m/z 337 for Se-cystine. The patterns observed for these two Se-amino acids agree with those previously presented by Kotrebai and co-workers[18] for one and two Se atoms. In this case, it is also possible to observe the association of Se-cystine with Na when this element is incorporated in the solution forming $(Se\text{-}Cys, Na)^+$ at m/z 359 simultaneously to $(Se\text{-}Cys, H)^+$. The common loss of NH_3 (17 Da) has been found also in the Se-methionine fragmentation pattern and is the most abundant fragment.[18]

The HCl leaf extracts were analyzed by HPLC−ES-MS and HPLC−ICPMS using the same chromatographic conditions. Figure 5 shows the chromatogram corresponding to ^{78}Se by ICPMS. The total ion chromatogram with ES detection for *juncea* leaves indicated a minor peak at 4.5 min where two clear selenium MS isotopic patterns could be observed. However, no other peak could be identified as Se-containing species with the ES-MS sensitivity available, even when such species can be observed in the ICPMS chromatogram such as selenomethionine. Also, the

(17) Casiot, C.; Vacchina, V.; Chassaigne, H.; Szpunar, J.; Potin-Gautier, M.; Lobinsky, R. *Anal. Commun.* **1999**, *36*, 77−80.

(18) Kotrebai, M.; Bird, S. M.; Tyson, J. F.; Block, E.; Uden, P. C., *Spectrochim. Acta B* **1999**, *54*, 1573−1591.

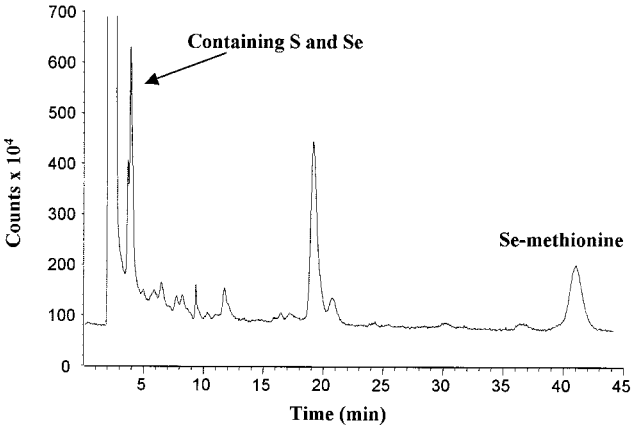

Figure 5. Chromatogram obtained by HPLC−ICPMS (^{82}Se) for the hydrochloric extract of *B. juncea* leaves. Chromatographic conditions involving 5% MeOH and 0.1% HFBA.

compound eluting at 19 min in the ICPMS chromatogram (see Figure 5) appears to be buried in an unresolved complex chromatographic peak as detected by ES-MS, therefore making difficult to distinguish a Se isotope pattern. A large, broad, and heavily fronted peak was also noted in the ES total ion chromatogram, indicating that the small-bore column was heavily overloaded and had a number of unresolved constituents. Further collection of this fraction, separation, and treatment with different reagents to liberate the Se-containing species is the objective of current research.

Figure 6A corresponds to the mass spectrum of one of the fragments identified from the chromatogrphic peakat 4.5 min and shows a single-ion selenium pattern at m/z 341, 342, 343, 345, and 347, suggesting the presence of a Se species. However, the major signal is seen in Figure 6B at m/z 287 (Se m/z 80) and also shows the appropriate Se isotope pattern. The peak observed at 345 amu may be the molecular ion $(MH)^+$ of a unique selenium species with a possible structure as suggested in Figure 7A. The fragment at m/z 287 may involve the loss of a glycine unit, similar to that reported by McSheeny et al.[14] (m/z 58 from the protonated molecular ion) or the loss of the CH_2O after the loss of CO as described by Kotrebai and co-workers.[18] It is also possible that these selenium signals could result from two independent Se species, which coelute on the LC column instead of being fragments of the same molecule. However, based on the experiments to date, the proposed structure seems the most probable. It involves a S−Se bond probably due to the substitution of Se for S in the metabolic pathway (see Figure 2). The presence of S−Se intermediates has been also described by other authors in samples such as selenized yeast.[14] In the same way, *B. juncea* species may incorporate some Se into cysteine, which disrupts the formation of disulfide bridges between adjacent polypeptide chains.

The structure proposed is also partially confirmed by the presence of other Se-containing residues such as m/z 109 $(Se-CH_2-CH_3)^+$ or the S analogue at m/z 61 $(S-CH_2-CH_3)^+$.

Additional characterization of the proposed structure was done by using dithiothreitol (DTT) to cleave the suggested S−Se bond, which should eliminate m/z 287 and 345 ions (and the surrounding Se isotopic peaks) from the mass spectra. The experiment was performed by collecting five different injections (100 μL each) of

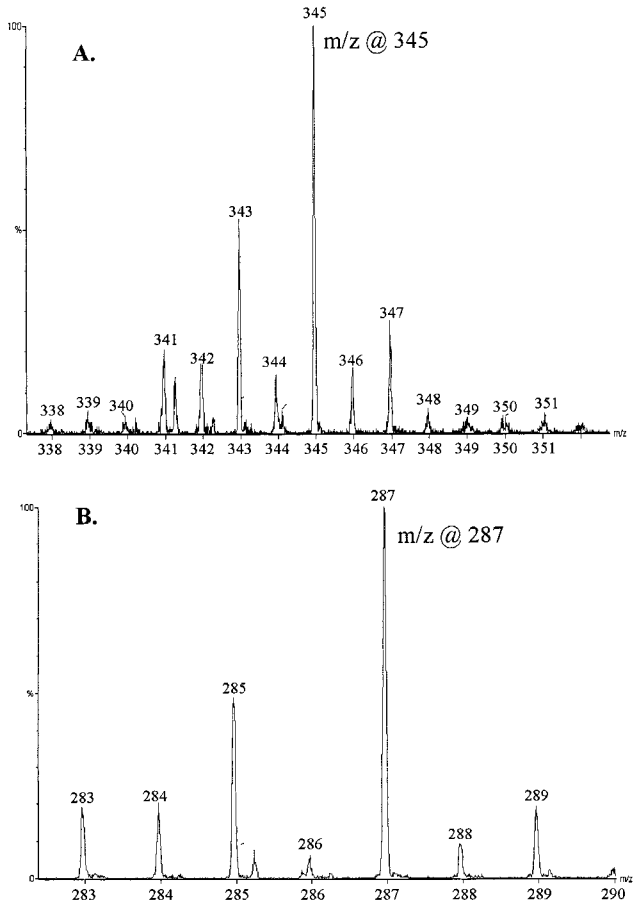

Figure 6. The two main fragments obtained by HPLC−ES-MS exhibiting Se pattern at t_r = 4.5 min: (A) *m/z* 345; (B) *m/z* 287 (most abundant).

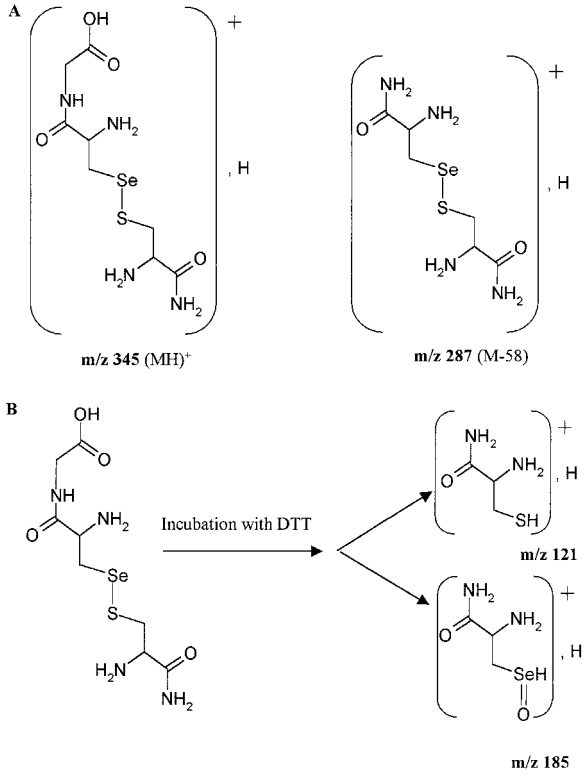

Figure 7. Proposed structures according to the obtained fragments by ES-MS: (A) *m/z* 345, 287, (B) Results obtained with the treatment of the collected fractions with DTT at *m/z* 121.

the chromatographic fraction eluting between 3 and 5 min, and the combined solution was freeze-dried. This sample was dissolved in 200 μL of 50% MeOH and 0.1 M acetic acid and divided into two different fractions; one of the fractions was incubated with a 5 mM solution of DTT, and the spectra were taken for both of these by ES-Q-TOF. The m/z 345 fragment disappears as does the one at m/z 287. A new fragment was obtained at m/z 121 corresponding to the protonated S-containing part of the molecule (345 − 119) + H^+ due to breaking the Se−S bond. The other expected fragment (m/z 227) was observed at very low intensity but insufficient to ensure a Se isotopic pattern. However, another peak that suggests a Se isotope pattern appears at m/z 185 (Se m/z of 80) and may represent the Se analogue of the S structure with the oxidation of Se (see Figure 7B) that could corroborate the proposed structure.

CONCLUSIONS

Within the scope of our experiments, *B. juncea* is a good Se accumulator over a short time period when grown under hydroponic conditions. The study shows that, depending on the extraction conditions, up to 73% of the Se present in the plant can be extracted (using proteinase K as extractant). The use of enzymatic hydrolysis allowed the extraction of Se-methionine as detected by ICPMS through matching retention times. The same experiments showed other unknown compounds that had no retention time matches with selenium species available in the laboratory and required further study. ES-MS was then used to characterize a minor peak observed in the ES total ion chromatogram. While not as capable as ICPMS for low detection limits, it does allow the detection of molecular ions and aids identification. The ES-MS spectra suggest a unique seleno amino acid, not reported for *B. juncea* until this time.

The species found or proposed to date with the two techniques can be summarized as inorganic forms of Se (eluting at the void volume of the chromatographic column), Se-methionine, and a selenium species containing a Se−S bond with a proposed structure similar to cystine. Further work is ongoing to characterize the structure of the other species present in the chromatograms obtained both by ICPMS and ES-MS. For the latter, a higher level of separation and preconcentration is underway.

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