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Studying the distribution pattern of selenium in nut proteins with information obtained from SEC-UV-ICP-MS and CE-ICP-MS

Sasi S. Kannamkumarath[a,*], Katarzyna Wrobel[b,1], Rodolfo G. Wuilloud[a,c]

[a] *Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221-0172, USA*
[b] *Instituto de Investigaciones Cientificas, Universidad de Guanajuato, L. de Retana No. 5, 36000 Guanajuato, Mexico*
[c] *U.S. Food and Drug Administration, Forensic Chemistry Center, 6751 Steger Drive, Cincinnati, OH 45237, USA*

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Abstract

In this work, size exclusion chromatography (SEC) with UV and inductively coupled plasma mass spectrometry (ICP-MS) detection was used to study the association of selenium to proteins present in Brazil nuts (*Bertholletia excelsa*) under five different extraction conditions. As expected, better solubilization of proteins was observed using 0.05 mol L^{-1} sodium hydroxide and 1% sodium dodecylsulfate (SDS) in Tris/HCl buffer (0.05 mol L^{-1}, pH 8) as compared to 0.05 mol L^{-1} HCl, 0.05 mol L^{-1} Tris/HCl or hot water (60 °C). Due to non-destructive character of Tris-SDS treatment, this was applied for studying molecular weight (MW) distribution patterns of selenium-containing nut proteins. Three different SEC columns were used for obtaining complete MW distribution of selenium: Superdex 75, Superdex Peptide, and Superdex 200 were tested with 50 mmol L^{-1} Tris buffer (pH 8), 150 mmol L^{-1} ammonium bicarbonate buffer (pH 7.8), phosphate (pH 7.5), and CAPS (pH 10.0) mobile phases. Using Superdex 200 column, the elution of at least three MW fractions was observed with UV detection (200–10 kDa) and ICP-MS chromatogram showed the co-elution of selenium with the two earlier fractions. The apparent MWs of these selenium-containing fractions were respectively about 107 and 50 kDa, as evaluated from the column calibration. For further characterization of individual selenium species, the defatted nuts were hydrolyzed with proteinase K and analyzed by capillary electrophoresis (CE) with ICP-MS detection. The suitability of CE for the separation of selenite, selenate, selenocystine and selenomethionine in the presence of the nut sample matrix is demonstrated. Complete separation of the above mentioned selenium species was obtained within a migration time of 7 min. In the analysis of nut extracts with CE-ICP-MS, selenium was found to be present mainly as selenomethionine.

Keywords: SEC-UV-ICP-MS; Brazil nuts; CE-ICP-MS; Selenomethionine; Selenocystine; Selenoproteins; Speciation

1. Introduction

Selenium is an essential trace element in the human body. This nutrient is an important part of antioxidant enzymes that protect cells against adverse effects of free radicals produced during normal oxygen metabolism. Selenium is also essential for normal functioning of the immune system and thyroid gland. Medical surveys show that increased selenium intake decreases the risk of breast, colon, lung and prostate cancer [1,2]. Furthermore, selenium contributes in better tissue elasticity; acts by slowing down aging processes and helps in the prevention and treatment of dandruff. There is a narrow range between the beneficial and toxic levels of selenium and, in both cases the biological effects are dependent on the actual chemical form of the element. Excessive selenium intake may cause a moderate to high health risk [3]. The principal manifestation of selenium toxicity is a condition called selenosis. Symptoms include gastrointestinal upsets, hair loss, white blotchy nails, and mild nerve damage.

Eating a healthy and nutritionally balanced diet is an important step towards achieving body's nutritional requirements. Within this context, appropriate doses of

* Corresponding author.
E-mail address: kannamkumass@ornl.gov (S.S. Kannamkumarath).
[1] At the University of Cincinnati while on the leave.

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supplemental selenium not only enhance cellular defense against oxidative damage but also may prevent certain types of cancers, according to human nutritional studies [4,5]. Organic selenium species (selenomethionine, selenocysteine and methylselenocysteine) have been shown more bioavailable than inorganic species. However, supplementation that provides more than three times the Daily Reference Intake (DRI) is likely to cause toxic manifestations [6]. The array of commercial selenium-fortified products has increased significantly in recent years, yet in most of the cases the exact composition as well as nutritional and toxicological data of these food supplements are not available or only partially known.

Brazil nuts, which contain exceptionally high levels of selenium can be considered an alternative dietary source of the element [7]. Previous studies carried out in our group showed the association of selenium to proteins in nuts, yet protein fractionation was not undertaken [8]. The primary species found after protein hydrolysis was selenomethionine [7–9].

Size exclusion chromatography (SEC) has proved to be a convenient technique for protein fractionation. In combination with element specific detectors, like atomic absorption spectrometry (AAS) [10–13], emission (AES) [14–18] or inductively coupled plasma mass spectrometry (ICP-MS) [19–21] it has been applied for speciation analysis in biological matrices. In many applications, SEC has been used as the initial fractionation step for determining metalloproteins or metal bound to biomolecules [22–24]. Even though the advantages of SEC for determining association of elements to different MW fractions are already established, its use for fractionating selenium-containing nut proteins has not presented previously.

The main attributes of capillary electrophoresis (CE) involve the high efficiency, versatility and low cost. This technique offers the ability to separate a large number of electrically charged and non-charged compounds within a wide range of molecular weights in a single run and relatively short time. Moreover, CE has been classically employed for separating aminoacids and hence it could be a good choice for resolving selenoaminoacids mixtures. Coupling CE to ICP-MS has been used in a number of element speciation studies [25–27]. However, to the knowledge of the author its application for investigating selenium speciation in nut samples has been never reported in the literature.

In this work, SEC with on-line UV and ICP-MS detection was used to study the selenium binding to nut proteins. Five different extraction conditions and three SEC columns, specified for different MWs ranges were tested. The possibility of using CE for separating selenium species in a relatively complex matrix such as nuts is demonstrated in this study. The confirmation of selenomethionine as the primary species built into the protein structure of nuts was obtained by CE-ICP-MS analysis of the enzymatic protein digest.

2. Experimental

2.1. Instrumentation

The ICP-MS instrument used was an Agilent 7500 s (Agilent Technologies, Tokyo, Japan) equipped with a grounded "ShieldTorch" system. The instrument was tuned by introducing a solution of Se (10 $\mu g\,L^{-1}$) (SPEX CertiPrep, Inc., Metuchen, NJ, USA). The ion lenses and quadrupole parameters were set to assure maximum signal to noise ratio for ^{78}Se (23.6%) and ^{82}Se (9.2% abundant). ICP-MS was on-line coupled with the high performance liquid chromatographic system, Agilent series 1100 equipped with an autosampler, a diode array detector and Chemstation data acquisition system. The coupling was through the 0.25 mm i.d. PEEK tubing from the outlet of the UV detector directly to the inlet of the concentric nebulizer (MicroMist AR30-1-F02). SEC columns used were: Superdex Peptide HR 10/300, Superdex 7510/300 and Superdex 20010/300 (Amersham Biosciences Corp., Piscataway, NJ, USA).

The CE instrument was a Waters Quanta 4000 capillary ion analysis system (Waters Corporation, Milford, MA, USA). CE capillaries were 75 μm i.d., 365 μm o.d. (Polymicro Technologies), and with a total length of 75 cm to the stainless steel tee make-up buffer junction. CE-ICP-MS data and peak areas were calculated using "ICP-MS Chromatographic Software" (Agilent Technologies, Tokyo, Japan). The tuning solution was introduced through self-aspiration. Both ^{82}Se and ^{78}Se were monitored during each CE run to verify selenium isotope patterns in the electropherograms. The instrumental operation conditions are listed in Table 1.

A Thermix Model 610T stirring hot plate (Fisher Scientific, Pittsburgh, PA, USA) was used for the hot water extractions with a thermometer to monitor the temperature. A Model RC5C centrifuge (Sorvall Instruments, DuPont) operated at 3500 rpm for 5 min was used as needed to separate the supernatant from undissolved material after extractions. Sonication was performed using a Branson Model B-2200R-1 ultrasonic cleaner (Fisher Scientific). PVDF (polyvinylidene fluoride) low protein binding disposable syringe filters of 0.45 μm (Alltech Associates, Inc., Deerfield, IL, USA) were used for sample filtration.

2.2. Reagents and samples

All water used was doubly deionized (18 MΩ cm) prepared by passing deionized water through a NanoPure treatment system (Barnstead, Boston, MA, USA). Nitric acid, concentrated, certified ACS plus was purchased from Fisher Scientific. All glassware was cleaned prior to use by washing with soap and water and then soaking in 10% (v/v) nitric acid overnight. They were then rinsed with 2% (v/v) nitric acid; triple rinsed with water, and allowed to dry.

Selenomethionine (98% purity), and selenocystine (98%) were obtained from Aldrich (Milwaukee, WI, USA). Sodium

Table 1
ICP-MS, CE and SEC instrumental operation conditions

ICP-MS parameters			
Forward power	1190 W (with shield torch)		
Plasma gas flow rate	15.6 L min^{-1}		
Carrier gas flow rate	1.08 L min^{-1}		
Nebulizer	MicroMist AR30-1-F02		
Spray chamber	Scott-type double pass (glass)		
Sampling depth	5.6 mm		
Sampling and skimmer cones	Nickel		
Isotopes monitored	^{78}Se, ^{82}Se, ^{77}Se		
CE operating conditions			
Power supply	−25 kV		
Injection	30 s hydrostatic		
Capillary	i.d. 75 μm; o.d. 365 μm; 75 cm long		
Electrolyte solution	Ammonium buffer pH 9.25 with 2% (v/v) OFM anion-BT		
ICP-MS make-up buffer	Same as electrolyte solution without OFM		
SEC chromatographic parameters			
Column	Superdex 200	Superdex 75	Superdex Peptide
Separation range	600–10 kDa	70–3 kDa	14–0.18 kDa
Mobile phase	150 mmol L^{-1} $(NH_4)HCO_3$ (pH 7.8)	50 mmol L^{-1} Tris/HCl (pH 8)	50 mmol L^{-1} Tris/HCl (pH 8)
Flow rate	0.5 mL min^{-1}	0.70 mL min^{-1}	0.60 mL min^{-1}
Injection volume	100 μL	100 μL	100 μL

selenate, sodium selenite and proteinase K were purchased from Sigma (St. Louis, MO, USA). Calibration of SEC columns was performed using a standard mixtures of (1) lysozyme (14.4 kDa), aprotinin (6.5 kDa), substance P (1.35 kDa) and $(Gly)_6$ (0.36 kDa) and (2) ferritin (440 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), myoglobin (17 kDa) and aprotinin (6.5 kDa) (Sigma reagents). A 50 mmol L^{-1} Tris (hydroxymethyl)aminomethane (Tris) and 150 mmol L^{-1} ammonium bicarbonate (Fisher Scientific, Fairlawn, NJ, USA) mobile phase solutions were prepared by dissolving the respective reagents in deionized water and adjusting the pH to 8 and 7.8, respectively with HCl (Merck, Darmstadt, Germany) and aqueous ammonia solutions. Mobile phase solutions of 10 mmol L^{-1} 3-cyclohexylamino-1-propane-sulfonic acid (CAPS) (Aldrich, Milwaukee, WI, USA) and 100 mmol L^{-1} sodium phosphate (Aldrich, Milwaukee, WI, USA) were prepared by dissolving the individual reagents in deionized water and adjusting the pH to 10 and 7.5, respectively with NaOH (Merck, Darmstadt, Germany) solution. Electrolyte solution for CE separation was the ammonium buffer at pH 9.25 (pH adjusted by adding 25 mmol L^{-1} nitric acid to aqueous ammonia) (Sigma reagents). The electrolyte contained 2% (v/v) CIA-Pak Waters OFM anion-BT (Waters Corp., Milford, MA, USA) for inactivation of capillary surface. All reagents were of analytical reagent grade and the presence of trace elements was not detected in the working range.

Brazil nuts were purchased in the local market. Since no significant amount of selenium was found in lipid fractions, lipids were extracted with chloroform–methanol (2:1) and the fine powder of defatted material was obtained as described earlier [8]. Speciation analysis was carried out on defatted nuts.

2.3. Procedures

2.3.1. Protein extraction for SEC-ICP-MS analysis

The sample aliquots (0.5 g) were weighed in plastic tubes and 10 mL of the extraction solution was added (three replicates). The extraction agents tested were: (1) 0.05 mol L^{-1} NaOH, (2) 0.05 mol L^{-1} HCl, (3) 0.05 mol L^{-1} Tris–HCl buffer, pH 8.0, (4) 1% SDS in Tris/HCl, pH 8.0 and (5) hot water at 60 °C. The tubes with extracting agents ((1)–(4)) were agitated in a vortex for 30 min and centrifuged for 10 min at 3500 rpm. The supernatants obtained were filtered through a low protein binding 0.45 μm PVDF filters. In the case of hot water extraction (5), 10 mL of deionized water were added to the powdered nut samples and the mixture was kept at 60 °C (30 min). For the purification of extract (4), proteins were precipitated with 80% acetone (−14 °C, 30 min). After centrifugation (10 min, 3500 rpm), the supernatant was eliminated and the residue dissolved in 2 mL of 1% SDS in Tris/HCl, pH 8.0. The obtained solutions were centrifuged and filtered through a 0.45 μm PVDF low protein binding filters prior to their introduction to the chromatographic system.

2.3.2. Fractionation of nut extracts by SEC-UV-ICP-MS

Calibration of Superdex Peptide column was accomplished with a standard mixture of lysozyme (14.4 kDa), aprotinin (6.5 kDa), substance P (1.35 kDa) and $(Gly)_6$ (0.36 kDa), showing in this range a good linear response for logarithm of molecular weight versus retention time ($R^2 = 0.9864$). A standard mixture of ferritin (440 kDa), β-amylase(200 kDa), bovine serum albumin (66 kDa), myoglobin (17 kDa) and aprotinin (6.5 kDa) was used for calibration of Superdex 200 column ($R^2 = 0.9912$). For the analysis of nut extracts, the injection volume was 100 μL. The chromatographic conditions are given in Table 1. The column effluent passed first through the UV diode array detector (230 and 280 nm) and then directly introduced into the ICP-MS detector.

2.3.3. Enzymatic hydrolysis of proteins prior to CE-ICP-MS analysis

An aliquot of the ground sample (0.25 g) was suspended in 5 mL Tris–HCl buffer (pH = 7.5) and 0.025 g of proteinase K was added (three replicates). The mixtures were incubated at 37 °C for 20 h in darkness with shaking, filtered through a glass fiber filter to remove the nut particulate and then, through a 0.45 μm PVDF membrane filter. For further purification (elimination of polysaccharides and excess of proteinase K), the extracts were treated with 20% ethanol and ultrafiltered through 5 kDa centrifugal filters from Millipore (Millipore Corp., Bedford, MA). The CE separation condi-

tions were partially adopted from the work done by Bendahl et al. [28] (Table 1).

3. Results and discussion

In the previous work carried out in our laboratory, the selenium binding to HMW compounds present in nut extracts was demonstrated. Sodium hydroxide (0.1 mol L^{-1}) was used for protein solubilization and the separation was carried out on a Superdex Peptide HR 10/300 column. ICP-MS detection showed the elution of a single selenium-containing fraction corresponding to the elution of HMW compounds (MW > 10 kDa) [8]. Due to the limitations of the Superdex Peptide column in resolving HMW fractions, investigation of the association of selenium to different HMW fractions was not achieved in that work. The present study was undertaken for further characterization of selenium MW distribution in nut proteins with the aim of determining a possible association of selenium to fractions of different MWs especially in the HMW region. Therefore, different extracting agents were examined for protein solubilization from defatted nuts and the performance of three SEC columns was tested for separating the selenium-containing fractions.

Several protocols that assure efficient extraction of proteins from biological materials are available [29]. These include acid precipitation, alkaline solubilization, solubilization through electrofocussing (buffered urea, CHAPS, etc.) or by ion-pairing formation with sodium dodecylsulfate (SDS). On the other hand, it is well known that, for selenium speciation relatively mild chemical conditions should be used in order to avoid possible changes in native selenium distribution/speciation. Based on the previous experience with selenium-containing proteins in *Brassica juncea* [30], five different conditions were tested in this work to extract proteins from defatted nuts. For leaching of water soluble proteins, hot water and 0.05 mol L^{-1} Tris/HCl extraction media were compared [30,31]. Alkaline solubilization was carried out with 0.05 mol L^{-1} sodium hydroxide. Since 1% SDS in Tris/HCl (pH 8) was successfully applied for extraction of selenoproteins from plant and mammalian tissues [30,32], this extraction agent was also examined for nut samples. In the first approach, the extracts obtained were analyzed by SEC-UV-ICP-MS using Superdex Peptide column. To check for possible effect of the mobile phase composition on the protein elution profile, the separation was performed using Tris/HCl (pH 8.0), phosphate (pH 7.5), ammonium bicarbonate (pH 7.8) and CAPS (pH 10.0) buffers. Comparing chromatograms of MW standards and of nut extracts obtained with these four mobile phases, no significant differences of the peak shapes or positions were observed, which confirms a non-disturbed size exclusion mechanism of separation. Since Tris/HCl was present in most extracts, this mobile phase was selected for further experiments. The flow rate was evaluated in the 0.3–1.0 mL min^{-1} range. The baseline separation of MW standards within acceptable time (40 min) was achieved at 0.6 mL min^{-1} which is also compatible with sample introduction system of the ICP-MS instrument. Therefore, this flow was adopted for further experiments.

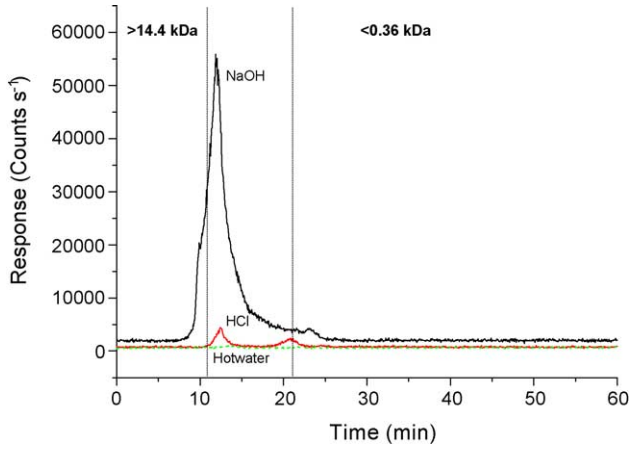

Fig. 1. Typical elution profiles of selenium obtained on Superdex Peptide column for hot water, 0.05 mol L^{-1} HCl and 0.05 mol L^{-1} NaOH nut extracts. The elution of molecular weight standards is marked and the detailed conditions are given in Table 1.

In Fig. 1, typical SEC-ICP-MS chromatograms obtained with the Superdex Peptide column of nut extracts are presented. In principle, similar elution profiles were obtained in each case: the major selenium peak eluted in the region corresponding to MW > 10 kDa, which confirms the binding of selenium to proteins. The elution of low abundant LMW selenium-containing compounds (MW < 1 kDa) can also be observed. On the other hand, different selenium amounts in HMW and LMW fractions were obtained using hot water, Tris/HCl (chromatogram almost identical to that of hot water extract, hence not shown in Fig. 1), diluted HCl, sodium hydroxide and SDS in Tris/HCl (chromatogram highly overlapped with that of NaOH extract, not shown in Fig. 1). As could be expected, low extraction efficiencies were achieved with diluted HCl, Tris/HCl and hot water (2–8%). For sodium hydroxide and SDS extracts, high and similar extraction efficiencies were obtained (85–90%). The percentage of extraction was evaluated referring selenium eluted from the column (two-point calibration with selenomethionine) to total Se in defatted nuts. It was verified that sonication of the mixtures over a period up to 12 h did not improve the efficiency of extraction. Worth mentioning that selenium abundance in LMW region of chromatogram was lower for SDS extract as compared to NaOH. Due to non-destructive aspect of SDS treatment (solubilization through the formation of ion pairs), this extract was used for further characterization of selenium in nut proteins.

The SEC-UV and SEC-ICP-MS elution profiles from Superdex Peptide were compared with those obtained on Superdex 75 and Superdex 200 columns. According to column specifications, the last two columns were used to enhance resolution of HMW compounds. The Superdex 75 (70–3 kDa) did not allow any improvement of resolution. A

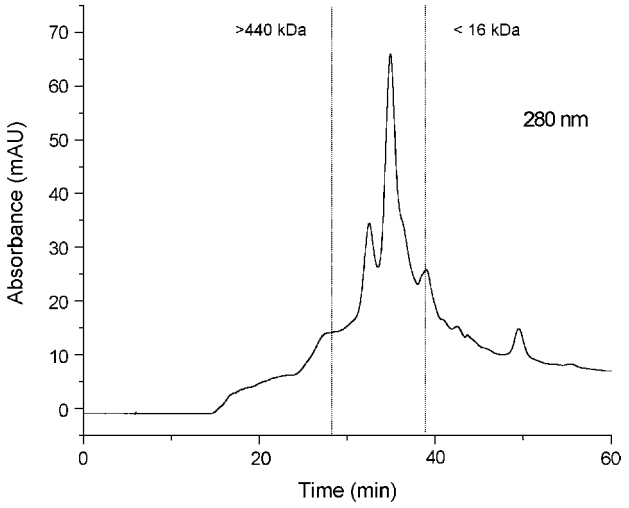

Fig. 2. SEC-UV-Vis chromatogram of SDS–Tris/HCl protein extract of Brazil nut obtained using Superdex 200 column. The absorbance was monitored at 280 nm. The apparent molecular weight ranges according to column calibration and other experimental conditions were as shown in Table 1.

single selenium-containing peak corresponding to the HMW region was obtained and this was similar to the results obtained with the Superdex Peptide column (Fig. 1). Typical SEC-UV and SEC-ICP-MS chromatograms of SDS nut extracts on Superdex 200 column (600–10 kDa) are presented in Figs. 2 and 3, respectively. Detection with the UV system showed that nut proteins were separated into at least three different fractions in the MW range of 200–10 kDa (Fig. 2). However, selenium was found to be associated to the first two MW fractions which were confirmed by detecting ^{78}Se and ^{82}Se with the ICP-MS system (Fig. 3). The apparent MWs of selenium-containing fractions (at the peak maximum) were

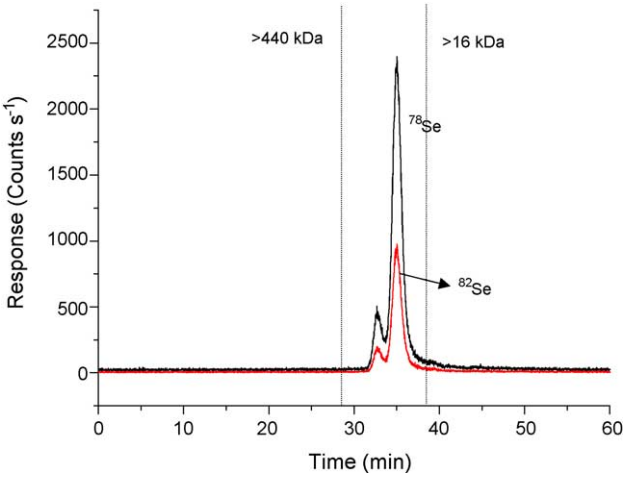

Fig. 3. SEC-ICP-MS chromatogram (^{78}Se, ^{82}Se) of SDS–Tris/HCl protein extract of Brazil nut obtained using Superdex 200 column. The apparent molecular weight ranges according to column calibration and other experimental conditions were as shown in Table 1.

about 107 and 50 kDa, as evaluated from the column calibration. These findings allow a proper explanation of the lack of resolution obtained with the Superdex 75 and Superdex Peptide columns as the selenium-containing fractions present in the nut extract were outside the separation range of these two SEC columns.

It is common knowledge that the speciation results can be affected by the experimental conditions applied during the entire analytical procedure. Qualitative and quantitative discrepancies observed in the analyses carried out on the same biological matrices, have been related to incomplete release of species from the sample, their loss and/or possible interconversion. For the enhanced reliability of speciation results, the use of different protocols, preferentially based on different physicochemical fundamentals has strongly been recommended [33]. The concordance between results obtained in such studies warrants that the "true" speciation pattern in the given sample has been approached. In the previous studies on selenium in nuts, we used two different sample pretreatments to release selenium species from protein structure and ion-pairing high performance chromatography was used for their separation prior to ICP-MS. The results obtained after enzymolysis and acid hydrolysis of proteins indicated selenomethionine as the primary element form incorporated to nut proteins [8,9]. To confirm this finding, in the present work selenium speciation in protein digest was carried out by capillary electrophoresis (CE) coupled with ICP-MS detection. The feasibility of CE-ICP-MS for two inorganic selenium forms, selenomethionine and selenocysteine was demonstrated by Bendahl et al. [28]. However, selenium speciation in nuts using CE separation is only demonstrated in the present work and reports have not been published so far. Optimization of both, hydrodynamic and electrostatic injections of the samples was performed to obtain the maximum sample transfer into the capillary without affecting the resolution of the peaks. Electrostatic injection was selected, as it was less affected by the backpressure originated in the nebulization system of the ICP-MS instrument and sample viscosities. An injection time of 30 s at −20 kV allowed the best resolution for the given capillary dimensions. Final separation conditions applied in this work are shown in Table 1. A typical electropherogram for the separation of four selenium standard compounds (Se(VI), Se(IV), selenocysteine and selenomethionine) is shown in Fig. 4 (dashed line). Sharp and baseline separated peaks were achieved within a total separation time of 7 min. Also, the electropherogram of the enzymatic nut digest is shown in same Fig. 4 (solid line). Selenium eluted in a single peak with a migration time matching the elution of the selenomethionine standard. The species identity was confirmed by spiking experiments (1 mg L^{-1} Se as selenomethionine). As can be observed in Fig. 4, the migration time of selenomethionine was increased in the analysis of nut extract as compared with standard solution. Furthermore, quantitative data could not be obtained, owing to significant signal suppression in the sample after addition of selenomethionine standard. Similar effects have often been observed in CE

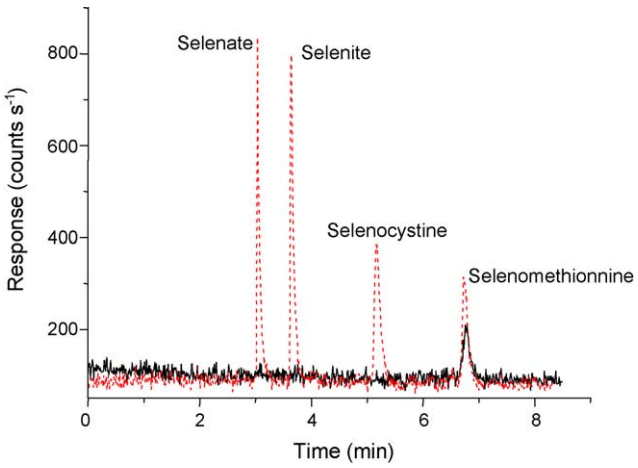

Fig. 4. Typical electropherograms of (- - -) selenium standards (100 μg L^{-1} as selenium) and (—) extract of Brazil nut after proteolytic digestion. Analytical conditions were as shown in Table 1.

separations performed on real-world samples. These should be ascribed to interferences caused by different conductivity of the sample with respect to electrolyte and standard solutions [27]. Even though some limitations were observed in the application of CE-ICP-MS to enzymatic nut digest, this technique provided additional evidence on the presence of selenomethionine as the primary selenium-containing species incorporated to nut proteins.

4. Conclusions

The characterization of selenium binding to nut proteins was undertaken in this work. Five different extraction procedures were tested. The results obtained showed non-destructive and efficient extraction with 1% SDS in Tris/HCl buffer (pH 8) which assured the stability of the selenium species and the reliability of the results obtained in this work. Also, the possibility of using size exclusion chromatography sequentially coupled to both UV and ICP-MS detection for fractionation of proteins extracted from nut samples was demonstrated. Three different SEC columns allowed covering a wide MW range (600–0.18 kDa) and lead to a more exhaustive study of the association of selenium to two proteins fractions of different MW. Selenium co-elution with two molecular weight fractions was observed in the region 200–10 kDa. The apparent MWs of these selenium-containing fractions were 107 and 50 kDa, respectively. In the application of capillary electrophoresis coupled to ICP-MS, selenomethionine was confirmed as the primary species incorporated to nut proteins. The information obtained with SEC-UV-ICP-MS and CE-ICP-MS combined gave confirmation of the association of selenium to proteins in nut extracts. However, the nature of those selenoproteins still require further characterization and future works will be developed to elucidate these compounds.

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