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Speciation of Se in *Bertholletia excelsa* (Brazil nut): A hard nut to crack?

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Abstract

A separation method based on ion-pairing liquid chromatography was combined with both elemental (inductively coupled plasma mass spectrometry (ICP-MS)) and molecular (electrospray tandem mass spectrometry (ES-MS-MS)) mass spectrometry in order to unravel the identity of the Se-species present in the complex matrix of Brazil nuts rich in Se. Via enzymatic digestion, Se-species were released from the matrix. Subsequently the species were separated and the Se was monitored on-line by ICP-MS. By HPLC–ES-MS-MS, the species were identified based on their molecular mass and their specific product ions. The main compound was identified as Se-Methionine. Another compound was identified as Se-Cystine, partly on the basis of the isotopic pattern of Se. This research was further extended to the analyses of in vitro gastrointestinal digests of the Brazil nuts. These digests were analyzed for their Se-content and screened for the presence of the different Se-species by HPLC–ICP-MS. In both the gastric and the intestinal digests, we were able to identify the Se-species as Se-Methionine and Se-Cystine by HPLC–ES-MS-MS. By coupling HPLC to both elemental and molecular mass spectrometry, the species present in Brazil nuts and supposedly extractable by our body were fully characterized.
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Keywords: Brazil nuts; Se; HPLC; ICP-MS; ES-MS-MS; Gastrointestinal digestion

1. Introduction

Being aware of the favourable influence Se may have on our organism, it is important to know which dietary sources of this element are most beneficial. Although there is an ample choice of Se-supplements, we focus on natural sources of Se.

A study by Ip and Lisk (1994) demonstrated that cancer mortality was reduced by 50% by supplementing people with Se-enriched Brewer's yeast (*Saccharomyces cerevisiae*). Many studies have been devoted to the speciation of Se in this Se-enriched supplement (Dumont et al., 2004a; McSheehy & Mester, 2003; Encinar, Sliwka-Kaszynska, Polatajka, Vacchina, & Szpunar, 2003). The main compound present in this matrix was identified as Se-Methionine (Dumont et al., 2005; Yoshida et al., 2002). Nearly every item of our diet has been screened for its total Se-content. Fruits and vegetables contain less than 0.01 μSe g^{-1}. The Se content in grain products varies between 0.02 and 0.4 μg Se g^{-1}. Meat is known to be an even more important source of Se: 0.1–0.4 μg Se g^{-1} (Ip, 1998). Researchers started to measure Se-species in different natural products containing high amounts of Se. Brazil nuts (*Bertholletia excelsa*) are known to be one of the products with the highest Se content. These nuts are the fruits from the Brazil nut tree, which grows in the Amazon

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River basin. The soil on which the tree grows is of great importance, since the Se-content of the nuts is highly dependent on the amount of Se present in the soil. It was shown that Brazil nuts originating from the central part of Brazil contained up to ten times more Se than the nuts exploited from the Western part of Brazil (Chang, Gutenmann, Reid, & Lisk, 1995). Some attempts have been made to identify the species present in Brazil nuts. A study by Chunhieng et al., concentrated on the distribution of Se in Brazil nuts among the different protein fractions (Chunhieng et al., 2004a; Chunhieng et al., 2004b). The nuts were investigated in search for a candidate reference material for the speciation of Se-compounds. Se-Methionine was demonstrated to be present be it only on the basis of HPLC–ICP-MS, lacking molecular information to prove this (Bodo et al., 2003). In the studies by Wrobel, Kannamkumarath, Wrobel, and Caruso (2003) the distribution of Se in different nut types, amongst which Brazil nuts, was examined by HPLC–ICP-MS. The identity of one peak was verified as Se-Methionine by spiking with the corresponding selenium standard. To our knowledge, only one research group attempted to characterize the Se-species by obtaining molecular information. In that study the enzymatic hydrolysate of the Brazil nut was examined by ion-pairing chromatography combined with ICP-MS. Fractions with the Se-containing species were pooled and further examined by ES-MS. On the basis of the results thus obtained a peptide structure was proposed (Vonderheide et al., 2002).

The Se bioavailability is dependent on the digestibility of the different Se-containing proteins (Combs, 2001). The matrix of Brazil nuts is quite complex (66–67% fat, 14% proteins and 13% of carbohydrates). It is surely interesting to examine the behavior of the Se-species in our gastric and intestinal digestive tract.

In the present paper a method, previously developed in our laboratory, and already shown to be successful for the speciation of Se in different types of Se-supplements was applied to identify the Se-species present in Brazil nuts (Dumont et al., 2004a; Dumont et al., 2005). The complexity of the matrix of the Brazil nuts and the lower Se-concentration hampered the application of this method. The Se-species were extracted after appropriate sample clean-up. The method is based on the combination of an efficient HPLC-method with both elemental and molecular mass spectrometry. The Se-species were characterized based on retention time matching and on-line detection of the molecular ions and their product ions. When molecular information is lacking, the isotopic pattern of Se was used in the identification. The research was further extended to the analysis of in vitro gastrointestinal digests of the nuts.

2. Materials and methods

2.1. Instrumentation

The microwave digester was a Milestone mls 1200 mega from Analis, Namur, Belgium. The Inductively Coupled Plasma Mass Spectrometer (ICP-MS) was a quadrupole based Perkin–Elmer SCIEX Elan 5000 (Glendale, Ontario, Canada). The electrospray tandem mass spectrometer (ES-MS-MS) was a Quattro Micro system (Micromass, Manchester, UK), equipped with a Z-spray source. High Performance Liquid Chromatography (HPLC) experiments with hyphenation to the ES-MS-MS were done on a Waters Alliance 2690 model equipped with an autosampler. For hyphenation to the ICP-MS an HPLC pump, model 625 from Alltech (Deerfield, IL, USA), equipped with a 6-way injection valve model 7161 from Rheodyne (Cotati, CA, USA) and a 10 μl loop, was applied. The mobile phase was degassed and flushed with argon prior to analysis. Two XTerra columns with appropriate guard columns were used for separation: an analytical column (*L*: 250 mm, i.d. 4.6 mm, 5 μm particles) with guard column (*L* 20 mm, i.d. 3.9 mm, 5 μm particles), and a narrowbore column with the same packing material (*L*: 250 mm, i.d. 2.1 mm, 5 μm particles) with guard column (*L*: 20 mm, i.d. 2.1 mm, 5 μm particles) for HPLC–ICP-MS and HPLC–ES-MS-MS, respectively. All columns were from Waters Corporation, (Milford, MA, USA).

2.2. Reagents and materials

All chemicals used were of analytical grade purity. Ultrapure Milli Q water was produced in the laboratory by using a Millipore system (Bedford, MA, USA). The Se-standards Se-Methionine (Se-Met) and Se-Cystine (Se-$(Cys)_2$), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, protease XIV from *Streptomyces griseus* and tetraethylammonium-chloride (TEACl) were from Sigma (Bornem, Belgium). Methanol, KH_2PO_4 and formic acid were purchased from Vel (Leuven, Belgium). NaOH was bought from Carlo Erba (Milan, Italy). H_2O_2 (30%) was from Merck (Darmstadt, Germany). $CHCl_3$ and CH_2Cl_2 were from UCB (Brussels, Belgium). Nitric acid (14 M) was purified by sub-boiling distillation in quartz equipment. The 5 ml polypropylene test tubes used in the simulation of the digestion are from Falcon, Becton Dickinson labware (Meylan, France). The 0.22 μm pore PVDF syringe filters to filter the samples prior to analysis were from Millipore (Bedford, MA, USA). The PEEK tubing for all couplings had an i.d. of 0.1 mm. The length of the tubing was limited to 40 cm in order to reduce peak broadening due to diffusion of the separated species in the tubing.

2.3. Total Se determination

Two types of Brazil nuts were examined: one type originating, according to the labelling, from Bolivia was bought in a local Belgian supermarket. These nuts were sold without the shell in packages of 250 g. Another type originated from Brazil (Diamond nut type) and was bought in a local supermarket in the United States in packages of approximately 450 g. These nuts were still in their shell. Prior to analysis the nuts were shelled if necessary. For total Se determination, an amount of nuts equalizing approximately 20 g were peeled, grinded in a coffee mill and mixed to obtain a homogenized mixture for further analysis. An 0.4 g aliquot of the mixture was transferred into a teflon vessel, 5 ml of concentrated HNO_3 and 1 ml of H_2O_2 were added. Gallium (50 $\mu g l^{-1}$) was added as an internal standard for ICP-MS measurements. The microwave oven program was as follows: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min at 400 W and finally 5 min at 650 W.

The digests were diluted with Milli Q water prior to analysis with ICP-MS.

2.4. Enzymatic and in vitro gastrointestinal extraction

The high fat content required that the nuts needed to be defatted prior to the enzymatic extraction. This was done with a $MeOH/CHCl_3$ mixture according to the method of Vonderheide et al. (2002). All fat could be removed. This was done for approximately 50 g of nuts in order to obtain a homogenized mixture. To an 0.4 g aliquot of defatted nut 40 mg of protease XIV and 3 ml Milli Q was added. The mixture was placed in a shaking hot water bath at 37 °C for 24 h in dark at 150 rpm and subsequently centrifuged. The supernatant was kept at −20 °C until analysis

For the in vitro experiments, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described earlier (Dumont, Vanhaecke, & Cornelis, 2004b; US Pharmacopeia XXIV & national formulary, 2000). To an 0.4 g aliquot of Brazil nuts, 3 ml of SIF or SGF was added. The mixture was placed in a shaking hot water bath at 37 °C for 0 min, 15 min, 30 min, 1, 2, 4, 8, 16 and 24 h in dark at 150 rpm. The solutions were centrifuged and the supernatant was kept at −20 °C until analysis.

2.5. (HPLC)-ICP-MS measurements

The separation method and the ICP-MS conditions for total Se measurements were as described earlier by the authors (Dumont et al., 2004b). Total Se-concentration in the extracts and digests of the nuts were determined by simple standard addition. The $^{82}Se^{+}$ isotope was measured to ensure interference free conditions. The enzymatic digests were diluted 20-fold, the SIF and SGF digests were diluted 10-fold in 0.14 M HNO_3. Se(IV) (10 $\mu g\ l^{-1}$) was added to the samples for calibration purposes.

2.6. HPLC–ES-MS-MS measurements

The method applied for obtaining molecular information was as described earlier by our research group (Dumont et al., 2005). The samples were dissolved in a 1% formic acid solution prior to analysis. A 10 µl aliquot of the extract was brought onto the column.

3. Results and discussion

3.1. Total Se-concentration in the Brazil nuts and their extracts

Determination of the total Se-concentration in both types of Brazil nuts was done in 10-fold. Calibration was accomplished via simple standard addition. The nuts without shell contained 5.1 µg Se $g^{-1} \pm 10.6\%$. The nuts with shell contained 49.9 µg Se $g^{-1} \pm 6.4\%$. The shells were not examined, this was already done by Vonderheide et al. (2002). It seemed from their experiments that the shells were very low in Se content. Furthermore they are not edible. The nuts from Brazil (those bought in their shell) thus contained 10 times more Se compared to those from Bolivia. It was decided from these experiments that only these Brazil nuts (Diamond type) would be used for further experiments.

The total Se content of the supernatant of the enzymatic digests was determined by ICP-MS in 3-fold. The extraction efficiency was calculated against the total Se content of the nuts determined after microwave digestion and yielded 73.7 ± 2.5%.

The bioavailability of an element is very much dependent on its species. Another important parameter is the conditions to which the species is submitted. When consuming food, the species are subjected to extreme conditions. Species transformation is at risk and should be brought into chart. A way to do so is by in vitro gastrointestinal digestions, as has been done in search of Se-species in cooked cod (Crews et al., 1996) and in different Se-supplements (Dumont et al., 2004b). Conditions in our digestive tract are quite extreme (low pH of the stomach: pH 1.2, presence of enzymes and microbial activity) and might not only lead to species transformation but also to degradation, so that our body may no longer absorb Se in its original form.

In Fig. 1, the amount of Se extracted from the Brazil nuts during gastric and intestinal digestion, are shown. For the gastric digestion, we can see that the longer the Brazil nut is treated with SGF, the more Se is extracted. For the intestinal digestion, results are quite

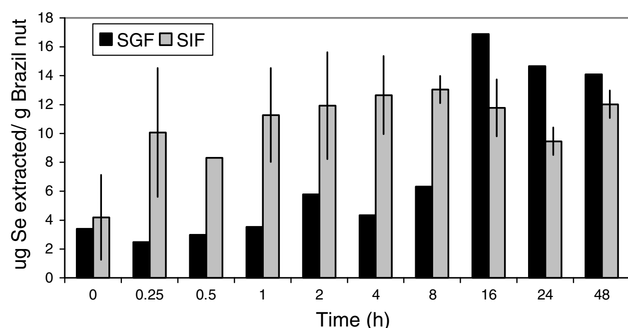

Fig. 1. Extraction of Se by SGF and SIF as a function of time.

different. The amount of Se extracted reaches a maximum very soon. The experiments with SIF were repeated three times and the results remained the same. A possible explanation can be the presence of the high fat content. In our body fats are digested through the action of the liver and bile. These steps were not included in this simulated digestion process and hence fats were not readily digested as in our body. As it was the intention of this research to mimic the conditions in our body as much as possible, it seemed pointless to defat the nuts prior or after digestion.

3.2. HPLC–ICP-MS of the proteolytic digest

A 10 μl aliquot of the extract was brought on the column and eluted. The $^{82}Se^{+}$ isotope was monitored. In the chromatogram, two peaks could be distinguished: one at t_R = Se-$(Cys)_2$ and one at t_R = Se-Met. This was verified by standard addition of Se-Met and Se-$(Cys)_2$. The result can be seen in Figs. 2(a) and (b). It is clear that these two species of the nuts coelute with the respective standards. However, as we only have elemental information, molecular information is needed to identify these compounds unequivocally.

3.3. HPLC–ES-MS-(MS) of the proteolytic digest: presence of Se-Met

At first, an attempt was made to monitor the mass to charge ratio (*m*/*z*) of the molecular ions of Se-Met and Se-$(Cys)_2$ in the extracts. A 10 μl aliquot of the extract was brought on the narrowbore column which was coupled to the electrospray probe. ES and MS parameters were as described earlier (Dumont et al., 2005). We tried to demonstrate the presence of Se-Met ($^{+}NH_3CH(COOH)CH_2CH_2SeCH_3$) by monitoring *m*/*z* 198 in the positive ion mode. The HPLC–ES-MS chromatogram is shown in Fig. 3(a). In this chromatogram we can see a peak of *m*/*z* 198 at t_R = Se-Met, but there are many other peaks present with the same *m*/*z* at other retention times. It is obvious that the matrix is very complex and that further molecular information is necessary. The product ions of Se-Met could be demonstrated in ES-MS-MS mode. The five possible fragment ions of Se-Met formed in the collision cell were found at *m*/*z* 181, 152, 135, 109 and 102. These product ions were measured by measuring the transitions of 198–181: loss of NH_3; 198–152: loss of CO and H_2O; 198–135: loss of CO, NH_3 and H_2O; 198–109: corresponding to the ion $CH_3SeCH_2^{+}$; 198–102 formation of the ion $NH_3CH(COOH)CH_2CH_2^{+}$, in multiple reaction monitoring (MRM) mode. From all these transitions, the transition of 198–102 is specific for a Se-molecule since a Se-fragment is lost. The other fragments are common fragments to be lost in biomolecules. In Fig. 3(b), the chromatogram of all transitions is shown. From this

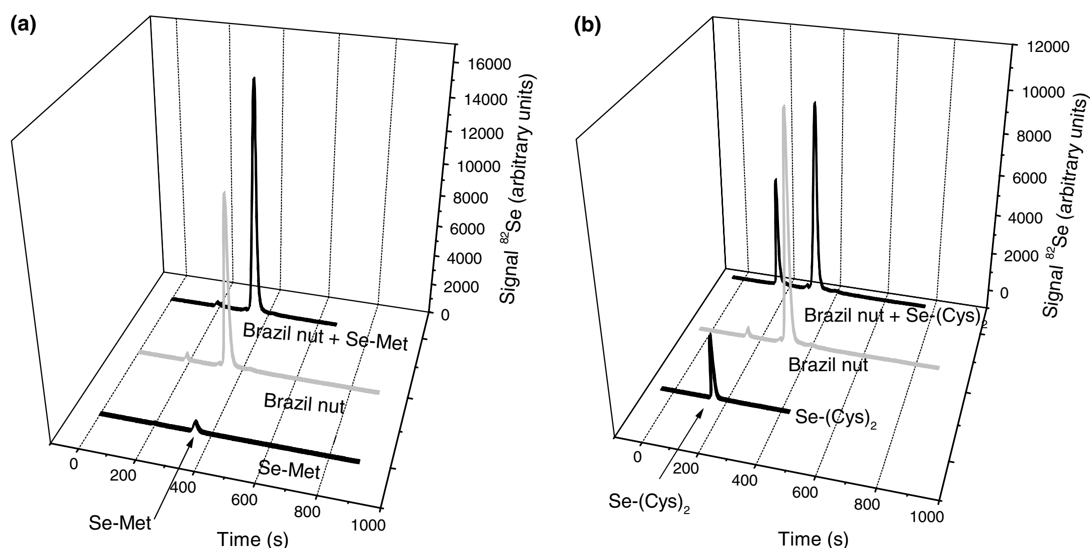

Fig. 2. HPLC–ICPMS chromatogram of the proteolytic digest of Brazil nut: standard addition of Se-Met (a) and Se-$(Cys)_2$ (b).

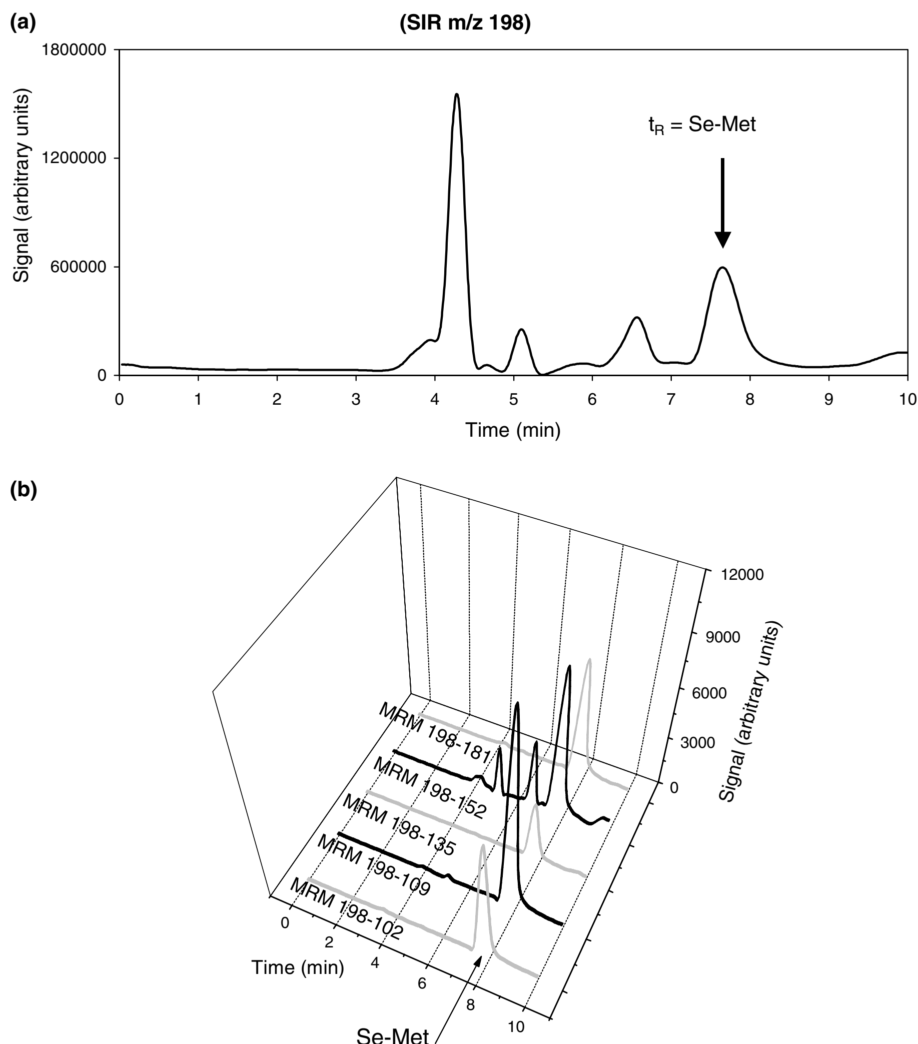

Fig. 3. (a) HPLC–ES-MS chromatogram of Brazil nut: SIR of *m/z* 198; (b) HPLC–ES-MS-MS: MRM of all product ions of Se-Met in Brazil nut.

chromatogram, we can see that all product ions can be monitored on-line by narrowbore HPLC–ES-MS-MS. The main compound present in the proteolytic digest of Brazil nut was Se-Met.

3.4. HPLC–ES-MS-(MS) of the proteolytic digest: presence of Se-(Cys)$_2$

It was demonstrated that Se-(Cys)$_2$ (*m/z* 337) ($NH_2(COOH)CHCH_2Se-SeCH_2CH(COOH)NH_3^+$) gives rise to only one product ion in ES-MS-MS (Dumont et al., 2005). Due to the complexity of the matrix, this might be insufficient to unequivocally characterize the molecule. Therefore, in the attempt to demonstrate the presence of Se-(Cys)$_2$, the isotopic pattern of Se was used. To monitor the *m/z* of Se-(Cys)$_2$, two different masses were measured: *m/z* 337 and *m/z* 335. *m/z* corresponds to $^{80}Se^{80}Se$ or to $^{78}Se^{82}Se$. *m/z* 335 corresponds to $^{78}Se^{80}Se$ or $^{76}Se^{82}Se$. The abundance of these two molecular ions was calculated to be 28,76% for *m/z* 337 (relative abundance of $^{80}Se)^2$ + (relative abundance of ^{78}Se multiplied with relative abundance of ^{82}Se multiplied by 2 (possible positions of the isotopes in the molecule)). For *m/z* 335 the abundance was calculated to be 25,22% (relative abundance of ^{78}Se multiplied with relative abundance of ^{80}Se multiplied by 2 (possible positions of the isotopes in the molecule)) + (relative abundance of ^{76}Se multiplied with relative abundance of ^{82}Se multiplied by 2 (possible positions of the isotopes in the molecule)). The abundance of C did not influence the abundance of these isotopes. The HPLC–ES-MS chromatogram in which *m/z* 337 and 335 are measured are shown in Fig. 4 (top). In this chromatogram we can see more peaks of *m/z* 337 and 335, amongst which two peaks at the retention time of Se-(Cys)$_2$, corresponding to *m/z* 337 and 335. More information was obtained by measuring in MRM-mode the transition of 337–248 and 335–246, corresponding to the loss of $NH_2CH(COOH)CH_3$. In the chromatogram in Fig. 4 (bottom), we observe the presence of both transitions

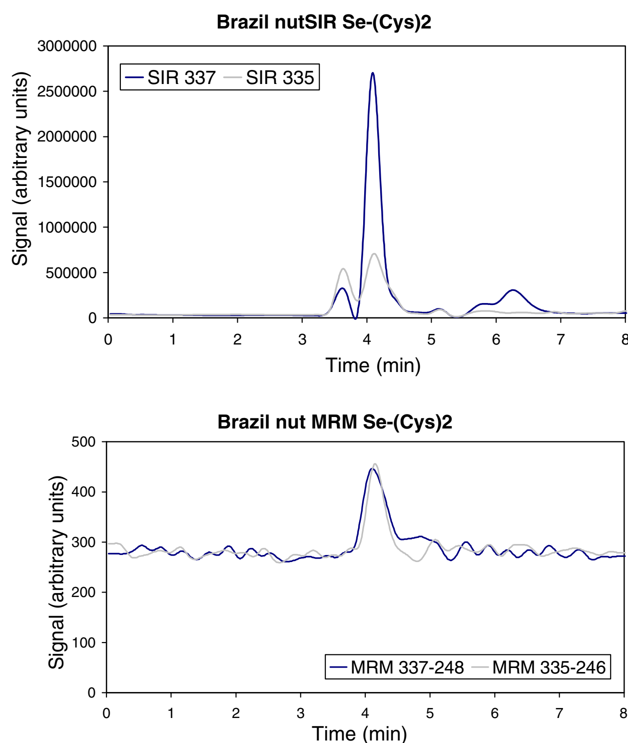

Fig. 4. HPLC–ES-MS-MS chromatograms: SIR and MRM of Se-$(Cys)_2$ in proteolytic digest.

at the retention time of Se-$(Cys)_2$. From this figure we can also see that the peak surfaces are almost equal to each other according to what should be expected from the abundance calculations. This was not the case for the selected ion recording (SIR) measurements. This might be due to the presence of another compound with *m*/*z* 337, which does not give rise to the same product ion.

The ability to measure Se on different isotopes offers opportunity to use HPLC–ES-MS-MS to monitor Se species in different matrices, even when molecular information is scarce. The method described here opens perspectives to measure and monitor Se-species in other foodstuffs (e.g., garlic, onion, etc.) or Se-enriched food. This combination of analytical techniques can be applied to detect fraud in produced Se-supplements, in that species present or absent in a product can be easily detected.

3.5. HPLC–ICP-MS of the gastrointestinal digests of Brazil nuts

The 24 h SGF and SIF extracts were filtered through an 0.22 μm pore filter prior to injection onto the column. In order to mimic the real conditions as much as possible, an aliquot of the Brazil nuts was submitted to gastric digestion during 3 h. The mixture was filtered and the filtrate was kept for analysis with HPLC–ICP-MS. The residue was dried and afterwards submitted to intestinal digestion. The filtrate was collected and kept for analysis with HPLC–ICP-MS. The chromatograms for SGF and SIF digestions are shown in Fig. 5. The same results were observed for the separate extractions and for the stepwise digestion (first SGF followed by SIF digestion). Two main compounds were observed eluting at t_R = Se-Met and Se-$(Cys)_2$. This was in accordance to the results of the proteolytic digests. From the chromatograms, we can see that in the SGF digest, almost the same amount of Se is extracted as Se-$(Cys)_2$ as under the Se-Met form. In the SIF digest much more Se-Met is extracted, compared to the SGF digest. Se-Met is thus mostly extracted in the intestinal tract.

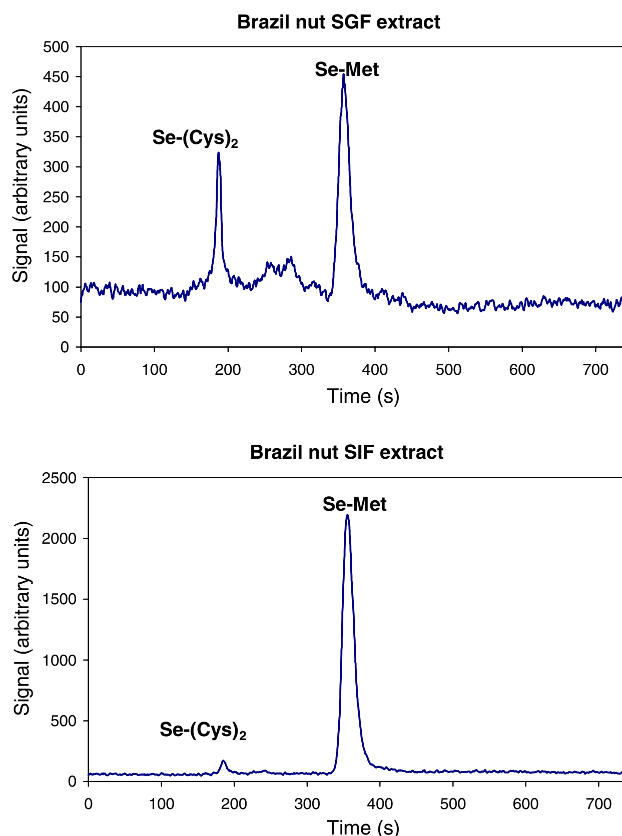

Fig. 5. HPLC–ICP-MS chromatogram of the SGF and SIF extracts of Brazil nuts.

3.6. HPLC–ES-MS-(MS) of the in vitro gastrointestinal digests of Brazil nuts

A 10 μl aliquot of the filtered SGF and SIF digests was brought onto the narrowbore column. First, the molecular ion was monitored at *m*/*z* 337 and 335 for Se-$(Cys)_2$ and at *m*/*z* 198 for Se-Met. Problems arose when adding formic acid to these extracts, because of precipitation of the compounds. An additional sample clean-up step was required. The proteins were removed from the extracts in order to simplify the

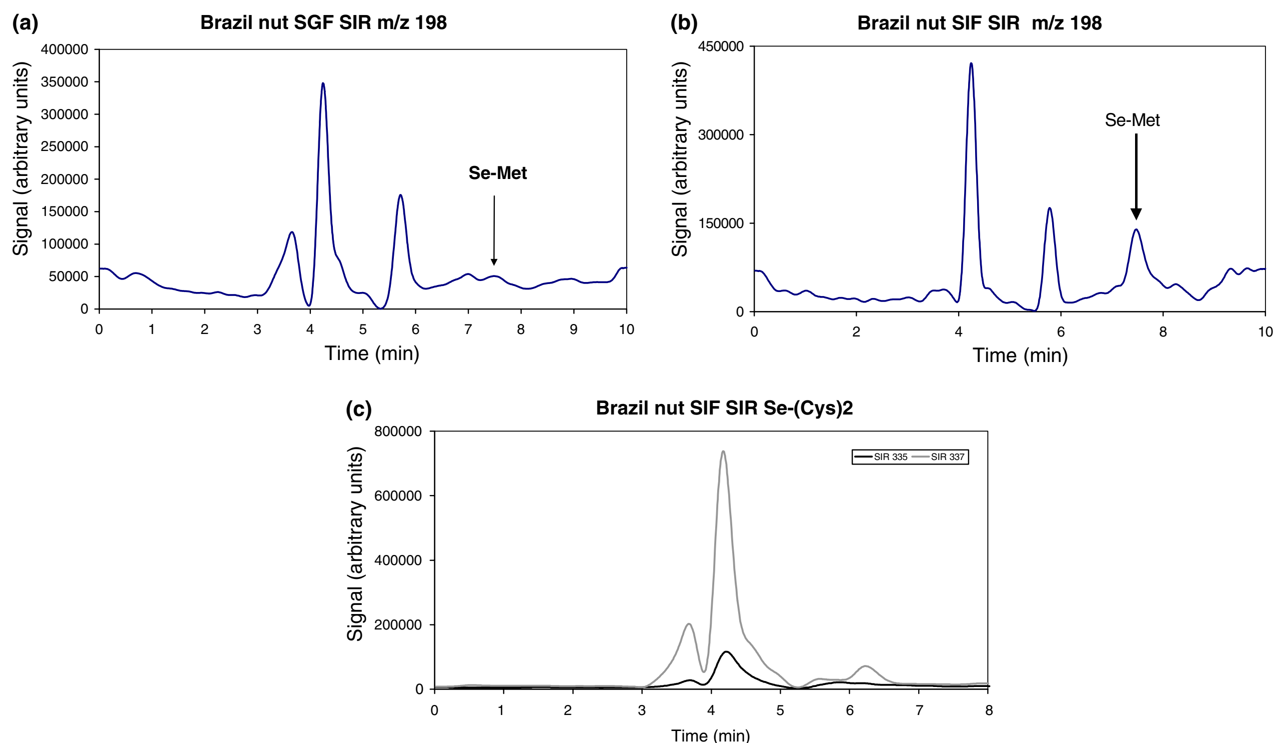

Fig. 6. HPLC–ES-MS chromatograms: (a) SIR of *m*/*z* 198 in SGF extract; (b) SIR of *m*/*z* 198 in SIF extract; (c) SIR of *m*/*z* 337 and 335 in SIF extract.

matrix. This was done by addition of 1 ml ethanol to 3 ml of extract. The mixture was placed at 4 °C for 30 min. The mixture was centrifuged and the precipitated proteins were removed. The extract was then placed under an argon stream to remove the ethanol. After addition of formic acid, precipitation was no longer observed. Additional attempts were made to remove the fat from the extracts by $CHCl_3$ and by CH_2Cl_2, but no improvements were observed in the HPLC–ES-MS-(MS) experiments. The results are shown in Fig. 6. In the chromatogram of the SGF extract (a), in which Se-Met was monitored, different peaks can be distinguished, but at the retention time of Se-Met it is hard to distinguish a peak. In the SIF extract of the Brazil nut (b), we can easily see a compound of *m*/*z* 198 at t_R = Se-Met. In Fig. 6(c) we see the SIR measurements of *m*/*z* 337 and 335, both masses could be monitored in the SIF extract,

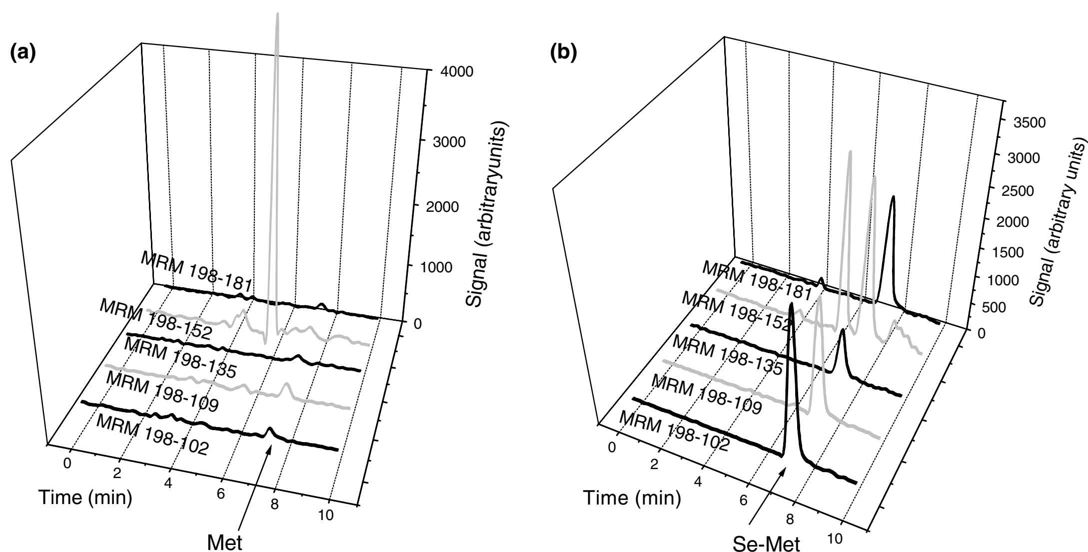

Fig. 7. HPLC–ES-MS-MS chromatograms: MRMs of Se-Met in SGF (a) and SIF (b) extract of Brazil nuts.

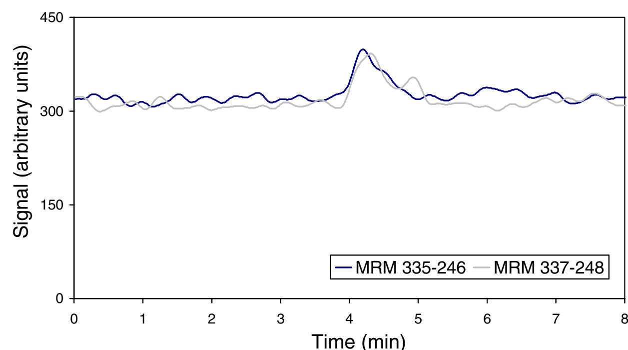

Fig. 8. HPLC–ES-MS-MS chromatogram: MRMs of Se-$(Cys)_2$ in SIF extract of Brazil nuts.

although not in the SGF extract. The underlying reason might be the very low concentration of Se-$(Cys)_2$ or the complexity of the matrix. Further analysis was necessary to examine the Se-species extracted during gastric and/or intestinal digestion. By applying tandem mass spectrometry we were able to detect the product ions of both Se-Met and Se-$(Cys)_2$ in this complex matrix. The results are shown in Fig. 7(a) we can see the MRMs of the five transitions of the product ions characteristic for Se-Met in the SGF extract of the Brazil nut. The peaks are difficult to distinguish as was to be expected from the low intensity of the peak in the HPLC–ICP-MS chromatogram. Fig. 7(b) shows the results of the MRM measurements of the transitions for Se-Met in the SIF extract. In the SIF extract the five transitions were easily observed. In Fig. 8 the results are given of the MRMs of transitions 337–248 and 335–246 in the SIF extract. Again we can see that the peak surfaces correspond to what was expected from the calculation of the abundances.

The data on the SIF and SGF experiments demonstrate that Se-Met and Se-$(Cys)_2$ can readily be extracted in the gastrointestinal tract. Furthermore it is obvious that, even though the extreme conditions, no species transformation occurs. The conditions simulated here do not influence the Se-species and that Se is extracted in its original form. If metabolisation of Se happens, it will probably be at another level. It is possible that microbial activity in our intestines is responsible for the transformation of species. The application of in vitro gastrointestinal digestion experiments opens perspectives in nutritional sciences. They can be used to check the digestibility and bioavailability of certain (Se)-compounds. They give an indication as to the relevance of the intake of the different (Se)-species.

4. Conclusions

In this paper the power of hyphenating HPLC to both elemental and molecular mass spectrometry is demonstrated. Indeed the speciation of Se in Brazil nuts seems a hard nut to crack, but there are ways to overcome the specific problems. The main compounds present in the matrix after proteolytic digestion are Se-$(Cys)_2$ and Se-Met, the latter being the major compound. By simulating the conditions in our gastric and intestinal tract, we were able to identify the two species extracted as Se-$(Cys)_2$ and Se-Met. HPLC–ICPMS and HPLC–ES-MS-MS are complementary techniques in speciation analyses. This method can further be applied to other complex matrices to fully characterize their Se-species. The application of these methods can also aid in the study of the metabolisation of Se in our body.

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