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ORIGINAL PAPER

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HPLC–ICP–MS determination of selenium distribution and speciation in different types of nut

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Abstract In addition to determination of total selenium in nuts, the element distribution among different fractions (lipid extract, low molecular weight, and protein fractions), and speciation analysis were studied. Improved precision for total selenium determination was observed after elimination of lipids. Because selenium was not detected in any of the lipid extracts obtained from the different types of nuts (ICP–MS), in each determination and/or speciation procedure used in this work lipids were extracted (chloroform–methanol, 2:1) and discarded before analysis. In agreement with previously reported data, high selenium levels were found in Brazil nuts (those purchased without shells contained approximately a quarter the content than those purchased with shells) and significantly lower levels in walnuts, cashews, and pecans nuts. Low-molecular-weight compounds were extracted with perchloric acid (0.4 mol L^{-1}) to furnish a fraction containing 3 to 15% of the total selenium in different types of nuts. The proteins were isolated from nut samples by dissolution in 0.1 mol L^{-1} sodium hydroxide and subsequent precipitation with acetone. They were then dissolved in phosphate buffer pH 7.5. Analysis of protein fractions focused on selenium in two possible states – weakly and firmly bound to proteins. Results obtained for Brazil nuts by size-exclusion chromatography with on-line ICP–MS detection, in the absence and in the presence of β-mercaptoethanol, showed that approximately 12% of total selenium was weakly bound to proteins. To obtain information about firmly bound selenium, the protein extracts were hydrolyzed enzymatically with proteinase K. Speciation was performed by means of ion-pairing HPLC–ICP–MS. The primary species found in all types of nuts was Se-methionine (19–25% of total selenium for different types of nuts).

Keywords HPLC–ICP–MS · Nuts · Proteinase K · Selenomethionine · Speciation

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Introduction

Selenium has long been recognized as an essential micronutrient, playing an important role as an antioxidant and involved in thyroid metabolism, and evidence found suggesting bioactivity in cancer prevention [1]. The selenium reference nutrient intake (RNI) has been 75 and 60 μg day^{-1} for adult males and females, respectively. In some geographical regions (Keshan in China, New Zealand, Finland, Egypt, UK) the estimated daily intake is as low as 10–20 μg day^{-1} [2]. Because the bioavailability, retention, and fate of selenium in the human body is selenium-species-dependent, natural products rich in selenium are recommended as supplements [3]. Although levels of selenium in plant foods are strongly related to the content of the element in soils, cereals and seeds have been reported to be good dietary sources [4]. Exceptionally high levels of selenium have been found in Brazil nuts (*Bertholletia excelsa*) and when these nuts were included in rat diets, protection against tumor formation was observed [5]. In the central region of Brazil (Manaus to Belem), where the soils are rich in Se, concentrations above 500 μg g^{-1} have been reported whereas nuts from western Acre and Rondonia areas contained up to 30 μg g^{-1} of the element [6]. Other types of nuts have also been analyzed for selenium; lower levels were found in cashews (*Anacardium occidentale*, 0.27 μg g^{-1}), walnuts (*Juglans regia*, 0.03 μg g^{-1}), hazelnuts (*Corylus avellana*, 0.02 μg g^{-1}), peanuts (*Arachis hypogea*, 0.04 μg g^{-1}) pecan nuts (*Carya pecan*, 0.02 μg g^{-1}), and macadamia nuts (*Macadamia whelanii*, 0.07 μg g^{-1}) [2, 7, 8].

The complex chemical composition of nuts (lipids 50–70%, proteins 10–20%, and carbohydrates 10–20%)

makes trace elemental analysis difficult. Several wet-digestion procedures and detection systems have been used for analysis of total selenium in nuts; these experiments showed that incomplete digestion of the sample was the main source of possible analytical error [9]. Logically, the pretreatment procedure becomes much more critical if speciation analysis is to be performed, because care must be taken to preserve the natural composition of the species of the element in the sample. This restricts selection of reagents and conditions.

As already mentioned, nuts contain relatively high levels of protein compared with other plant materials. It is also known that during the reproductive stage of growth, selenium is stored in seeds while the amount in leaves is substantially reduced [10]. Consequently, characterization of selenium distribution in nuts with emphasis on the protein fraction seems to be of primary importance. Non-specific incorporation of selenium in proteins has been reported for non-accumulator plants treated with different forms of the element [11]. It might, therefore, be expected that selenium in nuts is firmly bound to proteins (in the form of seleno amino acids incorporated into proteins during their synthesis) and/or weakly associated through of the selenodisulfides (RS–Se–SR′) or methylselenyl sulfides ($RS–SeCH_3$), formed during post-translational modification of the proteins (these forms are easily reduced, releasing low-molecular-weight selenium compounds) [10, 12]. As far as we are aware, there have been only two reports on selenium speciation in materials of chemical composition similar to that of nuts. Hammel et al. focused on analysis of protein-bound selenium in the seeds of the selenium-accumulator plant coco de mono (*Lecythis ollaria*). Extraction was performed at pH 4.5 and at pH 7.5 and the extracts were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); results showed that 9% and 29% respectively, of total selenium in the seeds was firmly bound to proteins in the two extracts [13]. In the second work, Se-methionine bound to proteins in rice and wheat was determined after protein degradation with cyanobromide. The CH_3SeCN formed was derivatized to the piazselenol compound and determined by gas chromatography with electron-capture detection (GC–ECD). The results obtained showed that approximately 14% of the total selenium was in the form of protein bound Se-methionine [14].

In this work, total selenium was determined in Brazil nuts, walnuts, cashews and pecans, and the distribution of the element among different fractions (lipid extract, low molecular weight and protein fractions) and speciation analysis were undertaken. Hyphenated techniques coupling chromatographic separation with inductively coupled mass spectrometry (ICP–MS) were used as the primary analytical tools for selenium speciation. Separation of proteins was accomplished by size-exclusion chromatography (SEC) and, to obtain information about firmly bound selenium, the protein extracts were hydrolyzed enzymatically with proteinase K and separation was performed by ion-pair high-performance liquid chromatography–ICP–MS.

Experimental

Instrumentation

High-performance liquid chromatography (HPLC) was performed with an Agilent Technologies (Palo Alto, CA, USA) series 1100 instrument equipped with an autosampler, a diode-array detector, and Chemstation. The chromatography columns were C8 Altima (150 mm×4.6 mm, 5 μm particle) and Superdex Peptide HR 10/30 (Pharmacia Biotech).

An Agilent 7500s inductively coupled plasma–mass spectrometer (ICP–MS) connected to a concentric nebulizer and Scott-type double-pass spray chamber (cooled to 2 °C) was used for selenium-specific detection. The solution eluting from the column was introduced on-line to ICP–MS. The instrumental operation conditions are given in Table 1.

In addition to the equipment described above a model NES 1000 closed vessel microwave digestion system (CEMCa Matthews, NC) Chermle Z 230 centrifuge (National Labnet, Woodbridge, NJ, USA) Rotavapor RE 111 (Buchi Laboratoriums Technik AG, Schwitzerland) and a model RC5C centrifuge (Sorvall Instruments, DuPont) were used.

Reagents and samples

Water was doubly deionized (18.2 MΩ cm), prepared by passing deionized water through a NanoPure treatment system (Barnstead, Boston, MA, USA). Analytical-reagent-grade chemicals and HPLC-grade methanol and acetone (Fisher Scientific, Pittsburgh, PA, USA) were used.

Elemental standards and solutions were prepared from SPEX Claritas PPT 1000 μg mL^{-1} stock solutions of germanium and selenium (SPEX CertiPrep, Metuchen, NJ, USA). L-selenomethionine, selenocystine, selenoethionine, and sodium selenite were obtained from Aldrich (Milwaukee, WI, USA). The stock solutions containing 1 mg mL^{-1} selenium compound were prepared in 10 mmol L^{-1} hydrochloric acid (Sigma, St Louis, MO, USA) and were stored frozen. Working solutions were prepared daily by appropriate dilution.

Phosphate buffer (pH 7.5, 100 mmol L^{-1}), acetic acid–ammonium acetate buffer (pH 4.5, 50 mmol L^{-1}), and citric acid (5 mmol L^{-1})

Table 1 Instrumental operating conditions for HPLC–ICP–MS

HPLC parameters	
Column	C8 Altima, 150 mm×4.6 mm, 5 μm particle
Mobile phase	(5 mmol L^{-1} citric acid NaOH, 5 mmol L^{-1} hexanesulfonic acid, pH 4.5): methanol (95:5)
Flow	0.9 mL min^{-1}
Volume injected	50 μL
SEC parameters	
Column	Superdex Peptide HR 10/30
Mobile phase	50 mmol L^{-1} acetic acid ammonium acetate buffer (pH 4.5) containing 0.02% SDS
Flow	0.6 mL min^{-1}
Volume injected	100 μL
UV detection (λ)	214 nm
ICP–MS parameters	
Forward power	1300 W
Nebulizer gas flow	1.07 L min^{-1}
Isotopes monitored	^{77}Se, ^{78}Se

Table 2 Microwave digestion program for determination of total selenium

Step	Power[a], %	Ramp time, min	Hold time, min
1	25	5	2
2	45	5	2
3	55	5	2
4	65	5	2

[a]Maximum power 950 W

containing 5 mmol L^{-1} hexanesulfonic acid (pH 4.5 adjusted with sodium hydroxide) were prepared from Sigma reagents (except hexanesulfonic acid, which was purchased from Fisher).

Solutions of following reagents were used: Phosphoric acid, perchloric acid, nitric acid, acetic acid, trichloroacetic acid, sodium chloride, sodium hydroxide, sodium dodecylsulphate, β-mercaptoethanol, proteinase K, protease XIV, bovine serum albumin, apoprotein, and $(Gly)_6$ were obtained from Sigma.

Brazil nuts with shells, brazil nuts without shells, "white" and "black" walnuts without shells, roasted and salted cashew nuts and pecans nuts without shells were purchased in a local market.

Procedures

Determination of total selenium

Each type of nut (approximately 20 g) was ground in a coffee-mill (the shells from brazil nuts were first removed), chloroform–methanol (2:1, 100 mL) was added, and the mixture was shaken vigorously. After filtration and drying (room temperature) the nuts were again ground. The procedure was repeated three times and the chloroform–methanol fractions were combined. The dried, ground sample was weighed to determine the lipid content. Three sub-samples were precisely weighed (0.5 g for brazil nuts, 1.0 g for other nuts), transferred to PTFE vessels, and nitric acid (50%, 10 mL) was added. Digestion was performed in a microwave oven (detailed program is given in Table 2). Total selenium was determined by ICP–MS (^{78}Se), using internal calibration with germanium (10 μg L^{-1}) for analytical signal correction and the two-point standard addition technique.

Analysis of selenium in the lipid fraction

Solvents from the lipid extracts were evaporated the samples were mineralized, and determination of selenium was performed as for determination of total selenium (Table 2).

Separation and analysis of the low-molecular-weight fraction

Perchloric acid (0.4 mol L^{-1}, 4 mL) was added to the nut sample (0.2 g, without lipids). The mixture was sonicated in ultrasonic bath (2 h), centrifuged (10 min, 5000 rpm), and filtered. The solution obtained (50 μL) was analyzed by HPLC–ICP–MS (Table 1).

Isolation of proteins

A lipid-free nut sample (approximately 1 g) was precisely weighed in a plastic tube and sodium hydroxide solution (0.1 mol L^{-1}, 10 mL) was added. After agitation (Vortex) the mixture was centrifuged (10 min, 5000 rpm), the supernatant was transferred to an Erlenmeyer flask, and the solid residue was again treated with sodium hydroxide solution. The combined solutions (20 mL) were neutralized to pH 7.0 with 4 mol L^{-1} phosphoric acid and acetone was added so that the final concentration of the solvent was 80%. The sample was kept at –14 °C for 30 min and the precipitate obtained was separated by centrifugation (10 min, 5000 rpm), washed with acetone, and dried (nitrogen gas stream, room temperature).

Size-exclusion chromatography (SEC) of the proteins

The proteins precipitated as described above were solubilized in acetic acid ammonium acetate buffer (50 mmol L^{-1}, pH 4.5) containing 5% sodium dodecylsulfate (water bath 60 °C, 5 min) and filtered through a hydrophilic low-protein-binding polysulfone filter (0.45 μm; Gelman Science). A sample (100 μL) of the filtrate was analyzed by SEC–ICP–MS (Table 1).

Enzymatic hydrolysis of proteins and HPLC–ICP–MS analysis

The precipitate containing the proteins was transferred to a new plastic tube and the proteins were solubilized in phosphate buffer (pH 7.5, 6 mL). The solution obtained was incubated with 1 ml of proteinase K (20 mg mL^{-1}, 37 °C, overnight). Acetone was then added (final conc. 80%), the mixture was centrifuged (10 min, 5000 rpm), the supernatant evaporated to dryness, and the residue was solubilized in the mobile phase (10 mL for Brazil nuts and 1 mL for other nuts). The solution (50 μL) was analyzed by HPLC–ICP–MS (Table 1).

Results and discussion

In the first approach to total selenium analysis, different types of B nuts were dried and ground. This procedure did not, however, result in a fine and dry powder, because of the hardness of the nuts and the high lipid content. To im-

Table 3 Results from determination of selenium distribution and speciation in different types of nuts

Type of nuts	Results for total selenium			% Se in different nut fractions	
	Mean, μg g^{-1}	RSD_1, %	RSD_2, %	Se-LMW	Se-Met
Brazil with shells	35.1	3.2	5.7	3.1	25
Brazil without shells	8.3	3.1	4.8	5.0	21
Walnuts (black)	0.38	6.5	29	15	19
Walnuts (white)	0.20	31	45	10	23
Cashews	0.27	3.1	12	12	22
Pecans	0.10	33	50	nd	25

RSD_1– relative standard deviation for the results obtained after extraction of lipids (n=3); RSD_2 – relative standard deviation for the results without elimination of lipids; Se-LMW – selenium in the form of low-molecular-weight compounds observed by extraction with perchloric acid; Se-Met – fraction evaluated by ion-pairing HPLC; nd – not detected

prove sample preparation the lipids were removed by extraction. For each type of nut the lipid fraction was analyzed by ICP–MS for total selenium and on no occasion was selenium detected. Then, as described above, selenium was determined in the lipid-free nuts and the results obtained were related to the total mass (correction for lipid content). In agreement with the data previously reported [15], the highest selenium level was found in Brazil nuts purchased with shells (35.1±1.1 μg g^{-1}); Brazil nuts without shells contained lower levels (8.3±0.3 μg g^{-1}) (Table 3). The results obtained for other nuts – walnuts (0.38±0.03 μg g^{-1} white and 0.20±0.06 μg g^{-1} black), cashews (0.27±0.01 μg g^{-1}), and pecans (0.10±0.03 μg g^{-1}) – were significantly lower than for Brazil nuts, also confirming earlier reports [2, 7]. Eliminating the lipids improved precision in the determination of total selenium (Table 3).

To study selenium binding in nut proteins, a procedure for isolating proteins, was adopted. By use of the Biuret spectrophotometric test [16], the most efficient solubilization was achieved with 0.1mol L^{-1} sodium hydroxide, rather than 0.1 mol L^{-1} sodium chloride, 0.1 mol L^{-1} acetic acid, or 0.1 mol L^{-1} phosphate buffer (pH 7.2). The proteins were separated from other compounds present in the solution (low-molecular-weight selenium species) by precipitation with acetone at –14 °C.

Results obtained by SEC–ICP–MS

The SEC column was calibrated with a mixture of bovine serum albumin (67 kDa), apoprotein (6.5 kDa), and $(Gly)_6$ (360 Da) standards; these were eluted with the retention times 10.8 min, 12.8 min and 24.8 min, respectively (UV detection at 214 nm). After solubilization in mobile phase containing 5% SDS, the protein extract of Brazil nuts (with shells) was injected into the SEC–ICP–MS system. The main selenium fraction was eluted in a peak with a retention time of approximately 10 min, that corresponded to the region of elution region of high-molecular-weight (HMW) compounds (Fig. 1a). This result indicates that selenium in Brazil nuts is bound to proteins. A second, small elution peak with a retention time of 25 min suggested the presence of low-molecular-weight (LMW; <360 Da) selenium compounds. With further development, the same chromatographic technique was used to distinguish between selenium firmly and weakly bound to proteins in nuts. The term weakly bound has been ascribed to selenodisulfides (RS–Se–SR′) or methylselenyl sulfides (RS–$SeCH_3$) that are formed during post-translational modifications of proteins and that can be easily reduced, releasing LMW selenium compounds [10, 12]. To ensure reducing conditions during the separation, in this work β-mercaptoethanol was added to the mobile phase (final concentration 0.1 mol L^{-1}) and the same sample was analyzed. A typical chromatogram is presented in Fig. 1b; the peak of bulk selenium eluted again in the region of HMW compounds, suggesting that the major fraction of the element was firmly bound to proteins. It should be remarked that this chromatographic peak was slightly delayed and broadened as compared with the peak in Fig. 1a. This might be because of the change in mobile phase composition, and possibly a change in protein structure, owing to addition of the reducing agent (some of the sulfur bridges break down).

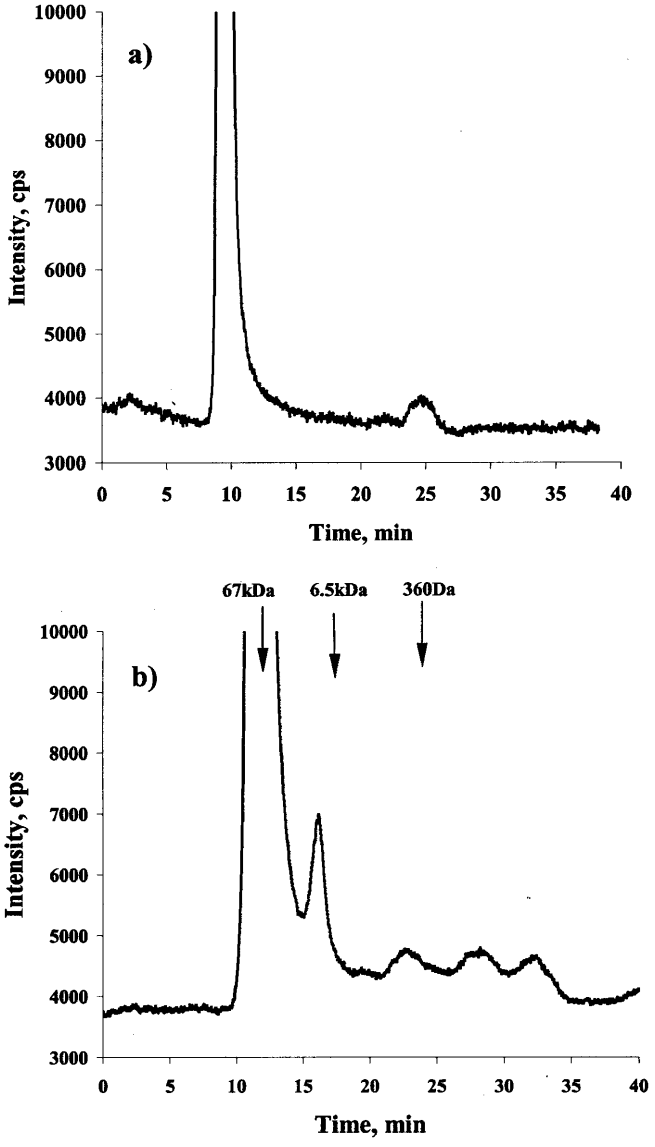

Fig. 1 Typical chromatograms of protein extracts from Brazil nuts (with shells) obtained by SEC–ICP–MS (^{78}Se) in the absence (**a**) and in the presence (**b**) of reducing agent (β-mercaptoethanol, 0.1 mol L^{-1})

In the presence of β-mercaptoethanol, the elution of selenium was also observed in the region of lower molecular weight compounds (one peak with a retention time 16 min and a few broad peaks eluting in the <400 Da region) (Fig. 1). The appearance of these new selenium peaks after addition of the reducing agent implies that some of the selenium in Brazil nut protein extract was only weakly bound to proteins. On the basis of peak-area measurements the percentage of selenium eluting with re-

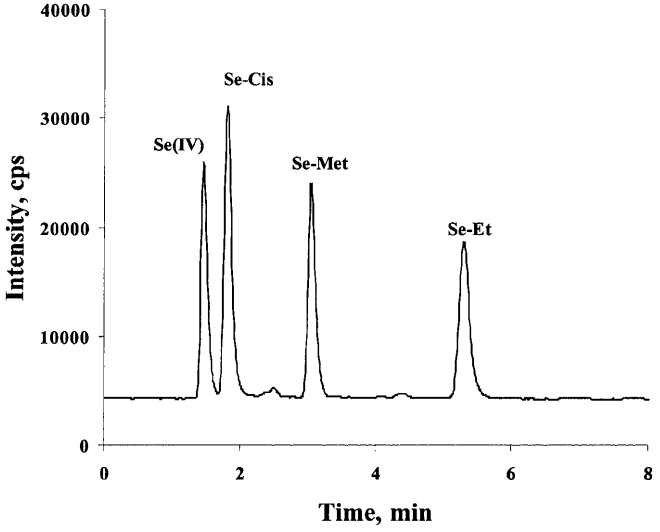

Fig. 2 Ion-pairing HPLC–ICP–MS (^{78}Se) chromatogram of selenite, Se(IV), selenocystine, SeCis, L-selenomethionine, SeMet, and selenoethionine, SeEt, (100 μg L^{-1} Se in each compound)

tention times >15 min accounted for approximately 12% of the selenium eluting from the column (total area under the chromatogram). The other types of nuts under study (walnuts, cashews, and pecans) contained lower levels of selenium and, using the same procedure as for Brazil nuts, selenium was barely detected by ICP–MS. When the sample volume introduced on to the column was increased, peak broadening and column overload were observed.

Results obtained by ion-pairing HPLC–ICP–MS

Ion-pairing HPLC with hexanesulfonic acid was used to separate LMW selenium compounds [17]. In Fig. 2 the chromatogram of four laboratory available selenium standards is presented: it shows that baseline resolution was obtained for sodium selenite (t_{ret}=1.5 min), selenocystine (t_{ret}=1.8 min), selenomethionine (t_{ret}=3.1 min), and selenoethionine (t_{ret}=5.3 min). The proteins isolated from the nuts were solubilized in phosphate buffer and enzymatic hydrolysis with proteinase K was performed (details given above). To avoid column loading with HMW compounds, the enzyme (proteinase K) was precipitated with 80% acetone (temperature –14 °C) before introduction of the hydrolysate to the ion-pairing HPLC–ICP–MS system.

As is apparent from Figs. 3 and 4a, similar selenium elution profiles were obtained from different types of nuts. To assign the chromatographic peaks, the retention times were matched with those of laboratory-available selenium standards and the spiking experiments were performed. By use of this procedure Se-methionine was found as the primary selenium species; the other selenium peaks (more pronounced in Fig. 4a, obtained from Brazil nuts with shells) remain unidentified. Selenium species eluting with retention times lower than that of Se-methionine (from 2 to 3 min in the chromatograms in Figs. 3 and 4a) could be oxidized forms of Se-methionine that are less hydrophobic [18]. It has been reported that these species are reduced to Se-methionine in the presence of β-mercaptoethanol [19]. The chromatogram obtained by following this procedure is presented in Fig. 4b; in the presence of reducing agent, fewer peaks can be observed between 2 and 3 min (Figs. 3 and 4a compared with Fig. 4b). An important aspect of enzymatic hydrolysis is that, depending on the cleavage specificity of the enzyme, some peptide bonds remain intact and this can affect the diversity of seleno compounds observed in the hydrolysate [20, 21]. It has been demonstrated that proteinase K mainly cleaves peptide bonds containing the amino group of hydrophobic amino acid residues (such as L-leucine or L-tyrosine) [22]. Thus forms of selenium eluting with retention times higher than that of Se-methionine possibly were more hydrophobic peptides, present because of the incomplete decomposition of proteins to amino acids [21].

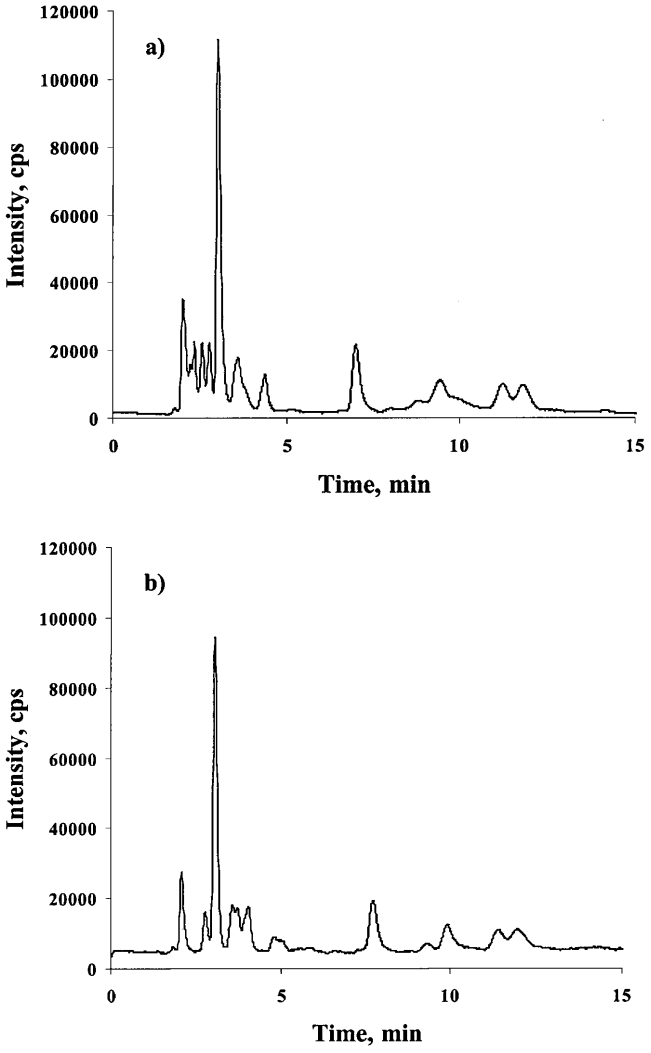

Fig. 3 Typical HPLC–ICP–MS (^{78}Se) chromatograms of protein extracts after enzymatic hydrolysis of (**a**) pecans and (**b**) cashew nuts

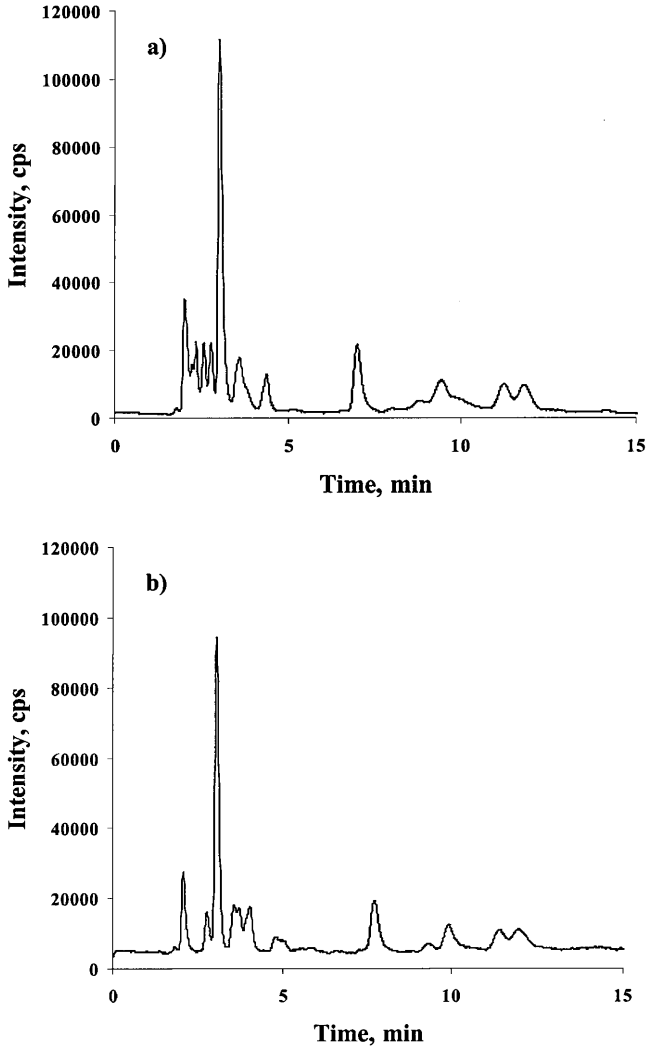

Fig. 4 Typical HPLC–ICP–MS (^{78}Se) chromatograms of an enzymatically hydrolyzed protein fraction from Brazil nuts (with shells) (**a**) in the absence and (**b**) presence of β-mercaptoethanol

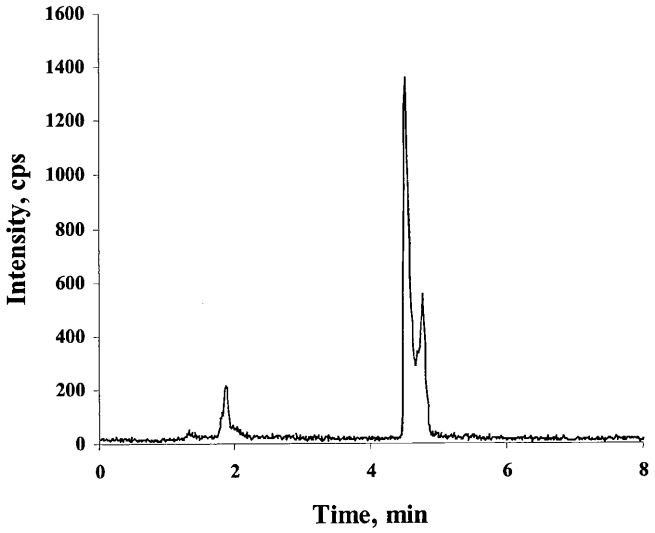

Fig. 5 Typical chromatogram of the cytosol fraction of Brazil nuts (with shells) obtained by HPLC–ICP–MS (^{78}Se)

Moreover, for Brazil nuts, recovery of selenium from the column was approximately 60% with respect to total Se content in nuts. Abraham et al. [20] used an acetonitrile gradient to separate peptides in a proteolytic digest of proteins (HPLC with off-line electrospray MS detection) and elution of non-polar peptides was observed with 80% acetonitrile. Thus under the isocratic conditions used in this work (on-line HPLC–ICP–MS), the highly non-polar selenium-containing peptides were possibly retained on the column. The percentage of selenium, as Se-methionine, with respect to total selenium was evaluated in different types of nuts by peak-area measurement. The results obtained (19 – 25% of the total selenium in the nuts, Table 3) are in agreement with the data reported previously for similar samples [13, 14], although the contribution of Se-methionine bound to proteins seems to be underestimated, because of incomplete enzymatic hydrolysis of proteins.

Finally, to determine the amounts of low molecular weight selenium compounds present in nuts, the lipid-free samples were treated with 0.4 mol L^{-1} perchloric acid and the mixture was centrifuged, filtered, and the solution obtained was analyzed by ion-pairing HPLC–ICP–MS (Table 1).

The elution of three selenium peaks was observed with the retention times 1.9 min, 4.4 min, and 4.8 min (in Fig. 5 the results obtained for Brazil nuts are presented). The peaks were identified by spiking the sample with selenium standards. The peak eluted at 4.4 min was assigned to Se-methionine. It should be remarked that elution of Se-methionine in the LMW fraction was delayed (Fig. 2, Fig. 5), most probably because of different sample composition (presence of perchloric acid in LMW fraction). The amount of selenium in the LMW fraction, relative to the total content of the element, was evaluated on the basis of peak-area measurements for different types of nut; the results obtained are presented in Table 3.

Conclusions

In this work, total selenium, its distribution and speciation in different types of nuts, was undertaken. In agreement with previously published data, high levels of the element were found in Brazil nuts purchased with shells, approximately four times higher than in nuts purchased without shells, and approximately 150 times higher than the selenium content in walnuts (white and black), cashews, and pecans. The results obtained from studies on selenium distribution showed that in each type of nut, selenium was present in the form of LMW compounds (cytosol fraction), bound to proteins, but was not detected in lipid extracts. The main species identified in cytosol and protein fractions was Se-methionine, confirming that Se is non-specifically incorporated to nut proteins (following the sulfur pathway). In samples of Brazil nuts, a weak selenium bond to proteins was also demonstrated.

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