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Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (*Allium sativum* and *Brassica juncea*) and Se speciation by HPLC-ICP-MS

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Abstract

Several sample extraction techniques have been evaluated in order to obtain highest selenium (Se) extraction efficiency in two types of selenium-enriched plants (*Allium sativum* and *Brassica juncea*). Three extracting solutions have been studied for this purpose: 0.1 M HCl, 25 mM ammonium acetate buffer (pH 5.6) and protease in aqueous solution. In each case, the effect of the ultrasonic probe during extraction was also evaluated. Selenium extraction yields were calculated based on the ICP-MS determination of the total selenium content in the corresponding extracts and in the plant tissue after its microwave digestion. The action of ultrasounds allowed the reduction on the extraction time while maintaining good Se recoveries (which ranged from 75 to 120% of the total Se in the plant). The accuracy of total Se determination was controlled by analyzing a reference material (aquatic plant, BCR-670). On the other hand, speciation studies of the extracts were carried out by using ion-pairing reversed phase and size exclusion/ion exchange (Shodex Asshipak) liquid chromatographic columns. The two separation mechanisms were suitable to isolate the main extractable Se species which were identified as Se-methyl selenocysteine and Se-methionine in both systems. The extracts of both plants (*A. Sativum* and *B. juncea*) exhibited also the presence of several unknown Se-species.

Keywords: Se-amino acids; Se-enriched plants; Se extraction methods; Ultrasonic probe; HPLC-ICP-MS; Speciation

1. Introduction

Selenium (Se) is an essential micronutrient for humans that enters the metabolism through the diet as constituent of vegetables and cereals [1]. Anti-carcinogenic properties of selenium have been evaluated in various studies [2] at supra-nutritional levels. However, the low abundance of this element in everyday food restricts the adequate Se intake level for a possible cancer preventive effect, according with the studies published by Clark et al. [2]. For this reason, there is a growing market on development of selenium-enriched nutritional supplements in which selenized yeast (generally *Saccharomyces cerevisiae*) has been the most widely studied [3,4]. The majority of the Se present in selenized yeast samples (about 70%) is in the form of Se-methionine (Se-met) [5,6]. Some studies have revealed that this compound tends to accumulate in tissues, and this represents its main limitation for cancer inhibition [7]. One alternative to selenized yeast is the enrichment of certain vegetables with Se and, in this regard, previous studies have shown that Se-enriched onions and garlic have an important reducing effect in chemically induced mammary tumours in rats, without accumulation of Se in the tissues [8].

The primary forms of Se found in selenium-enriched vegetables from *Allium* family (garlic, onions, broccoli and sprouts) are Se-methyl selenocysteine (SeMC) and derivatives like γ-glutamyl-Se-methylselenocysteine [1]. These non-protein Se-amino acids have been also reported to be present in Se-accumulator plants of the genus *Brassica* and *Astragalus* [1]. It has been hypothesized that γ-glutamyl-Se-

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methyl selenocysteine serves primarily as carrier of SeMC, [9] which is a good precursor for generating methylselenol when enzymes like *β-lyase* are present. This Se-metabolite seems to be the most active species for cancer reduction [10]. Therefore, Se-methyl selenocysteine has been widely studied as a potential anti-carcinogenic compound [11]. Thus, information on the speciation of selenium in plants, selenium-enriched food, and nutritional supplements is of paramount importance.

The most frequently used technique for Se speciation analysis is today a chromatographic separation coupled to inductively coupled plasma mass spectrometry (ICP-MS) detection. Before the chromatographic separation, however, the analytes must be extracted from the solid samples where identification and determination of the selenium species is intended. This solid–liquid extraction procedure is probably the most important and critical step concerning selenium speciation. Thus, several extraction methodologies have been studied in order to improve Se species recoveries from natural products, in particular, from plant material and dietary supplements [12]. In the case of selenized yeast, best results have been obtained when using enzymatic extractions that liberate Se-methionine (the most abundant Se species in this sample), present as an amino acid non-specifically incorporated into proteins or weakly associated to other biomolecules [13]. Such enzymatic hydrolysis, when assisted by ultrasounds, has provided a dramatic reduction of the extraction time [14].

However, fewer studies have been performed to evaluate extraction procedures for other amino acids such as Se-methyl selenocysteine. In this case, no important differences are expected to be found between enzymatic and non-enzymatic hydrolysis since Se-methyl selenocysteine does not seem to be incorporated into proteins [15]. Therefore, this work studies the possible quantitative differences encountered in the extraction of Se from plants when using three different extracting solutions: HCl (0.1 M), ammonium acetate buffer (pH 5.6) and protease in aqueous solution. Every extracting solution will be applied with and without the action of ultrasounds through the use of a titanium probe. These studies have been performed in two different plant species grown in the presence of Na_2SeO_3: garlic (*Allium sativum*) and Indian mustard (*Brassica juncea*). Total Se extraction efficiency (for each extraction procedure) are calculated based on the quantitative Se results obtained by microwave digestion of the plant and total Se determinations, both in the digest and the corresponding extracts, by ICP-MS.

In order to study species integrity (mainly Se-methyl selenocysteine) during the different extractions (especially in the case of applying ultrasounds), initial Se speciation studies have been performed on the extracts. For this purpose, two different chromatographic mechanisms have been applied to the extracts in order to asses the identity of the extracted species: reversed phase ion-pairing chromatography (RP-IP-HPLC) with heptafluorobutyric acid (HFBA) as ion-pairing agent [16] and an HPLC new column combining size exclusion and ion exchange mechanisms (Shodex) [17].

2. Experimental

2.1. Instrumentation

An inductively coupled plasma mass spectrometer equipped with a collision cell system, model 7500 from Agilent Technologies (Agilent, Tokyo, Japan) was used for the determination of total selenium as well as HPLC detector. The instrument was fitted with a PFA micro-nebulizer (Agilent microflow nebulizer). Hydrogen was used as collision gas at 2 mL min^{-1} for eliminating Ar polyatomic interferences at *m/z* 78 and 80. Other instrumental conditions were as follows: rf forward power, 1500 W; plasma flow rate, 15.0 L min^{-1}; auxiliary flow rate, 1 L min^{-1} and nebulizer flow rate, 1.14 L min^{-1}.

Microwave digestion of the samples was performed in a closed vessel Milestone instrument (Model MLS 1200, Sorisole, Italy). The samples were freeze–dried at −54 °C for 48 h (Heto, Lyolab 3000, JOUAN S.A., France). A mechanical shaker was used to homogenize the plant material and the extracting solution while maintaining the samples in a chamber at 37 °C for 20 h. Ultrasounds were applied through a titanium probe (Sonics and Materials Inc., Danbury, CT, USA) for 3 min (80% duty cycle) in order to avoid the overheating of the mixture. 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.

A Shimadzu HPLC pump was used as the solvent delivery system (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) and injections were made using an injection valve (Model 7125) with a 20 μL injection loop (Rheodyne, Cotati, CA, USA). The column was a C_8 (250 mm × 2.0 mm i.d., 5 μm) (Alltima, Alltech Associates, Deerfield, IL, USA) 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.). The mobile phase composition for ion-pairing reversed phase was as follows: 0.2% (v/v) heptafluorobutyric acid, 10% (v/v) methanol (pH 2.5) and it was pumped at a flow of 0.2 mL min^{-1}. In the case of the SEC column, the used mobile phase contained 10 mM ammonium acetate buffer (pH 6.5) and it was pumped at 0.6 mL min^{-1}.

2.2. Reagents and materials

All the reagents were of analytical grade and used without further purification. Hydrochloric acid (0.1 M) was prepared by diluting an adequate volume of 12 M HCl (Merck, Darmstadt, Germany) in 18 MΩ cm distilled de-ionized water (Millipore, Bedford, MA, USA). Similarly, 25 mM ammonium acetate buffer was prepared by dissolving 0.96 g of the salt (Merck) in Milli-Q water (0.5 L.) and the pH adjusted to 5.6 with acetic acid (Merck). Protease for the enzymatic extraction was obtained from Sigma–Aldrich (Madrid, Spain).

HPLC mobile phases were prepared with heptafluorobutyric acid (HFBA) from Sigma–Aldrich and HPLC grade methanol from Merck. Nitric acid (Suprapure) 68% and H_2O_2 30% from Merck were used for sample digestion. The selenium standards (Na_2SeO_3, Na_2SeO_4, Se-methyl selenocysteine and Se-methionine) were purchased for Sigma–Aldrich and diluted in 0.1 M HCl (Merck) to prepare the stock solutions of 10 ppm as Se. These stock solutions were stored at 4 °C. Working standards were prepared daily by appropriate dilution of the stock solutions. For the determination of total selenium content, working solutions were prepared daily by appropriate dilution of 1 mg mL^{-1} Se (IV) standard solution. The reference material aquatic plant (BCR-670) with a recommended Se content range was used for method evaluation. Distilled de-ionized water (18 MΩ cm) obtained with a Milli-Q system (Millipore) was used throughout.

2.3. Procedures

2.3.1. Plant culture

Plant species used in this study include garlic (*A. sativum*) and Indian mustard (*B. juncea)*. Garlic plants were cultured hydroponically in a greenhouse at temperatures between 16 and 25 °C and treated with Na_2SeO_3 which was added to the nutrient solution to a final concentration of Se of 25 and 50 μM, respectively. Then, the plants were left stand for 1 month. After this time, the plants were harvested and the cloves were washed with water and freeze–dried. The dry material was then ground with pestle and mortar and kept at 4 °C until their analysis. *B. juncea* plants were grown as described elsewhere [18] and exposed to 25 μM Na_2SeO_3 for one week. The green plant tissues (stems and shoots) were frozen with liquid nitrogen to break the cell walls, ground with pestle and mortar and stored at −18 °C until analysis.

2.3.2. Se extraction from plant tissues

Three extraction procedures: acid extraction (0.1 M HCl), buffer extraction (25 mM ammonium acetate buffer, pH 5.6) and enzymatic hydrolysis were studied. For the acid and buffer extraction, 2.5 mL of the corresponding solution were added to 0.2 g of the dried plant tissues in 10 mL centrifuge tube. In the case of the enzymatic hydrolysis, 2.5 mL of Milli-Q water were added to 0.2 g of the sample and 0.05 g of protease in 10 mL centrifuge tube. The mixtures were thoroughly homogenized by mechanical shaking in a chamber kept at 37 °C for 20 h. The mixtures were afterwards centrifuged at 2000 × *g* for 20 min and the supernatant removed and filtered through 0.45 μm. The extracts coming from the enzymatic hydrolysis were passed through a 3000 Da ultracentrifugation membrane in order to remove the excess of protease which might interfere in the chromatographic separation.

Three independent extractions were made, in each case from the same sample and aliquots of each extract were taken for total Se determination by ICP-MS as well as Se speciation by HPLC-ICP-MS. In this latter case, the sample aliquots were diluted in 0.1 M HCl for the buffer and protease extracts and in Milli-Q water in case of HCl extraction before injection.

2.3.3. Ultrasonic assisted extraction of Se from plant tissues

For ultrasonic extraction of the samples the same extracting solutions detailed above were used, but the mixtures were homogenized through the action of ultrasounds applied through a titanium probe. The action of ultrasounds for longer than 3 min provided an important over-heating of the mixture and therefore 3 min was selected as extraction time. Similarly to the case of conventional extraction, the experiments were carried out on triplicate and total Se determination as well as Se speciation was performed on each sample extract.

2.3.4. Total selenium determination

The total Se concentration in the investigated plant tissues (garlic cloves and Indian mustard green tissues) was determined by ICP-MS after microwave digestion of the samples. About 0.2 g of dried tissue was digested with 1.5 mL of HNO_3 and 1.5 mL of H_2O_2 under the following microwave digestion program described elsewhere [19]. The solutions obtained were diluted with Milli-Q water up to 10 mL. The total Se concentration was determined by ICP-MS (working conditions under experimental) with external calibration using internal standards (Ga, at 5 ng mL^{-1}). The performance of the method was tested by analyzing the available aquatic plant certified reference material BCR-670.

3. Results and discussion

3.1. Efficiency of extraction

The goal of our study was to obtain the maximum extraction efficiency as well as to study possible changes on the species integrity depending on the extraction procedure used. Thus, total selenium concentration in extracts obtained from acid, buffer and enzymatic extraction, of the two Se-exposed plants (*Brassica* and *Allium*) was determined. The results were compared with those obtained by MW digestion of the same samples in order to calculate Se extraction yields for each treatment. The suitability of the MW digestion method applied was addressed by analyzing a reference material (aquatic plant BCR-670). This plant material is certified for rare earths although an indicative value about the Se content (ranging from 0.149 to 0.273 mg kg^{-1}) is also provided. Using the proposed MW method (detailed on the Section 2.3), the total Se concentration found was 0.243 ± 0.06 mg kg^{-1} ($n = 3$). Therefore, the digestion method seems to be suitable for quantifying total Se in the tissues of the two Se-exposed plants.

Table 1 shows the obtained results for the extraction of Se in *B. juncea* by HCl, ammonium acetate buffer (pH 5.6) and protease, with and without ultrasonic probe action. As it

Table 1
Selenium extracted from *Brassica juncea* green tissues under different extraction conditions with and without ultrasonic probe ($n = 3$)

Extraction Se ($\mu g\,g^{-1}$)	HCl (0.1 M)	Ammonium acetate (25 mM, pH 5.6)	Protease
No ultrasounds	8.74 ± 0.27	9.80 ± 0.24	9.43 ± 0.15
Ultrasounds	6.80 ± 0.14	7.13 ± 0.17	10.61 ± 0.12

can be observed in Table 1, the differences in the extracted selenium are not statistically significant among extracting solutions when no ultrasonic probe is applied. However, if the extraction procedure involves the action of ultrasounds, it is possible to observe slightly higher yields with protease in respect to the buffer and HCl treatment. Table 1 also shows that, in all cases, the amount of selenium extracted does not increase upon the action of ultrasounds; however, the time for the extraction is substantially shortened (from 20 h to 3 min).

The results on Table 1 can be ascribed to the fact that in *B. juncea* the main Se species are non-protein amino acids (such as Se-methyl selenocysteine) and so they are not expected to be incorporated into protein structures. Therefore, the application of enzymatic hydrolysis that would cleave protein structures into peptides does not provide significant improvement in the Se extraction yields. This is not the case of the protein-rich selenized yeast where a significant part of the amino acids are incorporated into proteins [20].

Table 2 shows the amount of selenium extracted for *A. sativum* exposed to 25 and 50 μM Se using the three extraction methodologies under study, with and without sonication. Similarly to the results observed for *B. juncea* (Table 1), the amount of Se extracted is comparable for all the extracting solutions under study and only slightly higher Se yields can be obtained when using enzymatic hydrolysis. As before, the action of ultrasounds allows the reduction of the extraction time while maintaining the total amount of selenium extracted.

The results obtained in the case of exposing garlic to 25 μM of Se are very similar to those for 50 μM, although in this latter case, the total Se concentration in the sample is higher. As can be observed, Se extraction recoveries are above 75% for the HCl and buffer extractions showing that most Se is extractable with leaching procedures even in comparatively mild digestion conditions. In the case of protease, Se recoveries ranged from 103 to 127% showing that a very small part of Se could be associated to proteins/peptides in plant tissues (recoveries higher than 100% could be due to some contamination of protease used with selenium). Table 2 also shows that the use of the ultrasonic probe allows similar Se extraction yields in only 3 min to those obtained by 20 h mechanical shaking.

Table 2
Selenium extracted from garlic samples (exposed to 25 μM Se and 50 μM Se, respectively) under different extraction conditions

Extraction method	Extracted Se ($\mu g\,g^{-1}$) (25 μM Se)	Extracted Se ($\mu g\,g^{-1}$) (50 μM Se)
HCl (0.1 M)	5.99 (96%)	5.71 (62%)
25 mM Ammonium acetate (pH 5.6)	5.91 (95%)	9.87 (107%)
Protease	6.43(103%)	11.65 (127%)
HCl (0.1 M) (USP)	5.21 (84%)	7.00 (76%)
25 mM Ammonium acetate (pH 5.6) (USP)	5.20 (84%)	7.34 (80%)
Protease (USP)	7.59 (122%)	9.72 (106%)

Between brackets, the recoveries of the different extraction methods in respect to the total Se concentration obtained by MW digestion of the samples. For $n = 3$, R.S.D. ranged from 5 to 10%, USP: with ultrasonic probe applied.

In any case, it can be concluded that Se-exposed *Alliums* studied here do not seem to accumulate it in the form of Se-proteins since enzymatic hydrolysis did not provide Se extraction yields significantly higher than those observed with non-enzymatic extracting solutions.

3.2. Speciation studies

Considering the previously shown Se extraction yields, the action of ultrasonic probe is able to reduce dramatically the extraction time, which is of critical importance for speciation studies. However, in order to prove the suitability of the US assisted extraction method for future speciation studies, it is necessary to ensure species integrity during the whole extraction/separation/detection process. In this regard, matching retention times of standards in a single chromatographic method should not be used as conclusive evidence of the presence of a certain species in the sough sample. Therefore, all the extracts were run through two different chromatographic columns with different retention mechanism. First, RP-IP-HPLC was used to analyze the extracts since that chromatography has proved to be suitable for analyzing this type of samples [21]. The separation, in this case, is performed using a mobile phase containing 0.2% (v/v) HFBA and 10% (v/v) methanol and ICP-MS, coupled on-line for specific Se detection, is used. Similarly, a new chromatographic column with a combined mechanism of size exclusion and ionic exchange has been also evaluated for the same purpose. This chromatographic device has been successfully used in previous studies of Se speciation in mushroom samples [17] using a mobile phase of 10 mM ammonium acetate buffer (pH 6.5).

The RP-IP-HPLC-ICP-MS chromatograms (^{80}Se) of both the HCl and buffer extracts of *B. juncea* with the application of ultrasounds are shown in Fig. 1A and B, respectively. As expected, major known species in both sample extracts are Se-methyl selenocysteine and Se-methionine, identified by comparison with the retention times of standards and by spiking experiments. In any case, an important contribution to the total Se signal was also evident for unknown Se species. It was important to note that better separation of the Se species is obtained when using HCl as extractant (Fig. 1A). In the case of using a buffer extraction (Fig. 1B), an important number of unresolved Se species within the first 7 min of the chromatogram was apparent. The Se chromatographic profile obtained for HCl extraction without applying ultra-

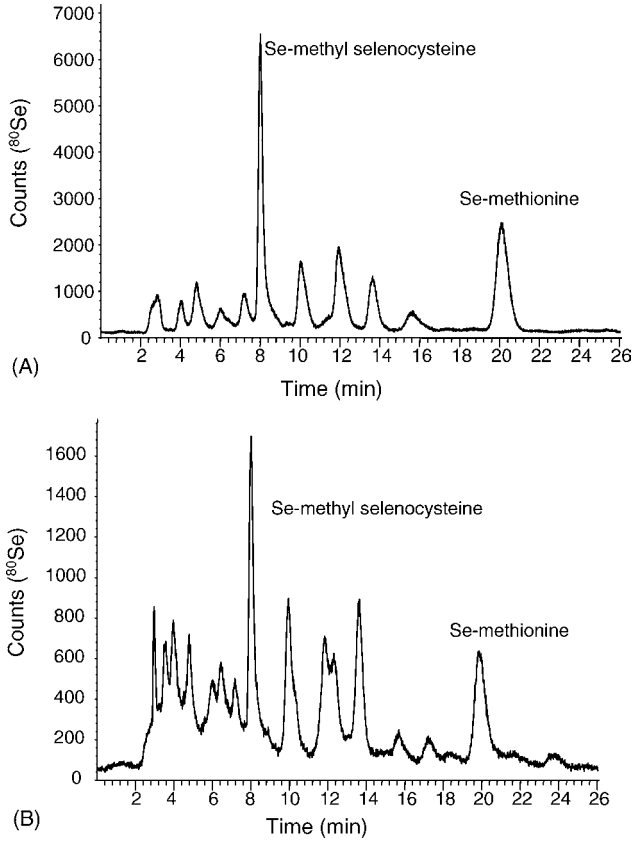

Fig. 1. Chromatographic profile obtained by HPLC-ICP-MS for ^{80}Se corresponding to (A) HCl extraction and (B) ammonium acetate buffer extraction both assisted by ultrasounds in *Brassica juncea* grown in the presence of Se (IV). Identification of Se-methyl selenocysteine and Se-methionine by matching retention times with the standards.

sounds (data not showed) is very similar to that in Fig. 1A. However, a rather different profile was observed in the case of the buffer extract where a much lower resolution among species was obtained when no ultrasounds were applied. Therefore, the use of ultrasonic probe does not provide any apparent species degradation in comparison to the leaching method.

For further confirmation of the obtained results, both *Brassica* extracts (HCl and buffer with no ultrasonic assisted extraction) were run through the Shodex column and the results can be observed in Fig. 2. As in the case of RP-IP-HPLC, a perfect matching with the retention time of the standards has been obtained for the identified species (Se-methyl selenocysteine and Se-methionie) in the HCl extract (Fig. 2). As in the case of RP-IP-HPLC, Se-methyl selenocysteine and Se-methionine seem to be more efficiently extracted by HCl although both species can also be detected in the buffer extract (Fig. 2).

In the case of protease extraction, the samples were previously passed through a membrane of pore size 3000 Da in order to eliminate the excess of protease that might disturb the chromatographic separation. Even so, the chromatographic profile by RP-IP-HPLC (data not showed) is completely different to those observed in Fig. 1. In this case, most Se elutes within the first 8 min of the chromatographic run. Unfortunately not peaks identification could be performed by retention time matching. Moreover, similar results were obtained when the extraction was carried out with the action of ultrasounds. The reason for this peak shifting is still unknown but the observed behaviour was reproducible in all cases.

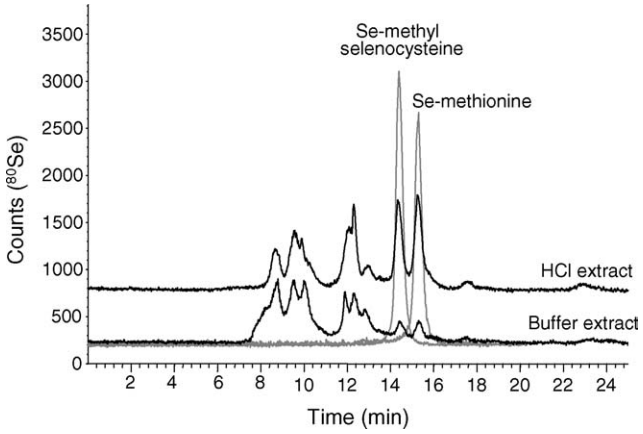

Fig. 2. Chromatographic profile obtained by HPLC-ICP-MS for ^{80}Se corresponding to HCl and ammonium acetate buffer extractions both assisted by ultrasounds in *Brassica juncea*. The standards of Se-methionine and Se-methyl selenocysteine are superimposed. The column, in this case, has a combined mechanism of ion exchange and size exclusion (Shodex Asahipak GS-220 HQ).

For garlic samples (exposed to 50 μM Se), the chromatographic profile corresponding to the HCl and buffer extracts of the samples achieved by RP-IP-HPLC can be seen in Fig. 3A and B, respectively. As in the case of *B. juncea* extracts, Se-methyl selenocysteine and traces of Se-methionine were positively identified in the chromatograms of Fig. 3 by matching retention times with the standards. Species distribution in this case, as in the case of *Brassica* samples, is different for HCl and buffer extracts. In the HCl extract, Se-methyl selenocysteine is the most prominent Se-species while in the buffer extract, an unknown Se-species eluting at about 3 min seems to be extracted almost as efficiently as Se-methyl selenocysteine (t_r = 7.8 min). However, no differences were observed in the chromatographic profiles when comparing the extracts obtained with and without ultrasounds, so species integrity seems to be preserved in both cases. In the case of the extraction with protease, a lack of resolution among Se species eluting between 2 and 9 min could be due to the presence of higher concentrations of sulphur species in the same elution time range (data not showed). Those abundant S species, well known in garlic samples, can overlap Se species separation (an important peak broadening for Se is observed as well as peak shifting to lower retention times).

When these extracts were run through the Shodex column, it was possible to observe a slightly different chromatographic profile. This is shown in Fig. 4 (HCl extract) where

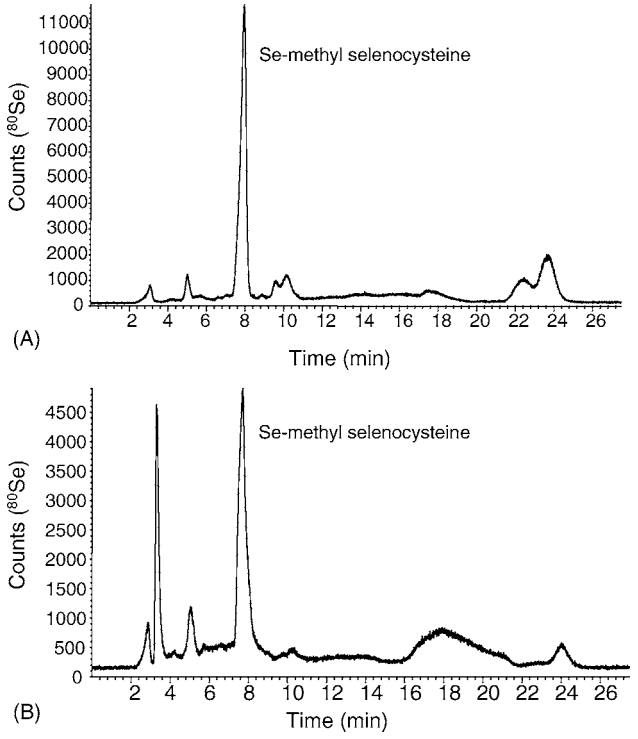

Fig. 3. HPLC-ICP-MS chromatogram for ^{80}Se extracted from garlic samples. (A) Using 0.1 M HCl and (B) using 25 mM ammonium acetate buffer without ultrasounds.

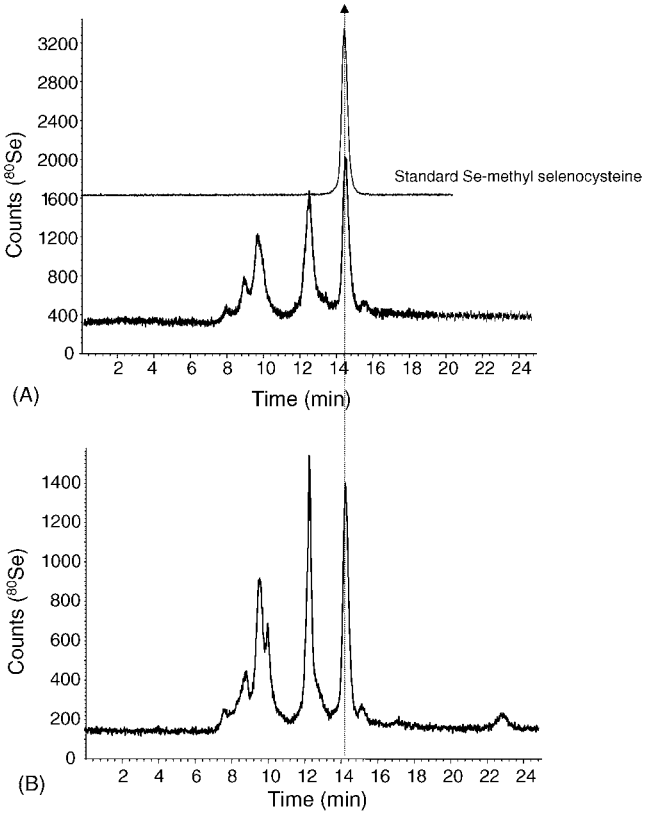

Fig. 4. Chromatographic profile obtained by HPLC-ICP-MS for ^{80}Se corresponding to HCl extraction of the garlic samples: (A) without ultrasounds and (B) with the use of ultrasounds. For clarity, the chromatogram of the standard of Se-methyl selenocysteine is shown off-axes. Shodex Asahipak GS-220 HQ column.

the chromatogram of a standard of Se-methyl selenocysteine has been also superimposed. Fig. 4A shows the HCl extract obtained with the use of ultrasonic probe and Fig. 4B without. Similarly to the RP-IP-HPLC, it is possible to observe Se-methyl selenocysteine as one of the main species extracted and traces of Se-methionine. However, there is also another Se-containing species (t_r = 12.5 min) that has not been identified by retention time with any of the standards available. This could be ascribed to γ-glutamil Se-methyl selenocysteine, another Se species often present in garlic extracts [22]. This species was either retained in the RP-IP-HPLC system or eluted in a broad elution profile (see Fig. 3B between 16 and 22 min) so it could not be clearly observed in the RP chromatogram. In any case, the same species were observed in the buffer extract of the garlic samples confirming the presence of Se-methyl selenocysteine as one of the main Se-containing compound.

As observed in the case of *Brassica* samples, no differences were found, in terms of the extracted species when applying ultrasounds to the extraction solution instead of mechanical shaking in any of the studied chromatographic methods. Therefore, the use of HCl or of the buffer, assisted by sonication seems to be an extremely interesting approach for quantitative extraction of non-protein Se-amino acids from plant tissues as it provides adequate efficiencies with very short extraction times with negligible species degradation.

4. Conclusions

Different extraction methodologies have been studied for the extraction of non-protein Se-amino acids such as Se-methyl selenocysteine and also Se-methionine from selenium-enriched plant tissues. Total Se extraction efficiencies ranged in all the experiments from 75 to 122% Se and no significant differences were found in the case of applying enzymatic hydrolysis of the plant tissues instead of acid or buffer extractions. A conclusion of those findings is that a minimum Se incorporation into proteins primary structures seems to occur in such plants. Also, the most remarkable aspect is that the action of ultrasounds allows a much faster extraction time with very similar extraction recoveries.

In the speciation of the sample extracts, HCl and buffer extraction methodologies with and without the use of ultrasounds have proved to be suitable for identification of Se-methyl selenocysteine and Se-methionine. Such species have been found in the *Brassica* and garlic samples by means of two different chromatographic mechanisms (RP-IP-HPLC and SEC/IE-HPLC). The most important finding of the study is that the use of ultrasonic probe allows a faster extraction of these species (using HCl or acetate buffer as extracting solutions). The application of ultrasonic probe provided neg-

ligible species degradation and this could be an extremely important approach for future speciation studies.

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