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Identification and characterization of Se-methyl selenomethionine in *Brassica juncea* roots

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Abstract

The present work shows the identification and characterization of Se-methyl selenomethionine (SeMMet) as an important Se species in *Brassica juncea* roots when grown in the presence of Se-methionine (SeMet) as the Se source. SeMMet was isolated by liquid chromatography employing two different liquid chromatographic mechanisms: reversed-phase ion-pairing using heptafluorobutyric acid as counter ion and cation exchange using a pyridinium formate gradient (pH 3). Inductively coupled plasma mass spectrometry was used for the detection of Se. SeMMet was characterized by electrospray quadrupole time-of-flight MS in both a synthesized standard and in the roots extract using collision-induced dissociation of the selected ion. Preliminary evidence suggests that *Brassica juncea* may also produce dimethylselenonium propionate, although to a much lesser extent.

Keywords: *Brassica juncea*; Plant materials; Organoselenium compounds; Selenium

1. Introduction

Selenium is an essential micronutrient for humans and animals [1]. However, malformations and reproductive defects due to environmental selenium contamination have been observed in wildfowl of certain regions of the Western USA [2]. Phytoremediation has been suggested as a low cost and environmentally friendly technology for the cleanup of soils and water contaminated with Se [3]. Plants can be classified as to their ability to accumulate Se according to this scheme: non-accumulators (less than 100 μg Se g^{-1} plant dry mass), accumulators (hundreds of μg Se g^{-1} plant dry mass), and hyperaccumulators (thousands of μg Se g^{-1} plant dry mass) [4]. *Brassica juncea*, Indian Mustard, is considered a Se accumulator and has been extensively studied for phytoremediation because of its fast growing cycle and high biomass [5]. A significant proportion of the Se removed by Brassica is volatilized into the atmosphere, the amount of which depends on the Se species supplied. The species formed in the phytovolatilization process are primarily dimethylselenide (DMSe) and dimethyldiselenide (DMDSe), which are about 500–700 times less toxic than inorganic selenium species [6]. The roots of these plants are known to volatilize about 20 times more Se than the shoots.

Previous studies have shown that Brassica plants volatilize more selenium when treated with Se-methionine (SeMet) than with inorganic forms [7]. Results obtained in our laboratory confirmed that the percentage of volatilized Se increases when the plant takes up SeMet rather than inorganic Se sources. This is in agreement with the previously reported mechanism [3], since SeMet is more readily available for further methylation to form Se-methyl selenomethionine (SeMMet; the DMSe precursor) than any other Se species.

Although these processes are not fully understood, the volatilization process is believed to take place through the formation of selenonium ions that are then cleaved to DMSe in analogy to the sulfonium assimilation pathway [8]. It is suggested that involves an initial methylation of SeMet to Se-methyl selenomethionine using a methyl donor group (e.g. *S*-adenosylmethionine) and a methionine methyl

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transferase (MMT) enzyme [9]. In shoots further production of volatile DMSe could happen by the action of another enzyme (e.g. DMSP lyase). However, since volatilization occurs to a greater extent in the roots, SeMMet could be transported from the shoots to the roots in order to form the volatile DMSe [3].

However, the complete mechanism, metabolic intermediates and enzymatic reactions involved in Se assimilation in plants are not yet fully confirmed. The difficulty in determining trace-levels of organoselenium metabolites and the lack of commercially available standards have been major barriers to a complete characterization of the Se metabolic process [10]. Recent literature has addressed the use of analytical mass spectrometric techniques as interesting approaches to investigate Se biochemistry in plants [11,12]. Inorganic mass spectrometry, mainly inductively coupled plasma mass spectrometry (ICP-MS), has proved to be an excellent tool to perform Se speciation studies in plant material and other biological samples at sub-ng levels when coupled to a powerful separation technique [13,14] (e.g. HPLC or GC). ICP-MS provides also excellent selectivity. Because the harsh ionization ICP source destroys all molecular information, the simultaneous the use of a "softer" ionization source such as electrospray in conjunction with mass spectrometry (ESI-MS) allows for the complimentary characterization of Se species at the molecular level [15–17] The combination of these techniques provide powerful tools to perform Se metabolic studies in plants.

The present study demonstrates this strong potential for Se metabolic studies by analyzing *Brassica juncea* roots using HPLC separation with ICP-MS and ESI-MS detection. The use of different chromatographic mechanisms (reversed-phase ion-pairing and cation exchange) was evaluated in order to obtain a good separation of Se species in a short time. ESI-MS was used as molecular identification technique for the characterization of Se metabolites in *Brassica juncea* roots.

2. Materials and methods

2.1. Instrumentation and reagents

An Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, a thermostated column compartment, and a diode array detector formed the separation system. The ICP-MS system was an Agilent 7500s (Agilent Technologies, Tokyo, Japan). Both systems are connected through a remote cable that allows the simultaneous start of the chromatographic run.

The chromatographic columns used were an Alltech C_8 (Alltech Associates, Deerfield, IL, USA) (250 mm × 4.6 mm i.d. with a 5 μm particle size) and a silica based strong cation-exchange column (Phenomenex Phenosphere SCX, 150 mm × 3.2 mm i.d. with a 5 μm particle size). The ESI-MS instrument used for these studies was a quadrupole time-of-flight (Q-TOF) system from Micromass (Platform, Micromass, Manchester, UK). The instrument was operated in the positive ion mode. The applied voltage to the capillary was 3000 V, and the cone voltage was 30 V with N_2 as nebulizing gas. The spectra were scanned from 50 to 1000 *m*/*z*.

All reagents were analytical grade and used without further purification. For extraction of the Se species from plant tissues, 0.1 M hydrochloric acid was prepared by diluting an adequate volume of 12 M HCl (Fisher Scientific, Fair Lawn, NJ, USA) in 18 MΩ cm distilled deionized water (Sybron Barnstead, Boston, MA, USA). The mobile phase employed in the ion-pairing reversed-phase separation contained 0.2% heptafluorobutyric acid (HFBA) (Sigma, St. Louis, MO, USA) and 15% methanol (Fisher Scientific) at 0.8 ml min^{-1} with an injection volume of 50 μl. In the case of cation exchange, a gradient of pyridinium formate (as shown in Table 1) was employed. The selenium standards [Na_2SeO_3, Na_2SeO_4, Se-methyl-Se-cysteine(MeSeCys), and SeMet] were obtained from Sigma and diluted in 0.1 M

Table 1
Instrumental operating conditions for HPLC, ESI-MS and ICP-MS

	Values
ICP-MS parameters	
Forward power (W)	1300
External flow (l min^{-1})	15
Internal flow (l min^{-1})	1.0
Carrier gas flow (l min^{-1})	0.85
Selected isotopes	^{78}Se, ^{82}Se
Dwell time	0.1
Shield torch	Off
ESI-MS parameters	
Capillary voltage (kV)	3
Cone voltage (V)	30
Nebulizing gas	N_2
HPLC parameters	
Reversed-phase ion-pairing	
Column	C_8
Mobile phase	0.2% HFBA and 15% MeOH
Flow rate (ml min^{-1})	0.8
Injection volume (μl)	50
Cation exchange	
Column	Strong cation exchanger
Mobile phase	(A) 5.6 mM pyridinium formate (pH 3) and 3% MeOH (B) 100 mM pyridinium formate (pH 3) and 3% MeOH
Gradient	0–5 min 100% (A) 5–30 min from 100% (A) to 100% (B) 30–32 min 100% (B) 32 min 100% (A)
Injection volume (μl)	20
Flow (ml min^{-1})	0.8

HCl to prepare the stock solutions of 10 ppm as Se. These compounds were stored at 4 °C and used for 1 month after preparation. Working standards were prepared by dilution of the stock solutions into the mobile phase to obtain a final concentration of 100 ppb. Synthesis of SeMMet, trimethyl selenonium (TMSe), and dimethylselenonium propionate (DMSeP) were performed in-house with slight variation of the method described by Fan et al. [10]. Confirmation of the nature of these standards was accomplished by ^{1}H NMR and ESI-MS. Nuclear magnetic resonance (NMR) spectroscopy was done using a AC-250 MHz (Bruker Daltonics, MA, USA).

2.2. Sample treatment

Plant growth and sample treatment was accomplished as described elsewhere [15]. In summary, two different *Brassica juncea* lines, a transgenic line transformed with tDNA including the Se-cysteine methyl transferase gene and wild-type seeds were sterilized and then sown in a grid pattern in each magenta box. The nutrient solution contained half-strength MS medium (Sigma) with 10 g l^{-1} sucrose and 5 g l^{-1} phytagar (Sigma). After two days, they were placed in hydroponic solution containers and left to stand for 1 day. The composition of the hydroponic solution is described elsewhere [15]. The next day, SeMet solution was added to a final concentration of 50 μmol of Se l^{-1}. Plants were placed under constant light in a controlled environment room maintained at 25 °C for 2 weeks.

After this time the plants were harvested and separated into stems, shoots and roots. Every part was placed in liquid N_2 and ground with pestle and mortar. Extraction of the Se species is performed by shaking 0.2 g of the tissue in 2 ml of 0.1 M HCl (24 h, 37 °C).

3. Results and discussion

3.1. Optimization of ion-pairing reversed-phase chromatography

Reversed-phase ion-pairing HPLC has been widely used to speciate Se compounds in natural products and previous studies provide satisfactory results for speciation in plants [11]. The use of perfluorinated ion-pairing reagents such as trifluoroacetic acid (TFA), HFBA, or pentafluoropropanoic acid (PFPA) with C_8 columns has been a suitable methodology for the analysis of Se compounds in natural products [18] and biological samples [19]. This methodology was performed by using 0.1% HFBA as the ion-pairing reagent in 10% MeOH and was adequate to resolve most of the Se species found in the extracts. However, this initial methodology had to be modified accordingly for analysis of root extracts, and final conditions can be observed in Table 1. The volatilization pathway of Se in plants is

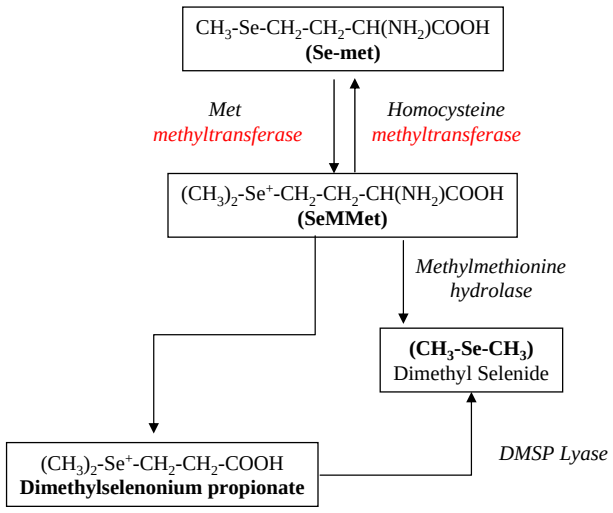

Fig. 1. Volatilization pathway of selenium in plants.

believed to be very similar to that of S and is suggested in Fig. 1.

Therefore, the production of DMSe could come through the formation of SeMMet and/or dimethylselenonium propionate. These species are not commercially available and had to be synthesized in the laboratory to be tested as possible components of the plant extract. The similarity in molecular structures of Se-amino acids and selenonium ions required careful optimization of the separation system. The concentration of the ion-pairing reagent and percentage of methanol in the mobile phases was optimized. Adequate separation of the species of interest can be accomplished by means of 0.2% HFBA and 15% MeOH, as can be seen in Fig. 2 (approximately 100 ppb as Se of all the standards except DMSe, with 20 ppb). Baseline resolution of SeMMet and MeSeCys can be observed as well as a reasonable separation of the two inorganic forms [Se(IV) and Se(VI)]. Under these chromatographic conditions, it is also possible to separate DMSe, normally determined by GC due to its high volatility, but probably present also in the extracts.

3.2. Optimization of cation-exchange chromatography

Species separation by ion exchange can also be applied successfully in this case due to the ionic nature of the compounds under study. For such optimization, the pH and concentration of the mobile phase buffers are critical for achieving good resolution between species with similar ionic characteristics. The similar dissociation constant of most of the species under study (Se-amino acids) requires the use of gradients in the ion-exchange separation. Since the other species of interest (selenonium ions) are cationic, cation-exchange chromatography was the preferred separation mechanism. Different mobile phases were studied in order to separate the analytes of interest. Initial studies were based on the work carried out by Larsen et al. [20] using a pyridinium formate gradient at pH 3.

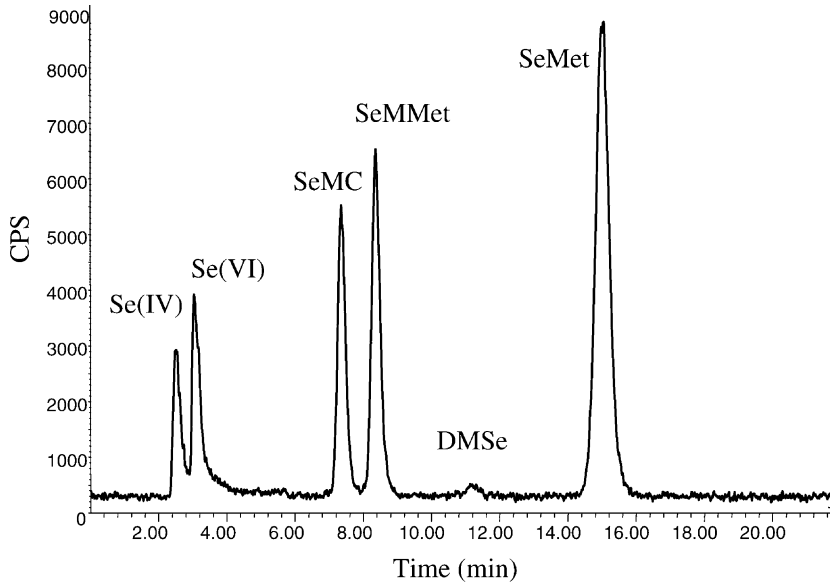

Fig. 2. Reversed-phase separation of selenium standards, using 0.2% HFBA and 15% MeOH. Each standard is 100 ng ml^{-1} except DMSe, which is 20 ng ml^{-1}.

The column used required a 5.6 mM buffer concentration (pH 2.9) to achieve good separation of SeMet and Se-methylselenocysteine in 3.3 and 5.2 min, respectively, in an isocratic run. Trimethylselenonium (TMSe) and SeMMet required a much higher buffer concentration to elute in a reasonable time and the final gradient steps appear summarized in Table 1. The chromatographic profile obtained for some of the species under study are given in Fig. 3. In general, the selenoamino acids elute first followed by the selenonium species. As documented by other authors [20], 3% MeOH added to the mobile phase resulted in an improved sensitivity with ICP-MS for Se determination.

3.3. Analysis of Se species and total Se concentration in Brassica juncea root extracts

Since volatilization takes place in the roots at a higher rate than in the shoots, this part of the plant was selected to perform our studies about the Se volatilization pathway and the production of selenonium ions. *Brassica juncea* roots exposed to Se in the form of 200 μM SeMet were extracted in 0.1 M HCl as described elsewhere and analyzed for total Se and for Se speciation. Wild type and genetically modified Brassica extracts (overexpressing the gene encoding for selenocysteine methyltransferase) were analyzed for total Se

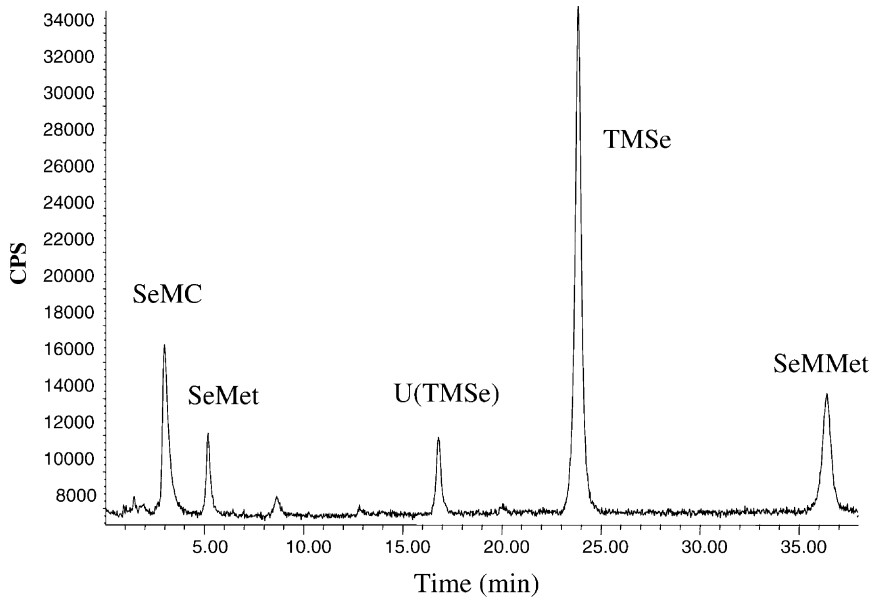

Fig. 3. Cation-exchange separation of selenium standards, using pyridinium formate gradient.

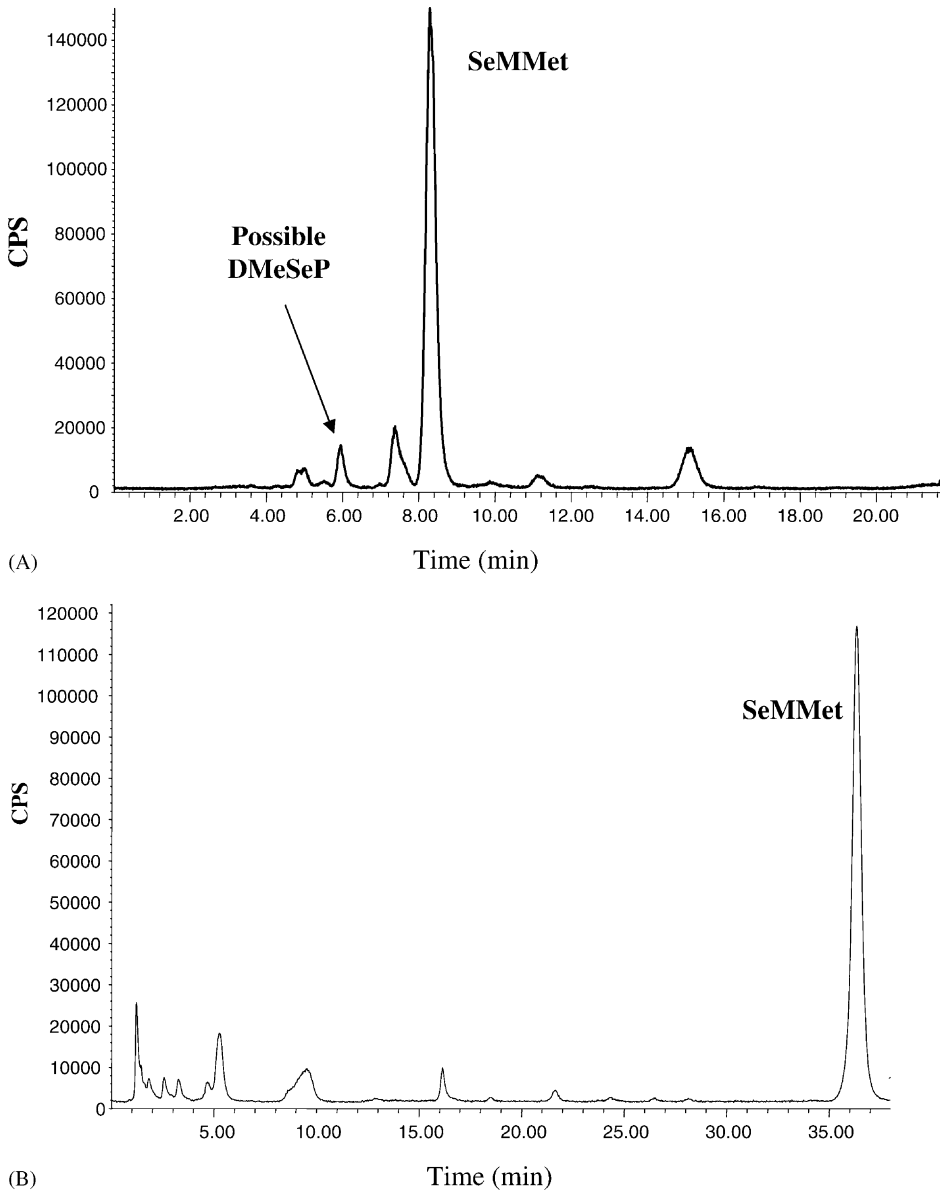

Fig. 4. Separation of selenium species in plant root extract by: (A) reversed-phase ion-paring chromatography; (B) cation-exchange chromatography.

by external calibration with an internal standard (Ge). The total Se concentration in the roots was $120 \pm 16\,\mu g\,g^{-1}$ in both plant lines. This is about 3 times higher than the total elemental concentration in the plant leaves (about $40 \pm 3\,\mu g\,g^{-1}$). The genetic modification does not seem to confer extraordinary capabilities to the plant in terms of SeMet uptake and accumulation [11].

Se speciation of plant extracts was performed by applying the previously described chromatographic techniques (reversed-phase ion-pairing and cation exchange). Fig. 4A and B show the separation of Se species in a plant extract by reversed-phased ion-pairing (A) and cation-exchange (B) chromatography. As can be observed, the primary species is, in both cases SeMMet. This is likely the precursor of the volatile DMSe, identified with both chromatographic systems. In the case of genetically modified Brassica, the peak area corresponding to SeMMet is about 73% of the total area of the Se species eluted in the chromatogram and in the case of the wild-type plant, this is about 60% (from the reversed-phase chromatograms). However, the compounds produced in both plants (wild type and genetically modified) are the same. The plant leaves were also analyzed using the same chromatographic separation, and Fig. 5 shows the presence of SeMMet as well as SeMC, but in different proportions relative to the results found for the roots. As said before, phytovolatilization takes place more efficiently in the roots so selenonium precursors are more likely present in that part of the plant.

Other minor species found in Brassica roots were SeMet and dimethylselenide. Since previous work characterizing

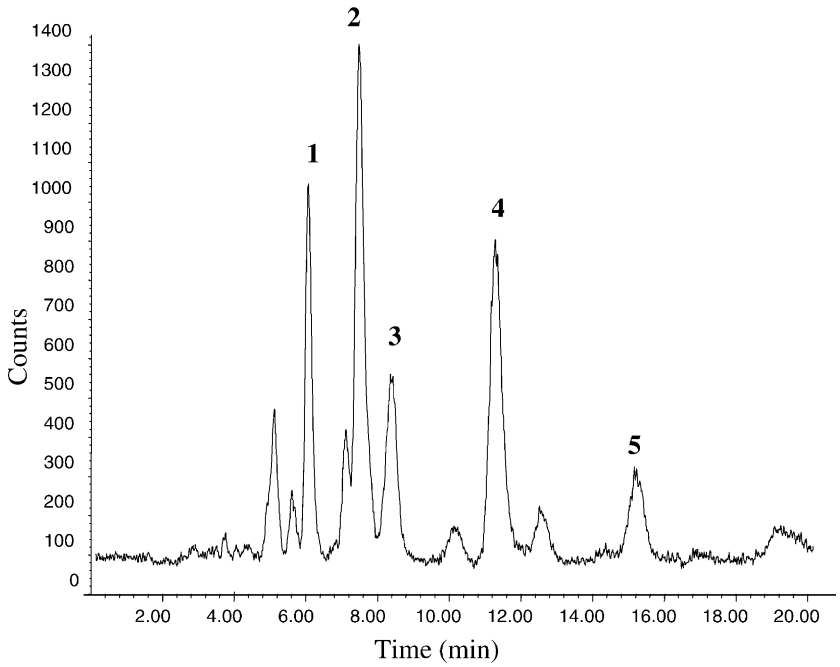

Fig. 5. Reversed-phase separation of selenium species in the extract of the plant leaves.

DMSe precursors showed dimethylselenonium propionate as a primary species in Spartina alterniflora [6] an attempt to synthesize DMSeP was made. The chromatographic data showed several peaks for the synthesized compound, including TMSe and a primary species eluting at about 6 min that might be the same as that observed in Brassica root extracts. Other studies have showed that in members of the Brassicaceae, like *Brassica juncea* (non-halophytes), SeMMet is more likely to be the precursor of DMSe rather than DMSeP [9]. According to the obtained results, this seems to be the case since no evidence of the presence of DMSeP has been observed.

3.4. Electrospray-Q-TOF-MS analysis of the extracts

The use of HPLC–ICP-MS provides highly valuable information enabling the quantification of very low levels of some Se species in plants. Also, the use of two different chromatographic types confirm the presence of SeMMet as the major species found in Brassica roots under these sample preparation conditions. Nevertheless, further confirmation of the nature of this species was accomplished by ESI-Q-TOF-MS. The first step was the characterization of the synthesized standard of SeMMet to establish the molecular ion and possible fragmentation pathways. This study was performed by continuous insertion of the solution into the ESI with pneumatically assisted nebulization using N_2 as nebulizing gas.

Fig. 6 shows the mass spectra of the synthesized SeMMet. As can be observed in Fig. 6, the molecular ion is seen at *m*/*z* 212, corresponding to the molecule $(CH_3)_2Se^+CH_2CH_2CH(NH_2)COOH$. It is also possible to observe the presence of *m*/*z* 198, ascribed to the excess of SeMet, used as reagent to produce SeMMet. An important signal at *m*/*z* 102 (from loss of CH_3SeCH_3 in $(CH_3)_2Se^+CH_2CH_2CH(NH_2)COOH$) is also seen. When collision-induced dissociation (CID or MS–MS) was taken at *m*/*z* 212, the mass spectrum showed in the inset of Fig. 6 was obtained. It is clear that the fragment at *m*/*z* 102 corresponds to the loss of the (CH_3SeCH_3, *m*/*z* 110) and therefore, does not contain the typical Se isotopic pattern that would be obtained if one Se atom were present in the fragment. Other than that, the minor species at *m*/*z* 158 has not been identified at this point.

Once the major fragments obtained in the standard were characterized, plant root extracts were analyzed. The ESI-MS data showed a complex spectrum that can be seen in Fig. 7A showing a minor signal that is observed at nominal *m*/*z* 212 (possibly ascribed to SeMMet). To further confirm the presence of this Se species on the extract, MS–MS was performed on the ion 212. Therefore, *m*/*z* 212 was selected in the first quadrupole and collisional induced dissociation was taken followed by fragment detection on the TOF (MS–MS). The resulting mass spectrum is shown in Fig. 7B. The obtained mass (212.0185) versus that calculated one for the proposed SeMMet (212.0190) is −2.2 ppm. The CID spectrum at this mass shows the primary fragment at *m*/*z* 102, as was seen for the standard. With this CID fragment the accuracy is −2.0 ppm for the calculated mass (102.0555) versus the obtained one (102.0553). It is possible to detect the presence of the fragment at *m*/*z* 158, also present in the standard, apparently associated to the molecule but still unidentified. Other minor species detected by HPLC–ICP-MS such as DMSe or SeMC could not be identified by ESI-Q-TOF-MS probably due to the

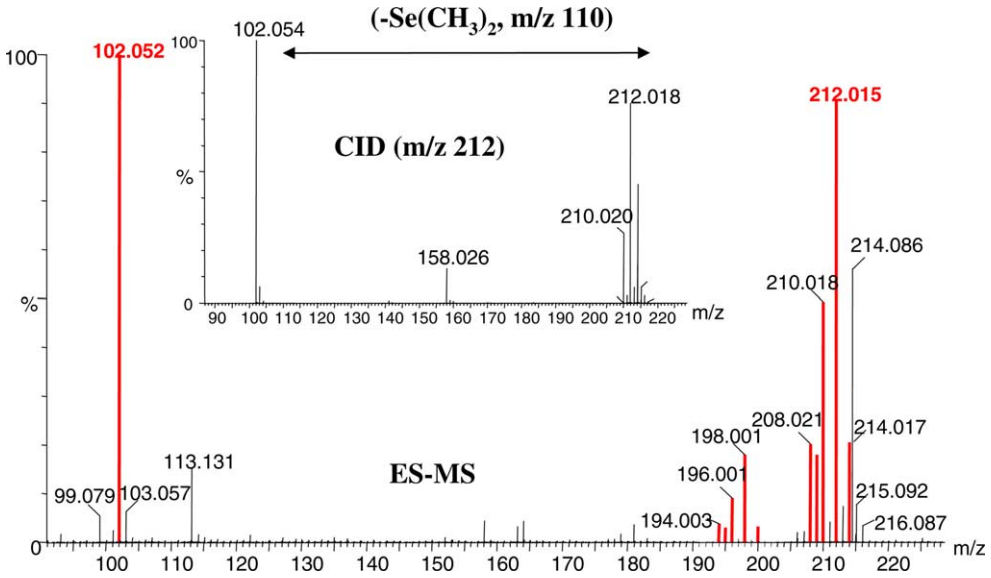

Fig. 6. Mass spectra of synthesized SeMMet standard.

low concentration of these species in the extract (ICP-MS allows low ppb detection level). Only SeMet seems to be detectable appearing at *m*/*z* 198 (protonated molecular ion) in Fig. 7A.

The mass spectra of the other synthesized species were also obtained and are shown in Fig. 8. The molecular ion appears at *m*/*z* 183 with the corresponding Se isotopic pattern $(CH_3)_2Se^+CH_2CH_2COO^-$. An important impurity from the

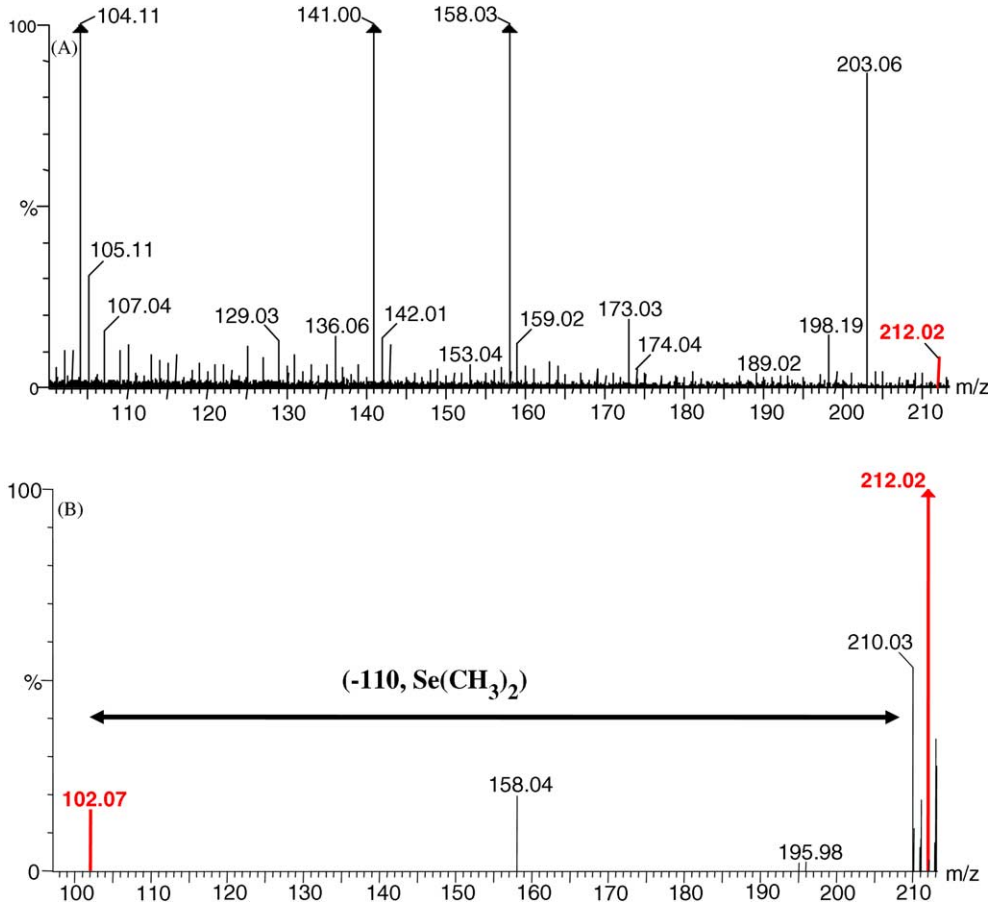

Fig. 7. (A) Mass spectra of plant extract showing *m*/*z* 212 possible corresponding to SeMMet. (B) MS/MS spectra of *m*/*z* 212, showing fragmentation of SeMMet.

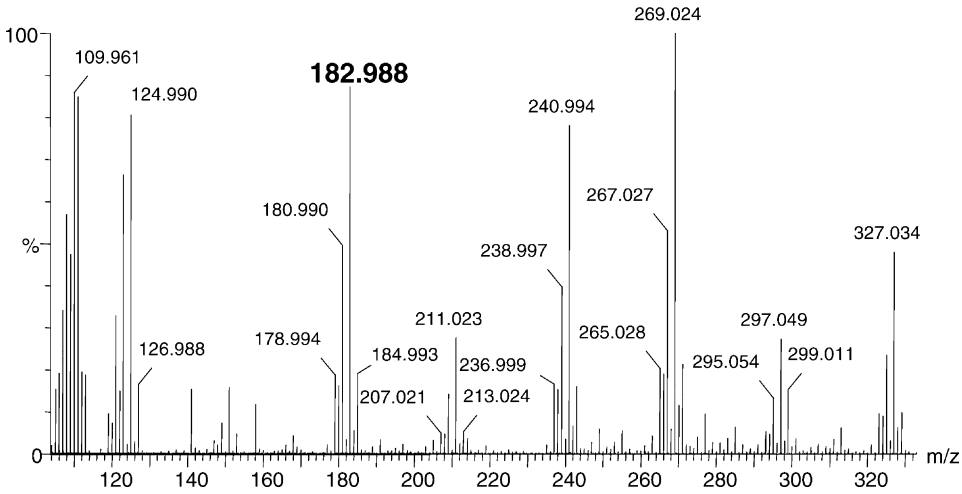

Fig. 8. Mass spectra of synthesized TMSe standard.

synthesis is TMSe (*m*/*z* 125) which can also be observed using HPLC–ICP-MS with reversed-phase ion-pairing chromatography. Higher molecular weight Se containing species can be also observed at *m*/*z* 241, 269, or 327, which may result from post-ionization association, since only three major peaks were observed in the chromatography.

4. Conclusions

Numerous pathways and precursors have been proposed to explain the volatilization of DMSe from soils and various plant species. In this manuscript, we have identified SeMMet in *Brassica juncea* roots as a molecule that appears to be the principal precursor of DMSe when plants are supplied with SeMet. To our knowledge, no definitive characterization of such species in *Brassica juncea* has been reported. Experiments including two chromatographic types with ICP-MS detection and augmented with the ESI-MS data, provides strong evidence for SeMMet identification. Clearly, the application of well-developed analytical techniques such as HPLC coupled to ICP-MS followed by ESI-MS has been effective in this study. The low detection limits of ICP-MS allows the detection of some minor species (possibly DMSeP), and the use of ESI-MS permitted further characterization of the synthesized standards as well as the major species in the plant extract.

Although initial characterization of standards can be done using just ESI-MS, the use of CID is generally required when dealing with plant extracts to strengthen the evidence for associating the fragment obtained to the parent ion precursor.

Acknowledgements

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