Talanta 110 (2013) 53–57

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Speciation analysis of selenium in plankton, Brazil nut and human urine samples by HPLC–ICP-MS

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ARTICLE INFO

Article history:
Received 26 November 2012
Received in revised form
1 February 2013
Accepted 5 February 2013
Available online 28 February 2013

Keywords:
Brazil nut
Urine
Selenium
Bioaccessibility
HPLC–ICP-MS

ABSTRACT

The HPLC (anion exchange)–ICP-MS technique was used for the identification (based on retention time of standards) and determination of four selenium species (selenite, selenate, selenomethionine and selenocystine) in plankton (BCR-414), Brazil nuts and urine samples. A recovery of 91% was attained for certified reference materials (BCR-414). Se(IV) was the predominant species in plankton, with the highest selenium concentration in the extract. The Brazil nuts showed only the organic species selenomethionine and selenocystine after water extraction, but after simulated gastrointestinal digestion, only selenomethionine was found as bioaccessible, corresponding to 74% of the total selenium ($54.8 \pm 4.6\ \mu g\ g^{-1}$). Analyses of the urine samples suggested the presence of selenocystine, and significant differences were observed between samples from men and women in terms of the concentration of this species after consumption of Brazil nuts (1 nut per day during 15 days).

1. Introduction

Although necessary, data on total concentration of an analyte are not sufficient to provide proper understanding of the behavior of the analyte relative to a system under study, since different species having the same chemical element do not present the same physical, chemical and toxicological characteristics. This fact makes studies involving chemical speciation increasingly important [1–3] to understand toxicity, biotransformation, and their effects on living organisms, among others [4]. Thus, chemical speciation presents a leap in quality in terms of analytical information. Speciation is defined as the distribution of an element in its chemical species, i.e., its isotopic composition, its oxidation or electronic state, and the nature of any substituents attached covalently or forming complexes [2].

In terms of selenium speciation, its importance is based on the fact that this element is an essential component for human health, acting in the cellular defense mechanism against oxidative damage by inhibiting toxic effects of other heavy metals. However, the range between deficiency, essentiality and toxicity is very narrow and depends on the chemical form [5]. The selenoamino acids derived from animals and plants are key compounds for obtaining dietary selenium as selenomethionine and selenocysteine [6,7]. In cases of selenium poisoning, this element metabolized via methylation is eliminated through urine, skin and breath. These processes are considered biomethylation steps of detoxification, because organo-senelium compounds vented and excreted are less toxic [7].

After these comments, it is then easy to rationalize the necessity in establishing methodologies presenting good figures of merit, which include accurate results, even though certified materials or standards are not available when focusing on the speciation analysis.

In this way, the proposal of this work was to determine organo-selenium species from plankton, Brazil nuts and human urine samples by using the coupling of HPLC to ICP-MS, focusing on selenium speciation and bioaccessibility. Some strategies for eliminating the inference of argon dimers in the selenium speciation are also discussed in the present work.

2. Experimental

2.1. Instruments

All analyses were performed inside a class 1000 clean room. The chromatographic analyses were carried out with a Perkin–Elmer Series 200 liquid chromatograph (Perkin–Elmer, Shelton, USA) equipped with a binary pump, degasser, autosampler,

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http://dx.doi.org/10.1016/j.talanta.2013.02.014

Table 1
Instrumental parameters.

Chromatographic conditions	
Column	PRP X100 (Hamilton, Reno, USA)
Column dimensions	4.6 × 250 mm, 7 μm particle size
Mobile phases	A: ammonium acetate buffer, 25 mmol L^{-1}, pH 5.17
	B: ammonium acetate buffer, 250 mmol L^{-1}, pH 5.17
Elution	Step gradient from 0% to 100% mobile phase B
Flow rate	1.0 mL min^{-1}
Injection volume	100 μL
ICP-MS conditions	
RF power	1200 W
Nebulizer gas flow rate	0.92 L min^{-1}
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1.2 L min^{-1}
RPq	0.5
Cell gas flow rate (DRC)—O_2	0.4 L min^{-1}
Monitored species	$^{96}SeO^+$

column oven, and diode array detector. Aqueous solutions were introduced into a PerkinElmer Elan DRC-e ICP-MS, equipped with a dynamic reaction cell (DRC) system. The samples were introduced using a glass Meinhard nebulizer and a glass cyclonic spray chamber. Instrumental parameters are listed in Table 1.

2.2. Reagents and standards

Deionized water from a Millipore Direct-Q water purification system (Bedford, USA) was used throughout this work. Ammonium acetate was from J.T. Baker (Phillipsburg, USA) and used for the mobile phase separations. Reagents used for gastrointestinal digestion were purchased from Sigma-Aldrich, as well as all the selenium species standards. HNO_3 (65%, w/v) and HCl (37%, w/v) were obtained from Merck (Darmstadt, Germany) and used to prepare standard solutions. H_2O_2 (30%, w/v) was obtained from Merck, and used for digestion procedures, together with HNO_3.

The certified reference material of plankton (BCR 414) was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Daily ICP-MS performance was checked using a multielemental standard (Perkin–Elmer, Shelton, USA) containing Mg, Al, Cr, Mn, Cu, Rh, Cd, In, Ce, Pb, Th, U and Ba at concentrations of 1 μg L^{-1}, except for Ba (10 μg L^{-1}).

2.3. Samples

Brazil nuts (*Bertholletia excelsa*) were acquired in a local market, and urine samples were collected from nine volunteers at day zero, before the ingestion of any Brazil nuts, and at day 15, after the ingestion of 1 Brazil nut per day. The sample collection was carried out in the morning by the volunteers and the samples were analyzed, at most, 10 h after collection. The analyses on these samples were approved by the Ethics Committee on Research (Certificate No. 00894712800005404), University of Campinas, Brazil.

2.4. Total selenium content analysis

Brazil nut samples were frozen in liquid nitrogen and ground with a mortar and pestle. Weighted amounts were then digested in a Provecto microwave oven (Jundiaí, Brazil), using closed vessels containing 3.0 mL of concentrated HNO_3 and 2.0 mL of 30% (w/v) H_2O_2. The microwave vessels were subjected to digestion following the program described in Table 2. The resulting digests were cooled, diluted to 50 mL with water and analyzed for the total content of selenium by ICP-MS. Three independent replicates were made, and the respective blanks were considered in the final results.

Table 2
Microwave oven program for Brazil nut digestion.

Step	Time (min)	Power (W)
1	2	300
2	2	0
3	5	330
4	5	460
5	4	590

2.5. Selenium species extraction

For Brazil nuts and plankton, 200 mg of the samples was extracted using 10 mL of water with sonication for 2 h. The supernatants were recovered using an Eppendorf 5804 R centrifuge at 1447 g for 20 min. This procedure was repeated three times, the supernatants were pooled, lyophilized, resuspended in 2 mL of water, filtered through a 45 μm membrane and analyzed by HPLC–ICP-MS [8].

The residues of Brazil nuts and plankton were digested with concentrated HNO_3 and 30% (w/v) H_2O_2 in a 2:1 ratio, using 3 and 2 mL of the mixtures, respectively, at 80 °C for 3 h in 15 mL PP tubes in a heating block. Final solutions were diluted with water to 100 and 25 mL, for Brazil nuts and plankton, respectively, and analyzed by ICP-MS. Three independent replicates were made for each extraction and digestion, and the respective blanks were considered in the final results.

2.6. Bioaccessibility test

The protocol used has been described elsewhere [9,10]. Briefly, for gastric extraction, 5 mL of a gastric solution (50 mg of pepsin with 5 mL of 150 mmol L^{-1} NaCl, pH 2.5) was added to 0.3 g of Brazil nuts and the mixture was incubated at 37 °C for 4 h. For simulating intestinal extraction, the pH of sample solutions was adjusted to 7.4 with concentrated NaOH solution and then 10 mL of an intestinal solution, containing solutions of 3.0% (w/v) pancreatin, 1.0% (w/v) amylase and 1.5 g L^{-1} bile salts, was added. The mixture was incubated at 37 °C for 4 h. The resulting supernatants were centrifuged in an Eppendorf 5804 R for 20 min at 1447 g, diluted 10 time with water, filtered through a 45 μm membrane and analyzed by HPLC–ICP-MS.

2.7. Urine sample preparation

A volume of 1 mL of each urine sample was centrifuged for 5 min at 8385 g. The supernatant was diluted two times with 25 mmol L^{-1} ammonium acetate buffer and filtered through a 45 μm membrane. Samples were analyzed using HPLC–ICP-MS, and selenium species identification was achieved by comparing standard retention times.

3. Results and discussion

3.1. Determination parameters and method accuracy using anion exchange chromatography

Anion exchange chromatography is particularly recommended for the identification and quantification of inorganic selenium species (selenite and selenate), as well as some of organic ones [11–14]. In this work, four species, which are among the main

species assessed in environmental and biological samples, were available as standards to study selenium speciation using HPLC–ICP-MS (Fig. 1). Selenium species identification was based on retention time, and quantification on peak areas and in terms of selenium.

To eliminate the interference of argon ($^{80}Ar_2^+$) when the $^{80}Se^+$ ion is monitored in the selenium determination by ICP-MS, a dynamic reaction cell (DRC) was applied using oxygen as the reaction gas, as described by Silva and Arruda [15]. The method consisted of reacting $^{80}Se^+$ ions with oxygen for forming $^{80}Se^{16}O^+$ (m/z 96), which is free of the interference of argon dimers.

The method showed good linear correlations for the four species, and detection limits in the range of micrograms per liter for SeCys2, SeMet, Se(IV) and Se(VI), as can be seen in Table 3. The detection and quantification limits were calculated based on the standard deviation of the analytical response and the slope of the regression line [16–18].

The accuracy of the method was verified using the certified reference material BCR-414 (plankton). A water extract was used for selenium speciation studies, and the residue was digested to attain mass balance results. Se(IV) was the main species found in the aqueous extract (Fig. 2), corresponding to 18% of the total selenium concentration certified in BCR-414, while SeCys2 corresponded to 2%, Se(VI) to 1%, and SeMet concentration was below the limit of detection. These results are in good agreement with previous results [8] for selenium speciation, where the authors found that less than 10% of the selenium in garlic was water soluble using a similar extraction procedure. Concentration in the residue corresponded to 71% and, although two selenium species remained unidentified (retention time of 2.98 and 15.14 min), recoveries of 91% (± 7) ($n=3$) were attained.

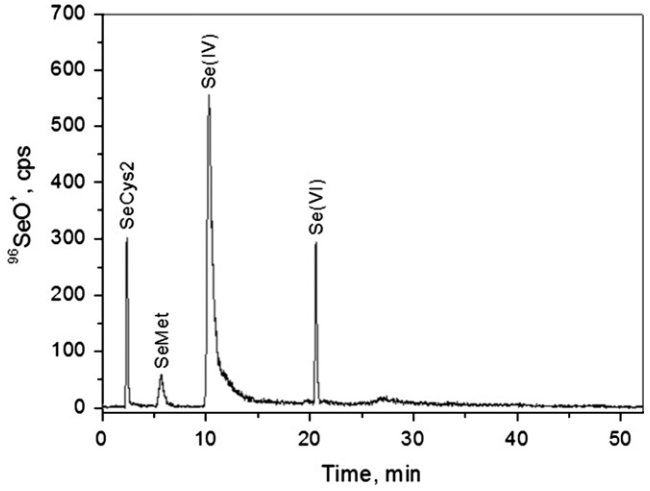

Fig. 1. Analysis of selenium species by anion exchange HPLC–ICP-MS. SeCys2—selenocystine; SeMet—selenomethionine, Se(IV)—selenite and Se(VI)—selenate. (Elution program: 7 min at 0% of B; 3.75 min at 25% of B; 3.75 min at 50% of B; 22.5 min at 100% of B; 7.5 min at 50% of B and 7.5 min at 0% of B). Chromatograms were smoothed with the Savitzky–Golay filter using the 2nd order polynomial and 50 data points.

Table 3
Analytical characteristics for selenium species determination by HPLC–ICP-MS.

Parameters	SeCys2	SeMet	Se(IV)	Se(VI)
Linear coefficient SD[a]	2.3974	0.7224	0.4053	1.1218
Slope ($\mu g\ L^{-1}$)	23.3197	5.8346	8.7542	13.0544
r[b]	0.9995	0.9995	0.9999	0.9996
LOD ($\mu g\ L^{-1}$)[c]	0.3	0.4	0.2	0.3
LOQ ($\mu g\ L^{-1}$)[d]	1.0	1.2	0.5	0.9

[a] Standard deviation.
[b] Correlation coefficient.
[c] Limit of detection.
[d] Limit of quantification.

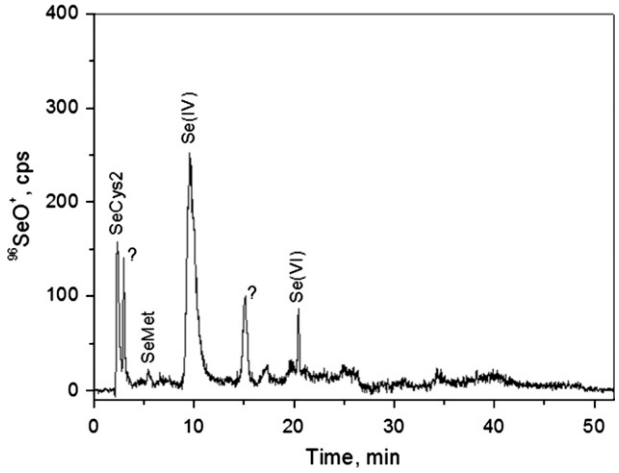

Fig. 2. Analysis of the aqueous fraction of certified reference material by HPLC–ICP-MS. Conditions and identifications as in Fig. 1.

3.2. Selenium species in Brazil nuts

Different species of selenium found in food do not show the same effect in the organism. When eaten, some of the selenium species present in food are effectively used by the organism and transformed to a bioactive form, and others are eliminated, especially via urine [19].

Brazil nuts have high naturally occurring selenium levels, since concentrations of 600 $\mu g\ g^{-1}$ have already been reported, this depends on the soil concentration of this element [20]. Daily ingestion of only one nut can be enough to make up the needs of an adult (55 $\mu g\ day^{-1}$) [21]; this information is demonstrated using data obtained in this work, where samples acquired in local markets had 54.8 (± 4.6) $\mu g\ g^{-1}$ ($n=3$) of selenium. It is important to highlight that this total concentration is not enough to infer the nutritional value of this food.

As in the plankton samples, a water extraction procedure was performed to check the species present in Brazil nuts, and only selenocystine and selenomethionine were found (Fig. 3a). The organic species are described in the literature as less toxic than the inorganic ones [22,23].

Trying to answer the question of which species is nutritionally important to the organism, an *in vitro* bioaccessibility test was performed (Fig. 3b). It should be pointed out that the bioaccessibility of a species is defined as the maximum soluble concentration in the simulated gastro-intestinal solution after filtration or centrifugation, as in this case [24–26]. As can be observed (Fig. 3b), only selenomethionine was found to be bioaccessible in Brazil nuts, corresponding to 74% of the total selenium present in the sample. This result is in agreement with others in the literature, which also showed this species as the most abundant in Brazil nuts, with concentrations ranging from 75% [27] to 96% [28] of the total concentration.

3.3. Urine samples

After the bioaccessibility tests, urine samples were analyzed before and after Brazil nut ingestion, to verify the species being excreted, since urine is the main elimination pathway of selenium

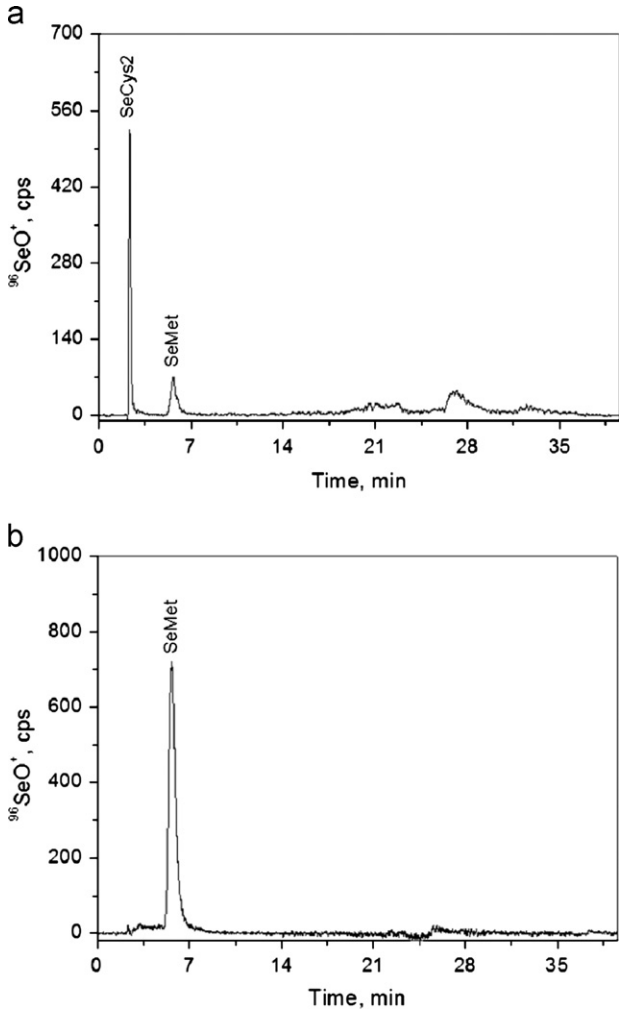

Fig. 3. Analysis of the aqueous (a) and bioaccessible (b) fraction of the Brazil nut by HPLC–ICP-MS. Conditions and identifications as in Fig. 1.

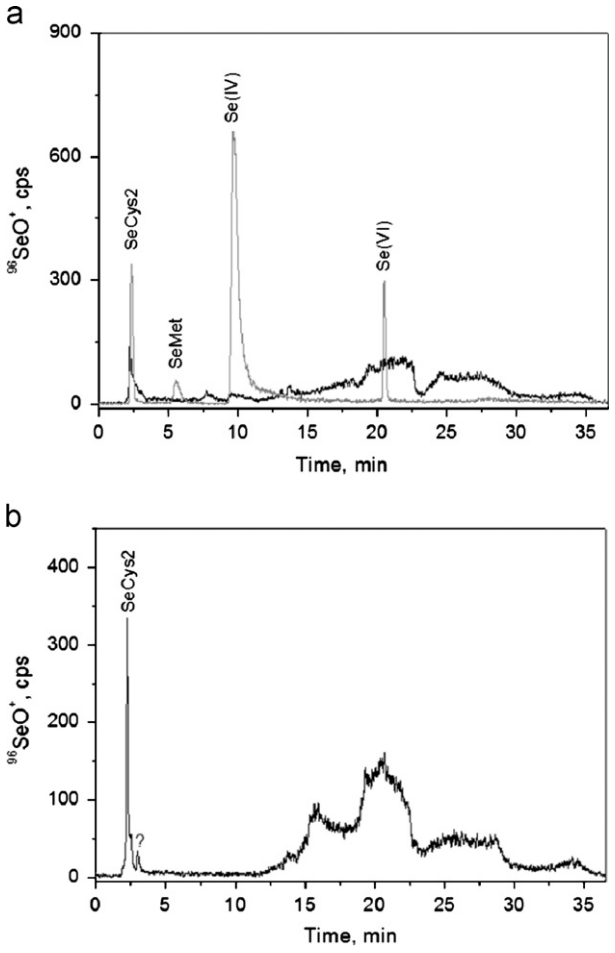

Fig. 4. Selenium species in human urine samples. (a) Standards (gray line) and urine sample before Brazil nuts ingestion (black line). (b) Urine sample (female) after Brazil nut ingestion. Conditions and identifications as in Fig. 1.

species. Before the ingestion of Brazil nuts, a peak with the same retention time as the selenocystine standard was observed (Fig. 4a), indicating the presence of this species in urine samples. Other researchers also reported the presence of this species in urine [29–34]. After ingestion of the Brazil nuts, an unidentified species was also observed in addition to SeCys2 (Fig. 4b).

Results for female and male volunteers are shown in Table 4. In terms of SeCys2 concentration, no significant differences were observed between urine samples from men and women before consumption of Brazil nuts. The average concentration found was 1.9 ($\pm$0.3) and 1.8 ($\pm$0.5) $\mu g\,L^{-1}$ for female and male groups, respectively. However, after 15 days of Brazil nut ingestion, the SeCys2 concentration increased only in urine samples from women, with an average concentration of 2.9 ($\pm$0.6) $\mu g\,L^{-1}$ for the female group. Significant variations were not observed in the average concentration of SeCys2 among men's urine samples, before and after Brazil nut ingestion. The average content was 1.7 ($\pm$0.5) $\mu g\,L^{-1}$ for the male group after Brazil nut ingestion.

The recommended dietary allowances of an element are influenced by gender, age, body measurements and physical activity [21,35], explaining the differences of recommendation for nutritional intakes. In the United Kingdom, different doses of selenium are recommended, being higher for men (75 $\mu g\,day^{-1}$) than for women (60 $\mu g\,day^{-1}$) [36]. Thus, to take more precise conclusions about the subject, a study should be performed with a greater number of volunteers, considering not only gender, but also the physical characteristics and habits (such as the practice of sports activity), while ingesting a higher number of Brazil nuts, because not all selenium present in this food can be metabolized by the organism.

Table 4
SeCys2 concentration as selenium in urine samples, before and after Brazil nut ingestion (1 nut per day for 15 days).

Samples	Concentration ($\mu g\,L^{-1}$) ($\pm$SD) (n=3)	
	Before ingestion	After ingestion
F1	1.8 ($\pm$0.03)	3.6 ($\pm$0.1)
F2	1.4 ($\pm$0.2)	2.9 ($\pm$0.2)
F3	1.9 ($\pm$0.1)	2.4 ($\pm$0.8)
F4	2.3 ($\pm$0.1)	2.8 ($\pm$0.1)
M1	< LOQ	< LOQ
M2	1.4 ($\pm$0.2)	1.4 ($\pm$0.3)
M3	1.9 ($\pm$0.05)	1.1 ($\pm$0.03)
M4	1.5 ($\pm$0.2)	2.1 ($\pm$0.1)
M5	2.5 ($\pm$0.1)	2.1 ($\pm$0.1)

Female (F) and male (M).

4. Conclusions

Using HPLC–ICP-MS and a dynamic reaction cell with oxygen for monitoring $^{80}Se^{16}O^+$ for the determination of selenium species, a recovery of 91% of the certified value in plankton

(BCR-414) was obtained. The main species identified in the Brazil nut samples were selenomethionine and selenocystine, and based on the simulated gastro-intestinal test, selenomethionine is the bioaccessible compound in this food. The speciation analysis by HPLC–ICP-MS also suggested the presence of SeCys2 in human urine. After Brazil nut ingestion, significant differences were observed between female and male samples. There was an increase in the selenium amount in female urine, while these changes were not significant in the male group.

Acknowledgments

The authors thank the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support. We also thank Prof. Carol H. Collins for language assistance.

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