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Characterization of Selenium Species in Brazil Nuts by HPLC−ICP-MS and ES-MS

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Brazil nuts have been classified as the foodstuffs that contain the highest level of unadulterated selenium, an essential trace element that appears to prevent cancer. To date, characterization of the selenium species in brazil nuts has not yet been investigated. In this work, various sample preparation approaches, including microwave extractions and enzymatic treatments, are examined with the goal of species preservation and subsequent selenium speciation; of these approaches, an enzymatic treatment with Proteinase K proved most effective. High-performance liquid chromatography (HPLC) separation strategies and inductively coupled plasma mass spectrometry (ICP-MS) detection schemes will also be presented. Extracts are evaluated against available standards for the commercially obtainable seleno-amino acids, selenomethionine (SeMet), selenoethionine (SeEt), and selenocystine (SeCys); selenomethionine was demonstrated to be the most abundant of these seleno-amino acids. Further characterization of unidentified selenium-containing peaks is attempted by the employment of several procedures, including electrospray-mass spectrometry (ES-MS). A peptide structure was identified; however, this was considered a tentative proposal due to the large background produced by the extremely complicated brazil nut matrix.

KEYWORDS: Selenium speciation; brazil nuts; HPLC-ICP-MS; ES-MS

INTRODUCTION

Selenium is an essential element that may exist in several chemical forms, both organic and inorganic, in foods and nutritional supplements. Studies have shown that its bioavailability, as well as how it is stored in the body, depends on its chemical form (*1*). Subsequently, there is great interest in the determination of the specific chemical species of selenium rather than just total element concentration (*2*).

Although selenium supplements have increased in popularity as of late, selenium is inherent in many of the foods we eat. It has been found that brazil nuts (*Bertholletia excelsa*, family *Lecythidaceae*) are the food that contain the highest levels of selenium by a substantial factor. Interest in different selenium-containing foods and supplements has elevated as research into the anticarcinogenic properties of selenium has increased (*3*, *4*). Selenium seems to prevent several different types of cancer by inducing apoptosis (cell death) in premalignant lesions (*5*). This element is also necessary for the optimal activity of an important selenoenzyme, glutathione peroxidase, which protects against oxidative stress by scavenging damaging peroxides.

Selenium levels in meats, grains, and vegetables depend on the selenium content of the soil where the food is raised (*6*). The selenium in the soil is absorbed by plants and subsequently by people and animals that eat them (*7*). Natural selenium levels in the soil are highly variable throughout the world and this is not an exception in Brazil. Brazil nuts are grown in two regions of Brazil: a central and a western region (*8*). The central region possesses selenium-rich soil and nuts from here are shipped with the shells intact. Nuts from the western region come without shells. Differences have been noted between the selenium levels of shelled nuts and those with the shells intact.

When incorporated in growing plants, selenium may replace the sulfur atom of the amino acids. Interestingly, dietary studies have shown that selenium in the form of amino acids is absorbed more readily than inorganic forms of the element (*9*). Brazil nuts have been determined to be rich in the sulfur-containing amino acids methionine (18%) and cysteine (8%) (*10*). Furthermore, evidence in protein studies indicates the existence of disulfide linkages between cysteine molecules, forming cystine entities (*11*). Subsequently, emphasis in the analysis of the brazil nut extracts was placed on the chromatographic identification of the available seleno-amino acids.

Most analytical methods for the analysis of the seleno-amino acids utilize high-performance liquid chromatography (HPLC) coupled with element-specific detection (*12*). Specifically, several methods employ ion-pair reversed-phase liquid chromatography (*13*−*15*). The main reason to use this technique is that amino acids are amphoteric: they are zwitterions that can

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act as either an acid or a base. The nonionic form does not occur in significant amounts in aqueous solutions and the zwitterion predominates at the isoelectric point. Therefore, the pH of the mobile phase determines the charge and, subsequently, the retentive behavior. At low pH, the predominant form is the protonated amino acid; for this application, two ion-pairing reagents were used as mobile phase additives. The first, hexanesulfonic acid, was used for the quantitation of the brazil nut extracts. The second, methanesulfonic acid, was employed as a more volatile alternative in the collection of fractions for analysis by electrospray mass spectrometry.

Inductively coupled plasma mass spectrometry is a sensitive and selective detector for selenium (*16*). Its coupling to HPLC is relatively straightforward and matching retention times with standards can yield identification of selenium species present in the sample (*17*−*19*). However, standards for many Se species are not available commercially or may not even be known (*20*). This makes identification based on retention time rather difficult and requires the employment of alternative techniques, such as electrospray mass spectrometry (ES-MS), which was ultimately utilized in this work.

EXPERIMENTAL PROCEDURES

Reagents and Standards. Commercial chemicals were of analytical reagent grade and were used without further purification. Se-methionine, Se-ethionine, and Se-cystine standards were obtained from Sigma (St. Louis, Missouri). Working standards were prepared fresh by serial dilution of stock standards. Proteinase K was purchased from Sigma. The mobile phase components, hexanesulfonic acid, methanesulfonic acid, and methanol, were obtained from Fisher Scientific (Fairlawn, NJ). Nitric acid and hydrochloric acid were also purchased from Fisher Scientific.

Instrumentation. *HPLC Conditions.* The Agilent 1100 liquid chromatograph was equipped with the following: a binary HPLC pump, an autosampler, a vacuum degasser system, a thermostated column compartment, and a diode array detector. The HPLC system was connected through a remote cable that allowed the simultaneous start of the chromatographic run on both instruments. The system is marketed by Agilent as a "metal speciation" option with the ICP-MS.

Ion pairing chromatography was chosen for the analysis of the seleno-amino acid; hexanesulfonic acid was selected as the ion-pairing reagent. Isocratic conditions utilizing 10% methanol were employed that were more adequate for the use of ICP-MS as the detector; large amounts of organic solvent destabilize the plasma and potentially may extinguish it (*19*). Specific chromatographic conditions for both ICP-MS and ES-MS are given in **Table 1**.

Inductively Coupled Plasma Mass Spectrometry. Total selenium levels were determined by using a Perkin-Elmer Elan (Norwalk, CT) 6000 ICP-MS. An Agilent 7500 ICP-MS was employed for chromatographic analysis. Instrument operating conditions are shown in **Table 1**. Selenium isotopes ^{77}Se, ^{78}Se, and ^{82}Se were monitored.

Electrospray Mass Spectrometry. The mass spectrometer employed was a quadrupole-time-of-flight (Q-TOF) from Micromass (Manchester, U.K.) in the ionspray mode with nitrogen as the nebulizing gas. The ionization source was operated in positive ion mode; specific parameters are shown in **Table 1**.

In addition to the equipment described above, a model NES 1000 closed vessel microwave digestion system manufactured by CEM Corporation (Matthews, NC), a Chermle Z 230 centrifuge (Woodbridge, NJ), and a Rotavapor RE 111 (Buchi Laboratoriums Technik AG, Switzerland) were used.

Samples. As mentioned previously, brazil nuts sold shelled are grown in the western area of Brazil bordered by Bolivia and Peru (Rondonia and Acre) (*8*). Those marketed with shells are grown along the tributaries of the Amazon River (near Manaus and Belam). Although this is the case, brazil nuts with shells and brazil nuts without shells were purchased in a local market and hence no definitive origin can be ascribed.

Table 1. Instrument Operating Conditions

HPLC Parameters for ICP-MS Detection	
column	Alltima C_8 with dimensions of 150 mm × 4.6 mm and a particle size of 5 μm
mobile phase	90:10 [5 mM citric acid and 5 mM hexanesulfonic acid (adjusted to pH 3.5)]:methanol
flow	1.0 mL/min
temp	30 °C
injection vol	20 μL
ICP-MS Parameters	
forward power	1300 W
plasma gas flow rate	15.0 L/min
carrier gas flow rate	1.08 L/min
dwell time	0.1 s per isotope
isotopes monitored	^{77}Se, ^{78}Se, ^{82}Se
HPLC Parameters for ES-MS Detection	
column	Alltima C_8 with dimensions of 150 mm × 4.6 mm and a particle size of 5 μm
mobile phase	90:10 [5 mM citric acid and 5 mM methanesulfonic acid (adjusted to pH 3.5)]:methanol
flow	0.7 mL/min
temp	30 °C
injection vol	100 μL
ES-MS Parameters	
capillary voltage	3000 V
cone voltage	45 V
nebulizing gas	N_2
mass range	*m*/*z* 50–1000

Sample Preparation. *Microwave Nitric Acid Digestion.* For the determination of the total selenium content, raw nuts were finely ground in a household coffee grinder. A 10-mL volume of 50:50 nitric acid−water was added to 0.5 g of the ground nuts in the microwave sample tube. Microwave power was increased over four steps at 5-min intervals, starting at 25%, 45%, and 55% and ending at 65%, where 100% power equals 1000 W. Temperature limits of 120, 140, 150, and 165 °C were set for each of the four steps. Pressure limits for the four steps were 20, 80, 120, and 170 psi. At the end of the digestion, the samples were diluted to 200 mL with 18 MΩ·cm deionized water. A portion of this solution was filtered through a 0.45-μm nylon membrane filter. Both indium and germanium were added as internal standards. Two standard addition solutions were prepared for each sample.

Lipid Removal. Ground nuts were sonicated with a 1:2 mixture of $CH_3OH-CHCl_3$. The solvent mixture was removed by vacuum filtration and collected, as discussed later. This procedure was repeated twice for each sample.

Water and Hydrochloric Acid Microwave Extractions (21). For the microwave extractions, a 10-mL volume of the appropriate solvent (water or 0.5 M HCl, respectively) was added to a weight of 0.5 g of the ground nut sample after lipid removal. This mixture was placed in the microwave sample tube. The microwave program was the same as for the total digestion. The extracts were then filtered with a glass fiber filter to remove the nut particulate. A portion of this solution was filtered through a 0.45-μm nylon membrane filter for HPLC analysis.

Proteinase K Enzymatic Hydrolysis (21−*23).* After lipid removal, a volume of 5 mL of Tris-HCl buffer (pH =7.5) was added to 0.25 g of ground sample and 0.025 g of Proteinase K. The samples were incubated at 37 °C for 20 h in darkness with shaking. The extracts were then filtered with a glass fiber filter to remove the nut particulate. A portion of this solution was filtered through a 0.45-μm nylon membrane filter for HPLC analysis.

Protein Removal after Enzymatic Hydrolysis. Residual proteins and any excess Proteinase K were removed from the extracts prior to chromatographic analysis. This was accomplished by the addition of 50 μL of a 20% solution of trichloroacetic acid (TCA) into 150 μL of the sample extract. The extract was then centrifuged and the supernatant removed from the precipitated proteins. This procedure was followed for both sample extracts and calibration standards. This was required

Table 2. Total Content of Selenium in Brazil Nuts (μg/g Wet Weight)

brazil nuts	av selenium content (μg/g) $n = 5$	rel standard deviation (% RSD)
with shell	35	5
shelled	8	5
shells only[a,b]	2	11

[a] One standard addition solution was prepared for each sample. [b] $n = 4$.

since the change in pH provoked by the addition of TCA changed the retentive behavior of the seleno-amino acids.

Protein Removal after Enzymatic Hydrolysis in Preparation for ES-MS. The TCA that was used in the original quantitation was considered too nonvolatile to use for those fractions to be analyzed by ES-MS. An alternate procedure was therefore employed. This was accomplished by the addition of a volume of 50 μL of ethanol to a volume of 150 μL of the sample extract. The extract was placed at −4 °C for 30 min to allow for protein precipitation. It was then centrifuged and the supernatant removed from the precipitated proteins.

Fraction Collection. The effluent of the HPLC column was collected for 10 injections at time intervals that represented the elution of the unidentified selenium-containing compounds. The time intervals for collection were offset by 0.1 min; this was to compensate for the travel time from the end of the HPLC column through the ICP-MS detection system. To verify this offset, the selenomethionine peak was collected as well. After collection, the samples were evaporated under a stream of nitrogen to effect further concentration. All collected extracts were then reanalyzed by HPLC-ICP-MS to confirm that the correct peaks were collected.

RESULTS AND DISCUSSION

Microwave Nitric Acid Analysis for Total Selenium. Nitric acid is an oxidizing reagent that dissolves most metals and nonmetals. It is most commonly used for the oxidation of organic matrixes. Of the three main components of food matrixes (carbohydrate, fat, and protein), a carbohydrate is the easiest structure to decompose because it is already partially oxygenated; it decomposes in nitric acid at approximately 140 °C. Fats typically decompose between 160 and 165 °C, while proteins decompose between 145 and 150 °C. Microwave temperature limits were therefore established accordingly. Also, the heating rate was set low to allow for safe and controllable decompositions, especially for easily oxidizable components such as carbohydrates.

The results for total selenium levels in brazil nuts are shown in **Table 2**. As mentioned earlier, differences in the total levels of selenium were noted between shelled nuts and those purchased with the shells intact. Additionally, the actual shells that were removed from brazil nuts purchased with shells intact were analyzed. Interestingly, low levels of selenium were found in these samples of shells only. Existing theories speculate on different selenium levels in the soil and/or various types of nut trees with differing efficiencies regarding the absorption of selenium.

Speciation. *Lipid Removal.* Brazil nuts are characterized as containing about 14% protein, 11% carbohydrates, and 67% fat or oil. The high oil content proved to be problematic in initial experiments. Early chromatographic runs showed increasing retention times of the seleno-amino acids over the course of a daily analytical sequence. The solution to this problem was found to be the removal of the lipids prior to chromatographic analysis.

However, to ensure that this step was not allowing the removal of any selenium species, the solvent fraction was collected as it was removed from the nuts. Because it is not feasible to analyze pure organic solvents by ICP-MS without extinguishing the plasma, the majority of the solvent mixture was evaporated through the use of a roto-vap apparatus and re-constitution was performed with a 2% nitric acid in water solution. Analysis of these extracts for total selenium by ICP-MS showed negligible selenium signal. Furthermore, and more importantly, removal of the lipids allowed for the accurate chromatographic analysis of the brazil nut extracts.

Table 3. Total Selenium Extracted with Different Extraction Procedures (μg/g Wet Weight)

extraction procedure	total selenium content (μg/g) of the extracts from brazil nuts with shells ($n = 5$)	selenium extracted (%)[a]
water microwave	3	9
0.5 M HCl microwave	13	37
Proteinase K enzymatic hydrolysis	29	83

[a] Based on the values obtained with the 50:50 nitric acid–water microwave digestion.

This procedure of removing the lipids served an additional purpose. Proteins may exist both outside and inside the cells of a plant. The cell membrane that separates these two areas is a lipid bilayer. Removing the lipids allowed the destruction of the cell membrane so that all proteins were accessible for hydrolysis by the Proteinase K of the enzymatic digestion.

Chromatographic Analysis. Selenium may be present either in the inorganic forms or in the form of amino acids. Furthermore, seleno-amino acids may be part of protein structures within the cell. Several extraction procedures were evaluated in terms of their ability to extract selenium species from the sample matrix. These included a water microwave extraction, a 0.5 M HCl microwave extraction, and a Proteinase K enzymatic hydrolysis. As in any speciation study, it remained of utmost importance that the procedures employed preserve the nature of the species.

The microwave extractions were chosen based on good results obtained in extracting the seleno-amino acids from yeast-based supplements (*21*). Microwave procedures provide a means by which to selectively and rapidly extract intact organometallic compounds (*24*, *25*). The enzymatic hydrolysis was selected as a means to evaluate whether the selenium was incorporated in proteins as seleno-amino acids. This was accomplished with Proteinase K, a protease that cleaves peptide bonds at the carboxylic sides of amino acids with very little cleavage specificity. It is thermally stable over a pH range of 7−10. Other work done in this laboratory showed Proteinase K to be the most efficient enzymatic treatment in extracting selenium species from plant materials, specifically *Brassica juncea* (*22*). Other work with yeast-based selenium food supplements showed similar results (*21*, *23*).

First, the extracts were analyzed for total selenium by ICP-MS. The results obtained were then compared to the total selenium content determined by the 50:50 nitric acid−water digestions. This was done to calculate the extraction efficiency of the procedures employed and these results are shown in **Table 3**. As can be seen from the data, Proteinase K was the most effective in extracting the Se species from the brazil nuts.

Table 4 shows the results obtained from the chromatographic analysis of the extracted brazil nut samples. The water and 0.5 M HCl microwave extractions showed no peaks in their respective sample chromatograms. This seems to indicate that

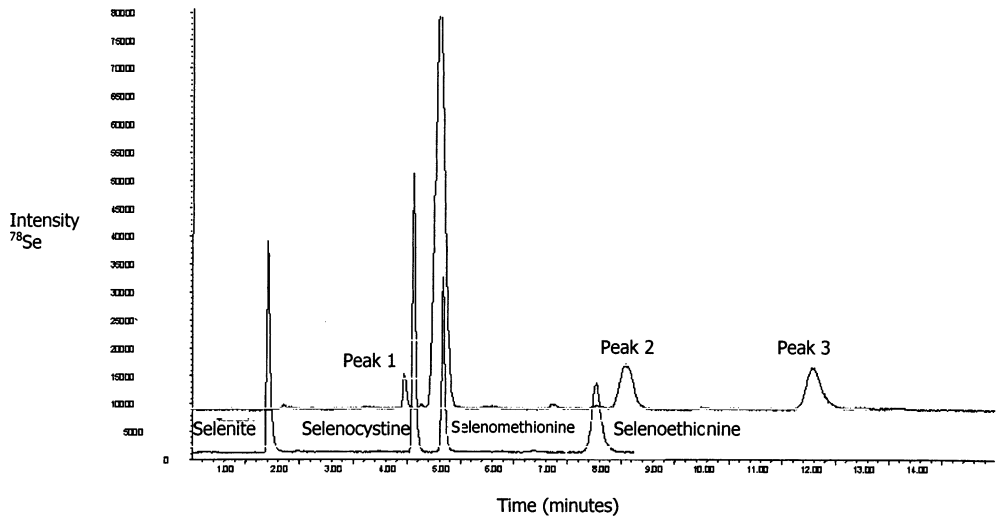

Figure 1. Chromatogram of Proteinase K extract of the brazil nut sample with the shell overlaid with the chromatogram of a 250-ppb standard of the seleno-amino acids.

Table 4. Concentration of Se-Amino Acids (μg/g Wet Weight) in Brazil Nuts with Shells and Those without Shells

	inorg Se	Se-cystine	Se-methionine	Se-ethionine
	Brazil Nuts with Shell			
water microwave	nd	nd	nd	nd
0.5 M HCl microwave	nd	nd	nd	nd
Proteinase K enzymatic hydrolysis	nd	nd	11 (7% RSD)	nd
	Shelled Brazil Nuts			
water microwave	nd	nd	nd	nd
0.5 M HCl microwave	nd	nd	nd	nd
Proteinase K enzymatic hydrolysis	nd	nd	2 (4% RSD)	nd

Table 5. Recoveries (%) of Matrix Fortification of Brazil Nuts Treated with Proteinase K

	inorg Se	Se-cystine	Se-methionine	Se-ethionine
with shell	nd	91	92	112
shelled	nd	74	105	87

very little of the selenium within the brazil nuts was in the form of inorganic selenium or free amino acids. It may be that proteins comprised of seleno-amino acids were extracted by these procedures. This would account for the fact that no peaks were observed within the time frame monitored (proteins would either elute much later or not at all). This would also explain the selenium levels found when the extracts were analyzed for total selenium by ICP-MS.

Of the three extraction procedures utilized, the Proteinase K enzymatic hydrolysis was the only procedure that allowed the recovery of any of the seleno-amino acids. From the data obtained with this enzymatic hydrolysis, it is demonstrated that one component of brazil nuts is selenomethionine. **Figure 1** shows a chromatogram of an extract of a brazil nut sample overlaid with that of a 250-ppb calibration standard. Although the enzymatic hydrolysis released mostly selenomethionine from the brazil nut sample, other selenium-containing species can also be observed in the chromatogram. Additionally, in a chromatogram obtained from a sample that was concentrated by evaporation prior to analysis it can be observed that brazil nuts are comprised of many selenium species at concentration levels that are quite low. Identification of the larger peaks, as shown in **Figure 1** at retention times of 4.3, 8.5, and 11.9 min (and referred to from this point as unidentified selenium-containing peaks 1, 2, and 3, respectively), will be considered shortly. It was speculated in work done by others in this laboratory that peaks 2 and 3 represented hydrophobic peptides that were a result of the incomplete decomposition of the proteins to their individual amino acids (*26*). This speculation stems from the knowledge that in the enzymatic hydrolysis of proteins, some peptide bonds can remain intact depending on the cleavage specificity of the enzyme (*27*).

Another point worthy of note concerns the protein removal steps described in the Experimental Section. These steps were performed to remove any remaining proteins or excess enzyme that could disrupt subsequent chromatographic analysis. However, unlike the steps taken to ensure that the removal of the lipids did not remove any selenium species, there was no guarantee that this procedure did not remove selenium-containing compounds. However, accurate chromatographic analysis necessitated these measures.

Chromatographic Figures of Merit. Method blanks were prepared for each type of extraction technique. The method blanks showed no detectable selenium species. Calibration curves were prepared with standards possessing levels that ranged from 5 to 250 ppb. All regression coefficients were acceptable, with the lowest value being 0.9997.

Matrix Fortification. Extracts obtained with all three extraction procedures were fortified with a solution containing inorganic selenium, selenocystine, selenomethionine, and selenoethionine. This work was done to determine any effect the matrix might exhibit toward the ability to recover the aforementioned analytes. The results of this experiment as performed with the Proteinase K extracts are shown in **Table 5**. All recoveries for the seleno-amino acids were fairly good when considering the difficult matrix; however, selenite was not recovered in the extracts obtained with the Proteinase K enzymatic digestion. Selenite was also not recovered after extraction with the 0.5 M HCl microwave procedure. It has therefore been speculated that the inorganic selenium, in the form of Se(IV), may have been reduced to a selenide form (Se^{2-}) or colloidal elemental selenium (Se 0) when placed in a biological matrix. These are further reduced forms of inorganic selenium that have been found in natural waters (*28*).

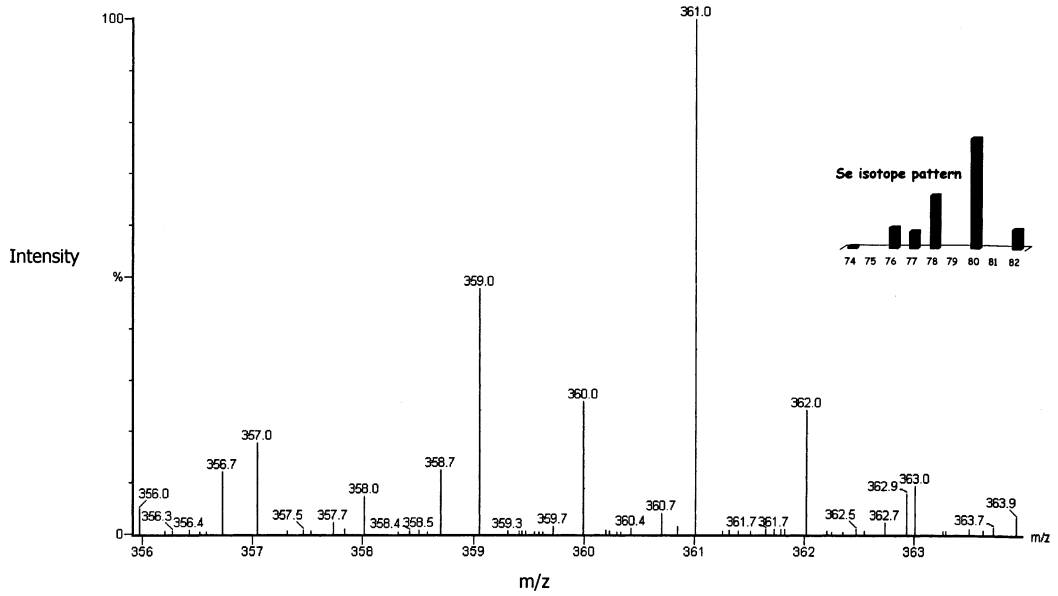

Figure 2. Spectrum of the molecular ion of fraction 3 obtained by electrospray-MS.

Attempts of Characterization of Unidentified Selenium-Containing Peaks. The lack of commercially available standards complicates identification based on retention time and requires the employment of other techniques. In the case of a plant material, it seemed logical to suspect that selenium would be contained in biological compounds. Subsequently, other selenium-containing biological compounds available in this laboratory, specifically, Se-methyl-Se-cysteine, Se-adenosyl-homocysteine, Se-adenosyl-methionine, and γ-glutamyl-Se-methyl-Se-cysteine, were analyzed under the ion-pairing chromatographic conditions. The retention times of these compounds were compared to those of the unidentified selenium-containing peaks. However, the retention times did not match and it was concluded that these compounds were not present in the brazil nut sample.

Several other qualitative experiments were performed in an attempt to identify the unknown selenium-containing species. First, a 200-μL volume of the extract was heated in the oven at 60 °C to determine if unidentified peaks represented volatile compounds such as CH_3SeCH_3 or $CH_3SeSeCH_3$. The sample was warmed overnight; the volume of the extract was then adjusted to its original value. In a second experiment, dithiothreitol (DTT) was added to a second aliquot of the brazil nut extract; if the unidentified peaks represented small peptides that contained a Se−Se bond, or potentially a Se−S bond, addition of this reagent would effect the breaking of any such bond A comparative analysis of the original extract and the manipulated extracts showed no appreciable difference in the peak areas (disappearance or reduction in peak areas) of the unidentified peaks. This allowed the conclusion that the peaks did not represent either volatile selenium-containing species or species that contained Se−Se or Se−S bonds.

A final procedure employed in the identification attempt involved the oxidation of selenomethionine. A calibration standard of selenomethionine was oxidized with hydrogen peroxide to form the oxidized form of SeMet, selenomethionine selenoxide. A second aliquot of the selenomethionine calibration standard was oxidized with performic acid to form the oxidized form of SeMet, selenomethionine selenone. The chromatogram obtained from the analysis of these standards showed two different peaks eluting prior to selenomethionine; the assumption was made that these two peaks corresponded to the two oxidized products. Analysis of the brazil nut extract showed that the first unidentified peak (peak 1) closely matched the retention time of selenomethionine selenone. However, adding an aliquot of the oxidized standard to an aliquot of the brazil nut extract showed that the retention times did not match exactly. It was therefore concluded that the first unidentified selenium-containing peak was not one of the oxidized forms of selenomethionine.

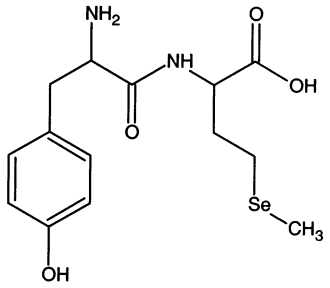

Figure 3. Proposed structure for the peptide of fraction 3.

ES-MS for Species Identification. Both the inductively coupled plasma and electrospray are utilized as ionization sources for mass spectrometry. However, electrospray is a much gentler ionization technique and therefore, the resulting molecular ion can be used to aid in the determination the original structure. On the negative side, ES-MS is typically less sensitive than ICP-MS. It was therefore required that fractions of the unidentified peaks be collected several times (specifically, 10) from the HPLC effluent and then concentrated by evaporation.

Three fractions were collected that corresponded to peaks 1, 2, and 3 in **Figure 1**. Although individual fractions were collected that correlated to single peaks on the chromatogram obtained with ICP-MS detection, monitoring of the UV spectrum showed large absorbances over the range monitored. Because one of the monitored wavelengths was 214 nm, it was speculated that this high background was due to the brazil nut matrix, specifically the amino acid components of this biological material. Unfortunately, ES-MS often has lower sensitivity in the presence of complex matrixes (*29*).

This large background proved to be problematic in the ES-MS analysis of fractions that represented peaks 1 and 2. Understandably, the high matrix prevented the distinguishing of a selenium pattern in the spectrum as the unidentified

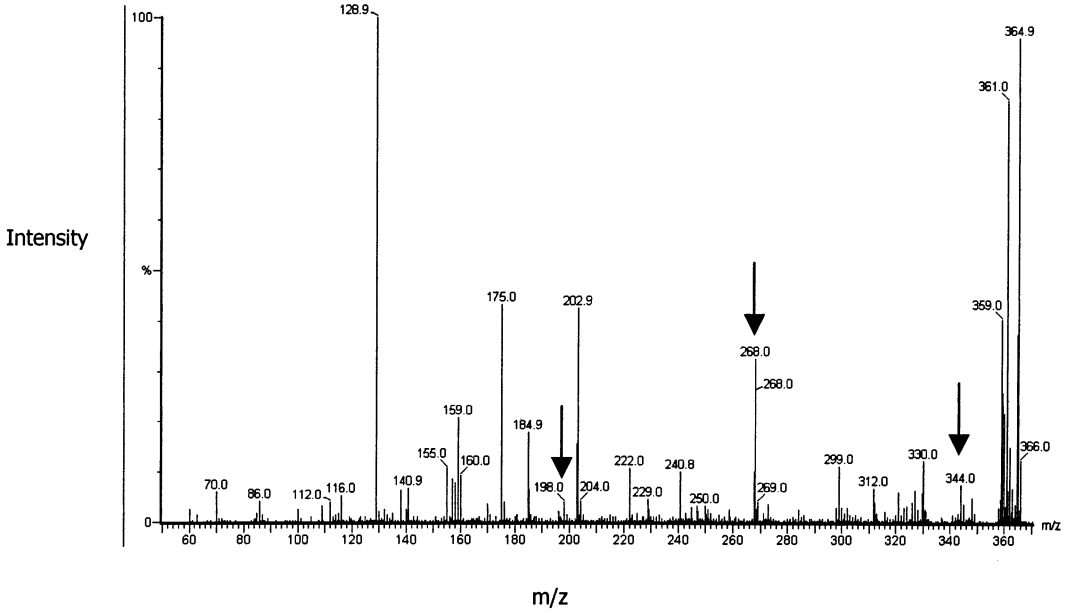

Figure 4. Spectrum of the fragments of Fraction 3 obtained by CID in ES-Q-TOF.

selenium-containing compounds represented only a small percentage of the fraction collected. The analysis of the fraction that represented peak 3, however, yielded more useable data. As can be viewed in **Figure 2**, the peaks at *m*/*z* 357, 358, 359, 361, and 363 showed the selenium isotope pattern. The inset of this figure shows the isotope pattern of one selenium atom.

As a further step in identification, *m*/*z* 361 (representing M + H) was chosen for collision-induced dissociation (CID) and further mass spectral analysis. From these data and the molecular ion identification, it was hypothesized that the unidentified compound represented a peptide consisting of the amino acids tyrosine and selenomethionine. The chemical structure of this peptide can be seen in **Figure 3**. The MS-MS data spectrum is shown in **Figure 4**. One of the fragments obtained, *m*/*z* 198, may likely represent protonated selenomethionine; additionally, another fragment at *m*/*z* 344 is hypothesized to represent the loss of the tyrosine hydroxyl group from the peptide. A third important fragment at *m*/*z* 268 is speculated to represent the loss of both the hydroxyl and phenyl group from the peptide.

CONCLUSIONS

The enzymatic treatment of brazil nut samples with Proteinase K was most effective in extracting selenium species. It appears that a good percentage of the selenium is in the form of selenomethionine; however, other unidentified peaks were also found to be present in the chromatograms obtained under the designated conditions. Attempts to identify those peaks were mostly unsuccessful and this was attributed mainly to the complicated matrix involved. Future work is planned to attempt to clean the first two fractions; if the unidentified selenium-containing compounds were purified, ES-MS spectra may be more helpful in identification as the substantial background would be less interfering. However, ES-MS and ES-MS-MS data were obtained for the latest eluting of the unknown fractions and a potential peptide structure was proposed. As a result, the conclusion is made that the contribution of selenomethionine as a percentage of the total selenium in the sample is underestimated as a result of the incomplete enzymatic hydrolysis of the proteins. This correlates well with conclusions drawn from other work done in this laboratory (*26*).

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