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Pretreatment procedure for selenium speciation in shellfish using high-performance liquid chromatography–microwave-assisted digestion–hydride generation-atomic fluorescence spectrometry

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A pretreatment procedure based on an enzymatic hydrolysis extraction followed by a two-step clean-up has been performed for selenium speciation in shellfish samples. Bivalve samples were extracted with protease XIV, lipase VII and protease VIII. By using a protease VIII–lipase VII mixture, quantitative recoveries were obtained for all the selenium species, except for selenocystine (59%). Owing to the complexity of the matrix, clean-up procedures were required to remove interferents that affected the chromatographic separation. The extracts were first partitioned in dichloromethane and then passed through a column with aminopropylsilane. Speciation of selenocystine, selenomethionine, selenoethionine, selenite and selenate was obtained using a high-performance liquid chromatography–microwave-assisted digestion–hydride generation-atomic fluorescence spectrometry coupling. The chromatographic system consisted of an anion exchange and a reversed-phase column, both connected through a six-port switching valve. On-line microwave-assisted digestion and hydride generation steps were performed prior to atomic fluorescence detection. The method was applied to clam and prawn samples collected from the southwest coast of Spain. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: selenium speciation; selenomethionine; selenocystine; selenoethionine; high-performance liquid chromatography; shellfish; enzymatic digestion; solid–liquid clean-up

INTRODUCTION

Selenium is an essential trace element for living organisms, but it is toxic at high levels. It exists in different chemical forms, as inorganic (selenite and selenate) and as organic species (selenoamino acids, selenoproteins), in environmental and biological matrices. The nutritional bioavailability and cancer chemoprotective activity of selenium depend on the concentration and the chemical form in which it is present.[1]

Selenium speciation involves the separation of species and the specific detection of the element. High-performance liquid chromatography (HPLC) has been reported as a suitable technique for the separation of non-volatile selenium species.[2] The separation of the inorganic selenium species, selenite and selenate, has been performed using anion-exchange stationary phases[3,4] or a reversed-phase column with ion-pair agents.[5] Reversed-phase columns have also been used to separate selenoamino acids.[1,6] Some authors proposed a phosphate buffer as the mobile phase for the simultaneous separation of inorganic selenium and selenoamino acids.[7] Others reported the use of a reversed-

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phase column modified with a vesicular mobile phase of dodecylammonium bromide (DDAB)[8] and ion-pair chromatography.[9] Another interesting approach was based on the successive use of an anion-exchange column and a reversed-phase column, both connected through a six-port switching valve.[10]

For the determination of these species, HPLC has been coupled to specific atomic detectors based on graphite furnace atomic absorption spectroscopy (GFAAS),[7] inductively coupled plasma-mass spectrometry (ICP-MS),[1,9,11] hydride generation ((HG)-AAS)[12] and HG-atomic fluorescence spectroscopy (HG-AFS).[10] Since only selenium(IV) forms SeH_2, a previous transformation of the other selenium compounds into selenium(IV) is required when AAS or AFS are used for detection. This can be done with $K_2S_2O_8$–NaOH or $KBrO_3$–HBr mixtures heated with microwave energy.[10,12,13]

Analytical techniques for organoselenium speciation in biological matrices usually need an extraction step followed by the chromatographic separation. Such extraction procedures involve the use of water,[14,15] water–methanol[14,16] or water–methanol–chloroform mixtures.[6,15,16] Selenocompounds are hydrolysed from the solid matrix using an MeOH–H_2O mixture with ammonia solutions or hydrochloric acid.[16] Enzymatic digestion is also used to fractionate the protein-containing materials. Proteolytic cleavage of selenoamino acids has been carried out on soybean proteins using successively pepsin, pancreatine and pronase.[5,7,14,15] However, one of the major drawbacks of these methods is the presence of a large amount of co-extracted lipids. The lipids must be removed, otherwise the chromatographic performance would deteriorate dramatically. Therefore, the extraction must be followed by a clean-up step to obtain extracts compatible with the HPLC[16] system. Other authors have just diluted the original extracts to reduce the matrix interference.[9]

In the present study, an extraction procedure for five selenium species based on enzymatic treatments of biota tissues was performed. Owing to the complexity of the sample matrix, clean-up procedures were required to remove interferences and prolong the lifetime of the column. This was carried out using several sorbents and solvent partitioning. The efficiency of these clean-up methods was evaluated with recovery experiments on spiked natural samples.

EXPERIMENTAL

Reagents

Stock standard solutions of selenium(IV) and selenium(VI) (selenium concentration: 100 mg l^{-1}) were prepared from analytical-reagent grade sodium selenite and sodium selenate (Sigma, Gillingham, UK) respectively. Selenoamino acids stock standard solutions were prepared at a concentration of 50 mg l^{-1} (as selenium) from seleno-DL-cystine (SeCyst), seleno-DL-methionine (SeMet) and seleno-DL-ethionine (SeEt) (Aldrich, Milwaukee, WI, USA). Stock solutions were stored in the dark at 4 °C. Diluted working solutions were prepared daily. Water obtained from a Milli-Q Gradient System (Millipore, Bedford, MA, USA) was used to prepare all the solutions. Concentrated HBr, HNO_3 and $HClO_4$ (Merck, Darmstadt, Germany) as well as $KBrO_3$, $NaBH_4$ and KCH_3COO (Panreac, Barcelona, Spain) were of analytical grade. Protease XIV (*Streptomyces griseus*), protease VIII (subtilisin Carlsberg) and lipase VII (*Candida rugosa*) obtained from Sigma (Gillingham, Dorset, UK) were tested for enzymatic hydrolysis.

Membrane filters of 0.45 μm and centrifuge filters of 10 000 molecular weight cut-off (MWCO) were obtained from Lida (Kenosha, WI, USA) and Supelco (Bellefonte, PA, USA) respectively. Hexane, pentane, chloroform and dichloromethane were pesticide grade (Romil, Waterbeach, Cambridge, UK). The sorbents as octadecyl (C-18), 2,3-dihydroxypropoxypropyl (Diol), benzenesulfonic (SCX), cyanopropyl (CN) and aminopropyl (NH_2) bonded silica (obtained from IST, Mid Glamorgan, UK) were tested for sample clean-up.

Instrumentation

The HPLC system consisted of a quaternary HPLC pump (Jasco 1580-PU) equipped with a Rheodyne 7125 injector and a 200 μl loop for sample introduction. The separation of the selenium species occurred in two columns, Nucleosil C_{18} ($100 \times 4\ mm^2$, 5 μm) and SAX ($20 \times 4.6\ mm^2$, 5 μm), connected by a switching valve and purchased from Supelco (Sigma–Aldrich, Gillingham, UK). The microwave-assisted digestion (MAD) of the selenium compounds was performed in a 6 m long PTFE loop placed inside a domestic microwave oven (Moulinex CY-1) operated at 150 W. HG of volatile selenium hydride prior to the detection was performed by on-line addition of a solution of $NaBH_4$ by means of a Gilson Minipulse-3 peristaltic pump (Minipuls 3, Gilson, Villiers, Le Bel, France).

AFS was undertaken with an Excalibur 10.33 spectrometer (PS Analytical, Orpington, Kent, UK) using a boosted-discharge hollow cathode lamp (Photron, Victoria, Australia). The separation of the gaseous selenium hydrides from the liquid stream was performed in a PS Analytical Type A gas–liquid separator, using argon as carrier gas. A hydrogen flow was also added to support the hydrogen–argon diffusion flame of the detector. The analog output signal was connected to a computer equipped with chromatography software (Varian, San Fernando, CA, USA).

Selenium speciation by HPLC–MAD–HG-AFS coupling

Separation of the selenium compounds was carried out in *ca* 15 min in both the anion-exchange and reversed-phase columns, connected through a six-port switching valve.[10] In position 1, the switching valve introduce 200 μl of sample

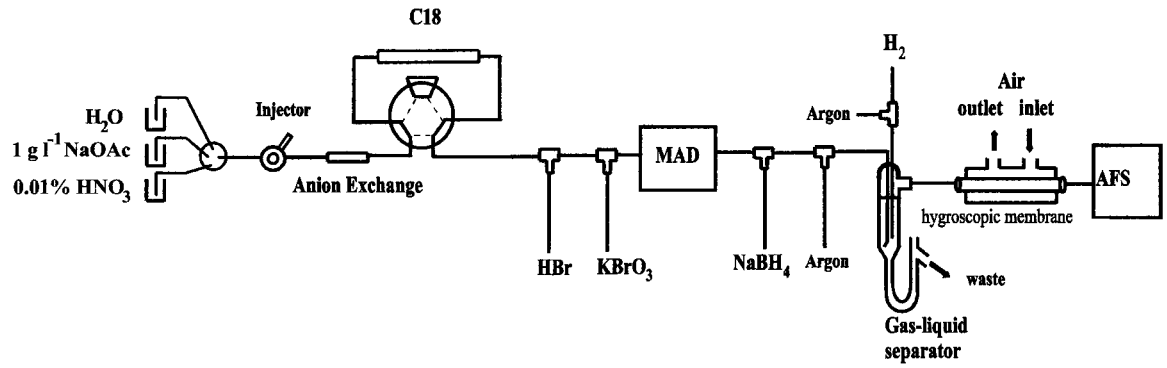

Figure 1. Scheme of the HPLC–MAD–HG-AFS coupling.

into the anion-exchange column, using water as mobile phase at a 1.0 ml min^{-1} flow rate. Selenium(IV) and selenium(VI) were retained in this column, whereas the selenoamino acids eluted as the dead volume and entered the reversed-phase column. After selenoamino acids detection, the column switching valve was changed to position 2, and the mobile phase changed to 0.4% (w/v) potassium acetate. The elution order was SeCyst, SeMet, SeEt, selenium(IV) and selenium(VI). After the last peak detection, the anion-exchange column was cleaned for 3 min with 0.01% (v/v) HNO_3 and for another 3 min with water. Then the switching valve was changed to position 1 for the next analysis.

MAD was achieved by on-line addition of 15 mM $KBrO_3$ (flow rate 0.6 ml min^{-1}) and 47% HBr (flow rate 1.2 ml min^{-1}). An ice-bath-cooled loop was placed after the microwave device for temperature reduction of the flow. HG was carried out by adding 1 ml min^{-1} of 1.5% (w/v) $NaBH_4$ in 1% (w/v) NaOH. Selenium hydride was separated from the liquid flow using a gas–liquid separator that introduced two argon flows: one to carry the hydrides to the separator (100 ml min^{-1}), the other, at 200 ml min^{-1}, to transport them to the AFS system. Before detection, the argon stream was dried with a hygroscopic membrane drier tube. Also, a 60 ml min^{-1} hydrogen flow was added at the gas–liquid separator in order to maintain the argon–hydrogen diffusion flame. The retention times were 2.2 min, 4.0 min, 8.6 min, 11.8 min and 13.5 min for SeCyst, SeMet, SeEt, selenium (IV) and selenium (VI) respectively. The scheme for the instrumental coupling is shown in Fig. 1.

Samples

Prawns (*Palaemonidae* sp.) and clams (*Donax* sp.) were collected from the southwest coast of Spain. The bivalve shells were opened, excess water in the mantle cavity was allowed to drain and all soft tissues removed from the shell with a disposable plastic knife and then frozen at −20 °C. Before analysis the specimens were freeze-dried for 48 h (Virtis, New York, NY, USA) at −60 °C and 20 mTorr. The freeze-dried tissue was pulverized to 100 μm, and stored at −20 °C.

Total selenium analysis

The freeze-dried samples were dissolved with 2 ml of HNO_3 and transferred to a Kjeldahl flask. After 1 h of predigestion at room temperature, 2 ml of $HClO_4$ were added and heated until appearance of white fumes. Then the samples were evaporated almost to dryness, transferred to a volumetric flask to a final volume of 5 ml with water. Total selenium determinations were performed by a flow injection (FI)-MAD–HG-AFS approach, using a manual valve for sample introduction.

As a quality control, the total selenium content was determined in the certified reference material TORT-1.

Extraction and clean-up procedure

Enzymatic hydrolysis

100 mg of freeze-dried tissue and 40 mg of a non-specific protease–lipase (1:1) mixture were added to 10 ml of water in a 50 ml Teflon centrifuge tube and shaken at 300 rpm in the dark for 24 h using a mechanical shaker at 37 °C. After extraction, the extract was separated from the sample by centrifugation for 10 min at 10 000 rpm. The supernatant was filtered to 0.45 μm to eliminate suspended solids.

Ultracentrifugation

The solution was passed through a 10 000 Da molecular weight cut-off filter by centrifugation for 30 min at 12 000 rpm.

Liquid–liquid partitioning clean-up

The ultracentrifuged solution was extracted with 10 ml of dichloromethane by shaking for 5 min and centrifuged at 10 000 rpm for 10 min.

Sorbent clean-up

5 ml of the aqueous phase was passed through a column with 1 g of aminopropyl sorbent. The column was previously conditioned with 5 ml of methanol and 5 ml of water. The

Table 1. Recoveries (%) of SeCyst, SeMet, SeEt, selenite [selenium(IV)], selenate [Selenium(VI)] from prawn tissue using different enzymatic digestion methods[a]

	SeCyst	SeMet	SeEt	Se(IV)	Se(VI)
Protease XIV	58 ± 2	105 ± 4	96 ± 4	ND	ND
Lipase VII	26 ± 1	52 ± 2	76 ± 3	89 ± 2	102 ± 3
Protease VIII	44 ± 2	101 ± 4	98 ± 3	88 ± 2	97 ± 3
Protease VIII–lipase VII	59 ± 2	107 ± 4	99 ± 3	98 ± 2	107 ± 3

[a] Sample was spiked to a final concentration of 4 μg g^{-1}, as Se, for each selenium species. ND: not detected.

selenium species were eluted with 20 ml of water at pH 9.8, adjusted with NH_4OH, at a flow rate of 1 ml min^{-1}. The eluate was then ready for injection for HPLC–MAD–HG-AFS.

Statistical analysis

Statistical analysis, consisting of a parametric test (Student's *t*-test), was performed on a personal computer using CSS: STATISTICA (StatSoft, Inc., Tulsa, OK). An α value of 0.05 was adopted as the critical level for all statistical testing, giving a 95% confidence level.

RESULTS AND DISCUSSION

Sample extraction and the clean-up procedures constitute crucial steps in the pretreatment of biota samples for selenium speciation. This is due to possible analyte losses, changes of the species or incomplete extractions of the selenium compounds. The study of the extraction efficiency and clean-up methods was carried out by using recovery experiments performed with prawn tissues spiked with SeCyst, SeMet, SeEt, SeO_3^{2-} and SeO_3^{2-} to a final concentration of 4 μg g^{-1}, as selenium, for each species, and subjected to the procedure described in the Experimental section. All the experiments were performed in triplicate.

Optimization of the extraction step

The extraction procedure was carried out by enzymatic hydrolysis. In this work, protease XIV, lipase VII, protease VIII and a protease VIII–lipase VII mixture were tested as enzymes following the studies of other authors on yeast,[5–7,9,15] urine,[6] pig kidney or white clover.[14]

Table 1 summarizes the results obtained with prawns. Quantitative recoveries for SeEt and SeMet were obtained using protease XIV, protease VIII and a protease VIII–lipase VII mixture. However, inorganic selenium species were only extracted quantitatively using the protease VIII–lipase VII mixture. Selenocystine was not recovered quantitatively with any treatment, and the best results (59%) were obtained with the enzymatic mixture, which was selected for further experiments.

It was observed that protease XIV yields low recoveries for SeCyst, and the inorganic selenium species were not detected. Moreover, this enzymatic treatment led to serious chromatographic problems because the inorganic selenium species were not retained on the anion-exchange column. This fact can be explained by the excess of acetate ions present in this enzymatic mixture. Using lipase VII, the extractions of SeCyst, SeMet and SeEt were not quantitative (less than 76%).

Optimization of the clean-up step

In order to evaluate the ability of the liquid–liquid partitioning clean-up to remove the lipids present in the sample, several solvents, such as hexane, pentane, chloroform and dichloromethane, were tested. The organic phase was also injected into the FI-MAD–HG-AFS coupling to check the absence of total selenium. Only a small amount (<1%) of selenium was detected in the organic phase in all cases. Dichloromethane was selected because it removed the highest amount of lipids. However, this clean-up procedure did not completely removed the lipids from the biota extract, which exhibited a dark yellow colour. The direct injection of such extracts onto the chromatographic column resulted in a shortened column life. Therefore, a further clean-up was advisable to protect columns and reduce interferences, and several types of sorbent were tested for this purpose. Of these, aminopropyl silane gave the best results. Water was used as eluent and some parameters controlling the extraction were studied, such as the amount of sorbent and the pH and volume of water. Amounts of sorbent ranging from 0.5 to 2.0 g were tested. At least 1.0 g was needed to obtain suitable extracts for the chromatographic analysis. Lower amounts did not remove the interferents totally, but amounts higher than 1.5 g increased unnecessarily the volume of water.

Values of pH ranging from 7 to 11 (adjusted with NH_4OH) were tested in the eluent. Analytes elution was favoured for high pH values, but for a pH higher than 9.8 interferents were also eluted. Therefore, this value was selected as optimum for further experiments.

Eluent volumes between 0 and 30 ml were tested. Results are summarized in Table 2. SeMet and SeEt were not retained at all. However, quantitative recoveries were obtained for SeCyst when at least a volume of 5 ml of water at pH 9.8 was used as eluent. Moreover, 10 ml of eluent were

Table 2. Recoveries (%) of selenium species in different fractions eluted from aminopropyl sorbent with H_2O at pH 9.8 (adjusted with NH_4OH) as solvent

	Fraction (ml)	SeCyst	SeMet	SeEt	Se(IV)	Se(VI)
Sample	5	76.5	95.0	100	0	0
H_2O at pH 9.8	5	18.4	5	0	23.6	12.9
	10	5.1	0	0	76.4	87.1

needed for the complete elution of the inorganic selenium species.

Other polar sorbents, such as CN, Diol and silica gel were also assayed. However, they did not remove the interferents. Similar results were obtained using the cationic sorbent, SCX.

The hydrophobic sorbents C-18 and end-capped C-18, used in the literature to remove interferents from urine, yeast and clover grown samples,[16–18] were tested. In our studies, methanol was needed as a solvent to elute the inorganic selenium species, but several interferents were also eluted.

Features of the method

In order to check the efficiency of the proposed method, calibration curves for each selenium species were constructed by the analysis of standards (selenium concentration from 1 to 50 μg l^{-1} for each selenium species), which were submitted to the whole extraction and clean-up method. Results are summarized in Table 3. They were compared with the calibration curves obtained by direct injection of the standards onto the chromatograph. Significant differences were found in the slopes of calibration curves for all selenium species (*t*-test, $p < 0.002$).

The slopes of the calibration curves for each selenium species submitted to both the extraction and clean-up methods were also compared with those obtained with the standard addition method for the extracts of prawn and the certified reference material TORT-1. Significant differences were found for SeMet, SeEt and selenium(VI) (*t*-test, $p < 0.004$) in prawns and TORT-1, and for SeCyst (*t*-test, $p < 0.001$) in TORT-1. Therefore, the standard addition method must be used for quantitative analysis.

To evaluate the repeatability of the instrumental method, five independent injections of 200 μl of a standard mixture containing known amounts of inorganic selenium [20 μg l^{-1} of each SeCyst, SeMet, SeEt, selenium(IV), selenium(VI)] were carried out. RSD values were always better than 3%.

The detection limits (evaluated as 3σ of blank) in prawn samples, including the extraction step, were: 0.06 μg g^{-1}, 0.07 μg g^{-1}, 0.08 μg g^{-1}, 0.05 μg g^{-1} and 0.06 μg g^{-1} for SeCyst, SeMet, SeEt, selenium(IV) and selenium(VI) respectively. The precision was evaluated by analysing five replicates of this sample spiked with SeCyst, SeMet, SeEt, SeO_3^{2-} and SeO_4^{2-} to a final concentration of 4 μg g^{-1}, and was lower than 7% of RSD.

Application to environmental samples

The procedure was applied to biota samples (prawns and clams) collected from the southwest coast of Spain, as well as to TORT-1. The chromatogram denoted (a) in Fig. 2 represents a pure extract obtained from TORT-1. Chromatograms denoted (b) and (c) in Fig. 2 correspond to the same extract spiked with 20 ng ml^{-1} and 40 ng ml^{-1} respectively for each selenium species.

Table 4 summarizes the results obtained for biota tissues by means of standard additions of selenium species. It can be

Table 3. Analytical features of the HPLC–MAD–HG–AFS method for standards submitted to the extraction and clean-up procedure

Species	Intercept	Slope	Linear range (ng l^{-1})	Correlation coefficient	DL[a] (ng ml^{-1})	Reproducibility[b] (% RSD)
SeCyst	9300	45 400	40	0.998	0.04	8
SeMet	7900	19 900	50	0.991	0.08	9
SeEt	9700	28 400	40	0.997	0.06	7
Se(IV)	2700	52 800	40	0.999	0.04	7
Se(VI)	3000	46 100	40	0.998	0.04	7

[a] DL: detection limit, computed as 3 × standard deviation of mean + value for mean standard blank, for $n = 7$ standard blank runs.
[b] Five repeated analyses using a selenium concentration of 10 ng ml^{-1} standard for each selenium compound.

Table 4. Selenium concentrations (μg g^{-1}, dry weight basis) ± standard deviation in biota samples[a]

Sample	SeCyst	SeMet	SeEt	Se(IV)	Se(VI)	Unknown	Se_{Total}	Se_{Total} certified value	Extraction (%)
Prawn	<DL	<DL	<DL	0.43 ± 0.02	<DL	ND	0.79 ± 0.03		60
Clam	<DL	<DL	<DL	<DL	<DL	0.86 ± 0.03	2.93 ± 0.88		29
TORT-1	0.56 ± 0.02	0.16 ± 0.01	<DL	2.38 ± 0.06	<DL	ND	6.88 ± 0.47	7.00 ± 0.21	44

[a] DL: detection limit.

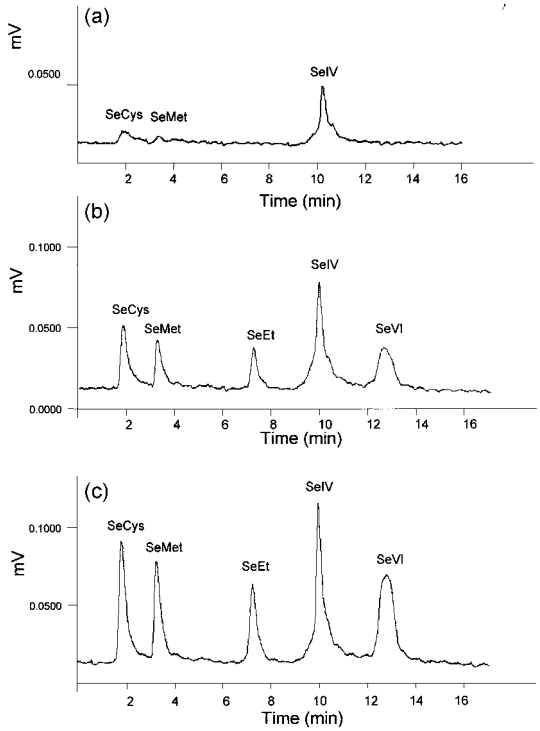

Figure 2. (a) Chromatogram of 200 μl injections of extracts from TORT-1. (b) Addition of five selenium species standards at concentrations of 20 μg l^{-1} (as Se). (c) Addition of five selenium species standards in concentrations of 40 μg l^{-1} (as Se). Retention times of selenium species: 2.2 min, SeCyst; 4.0 min, SeMet; 8.6 min, SeEt; 11.8 min, selenium(IV); 13.5 min, selenium(VI).

observed that the recoveries were low, between 29% and 60% of the total selenium for clams and prawns respectively. The clams only contained an unidentified peak at 2.3 min.

Selenite was identified as the major constituent in prawn and TORT-1, representing 54% and 34% of the total selenium amount respectively. On the other hand, selenate was not identified in the samples.

Of the three selenoamino acids considered in this work, SeEt was not detected in the samples. SeCyst (8%) and SeMet (2%) were only identified in TORT-1. These results are in agreement with previous work that identified SeMet and SeCyst in different biota samples, such as cockles, mullet[19] and mouse kidney,[14] SeMet being the major species of selenium present in living organisms.[14]

CONCLUSIONS

Few previous studies have considered the speciation of selenium in shellfish, and the extraction and clean-up pretreatment are critical steps in the complete speciation procedure. Enzymatic digestion is an easy way to release the selenium species from biota samples, although the extracts must be subsequently submitted to a clean-up procedure to be suitable for HPLC–AFS analysis. However, recoveries are low, possibly due to the incomplete extraction of the species from the complex matrix of shellfish. This hypothesis can explain the complete recoveries obtained, except for SeCyst (59%), in spiked samples. Therefore, further studies are necessary to optimize the extraction procedure. When the procedure was applied to shellfish samples, the presence of selenium(IV), SeMet and SeCyst was observed, but also an unidentified selenium species was found, which suggests the use of some additional technique for structure identification of the species.

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