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Studies of Various Elements of Nutritional and Toxicological Interest Associated with Different Molecular Weight Fractions in Brazil Nuts

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On-line hyphenation of size exclusion chromatography (SEC), UV, and inductively coupled plasma mass spectrometry (ICP-MS) was used to study the molecular weight distribution patterns of several elements in Brazil nuts (*Bertholletia excelsa*). This technique was used for the elemental speciation of different elements of nutritional and toxicological interests such as Mg, Fe, Co, Mo, Ag, Hg, and Pb. Elemental fractionation in Brazil nuts was studied using a Superdex peptide column with resolving capacity in the range of 14 to 0.18 kDa. Three different mobile phases, Tris buffer solution (pH 8.0), phosphate buffer (pH 7.5), and CAPS buffer solution (pH 10.0), were tried for the SEC fractionation. Size exclusion fractionation of all the extracted solutions was performed using a 50 mmol L^{-1} Tris buffer (pH 8) as the mobile phase at a flow rate of 0.6 mL min^{-1}. Three different extractions, 0.05 mol L^{-1} NaOH, 0.05 mol L^{-1} HCl, and hot water at 60 °C, were performed, and the association of elements with various molecular weight fractions was evaluated. Total elemental concentrations in the extracted samples were determined and compared with the values obtained after total digestion to calculate the recovery values. Generally, high extraction efficiency was obtained with the NaOH solution as compared with HCl and hot water except in the case of magnesium, for which HCl was found to be a good extractant. Chromatographic elution profiles for these extractions were quite distinct from each other in most cases. Most of the elemental species were found to be associated with high molecular weight fractions. To study the differences obtained during the sample-processing step, the results obtained for nuts with shell were treated differently from those obtained for nuts purchased without shell and were compared.

KEYWORDS: Brazil nuts; molecular weight fractionation; elemental speciation; metalloproteins; SEC-UV-ICP-MS

INTRODUCTION

Evaluation of food and related products for trace elements of nutritional and toxicological interest has attracted attention the world over in recent years (*1*, *2*). It is widely known that nuts are a rich source of nutrients, mainly protein, fat, vitamins, minerals, and dietary fiber (*3*). Nuts also constitute an important part of the Mediterranean and Asian diets. Nuts provide many of the same nutrients to the diet as meat and poultry and have potential health benefits (*4*), which is why nuts are classified as part of the USDA Food Guide Pyramid's meat/meat alternate group. Recent research in the field of nutritional epidemiology has linked protection against ischemic heart diseases (IHD) to nut consumption (*5*). It also suggested an inverse association between death from stroke and intake of vitamin E rich food sources such as nuts (*6*). Lower serum cholesterol and improved lipoprotein profiles are also related to the intake of nuts (*7*, *8*). Improved control of blood-glucose concentration, lower insulin requirements, and better weight control in the case of diabetic patients are some of the advantages of vegetarian foods, including nuts (*9*).

Elements are nutrients that can act as catalysts for many biological reactions within the human body and are crucial for various metabolic processes (*10*, *11*). Elements are found in organic and inorganic combinations in food. It is vital to know the concentration of essential and toxic elements in various food items and products of daily consumption, as food is the main source of these elements (*12*). Information on the association of trace elements with the different compounds present in the food is significant in evaluating the adequacy of the foodstuff consumed with respect to the intake of essential elements and in monitoring potential health risks due to the presence of toxic elements (*13*). There is extensive literature on the determination of trace element levels in various food and food-related products (*14*−*18*). Studies have shown that bioavailability of elements as well as the way in which they are stored in the body is dependent on their chemical form (*19*). Therefore, it is imperative to know the elemental distribution in various fractions and

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the chemical forms in which these elements are present to provide information on the bioavailability and/or toxicity of heavy metals consumed daily through foods (*20*). At present, only a few publications dealing with elemental speciation in food-related products exist (*21*−*24*). Moreover, speciation studies providing data of the different chemical species of elements in nuts are rather scarce. Most of the literature available on nuts deals with the determination of total elemental levels. The only literature available for speciation studies in nuts was for selenium and more recently for arsenic in nut oil (*25*). With the emergence of chromatographic coupling to analytical plasma sources, there have been vast improvements in speciation studies, which is apparent from the increasing number of publications in this area. The analytical approach involves a fractionation/separation step followed by on-line element-specific detection using atomic absorption spectrometry (AAS), emission (AES), or inductively coupled plasma mass spectrometry (ICP-MS). Size exclusion chromatography (SEC) is often used in speciation studies for determining metalloproteins or metals bound to biomolecules and can provide crucial information regarding the association of the elements with various molecular weight fractions in the sample (*26*, *27*). SEC coupled to an element-specific detector has been successfully applied to various matrices such as milk whey (*28*), vegetable food extracts (*29*, *30*), soybean flour and common white bean seeds (*22*), and seeds of legumes (*21*) for elemental fractionation. Therefore, SEC with on-line and sequential coupling of UV−vis detection plus ICP-MS is a very promising and useful analytical approach for studying the molecular weight distribution of elements in nuts.

In the present study, the chromatographic profiles of various elements associated with different molecular weight fractions present in Brazil nuts were analyzed and compared. Brazil nuts with shell were treated as different samples from the ones without shell because of the different geographic regions in which they are grown. SEC-UV-ICP-MS was used for the separation and identification of various elements associated with different fractions using different types of extractions. A comparative speciation study to determine the association of elements to fractions present in the different nut samples was performed in this work.

EXPERIMENTAL PROCEDURES

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

Microwave extractions were performed using a CEM model MES 1000 microwave solvent extraction system (CEM Corp., Matthews, NC). This closed-vessel system has a capacity of heating 12 samples simultaneously. A microwave transparent fiber-optic temperature probe was used to monitor and control temperature conditions (0−200 °C), whereas an internal pressure control system was used to monitor the pressure inside the extraction vessels. Advanced composite extraction vessels made of Teflon were supplied by CEM Corp.

The HPLC system used was a Shimadzu (Shimadzu Scientific Instrument Inc., Columbia, MD) system with an LC-6A pump. It was connected to a Rheodyne injector with a 100 μL loop (Rheodyne 7725 injection valve, Rheodyne, Cotati, CA) and an SPD-6A UV−vis spectrophotometric detector. Data acquisition was performed through an RS 232 port of a Dionex Interface connected to a 133 MHz PC equipped with AI-450 release 3.21 software.

Table 1. Inductively Coupled Plasma Mass Spectrometry and Size Exclusion Chromatography Instrumental Parameters

Inductively Coupled Plasma Mass Spectrometry Parameters	
forward power	1350 W
plasma gas flow rate	15.0 L min^{-1}
auxiliary gas flow rate	0.87 L min^{-1}
carrier gas flow rate	0.90 L min^{-1}
sampling and skimmer cones	nickel
dwell time	0.1 s per isotope
isotopes monitored	^{26}Mg, ^{54}Fe, ^{59}Co, ^{95}Mo, ^{107}Ag, ^{202}Hg, ^{208}Pb
Size Exclusion Chromatography Parameters	
column	Superdex HR 10/30 Peptide
mobile phase	50 mmol L^{-1} Tris-HCl buffer, pH 8.0
flow rate	0.6 mL min^{-1}
injection volume	100 μL
working UV–vis wavelengths	200–500 nm (studied 230 nm)

The ICP-MS instrument was a Perkin-Elmer SCIEX ELAN 6000 (Perkin-Elmer Corp., Norwalk, CT) equipped with a cross-flow nebulizer (gem-tip), a double-pass quartz spray chamber, and a standard quartz torch. The dwell time was 100 ms.

The acid-digested samples were introduced to the ICP-MS using a peristaltic pump (Perkin-Elmer Corp.) at a flow rate of 1 mL min^{-1}. For chromatography coupled to ICP-MS, the solution eluting from the UV detector was introduced on-line to ICP-MS. The SEC column was a Superdex Peptide HR 10/30 (Pharmacia Biotech). The instrumental operation conditions were as given in **Table 1**.

Reagents and Standards. All water used was doubly deionized (18 MΩ·cm) prepared by passing deionized water through a NanoPure treatment system (Barnstead, Boston, MA). Nitric acid, concentrated, certified ACS plus, was purchased from Fisher Scientific. Elemental standards and solutions for standard additions were prepared from SPEX Claritas PPT 1000 μg mL^{-1} stock solution of arsenic, cadmium, cobalt, molybdenum, and selenium (SPEX CertiPrep, Inc., Metuchen, NJ). Lower concentrations were prepared by serial dilution with ultrapure water. 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.

A 50 mmol L^{-1} Tris [tris(hydroxymethylamino)methane] (Fisher Scientific, Fair Lawn, NJ) mobile phase solution was prepared by dissolving the reagent in ultrapure water and adjusting the pH to 8 with HCl (Merck, Darmstadt, Germany) solution. Mobile phase solutions of 10 mmol L^{-1} 3-cyclohexylamino-1-propanesulfonic acid (CAPS) (Aldrich, Milwaukee, WI) and 0.1 mol L^{-1} sodium phosphate (Aldrich) were prepared by dissolving the individual reagents in ultrapure water and adjusting the pH to 10 and 7.5, respectively, with NaOH (Merck) solution. Lower concentrations were prepared by serial dilution with ultrapure water.

All reagents were of analytical reagent grade, and the presence of trace elements was not detected in the working range of these experiments.

Sample Collection and Preparation. Brazil nuts with shells and those without shells were purchased from the local market. However, the search for the origin of these nuts determined that Brazil nuts are grown in both central and western regions of Brazil. Brazil nuts sold with their shell removed are grown in the western area of Brazil bordered by Bolivia and Peru (Rondonia and Acre). Those marketed with shells are grown along the tributaries of the Amazon River (near Manaus and Belam). Hence, different trace element profiles are expected.

Determination of Total Elemental Concentration. About 20 g of each type of nut was ground in a coffee mill. Shells from Brazil nuts were removed, for those nuts purchased with their shells, before grinding. A solution of chloroform and methanol in the ratio 2:1 was added (100 mL), and the mixture was shaken vigorously. The solid part is collected after filtration and then dried before regrinding. The procedure was repeated three times to remove the lipid fraction. Three

Table 2. Percentage Distributions of Elements among Different Molecular Size Fractions of the Brazil Nut Extracts with Different Extraction Media

	molecular size fraction distribution of elements in Brazil nuts with shell[a] (%)			molecular size fraction distribution of elements in Brazil nuts without shell[a] (%)		
element	>14.4 kDa	14.4–0.36 kDa	<0.36 kDa	>14.4 kDa	14.4–0.36 kDa	<0.36 kDa
			NaOH Extract			
Mg	23.3	76.7	0	10.7	89.3	0
Fe	16.6	54	29.4	52	48	0
Co	52.1	47.9	0	69.1	30.9	0
Ag	63	27	0	33.4	66.6	0
Hg	69.2	30.8	0	33.2	66.8	0
Pb	78.9	21.1	0	68.5	31.5	0
Mo	0	100	0	–	–	–
			HCl Extract			
Mg	0	37.8	62.2	0	10.8	89.2
Fe	0	78.5	21.5	–	–	–
Co	5.7	94.3	0	0	100	0
Ag	6.7	93.3	0	–	–	–
Hg	–	–	–	–	–	–
Pb	0	74.5	25.5	0	9.4	90.6
Mo	0	100	0	–	–	–
			Hot Water Extract			
Mg	0	100	0	4.5	95.5	0
Fe	–	–	–	–	–	–
Co	71	29	0	79.2	20.8	0
Ag	–	–	–	–	–	–
Hg	–	–	–	–	–	–
Pb	27	73	0	0	100	0
Mo	–	–	–	–	–	–

[a] Results are given as percentage of the peak area under different size fractions with reference to the total area under the chromatogram.

subsamples were precisely weighed (0.5 g) and transferred to PTFE vessels, and 10 mL of 50% nitric acid was added. The digestion was carried out in a microwave oven according to the method of Kannamkumarath et al. (*23*). Total elemental concentrations were determined by ICP-MS using a two-point standard addition technique. Germanium (10 $\mu g \cdot L^{-1}$) and indium (10 $\mu g \cdot L^{-1}$) were used as internal standards.

Extraction Procedures for Nuts. Three different types of solutions were used for extracting the nut samples. For each of these extractions, the powdered samples (0.5 g) were weighed in separate plastic tubes and a specified volume (10 mL) of the extraction solution was added. The tubes were agitated in a vortex for 30 min at ambient temperature, and the mixture was centrifuged for 10 min at 3500 rpm. The supernatant is collected and then filtered through a 0.45 μm PVDF filter. A solution of 0.05 mol L^{-1} sodium hydroxide and 0.05 mol L^{-1} hydrochloric acid was used for alkaline and acidic extractions, respectively. Hot water extraction was carried out by adding 10 mL of deionized water to 0.5 g of the powdered nut samples, and the mixture was kept at 60 °C for the 30 min extraction. The rest of the procedure was the same as in the case of acidic and alkaline extractions.

Elemental Fractionation Profiles by SEC-UV-ICP-MS. The nut extracts were injected (100 μL) onto a Superdex Peptide HR 10/30 size exclusion column (in the range of 14 to 0.18 kDa). The extracts were also tried in a Superdex 75 (Amersham Biosciences, Inc., Piscataway, NJ) column, which has an optimum fractionation range of 3000−70000 Da. Calibration of the SEC 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 ($r^2 = 0.9914$). Because the standards used for column calibration may be structurally different from those eluting from injection of the nuts extracts, these values and ranges are only approximations and will be referred to as apparent molecular weights. The samples extracted were centrifuged and then filtered through 0.45 μm PVDF low protein binding filters. The extracted samples was injected onto the column using a Rheodyne 9025 injector fitted with a 100 μL polyether ether ketone (PEEK) sample loop. The mobile phase, 50 mmol L^{-1} Tris buffer (pH 8), was filtered and degassed using a sonicator, and the flow rate was fixed at 0.6 mL min^{-1}. The outlet of the SEC column was connected to the inlet of a diode array detector to collect the UV−vis spectra of chromatographic eluents. This system is coupled on-line to the ICP-MS system by connecting the outlet of the diode array detector to the cross-flow nebulizer using a PEEK tube. The on-line use of the UV−vis detector prior to ICP-MS did not produce significant dispersion of the analyte signal.

RESULT AND DISCUSSION

Although Brazil nuts are widely known as one of the best natural sources of selenium, the presence of other elements and their association with various molecular weight fractions need to be evaluated. In this study, element-specific ICP-MS detection was coupled to UV and SEC to obtain direct experimental evidence for distribution of various elements associated with different molecular size fractions.

Effect of Extraction Media on the Elemental Speciation in Nuts. Three different extraction media were used for the extraction of various nut fractions. The elemental compounds were extracted from nut samples using 50 mmol L^{-1} NaOH, 50 mmol L^{-1} HCl solution, and hot water (60 °C). The extractions were performed for 30 min with constant agitation. Typical extraction efficiencies for NaOH, HCl, and hot water are shown in **Table 3**. The extractability of elements for NaOH was found to be higher than for other extractant media and except in the case of Mg, for which HCl was found to be a better extractant. The chromatographic elution profiles of various extracts clearly indicate the differences in the extraction procedures. The acidic extraction selectively extracted low molecular weight (LMW) fractions, whereas the alkaline solution extracted high molecular weight (HMW) fractions along with some LMW fractions. Although the elution profiles of hot water extracts did not exactly match with any of the other types of extraction, it was clear that in most cases it did not extract the HMW fractions.

SEC Fractionation for Elemental Speciation in Nuts. Initially, two SEC columns, a Superdex 75 (working range of 3−70 kDa) and a Superdex Peptide (working range of 0.18−14 kDa), were compared for the separation of nut extracts.

Table 3. Total Contents of Elements and the Percentages Extractable with Different Extraction Media ($n = 6$)

	Brazil nuts with shell				Brazil nuts without shell			
element	total μg g^{-1}	NaOH extractability (%)	HCl extractability (%)	hot water extractability (%)	total μg g^{-1}	NaOH extractability (%)	HCl extractability (%)	hot water extractability (%)
Mg	2950 ± 94	8	82	38	2370 ± 56	11	84	48
Fe	251 ± 18	35	18	4	38 ± 2.6	28	8	7
Co	15 ± 0.8	98	9	13	0.9 ± 0.1	77	13	64
Mo	0.71 ± 0.06	105	32	12	0.12 ± 0.03	85	28	8
Ag	3.6 ± 0.9	89	12	3	0.32 ± 0.03	74	6	5
Hg	8.2 ± 0.6	82	5	4	0.4 ± 0.04	72	5	4
Pb	7.0 ± 0.8	64	59	8	3.4 ± 0.7	58	49	26

The elution profiles obtained were very similar in the HMW region, whereas the separation was better in the LMW region using the Superdex Peptide column. Because the separation was better in the LMW region, the Superdex Peptide column was selected for the SEC studies. Three different mobile phases, Tris buffer solution (pH 8.0), phosphate buffer (pH 7.5), and CAPS buffer solution (pH 10.0), were evaluated at different flow rates for resolution and retention times. The use of 50 mmol L^{-1} Tris buffer avoided possible protein precipitation and reduced hydrophobic interactions between these compounds with the column material. Different flow rates (0.3−1.0 mL min^{-1} range) of the mobile phase were also studied. Adequate resolution was obtained at 0.6 mL min^{-1}. Higher flow rates worsened the resolution, and lower flow rates did not improve the resolution. For fraction profiles in which the resolution was incomplete, deconvolution software was used for calculation of the areas.

Brazil Nuts with Shell. The chromatogram (230 nm UV) obtained for Brazil nuts with shell is shown in **Figure 1A**. It is clear that both NaOH and hot water show similar extraction profiles. However, NaOH seems to extract a little more than hot water, and the profiles obtained for different elements with ICP-MS were completely different. HCl, on the other hand, extracted mainly LMW compounds. The elution profile for different elements with NaOH extraction is shown in **Figure 2A−C**). The chromatographic profiles of Co, Ag, and Hg were similar. There was incomplete resolution between the various peaks. The molecular size fractions >14.4 kDa were found to be in the range of 52−69%, and the rest of them were in the region between 14.4 and 0.36 kDa. In the case of Fe there were three distinct peaks: one in the HMW region (17%) and two between 14.4 and 0.36 kDa. In a similar analysis of pea and lentil, Koplik et al. (*21*) found a major HMW peak (>150 kDa) and a minor LMW (10−20 kDa) peak for Fe using a Superdex 75 column. Molybdenum eluted as a single peak at the same time as a 0.36 kDa calibration standard [as in the case of white bean and soybean flour (*22*)], whereas Pb eluted in two peaks, a major HMW fraction (80%) and a small LMW fraction (20%). In the case of acidic and hot water extractions the elution profiles (**Figure 2D−H**) were very different. Compounds associated with Hg, Fe, and Mo were not extracted at all in hot water extractions. The cobalt elution profile showed 71% of the total chromatographic area under the HMW region with hot water extraction being completely different from the elution profile obtained for acidic extraction, where the HMW region (6%) was almost negligible. The presence of HMW and LMW cobalt compounds has already been established in similar matrices such as peas and lentil where there is high protein content (*21*). Silver also showed a profile similar to that of Co with acidic extraction but was not extracted with hot water. The elution profile for Mg is shown in **Figure 2F**,**H**. The elution profile for Pb showed well-resolved peaks in the HMW region (27%) and in the LMW region (73%) with hot water extraction. With acidic extraction, Pb showed a complex elution profile with unresolved peaks.

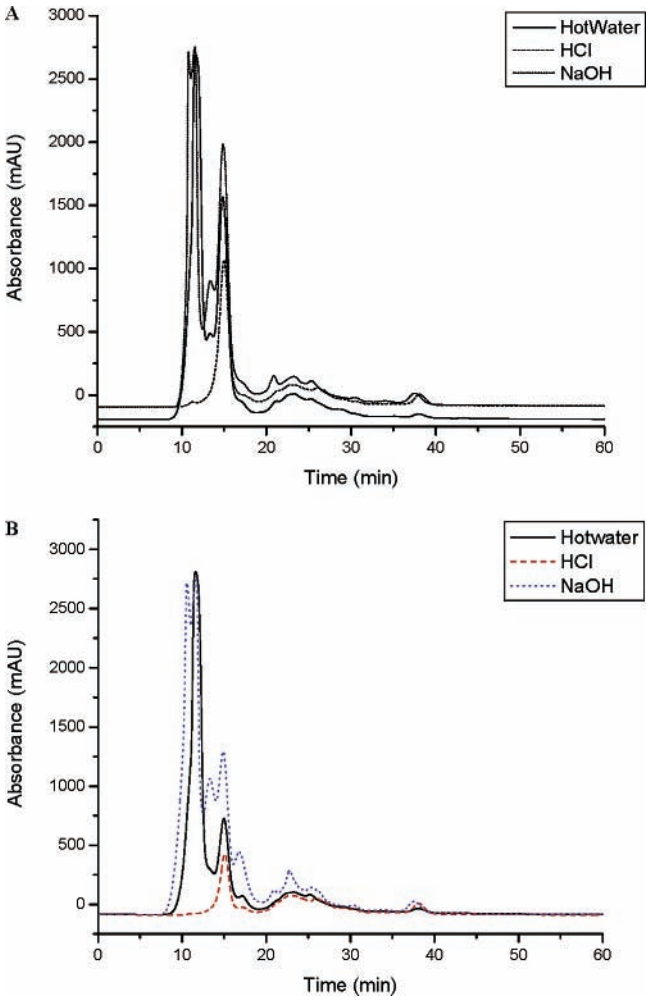

Figure 1. Typical UV–vis fractionation profiles obtained for Brazil nut samples using different extraction media: (**a**) Brazil nut with shell; (**b**) Brazil nut without shell. Absorbance was monitored at 230 nm. Other experimental conditions were as shown in **Table 1**.

Brazil Nuts without Shell. The chromatogram (230 nm UV) obtained for Brazil nuts without shell is shown in **Figure 1B**. It is clear from the chromatogram that the extractions obtained from these samples were very similar to the one obtained for Brazil nuts with shell. The NaOH extraction is a bit more than hot water, and the profiles obtained for different elements with ICP-MS were completely different. HCl, on the other hand, extracted mainly LMW compounds. From **Table 2** it is clear that the abundance of elements studied in this work was lower in Brazil nuts without shell compared to the ones with shell.

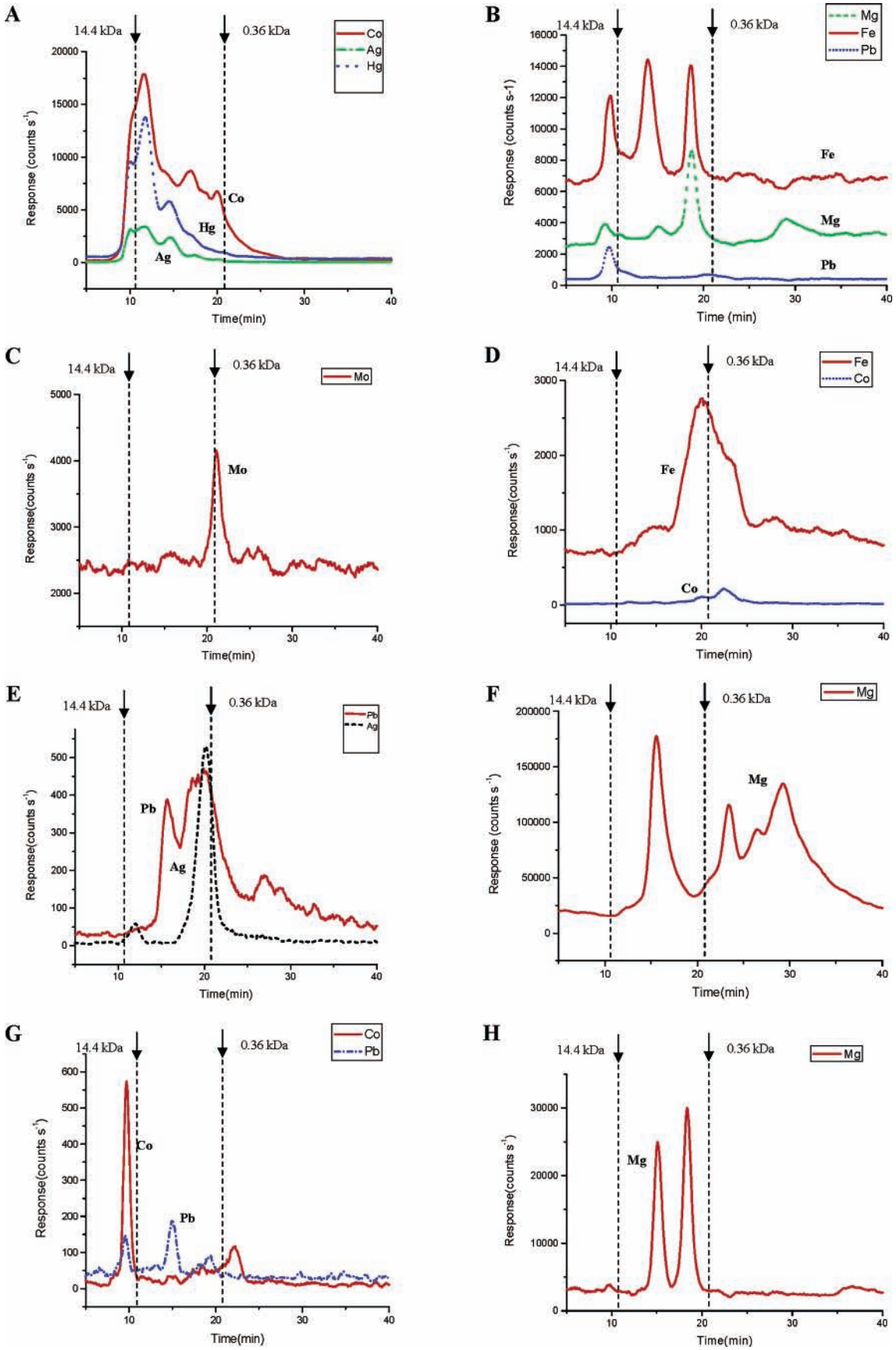

Figure 2. Fractionation profiles of different elements in Brazil nut with shell using different extraction media: (**A**, **B**, **C**) 0.05 mol L^{-1} NaOH as extraction medium; (**D**, **E**, **F**), 0.05 mol L^{-1} HCl as extraction medium; (**G**, **H**) hot water (60 °C) as extraction medium. Other experimental conditions were as shown in **Table 1**.

However, the extraction efficiency was not that different for the elements studied in both cases. As with the case of Brazil nuts with shell, NaOH gave the best extraction efficiencies. In the case of SEC, when alkali extract was injected, the elution

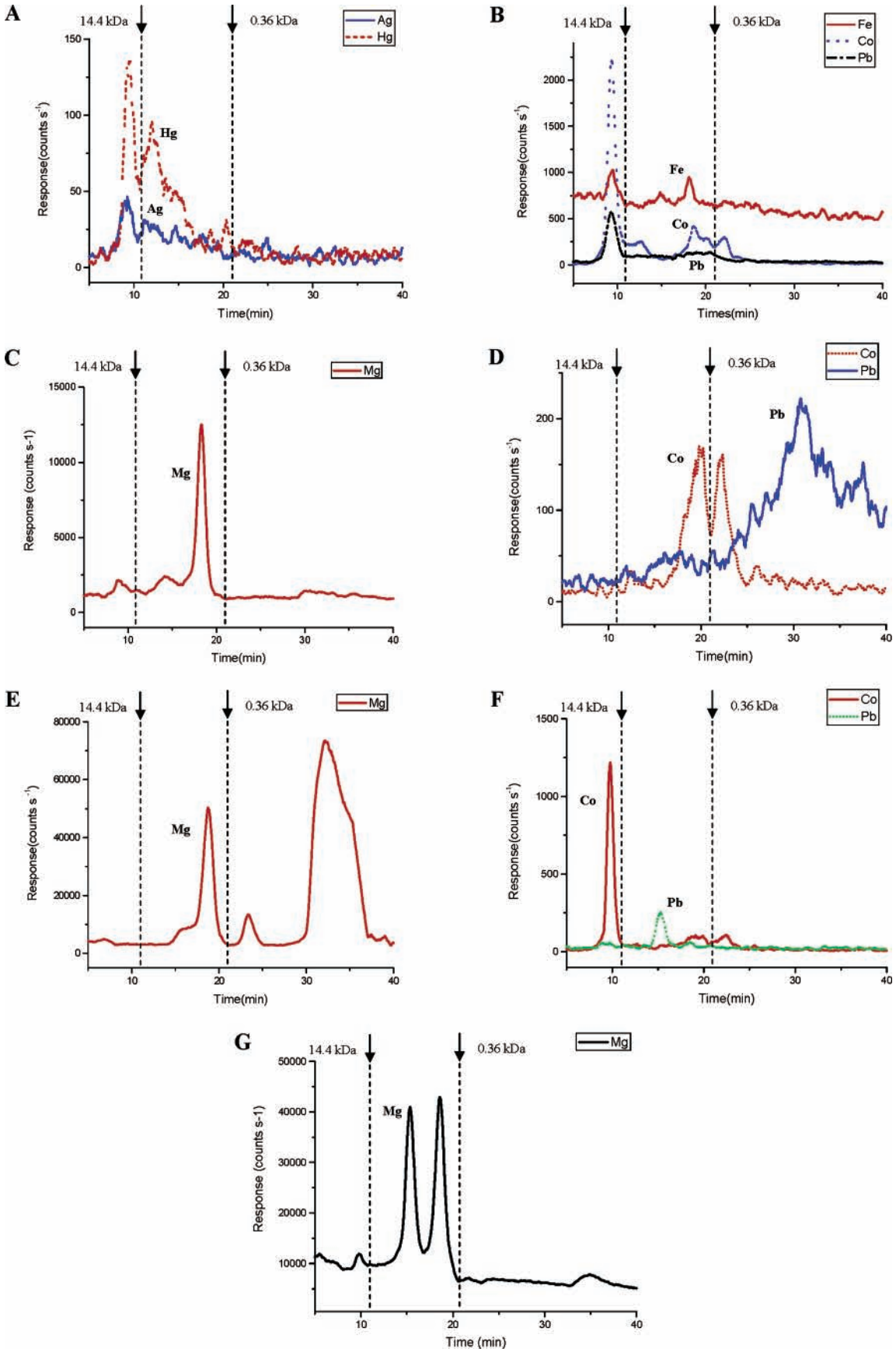

Figure 3. Fractionation profiles of different elements in Brazil nut without shell using different extraction media: (**A**, **B**, **C**) 0.05 mol L^{-1} NaOH as extraction medium; (**D**, **E**) 0.05 mol L^{-1} HCl as extraction medium; (**F**, **G**) hot water (60 °C) as extraction medium. Other experimental conditions were as shown in **Table 1**.

profiles for Ag and Hg looked similar. It showed 33% of the total area as HMW compounds, whereas the rest are in the 14−0.36 kDa region. Mo was absent in all of the extraction profiles. It is interesting to note that the extraction efficiencies for the

elements Fe and Mg, which are present in higher amounts compared to the other elements studied, were low. The SEC results of Co and Pb were similar, with 70% of the total area under HMW fraction and the rest in the region between 14 and 0.36 kDa. However, Mg did show a high peak around 18 min that also would fall under the region between 14 and 0.36 kDa. Even though the extraction efficiencies of all elements in Brazil nuts without shell with hot water and HCl extractions were similar to those of Brazil nuts with shell, extractabilities of Co and Pb were high with hot water extraction. In both of these types of extractions only Co, Pb, and Mg did show some peaks. In the case of HCl extraction, Pb showed as a broad peak outside the calibration range. Co and Mg did show some distinct peaks. In the case of Co there were two peaks eluting very close without separation and Mg eluted as three distinctive peaks. As in the case of Brazil nuts with shell, hot water extraction did indeed produce some well-refined peaks, which could help in the characterization of the compounds with which the elements could be associated.

Lipids are the major constituents in nuts (~60%), and defatting the nuts changes the amount of proteins and carbohydrates, the other constituents in nuts by weight. The higher percentage of HMW fraction with NaOH extraction can be explained by considering the solubility of protein in alkaline solution and the high content of proteins in nuts, which is ~10−20% in the whole sample and is higher in the defatted nut sample. The insolubility of protein in the acidic solution and the absence of peaks in the HMW region of **Figure 3** give additional support to the assumption that the HMW fraction obtained in alkaline extraction was mainly metalloproteins. Also, it needs to be taken into consideration that acidic solutions are more effective media for the extraction of LMW and inorganic elemental forms compared to basic solutions such as NaOH. The use of hot water as an extraction medium did not improve the extraction efficiency. However, the elution profile showed some very well-resolved peaks in the case of Mg, Co, and Pb that could help in the identification of the nature of these compounds in the future.

Conclusion. In the present study, SEC-UV-ICP-MS was used for the speciation studies of Mg, Fe, Co, Mo, Ag, Hg, and Pb in Brazil nut samples. Elution profiles of elements were different from each other in most of the cases and also differed depending on the extraction medium used. The association of elements with HMW compounds (most likely proteins) was evident from the chromatographic profiles as defatted nuts had high protein content (~50%). Because of the difference in the fractionation profiles obtained, it is important that the extraction procedure carried out be optimized depending on the type of compounds (HMW or LMW) to be studied. This work is only a preliminary step toward understanding the elemental contamination and the nutritional value of elements associated with various fractions. More work is needed to separate and purify these fractions and characterize the biomolecules. In that case the use of multidimensional separation techniques and identification tools such as molecular mass spectrometry will be inevitable. More work is in progress to characterize and determine the individual elemental species (mainly proteins) in the nuts analyzed in this work.

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