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Original Research Article

Determination and evaluation of element bioaccessibility in some nuts and seeds by *in-vitro* gastro-intestinal method

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ABSTRACT

Element bioaccessibility in some nuts and seeds has been determined by performing a physiologically based extraction test. Nine elements (B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn) in gastric and intestinal phase extractions of nuts and seeds were determined using inductively coupled plasma-atomic emission spectrometry and inductively coupled plasma-mass spectrometry. Hazelnuts, almonds, sunflower seeds, peanuts, cashew nuts, Brazil nuts, walnuts, chickpeas, pumpkin seeds and pistachio nuts were used as the materials in this study. The bioaccessible portions of magnesium and calcium were higher than for the other elements whereas B bioaccessibility was the lowest for each of the different types of nuts and seeds. Based on an ingestion rate of 10 g day^{-1}, the amounts of B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn from the nuts and seeds accessible to the body were found to be lower than the Tolerable Upper Intake Levels. The data were also subjected to chemometric evaluation using tools such as Principal Component Analysis (PCA) and Linear Discriminant Analyses (LDA) in an attempt to classify the nuts and seeds according to these elements bioaccessibility and to find out which elements are more bioaccessible in gastric and intestinal ingestions.

1. Introduction

Nuts and seeds are not only rich in fiber and protein, but they also contain numerous other nutrients. These include high levels of mono and polyunsaturated fats, omega-3 fatty acids as well as other bioactive compounds including several antioxidants, which are important for heart health. They can lower cholesterol levels and improve cardiovascular outcomes through their lipid lowering, anti-inflammatory, antioxidant, vasoactive, and anti-arrhythmic effect. The American Heart Foundation recommends including some nuts and seeds in the diet daily because of these apparent benefits to heart health (Tupper, 2012; Yang et al., 2009; Lee et al., 2011). Nuts and seeds are also rich in micronutrients such as folic acid and niacin, vitamins (E and B_6) and elements (Ca, Cr, Mg, Mn, Cu, Fe, Zn, Se, P and K). Vitamin E, folate, manganese, and selenium are very important in the body as they help fight damage-causing free-radicals and are therefore thought to protect against cancers. Nuts are also a good source of elements such as zinc and magnesium and the B vitamins which are essential for energy (Tupper, 2012; Nascimento et al., 2010; Naozuka et al., 2011). In addition, nuts and seeds also contain trace elements such as copper, chromium, iron, zinc and selenium that are essential for human health. Some toxic elements such as Pb, Cd and Hg can also be transferred to nuts and seeds through handling, including food processing and packaging and can cause potential health effects for humans (Rodushkin et al., 2008).

The determination of elements in food samples that are either essential or that have toxic effects in the human body is therefore very important for nutritional and toxic assessment. Total elemental concentrations of Ag, Al, Ba, Ca, Co, Fe, Hg, Mg, Mn, Mo, Pb, Se, Sr, and Zn in nuts and seeds have been determined in several types of nut, including the Brazil nut in numerous papers previously (Kafaoğlu et al., 2014; Naozuka et al., 2010, 2011; Rodushkin et al., 2008; Cabrera et al., 2003; Kannamkumarath et al., 2004; Wuilloud et al., 2004).

In terms of nutrition, it is not sufficient to measure only the total concentrations of element ions. Instead, it is also important to know the bioavailability, i.e. the amount adsorbed and used by the organism or the bioaccessibility; that is the fraction of an element which is solubilized from a sample under simulated gastrointestinal conditions (Nascimento et al., 2010; Intawongse and Dean, 2006a).

Information obtained for the bioavailable fraction from in vivo studies can be difficult to interpret because of physiological

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discrepancies between humans and the experimental animals adopted. Such problems led to the development of several *in-vitro* systems based on gastrointestinal extraction that give an indication of the levels of metals accessible to the body by either intentional or unintentional ingestion of foods or soils. These systems include the so called physiologically-based extraction test (PBET) (Intawongse and Dean, 2008). Several *in-vitro* methods have been developed and are reported in the literature (Miller et al., 1981; Crews et al., 1983; Ruby et al., 1993; Hack and Selenka, 1996). *In-vitro* testing methods have been used most for assessing oral bioaccessibility of total trace metals in soil and food samples. Soils containing contaminants such as some toxic elements can cause a particular hazard to children's health because of hand-to-mouth behavior and ingestion of the soil (Hamel et al., 1998; Schroder et al., 2003). The ingested amounts of elements can be very high when the ingested soils are near to mine areas (Karadas and Kara, 2011). *In-vitro* testing methods were reviewed by Intawongse and Dean (Intawongse and Dean, 2006b). *In-vitro* extraction procedures to assess bioaccessibility seek to mimic processes that occur in typically two (or occasionally three) distinct, but linked, areas of the human digestive system (i.e. stomach and small intestine and sometimes the mouth) (Intawongse and Dean, 2006a). Some recent studies were published to determine the bioaccessibility of different elements from different food stuffs using different *in-vitro* gastrointestinal methods (Da Silva et al., 2015; Horner and Beauchemin, 2013; Laird and Chan, 2013; García Sartal et al., 2013; Stelmach et al., 2014; Fu and Cui, 2013). Recently, the *in-vitro* digestion method combined with the Caco-2 cell model has been proposed and validated (Dhuique-Mayer et al., 2007; Yun et al., 2004) to obtain a more reliable approximation of the in vivo conditions to estimate bioavailability at the intestinal level (Ekmekcioglu, 2002; Trapecar and Cencic, 2012; Dhuique-Mayer et al., 2007; Tako and Glahn, 2010; Tako et al., 2011; Fu and Cui, 2013).

The present study will focus on the determination of the bioaccessible amounts of trace elements (B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn) in some nuts and seeds using an *in-vitro* gastrointestinal method that employs enzymes and dilute hydrochloric acid. This procedure was first described by Ruby et al. (1996). Two modifications have been adopted that make the test more reproducible and easier to undertake (Rodriguez et al., 1999; Ruby et al., 1999; Medlin, 1997). The original method used dialysis tubing containing sodium carbonate or bicarbonate which raised the pH of the digest ready for the small intestine extraction step. This was replaced by simply titrating the stomach extract directly with saturated sodium carbonate or bicarbonate solution to bring the pH to 7 (Rodriguez et al., 1999). Other workers (Medlin, 1997; Ruby et al., 1999) showed that it was not necessary to maintain anaerobic conditions in the extraction solutions and the extraction could be carried out in screw top polypropylene vessels. Agitation of the sample solution mixture could then be reproducibly carried out by end over end shaking in a water bath (Medlin, 1997). Since *in-vitro* gastrointestinal extraction methods have been used to determine the bioaccessibility of elements in different food samples and have also been correlated to bioavailability determined using in vivo studies for Pb and As (Ruby et al., 1996; Rodriguez et al., 1999), the physiologically-based extraction test (PBET) was selected for the determination of the bioaccessibility of elements from nuts and seeds samples in this work.

2. Experimental

2.1. Reagents and solutions

Doubly de-ionized water (18.2 MΩ cm), obtained from a combined Prima and Maxima water system (Elga, Buckinghamshire, UK) was used throughout the experiment. Nitric acid (Trace Analysis grade, Fisher Scientific, Loughborough, UK) was used to digest the nut samples prior to total metal concentration determination. Stock standard solutions of individual elements (1000 or 10,000 mg L^{-1}) were supplied by Fisher Scientific. Pepsin (>250 units/mg, Sigma, St. Louis, MO, USA), sodium malate (Aldrich, 99%, St. Louis, MO, USA), tri-sodium citrate (BDH, Aristar, Poole, UK), lactic acid (AnalaR, BDH), bile salts (cholic acid 50% and deoxycholic acid 50%, Sigma), pancreatin (from Hog pancreas, Sigma), acetic acid (Analytical reagent grade, Fisher Scientific) and hydrochloric acid (AnalaR Normapur, BDH) were used as supplied in the experiments.

2.2. Instrumentation

An inductively coupled plasma mass spectrometry (ICP-MS) instrument, X Series 2 (Thermo Scientific, Hemel Hempstead, UK) was used for the determination of Co and Ni. Operating conditions for the ICP-MS instrument were: forward power 1.40 kW, coolant gas flow rate 13 L min^{-1}, auxiliary gas flow rate 0.75 L min^{-1} and nebulizer gas flow rate 0.9 L min^{-1}. The dwell time per isotope was 10 ms and 50 sweeps were used. An inductively coupled plasma atomic emission spectrometry (ICP-AES) instrument Varian 725-ES (Varian Inc., Melbourne, Australia) was used for the determination of B, Ca, Cu, Fe, Mn, Mg, and Zn in the samples. Operating conditions for the ICP-AES instrument were: forward power 1.4 kW, plasma (coolant) gas flow rate 15 L min^{-1}, auxiliary gas flow rate 1.5 L min^{-1} and nebulizer gas flow rate 0.68 L min^{-1}; the viewing height was 8 mm above the load coil and the replicate read time was 4 s. For both instruments, the sample was introduced via a V-groove nebulizer and a Sturman-Masters spray chamber.

2.3. Procedure

2.3.1. Sample preparation

Hazelnuts, almonds, sunflower seeds, peanuts, cashew nuts, Brazil nuts, walnuts, chickpeas, pumpkin seeds and pistachio nuts were purchased from a shop in Bursa, Turkey. Some of the samples were imported: e.g. almonds from Spain, cashew nuts from India, walnuts from Chile and Brazil nuts from Brazil. Other samples were produced from different parts of Turkey. The samples were ground using a pestle and mortar. The pulverized and powdered or caked nut and seed samples were then transferred into plastic bags. All nuts and seeds were treated in an identical manner.

2.3.2. Modified PBET method

A modified procedure similar to that used in several other research studies (Palumbo-Roe et al., 2005; Intawongse and Dean, 2008; Wragg et al., 2007; Cave et al., 2002; Meunier et al., 2010) was adopted. A mass of 0.4 g of the pulverized nuts and seeds was weighed accurately into a wide-mouthed HDPE (high density polyethylene) bottle. A volume of 40 mL of simulated gastric solution (1.25 g pepsin, 0.50 g sodium malate, 0.50 g sodium citrate, 420 μL lactic acid and 500 μL acetic acid made up to 1 L with freshly prepared de-ionized water, adjusted to pH 2.5 with concentrated hydrochloric acid) was added to each bottle. The bottles were placed in an end over end shaker within a temperature controlled water bath set at 37 °C. After 1 h at 37 °C, a 5.0 mL aliquot was removed and centrifuged for 15 min at 1610 × *g*. The liquid phase was decanted into a 15 mL capacity polyethylene tube. This extraction sample is known as the *gastric phase* sample. Then, 5.0 mL of the original gastric solution was back-flushed through the filter into the HDPE bottle (to retain the original solid:solution ratio). The conditions in the vessel were then altered from those that simulate the stomach to those in the small intestine by titration to pH 7.0 with saturated sodium bicarbonate and the addition of 175 mg bile salts and 50 mg pancreatin. The samples were then incubated in the water bath for a further 4 h when a second 5 mL aliquot was removed and filtered. This sample is known as *intestinal phase sample*.

2.3.3. Preparation of samples for total element determination

The total digested element contents of nuts and seeds were determined after digestion with nitric acid. The full methodology was described in a previous study (Kafaoğlu et al., 2014). Briefly, nut and seed samples (0.2500 g) were weighed into long digestion tubes and 8 mL of concentrated nitric acid were added. The samples were left overnight at room temperature to digest slowly and then boiled gently in a laboratory hot-block until digestion was complete. The digests were then transferred quantitatively to pre-cleaned 25 mL volumetric flasks. To ensure that the results obtained from the analyses were accurate, two certified reference materials (Hay Powder, IAEA V-10 obtained from International Atomic Energy Agency, Vienna, Austria and GBW 07604 Poplar Leaves obtained from National Institute of Standards and Technology, Beijing, China), were prepared in the same way.

2.3.4. Sample analysis

The concentrations of Co and Ni were determined using ICP-MS and B, Ca, Cu, Fe, Mn, Mg, Ni and Zn concentrations were determined using ICP-AES for all sample types. As an internal standard for ICP-MS determinations, a mixture of indium and iridium was added to each extract to give a final concentration of 100 $\mu g L^{-1}$. Similarly, the internal standard mixture was also added to all blanks and standards. All results are expressed as the mean of the three replicates. All statistical calculations were made using the IBM SPSS Statistics version 21 software (1989–2012) package.

For *in-vitro* results, the bioaccessible metal concentrations for the stomach and intestinal digestions were calculated by dividing the metal ions' concentrations measured in the *in-vitro* gastric phase or the *in-vitro* intestinal phase solutions by the total concentrations of metal ions as described by the following equation (Intawongse and Dean, 2008):

$$\text{In vitro bioaccessible metal ion } (\%) = \frac{[\text{In-}\textit{v}\text{itro metal}]}{[\text{Total metal}]} \times 100$$

3. Results and discussion

The results for the analysis of the certified material (Hay Powder, IAEA V-10) from the ICP-AES and ICP-MS analyses were given in our previous paper (Kafaoğlu et al., 2014). The concentrations of B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn in Poplar Leaves (GBW 07604) and the results of the *t*-test to show the accuracy of the instrumental measurements using this certified reference material are given in Table 1. The Student *t*-test was used to determine whether or not there is a significant difference between the mean concentrations found using the proposed method and the certified values. The results show that the *t* values calculated using the equation are smaller than the critical value of *t* at the 95% confidence interval at 2 degrees of freedom which is 4.30, for all analytes except for Fe and Mg. The results of the *t*-test showed that the concentrations of Fe and Mg ions are significantly lower than the certified values. Although the organic matter of the samples was efficiently destroyed by the nitric acid digestion, the food samples like Poplar Leaves CRM also contains inorganic material perhaps a silicaceous backbone that is not soluble by boiling with nitric or perchloric acids even in the presence of hydrogen peroxide. Therefore, these elements "associated with" silicaceous material would give slightly lower concentrations than the certified values.

Table 1
The results of the certified reference material (GBW 07604 Poplar Leaves) ($n = 3$).

Element	Reference value, $\mu \pm \sigma$ ($\mu g\, g^{-1}$)	Found value, $\bar{x} \pm s$ ($\mu g\, g^{-1}$)	$t = \frac{\lvert\mu - \bar{x}\rvert\sqrt{N}}{s}$
B	53 ± 5	53.2 ± 2.3	0.15
Ca	18,100 ± 1300	18,002 ± 1745	0.10
Co	0.42 ± 0.03	0.47 ± 0.04	2.17
Cu	9.3 ± 1.0	9.88 ± 0.68	1.48
Fe	274 ± 17	201 ± 11	11.5
Mg	6500 ± 500	5780 ± 125	9.98
Mn	45 ± 4	41.1 ± 1.9	3.56
Ni	1.9 ± 0.3	1.62 ± 0.4	1.21
Zn	37 ± 3	32.9 ± 2.5	2.84

An *in-vitro* gastro-intestinal method was applied to determine the trace element concentrations that can dissolve in gastric and intestinal solutions from samples of different nuts and seeds. The "total" concentrations of elements (B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn) obtained using sample digestion (Kafaoğlu et al., 2014) and the *in-vitro* gastrointestinal experiments in the nuts and seeds (mean and standard deviation) as well as the proportion of the trace elements that are bioaccessible are given in Table 2. The data given in this table indicate that the vast majority of the trace elements are not bioaccessible. The bioaccessibilty is the highest for Zn in most of the nuts and seeds. The highest bioaccessibilty in gastric phase was observed from hazelnuts (20.6%) whereas 36.1% of Zn was bioavailable from pistachio nuts after the intestinal phase digestion. The lowest bioaccessibilty was generally obtained for B and Cu in most of the nuts and seeds samples where the highest bioaccessibilty for B was 11.3% in the gastric phase from walnuts and for Cu it is 11.1% in the intestinal phase from peanuts. Magnesium and calcium concentration in gastric and intestinal solutions were higher than other element's concentrations for most of the nuts and seeds. It was shown that nuts and seeds are very healthy snacks because of their magnesium and calcium contents. These results also show that when the nuts and seeds are digested, even though the bioaccessible fractions are not large, they are also still supplying very beneficial levels of Fe, Mn and Zn which are essential elements for humans. Therefore the nuts and seeds are very good food supplements. There are relatively few research studies that have determined the bioaccessibility of elements from nuts and seeds. However, similar low bioaccessibilities were found from Brazil nuts, cashews, hazelnuts and walnuts for Fe, Zn, Ca and Mg (Suliburska and Krejpcio, 2014) and from hazelnuts and walnuts for Cd, Cu, Fe, Mn, Pb, and Zn (Arpadjan et al., 2013).

Daily B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn ingestion amounts from nuts and seeds have been calculated based on an ingestion rate of 10 g of nuts or seeds day^{-1} using the *in-vitro* intestinal bioaccessibility. The results are given in Table 3 which also compares the levels found with the Dietary Reference Intakes (DRIs) given as Recommended Dietary Allowances and Adequate Intakes (USDA (United States Department of Agriculture) (a), 2010; WHO, 1996) and Tolerable Upper Intake Levels (USDA (b), 2010). Although no safe Recommended Dietary Allowance (RDA) for cobalt has been established yet, the average adult intake of cobalt is 5–8 $\mu g\, day^{-1}$ (University of Utah Health Care). If cobalt is present in nutritional supplements, it is usually given in micrograms (μg). Recommended intakes of cobalt have not been set as the only form of cobalt required by the body is vitamin B12, of which cobalt is an integral part (Food Standards Agency, 2003). In the UK, COMA (Committee on Medical Aspects of Food and Nutrition Policy) has set a RNI (Reference Nutrient Intakes) value for vitamin B12 of 1.5 $\mu g\, day^{-1}$ for adults, including pregnant women (COMA, 1991). The average daily intake of cobalt from food is estimated to be 5–40 $\mu g\, day^{-1}$ (EPA, 2000). As seen from Table 3,

Table 2
Concentrations of elements obtained using total digestion and the *in-vitro* gastrointestinal experiments in nuts and seeds (mean and standard deviation) ($n = 3$).

		B (mg kg^{-1})	Ca (mg kg^{-1})	Co (μg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	Ni (μg kg^{-1})	Zn (mg kg^{-1})
Hazelnut	Total[a]	16.0 ± 0.5	1436 ± 71	269 ± 10	13.7 ± 0.4	26.0 ± 1.0	1276 ± 19	53.5 ± 1.7	1497 ± 58	16.0 ± 0.2
	Gastric	1.29 ± 0.28	116 ± 9	22.4 ± 1.1	0.78 ± 0.08	1.76 ± 0.31	172 ± 12	5.98 ± 0.46	180 ± 8	3.31 ± 0.15
	Intestinal	1.57 ± 0.15	121 ± 6	35.0 ± 0.8	1.27 ± 0.09	3.05 ± 0.23	188 ± 6	6.02 ± 0.07	212 ± 22	5.40 ± 0.37
	Gastric phase (%)	8.1	8.1	8.3	5.7	6.8	13.5	11.2	12.0	20.6
	Intestinal phase (%)	9.8	8.4	13.0	9.3	11.8	14.7	11.3	14.1	33.7
Almond	Total[a]	27.8 ± 4.0	2709 ± 99	69.0 ± 8.8	11.6 ± 0.8	20.6 ± 1.9	2057 ± 135	21.1 ± 0.9	1040 ± 118	27.3 ± 1.0
	Gastric	0.95 ± 0.26	119 ± 39	2.76 ± 0.45	0.45 ± 0.11	1.15 ± 0.06	137 ± 23	1.10 ± 0.27	83.3 ± 7.6	3.35 ± 0.61
	Intestinal	1.40 ± 0.09	187 ± 17	10.6 ± 0.7	1.20 ± 0.10	3.03 ± 0.19	259 ± 18	2.09 ± 0.14	239 ± 91	6.31 ± 0.56
	Gastric phase (%)	3.4	4.4	4.0	3.9	5.6	6.7	5.2	8.0	12.3
	Intestinal phase (%)	5.0	6.9	15.4	10.3	14.7	12.6	9.9	22.9	23.1
Pistachio	Total[a]	10.8 ± 2.1	1793 ± 11	10.7 ± 1.0	10.2 ± 0.3	24.9 ± 3.9	1109 ± 23	9.11 ± 0.23	1245 ± 42	16.8 ± 0.6
	Gastric	0.50 ± 0.30	159 ± 9	1.94 ± 0.11	0.51 ± 0.11	0.80 ± 0.01	118 ± 7	0.78 ± 0.01	115 ± 11	3.05 ± 0.20
	Intestinal	0.90 ± 0.19	212 ± 8	3.21 ± 0.41	1.07 ± 0.18	1.14 ± 0.06	174 ± 7	1.16 ± 0.02	181 ± 6	6.07 ± 0.19
	Gastric phase (%)	4.6	8.9	18.2	5.0	3.2	10.6	8.6	9.2	18.2
	Intestinal phase (%)	8.3	11.8	30.1	10.4	4.6	15.7	12.7	14.6	36.1
Peanut	Total[a]	19.6 ± 1.4	676 ± 3	52.0 ± 7.6	7.00 ± 0.26	13.7 ± 0.6	1549 ± 30	16.4 ± 0.9	1598 ± 146	22.2 ± 1.4
	Gastric	0.53 ± 0.12	31.3 ± 3.2	2.54 ± 0.56	0.26 ± 0.10	0.74 ± 0.04	79.2 ± 6.6	0.54 ± 0.10	130 ± 39	2.18 ± 0.15
	Intestinal	1.78 ± 0.70	66.3 ± 5.5	9.96 ± 1.35	0.78 ± 0.04	2.69 ± 0.16	165 ± 11	1.59 ± 0.10	253 ± 27	5.62 ± 0.17
	Gastric phase (%)	2.7	4.6	4.9	3.7	5.4	5.1	3.3	8.2	9.8
	Intestinal phase (%)	9.0	9.8	19.2	11.1	19.6	10.6	9.7	15.9	25.4
Cashew	Total[a]	9.06 ± 0.30	386 ± 10	52.6 ± 6.4	16.0 ± 0.2	53.0 ± 3.2	2025 ± 39	12.4 ± 0.3	3812 ± 102	39.1 ± 0.6
	Gastric	0.37 ± 0.13	25.3 ± 2.1	4.10 ± 0.44	0.43 ± 0.05	1.06 ± 0.12	127 ± 13	0.68 ± 0.07	284 ± 22	3.48 ± 0.31
	Intestinal	0.53 ± 0.04	39.7 ± 1.7	10.8 ± 1.2	1.29 ± 0.10	5.60 ± 0.65	227 ± 2	1.30 ± 0.03	568 ± 39	6.45 ± 0.09
	Gastric phase (%)	4.0	6.6	7.8	2.7	2.0	6.3	5.5	7.4	8.9
	Intestinal phase (%)	5.9	10.3	20.5	8.1	10.6	11.2	10.5	14.9	16.5
Brazil nut	Total[a]	9.79 ± 0.29	1315 ± 17	927 ± 14	18.7 ± 0.7	21.3 ± 1.4	3323 ± 25	12.0 ± 0.2	5127 ± 40	32.1 ± 1.2
	Gastric	0.45 ± 0.11	90.5 ± 1.3	69.2 ± 7.4	0.71 ± 0.02	0.85 ± 0.02	285 ± 6	0.89 ± 0.02	504 ± 19	3.62 ± 0.08
	Intestinal	0.85 ± 0.06	62.9 ± 1.3	114 ± 14	1.86 ± 0.33	3.61 ± 0.33	345 ± 19	1.16 ± 0.12	673 ± 25	6.10 ± 0.69
	Gastric phase (%)	4.6	6.9	7.5	3.8	4.0	8.6	7.4	9.8	11.3
	Intestinal phase (%)	8.7	4.8	12.3	10.0	17.0	10.4	9.7	13.1	19.0
Walnut	Total[a]	15.5 ± 1.35	793 ± 67	29.1 ± 3.4	11.7 ± 0.6	22.1 ± 1.0	1035 ± 62	24.2 ± 1.6	719 ± 80	23.2 ± 0.5
	Gastric	1.75 ± 0.52	92.0 ± 4.0	4.33 ± 0.19	1.05 ± 0.15	0.34 ± 0.09	143 ± 9	3.18 ± 0.24	108 ± 16	4.51 ± 0.13
	Intestinal	1.37 ± 0.07	93.1 ± 5.4	7.57 ± 0.58	1.08 ± 0.18	1.32 ± 0.13	139 ± 8	2.12 ± 0.03	145 ± 49	4.06 ± 0.20
	Gastric phase (%)	11.3	11.6	14.9	9.0	1.5	13.8	13.1	15.0	19.4
	Intestinal phase (%)	8.8	11.7	26.0	9.2	6.0	13.4	8.8	20.2	17.5
Chickpea	Total[a]	7.26 ± 0.76	515 ± 12	102 ± 6	7.42 ± 0.12	29.0 ± 3.2	806 ± 15	14.4 ± 0.4	1905 ± 58	20.0 ± 0.4
	Gastric	0.71 ± 0.28	60.1 ± 7.2	11.5 ± 1.7	0.35 ± 0.03	1.02 ± 0.10	103 ± 14	1.53 ± 0.22	177 ± 30	3.64 ± 0.34
	Intestinal	0.37 ± 0.08	73.0 ± 1.7	17.6 ± 1.2	0.71 ± 0.11	4.23 ± 0.27	116 ± 4	1.72 ± 0.07	284 ± 5	5.29 ± 0.23
	Gastric phase (%)	9.8	11.7	11.2	4.8	3.5	12.8	10.7	9.3	18.2
	Intestinal phase (%)	5.1	14.2	17.2	9.6	14.6	14.4	12.0	14.9	26.5
Pumpkin seed	Total[a]	12.4 ± 1.9	315 ± 10	117 ± 11	10.6 ± 0.3	61.3 ± 2.3	4112 ± 155	42.7 ± 1.0	1930 ± 126	59.2 ± 2.3
	Gastric	0.25 ± 0.07	16.0 ± 1.1	2.09 ± 0.24	0.15 ± 0.02	0.66 ± 0.09	105 ± 14	0.79 ± 0.10	91.5 ± 8.8	2.15 ± 0.17
	Intestinal	0.72 ± 0.12	36.2 ± 2.3	16.1 ± 1.3	0.80 ± 0.15	4.00 ± 0.31	276 ± 21	2.23 ± 0.14	313 ± 11	6.79 ± 0.08
	Gastric phase (%)	2.0	5.1	1.8	1.4	1.1	2.6	1.8	4.7	3.6
	Intestinal phase (%)	5.8	11.5	13.8	7.5	6.5	6.7	5.2	16.2	11.5
Sunflower seed	Total[a]	20.3 ± 2.6	901 ± 1.6	92.7 ± 8.2	18.2 ± 0.8	45.8 ± 2.2	2826 ± 59	25.1 ± 0.1	5535 ± 85	45.8 ± 0.9
	Gastric	1.12 ± 0.77	50.4 ± 8.3	6.19 ± 1.76	0.45 ± 0.02	1.30 ± 0.27	138 ± 13	0.10 ± 0.03	342 ± 84	3.40 ± 0.37
	Intestinal	1.61 ± 0.39	98.1 ± 2.1	12.5 ± 0.6	1.89 ± 0.07	5.34 ± 0.09	327 ± 2	2.28 ± 0.09	702 ± 45	7.87 ± 0.44
	Gastric phase (%)	5.5	5.6	6.7	2.5	2.8	4.9	0.4	6.2	7.4
	Intestinal phase (%)	7.9	10.9	13.5	10.4	11.6	11.6	9.1	12.7	17.2

[a] These data were obtained from Kafaoğlu et al. (2014).

Table 3
Amounts (μg day^{-1}) of metals ingested from nuts and seeds assuming an ingestion rate of 10 g day^{-1} calculated from values taken by *in-vitro* intestinal bioavailability results.

	B	Ca	Co	Cu	Fe	Mg	Mn	Ni	Zn
Hazelnut	15.7	1211	0.35	12.7	30.5	1877	60.2	2.12	54.0
Almond	14.0	1873	0.11	12.0	30.3	2586	20.9	2.39	63.1
Pistachio	8.97	2122	0.03	10.7	11.4	1743	11.6	1.81	60.7
Peanut	17.8	663	0.10	7.76	26.9	1649	15.9	2.53	56.2
Cashew	5.33	396	0.11	12.8	55.9	2274	12.9	5.68	64.5
Brazil nuts	8.52	628	1.14	18.6	36.1	3452	11.6	6.73	61.0
Walnut	13.7	930	0.08	10.8	13.2	1392	21.2	1.45	40.6
Chickpea	3.72	730	0.18	7.08	42.3	1160	17.2	2.84	52.9
Pumpkin seed	7.21	362	0.16	7.96	40.0	2762	22.3	3.13	67.9
Sunflower seed	16.1	980	0.13	18.9	53.4	3268	22.8	7.02	78.7
Recommended Dietary Allowances and Adequate Intakes	[b]1.52 mg day^{-1}	[a]1000 mg day^{-1}	[a]2.4 μg day^{-1} as Vitamin B12	[a]900 μg day^{-1}	[a]8 mg day^{-1} for male and 18 mg day^{-1} for female	[a]420 mg day^{-1} for male and 320 mg day^{-1} for female	[a]2.3 mg day^{-1} for male and 1.8 mg day^{-1} for female	[b]150 μg day^{-1}	[a]11 mg day^{-1} for male and 8 mg day^{-1} for female
Tolerable Upper Intake Levels[a] (mg day^{-1})	20	2500	ND	10	45	350	11	1	40

ND, not determinable due to lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake (USDA(b)).
[a] Dietary Reference Intakes (DRIs) were given as Recommended Dietary Allowances and Adequate Intakes by USDA(a) and Tolerable Upper Intake Levels by USDA(b).
[b] These values are given by World Health Organization Geneva 1996 as average daily intakes (WHO, 1996).

the amounts of these elements obtained assuming an ingestion rate of 10 g day^{-1} of nuts and seeds using the *in-vitro* intestinal bioavailability are much lower than the Tolerable Upper Intake Levels. For most of the elements, consumption of 1 kg of seeds and nuts in a day would still be insufficient to reach the Tolerable Upper Intake Levels.

Further investigations were performed using Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) to investigate whether there is a relationship between metal ions in gastric and intestinal phases and nuts and seeds.

3.1. Principal component analysis

PCA was applied to the average concentrations for each element in each matrix (nut or seed) for both gastric and intestinal ingestions (data given in Table 2). The principal components which have eigenvalues higher than 1 were extracted for each gastric and intestinal ingestion separately. The observations from PCA analyses were described as the score values for the matrix (nut or seed) and loading values for each trace element. The components were rotated using Varimax rotation. This led to the formation of three principal components for the *in-vitro* gastric extraction step. The first component accounted for 41.6%, the second for 27.0% and the third for 13.6% of the total variation of the data. The first three components account for 82.2% of variances for all of the data given for *in-vitro* gastric ingestion. Table 4 gives the loadings and the scores of the three rotated principal components for *in-vitro* gastric ingestion of these nuts and seeds. Table 4 shows that the concentrations of the first group of elements, B, Ca, Cu, Mn and Zn on the first principal component are higher for walnut and hazelnut than for the other nuts and seeds. This is because these samples have higher score values for the first principal component. The score and loading values of the second principal component were evaluated and demonstrated that the Brazil nuts have higher concentrations of Co, Mg and Ni than the other nuts and seeds. When the third principal component is interpreted, it is evident that the concentrations of Fe and Mn are higher in hazelnut than other nuts and seeds for *in-vitro* gastric ingestions of these nuts and seeds.

The classification of the different nuts and seeds in the gastric phase from the viewpoint of metal contents can be made using three ways PC loading and score graphs. Fig. 1a shows the two way PCA loadings graphs (PC 1-2) and Fig. 1b shows the two way PCA score graphs (PC 1-2). The PC 1-2 graph shows the highest percentage of total variance of about 68.6%. Using Fig. 1a and b, the nuts and seeds can be classified into five groups in gastric phase. These groups are:

Group 1: Brazil nuts,
Group 2: Walnuts, hazelnuts,
Group 3: Almonds, pistachio nuts, chickpeas,
Group 4: Sunflower seeds, cashew nuts,
Group 5: Pumpkin seeds, peanuts.

For *in-vitro* intestinal ingestions, the first component accounted for 44.1%, the second for 19.6%, the third for 13.0% and the fourth for 12.0% of the total variation of the data. The first four components

Table 4
The loadings and the scores of the three rotated principal components for gastric phase.

The loadings				The scores			
Element	PC1	PC2	PC3	Nut or seed	PC1	PC2	PC3
B	**0.88**	−0.19	0.08	Hazelnut	**1.03**	0.02	**2.44**
Ca	**0.60**	0.06	0.25	Almond	0.22	−0.52	0.36
Co	0.09	**0.94**	0.09	Pistachio	0.06	−0.48	−0.17
Cu	**0.93**	0.29	−0.05	Peanut	−1.05	−0.71	−0.25
Fe	0.00	0.13	**0.93**	Cashew	−0.62	0.18	−0.21
Mg	0.30	**0.92**	0.08	Brazil nut	−0.06	**2.67**	−0.45
Mn	**0.68**	−0.08	**0.56**	Walnut	**2.14**	−0.54	−1.57
Ni	−0.11	**0.93**	−0.01	Chickpeas	−0.16	−0.26	−0.02
Zn	**0.81**	0.28	−0.22	Pumpkin seed	−1.33	−0.63	−0.31
				Sunflower seed	−0.22	0.26	0.17

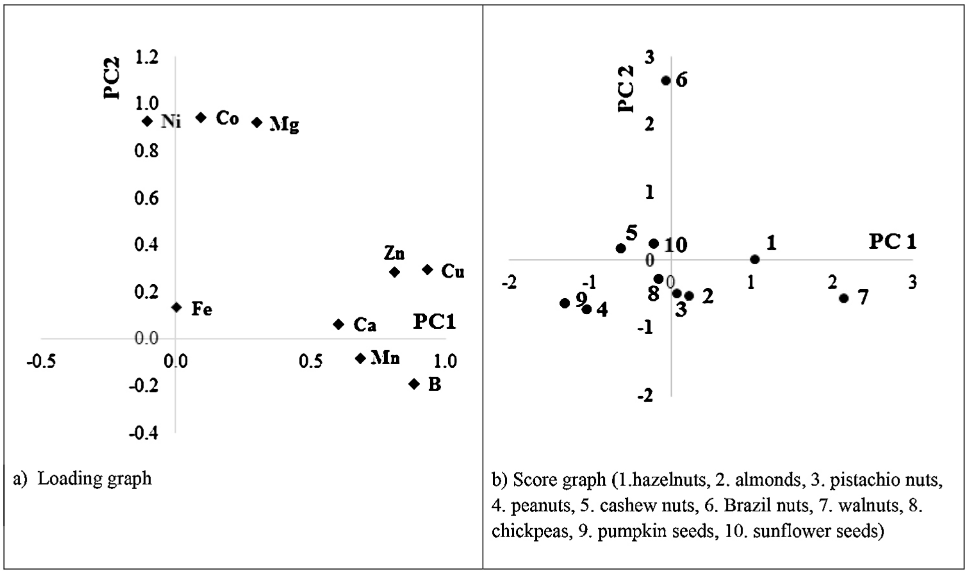


Fig. 1. Two way Principal Component Analysis loadings and score plot for gastric ingestions.

account for 88.7% of variances for all of the data given for *in-vitro* intestinal ingestion. The first component represents the maximum variation of the data set. Table 5 gives the loadings and the scores of the four rotated principal components for *in-vitro* intestinal ingestion of these nuts and seeds. From Table 5, when the score and loading values of the first principal components were evaluated, it was demonstrated that Cu, Fe, Mg, Ni and Zn are higher for white sunflower seeds (the highest), and cashew than for the other nuts and seeds. The concentrations of Co and Cu on the second principal component are higher in Brazil nuts than the other nuts and seeds. When the third principal component is interpreted, it is evident that the concentrations of Ca are higher in almond and pistachio than in other nuts and seeds for *in-vitro* intestinal ingestions. Interpretation of the fourth principal component demonstrates that the concentrations of B and Mn are higher in hazelnut (the highest) and sunflower seeds. Three ways PC loading and score graphs were made for the classification of the different nuts and seeds in intestinal phase from the viewpoint of metal contents. Fig. 2a shows the three way PCA loadings graphs (PC 1-2-3) and Fig. 2b shows the three way PCA score graphs (PC 1-2-3). The PC 1-2-3 graph shows the highest percentage of total variance of about 76.7. Using Fig. 2a and b, the nuts and seeds can be classified into four groups in the intestinal phase. These groups are:

Group 1: Chickpeas, pumpkin seeds, cashew nuts, sunflower seeds,
Group 2: Brazil nuts,
Group 3: Hazelnuts, walnuts,
Group 4: Almonds, pistachio nuts, peanuts.

The recognition of the groups made to classify the nuts and seeds for gastric and intestinal ingestions using PCA were done by introducing these groupings to LDA, in the next section.

3.2. Linear discriminant analysis

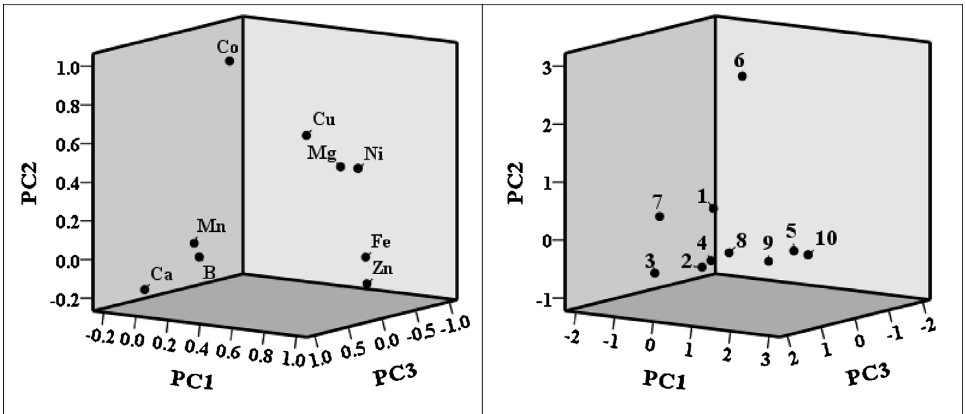
LDA can be used to show how these group members made by PCA, above, for gastric and intestinal ingestions may correctly be classified as a percentage of the original group. LDA was performed for the 9 elements on each of the groups for gastric and intestinal ingestions resulting from PCA as discussed above. The recognition of the groups was highly satisfactory for gastric digestions using LDA; with all the group members determined using PCA being predicted to be 100.0% correctly classified. Also, cross-validation segments for the LDA model validation were performed for all the data sets for nuts and seeds for the gastric digestions with 90.0% of the cross-validated grouped cases being correctly classified using PCA. From the results of the cross-validation, only the Brazil nuts were mis-classified using the PCA interpretations made above. This nut should have been included in Group 2 according to the cross-validated groups.

Four canonical discriminant functions with eigenvalues greater than 1 were obtained from the data. The first canonical discriminant function explains 95.7% of the variance. The discriminant equation of the first function is:

$$Z = -10.06 + (3.10 \times \text{Co}) + (53.01 \times \text{Cu}) + (-9.51 \times \text{Mn}) + (-0.21 \times \text{Ni})$$

Table 5
The loadings and the scores of the three rotated principal components for intestinal phase.

The loadings					The scores				
Element	PC1	PC2	PC3	PC4	Nut or seed	PC1	PC2	PC3	PC4
B	0.10	−0.01	0.41	**0.75**	Hazelnut	−0.62	0.32	−0.27	**2.32**
Ca	−0.03	−0.15	**0.91**	0.09	Almond	0.46	−0.38	**1.30**	0.12
Co	0.05	**0.95**	−0.16	−0.06	Pistachio	−0.23	−0.45	**1.91**	−1.00
Cu	**0.65**	**0.65**	0.13	0.17	Peanut	−0.49	−0.54	−0.05	0.34
Fe	**0.67**	−0.05	−0.70	−0.05	Cashew	**0.62**	−0.45	−1.23	−0.77
Mg	**0.80**	0.49	−0.02	0.00	Brazil nut	0.24	**2.69**	−0.14	−0.71
Mn	−0.15	−0.01	−0.11	**0.90**	Walnut	−1.47	0.20	0.35	0.19
Ni	**0.77**	0.45	−0.35	−0.17	Chickpeas	−0.90	−0.58	−1.05	−0.90
Zn	**0.96**	−0.10	−0.03	−0.07	Pumpkin seed	0.23	−0.62	−0.93	−0.29
					Sunflower seed	**2.16**	−0.20	0.11	0.70



a) Loading graph

b) Score graph (1.hazelnuts, 2. almonds, 3. pistachio nuts, 4. peanuts, 5. cashew nuts, 6. Brazil nuts, 7. Walnuts 8. chickpeas, 9. pumpkin seeds, 10. sunflower seeds)

Fig. 2. Three way Principal Component Analysis loadings and score plot for intestinal ingestions.

The concentrations of each of the elements were inserted to this equation and the figure obtained for each nut or seed type is given in the parentheses below enabling the first canonical discriminant function for each of the nuts and seeds from gastric indigestions to make clear groupings:

Group 1: Brazil nuts (129.3),
Group 2: Walnuts (6.43), hazelnuts (6.55),
Group 3: Almonds (−5.40), pistachio nuts (−8.00), chickpeas (−6.95),
Group 4: Sunflower seeds (−38.9), cashew nuts (−39.9),
Group 5: Pumpkin seeds (−22.3), peanuts (−20.8).

A similar trend was obtained using LDA for the intestinal digestions, with 100% of the groups predicted using PCA being correctly classified. Cross-validation again showed that 90% were correctly classified with Brazil nuts again being mis-classified. This nut type should have been included in Group 1 according to the cross-validated groups. Three canonical discriminant functions with eigenvalues greater than 1 were obtained by linear discriminant analyses. The first canonical discriminant function explains nearly 100% of the variance. The discriminant equation of the first function is:

$$Z = -79.0 + (0.025 \times \text{Ca}) + (2.73 \times \text{Co}) + (-22.7 \times \text{Mn}) + (10.2 \times \text{Zn})$$

The concentrations of each of the elements in the intestinal digestions were inserted to the equation above and the figure obtained for each nut or seed type is given in the parentheses below. The calculated values given in the parentheses from first canonical discriminant function for each of the nuts and seeds enables clear groupings to be made:

Group 1: Chickpeas (−14.2), pumpkin seeds (−15.7), cashew nuts (−12.4), sunflower seeds (−4.2),
Group 2: Brazil nuts (269.0),
Group 3: Hazelnuts (−62.3), walnuts (−62.9),
Group 4: Almonds (−28.7), pistachio nuts (−29.5), peanuts (−29.1).

The linear discriminant analyses therefore proved that the groupings for gastric and intestinal digestions made by PCA are highly accurate because the figures for each of the nuts in each of the groups are very close to each other.

4. Conclusions

The trace element concentrations that can dissolve in gastric and intestinal solutions obtained from different nuts and seeds were determined using an *in-vitro* gastro-intestinal method. The bioaccessible proportions of the elements (B, Ca, Co, Cu, Fe, Mn, Mg, Ni and Zn) in nuts and seeds were calculated using concentrations obtained using total digestion and the *in-vitro* gastrointestinal experiments in nuts and seeds. The bioaccessible proportion as expressed as a percentage was highest for Zn compared with other elements while the proportion was the lowest for B in gastric and intestinal solutions for each of the nuts and seeds. Magnesium and calcium concentrations in gastric and intestinal solutions were higher than other elements' concentrations for most of the nuts and seeds. The concentrations of B were the lowest for the other elements in gastric and intestinal solutions for each of the nuts and seeds.

Relationships between nuts and seeds from the perspective of metal concentrations in gastric and intestinal solutions were demonstrated using PCA interpretations. The chemometric tool of LDA demonstrated that these groupings made using PCA in gastric and intestinal digestions were 100% correctly classified. The interpretations between nuts and seeds using their heavy metal concentrations are based solely on the statistical analysis of the analytical data obtained.

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