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In vitro evaluation of Cu and Fe bioavailability in cashew nuts by *off-line* coupled SEC–UV and SIMAAS

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ABSTRACT

In this work Cu and Fe bioavailability in cashew nuts was evaluated using *in vitro* method. Extractions with simulated gastric and intestinal fluids and dialysis procedures were applied for this purpose. The proteins separation and quantification were performed by size exclusion chromatography (SEC) coupled *on-line* to ultra-violet (UV) and *off-line* to simultaneous multielement atomic absorption spectrometry (SIMAAS). The SEC–UV and SIMAAS profiles of the protein fractions obtained by alkaline extraction (NaOH) and precipitation with HCl indicated the presence of high and low molecular weight species in the range between >75 kDa and 9.3 kDa. Almost 83% of Cu and 78% of Fe were extracted during cashew nut digestion and 90% of both elements were dialyzed. With these results it is possible to assume that 75% of Cu and 70% of Fe present in cashew nut could be bioavailable. The SEC–UV and SIMAAS chromatographic profiles obtained after *in vitro* gastrointestinal digestion reveal that Cu and Fe not dialyzed can be associated to a compound of 9.2 kDa.

1. Introduction

Nuts are a rich source of nutrients, mainly protein, fat, vitamins, minerals such as phosphorus, potassium, magnesium, iron, and dietary fiber [1,2]. With the increasing recognition of nuts as important sources of some essential elements (e.g. B, Se, Cu, Zn, Fe and Mn) in human nutrition, especially for vegetarians, as well as the growing popularity of various healthy diets, the use of these constituents in diet became more frequent [3].

In terms of nutrition it is not sufficient to measure the total content of nutrients, but it is important to know the bioavailability or the amount absorbed and used by the organism. The nutrients bioavailability depends not only on its absorption in the intestine but also on its conversion to biologically active form [4]. So, the nutrients must be transformed into simpler molecules through digestion process that could be absorbed to the human body [1,2,4–8].

Bioavailability studies of nutrient in foods can be performed using *in vivo* and/or *in vitro* methods. Indeed the combination of these methods can provide information that can help during results interpretation. In the *in vivo* method, the bioavailability amount of an element is estimated as the difference in the concentration of the element in ingest and excreta, using radiotracers and stable isotopes [9,10]. The hazards of ionizing radiation of using radiotracers have caused the shift towards stable isotope techniques. Enriched stable isotopes provide scientifically rigorous tools for investigating the absorption and metabolism of mineral nutrients in human subjects [10]. The *in vitro* method offers an appealing alternative to *in vivo* studies and is widely performed by using a simulated gastrointestinal digestion, with pepsin during the gastric stage and a mixture of pancreatin and bile salts during the intestinal stage. The element diffusing across a semi-permeable membrane during the intestinal stage is used as a measure for element bioavailability [11–15].

An *in vitro* procedure to determine calcium binding capacities in different foods was proposed by Dendougui and Schwedt [16]. The samples were extracted using pepsin in order to simulate the gastric digestion process. Calcium and phytic acid were determined at two pH (2 and 8) to simulate the digestion process when food moves from the acid of stomach to the alkaline medium of intestine. The results were used to calculate the calcium binding capacities with phytic acid and to estimate the calcium available to absorption during this process. Kapolna and Fodor prepared Se-enriched green onions and chives to evaluate the bioavailability of Se from selenized *Allium* plants by three sample preparation methods: using proteolytic, gastric and intestinal digestions [17]. The proteolytic digestion was done using a mixture between sample and Pronase E enzyme, which was used to assess the initial species distribution of the selenized green onions and chives. Simulated gastric and intestinal fluids were used to imitate the gastrointestinal digestion. In all extracts obtained the total concentration of Se and speciation was achieved by liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS).

Cashew nut fruit (*Anacardium occidentale*, *L.*) is a tropical fruit valued not only for its high ascorbic acid content but also for the nut, which is the principal consumer product. The cashew nuts have kernels, which are rich in lipids (43%) and proteins (20%) and inorganic constituents [1,3]. The processed kernels are consumed as snacks in roasted and

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salted form [8]. Additionally, it is especially consumed as the breakfast cereal. The copper and iron concentrations are around 20 μg g^{-1} and 67 μg g^{-1}, respectively [3].

These elements act as cofactors for many physiological and metabolic functions. The Cu deficiency leads to high blood pressure and fertility. The essentiality of copper is mediated through specific copper proteins. A deficiency of Fe caused anemia whilst an excess may increase the risk of developing cancer or heart attacks. Biologically available forms of iron may be put into two categories: haeme, where iron is chelated by porphyrin, and iron pool, consisting of non-haeme iron chemically very heterogeneous and largely bound to protein [6,18,19].

Regarding the nutritional importance of the elements of interest, the objective of this work was to evaluate Cu and Fe bioavailability in cashew nuts, using *in vitro* method. The extractions with simulated gastric and intestinal fluids and dialysis procedure were used in this study. The elemental determination was done by simultaneous multielement atomic absorption spectrometry (SIMAAS). The proteins separation and quantification were performed by size exclusion chromatography and UV spectrophotometry detector.

2. Experimental

2.1. Instrumental

A SIMAA 6000 simultaneous atomic absorption spectrometer with longitudinal Zeeman-effect background correction, Echelle optical arrangement, solid state detector and standard THGA (transversely heated graphite atomizer) tube with pyrolitically coated integrated platform (Perkin-Elmer, Norwalk, CT) was used for the determination of multi (Cu/Fe) elemental mode. The spectrometer was operated using hollow cathode lamps. Solutions were delivered into the graphite tube by means of an AS-72 autosampler. The instrumental conditions and the heating program used for Cu and Fe determination are shown in Table 1.

A Spectro Ciros^{CCD} ICP optical emission spectrometer (Spectro Analytical Instruments, Kleve, Germany) equipped with axially-viewed plasma and solid state charged-coupled device (CCD) detector was used to obtain the total concentration of Cu and Fe in cashew nuts. The ICP instrumental and operational parameters are described in Table 2.

The digestion of samples was carried out in a closed vessel microwave oven, model Microwave 3000 (Anton Paar, Graz, Austria).

A chromatographic system (CBM-20A, Shimadzu, Japan) equipped with degassing, piston pump, UV–Vis spectrophotometer detector and an autosampler with 110 sampling positions was used to separate molecular weight compounds by size exclusion chromatography (SEC). The Superdex 75 10/300 GL (3–70 kDa) (Amersham Biosciences, Sweden) was used as stationary phase and 0.2 mol L^{-1} Tris–HCl buffer (pH = 7.5) as mobile phase. The column was percolated with 36 mL of buffer and aliquots of 0.5 mL were collected every 1 min. The flow rate, injection volume and UV wavelength parameters were 0.5 mL min^{-1}, 100 μL and 280 nm, respectively. The Tris–HCl buffer was chosen to avoid possible protein precipitation and to reduce hydrophobic interactions between the compounds present in the sample. The flow rate and the wavelength were chosen according to the manufacturer's recommended and previous experience of our group [20].

Table 1
Instrumental conditions and heating program for simultaneous determination of Cu and Fe by SIMAA 6000.

Element	λ (nm)	Source	*I* (mA)	Band pass (nm)
Cu	324.8	HCL	12	0.8
Fe	248.3	HCL	25	0.8

Graphite furnace heating program

Step	Temperature (°C)	Ramp (s)	Hold (s)	Ar flow (mL min^{-1})	Stop flow
Drying I	110	1	30	250	No
Drying II	130	15	30	250	No
Pyrolysis	1200	10	20	250	No
Atomization	2300	0	5	0	Yes
Cleaning	2500	1	3	250	No

HCL (hollow cathode lamp).

Table 2
Instrumental conditions for the determination of Cu and Fe by ICP OES.

Power (R.F.)	1400 W
Plasma flow	12 L min^{-1}
Auxiliar flow	1 L min^{-1}
Nebulizer flow	1 L min^{-1}
Analytical emission line (λ)	Cu (I): 324.754 nm
	Fe (II): 259.941 nm

A 700 S UV–visible spectrophotometer (Fento, Sao Paulo, Brazil) equipped with xenon lamp and wavelength range from 190 to 900 nm was used for protein determination at 595 nm.

The nitrogen determination in cashew nuts was performed using CHNS Elemental Analyzer (Perkin-Elmer-2400-series II—USA) using helium as driving gas and oxygen as combustion gas.

For proteins extraction an orbital shaker (Quimis, São Paulo, Brazil) to mix the samples and extract was used at 300 rpm for 30 min.

For simulated digestion procedure a water bath at 37 °C was used at 90 rpm for 120 min, model Q226M2 Dubnoff Water Bath (Quimis, São Paulo, Brazil).

A model Q222TM centrifuge (Quimis, São Paulo, Brazil) was used to separate the phases.

2.2. Reagents and samples

All solutions were prepared from analytical-grade chemicals and using high purity deionized water obtained from a Milli-Q water purification system (Millipore, Belford, MA, USA). Tritisol® standard analytical solutions of 1000 mg L^{-1} of Cu(II) [$Cu(NO_3)_2$] and Fe(III) [$FeCl_3$] (Merck, Darmstadt, Germany) were used to prepare the reference analytical solutions by serial dilutions with Tris–HCl buffer solution (pH = 7.5).

Nitric acid (Merck), distilled in a quartz sub-boiling still (Marconi, Piracicaba, SP, Brazil) and 30% (v/v) H_2O_2 (Merck) were used to prepare the oxidant mixture for digestion of samples in a microwave oven. Sodium hydroxide and hydrochloric acid (Merck) were used for the extraction and precipitation of proteins, respectively.

The total protein concentration was determined by the Bradford method [21]. The Bradford reagent was prepared using 10 mg of Coomassie Blue G-250, 5.0 mL of methanol and 10 mL of 85% v/v phosphoric acid (Sigma-Aldrich). Finally, this mixture was diluted to 100 mL with deionized water. The protein standard was prepared dissolving 4.0 mg of ovoalbumin (BioAgency, São Paulo, Brazil) in 2.0 mL of deionized water, Vortex stirring for 2 min and 10 times diluted with deionized water. The spectrophotometer calibration was performed using analytical reference solutions of 4, 6, 8, 10, 12, 16 and 20 μg mL^{-1} of ovoalbumin in Bradford Reagent.

The buffer solution of 0.2 mol L^{-1} Tris–HCl was prepared by dissolving the appropriate mass of Tris(hydroxymethyl)aminomethane (USB Corporation, Cleveland, USA) in deionized water and adjusting the pH to 7.5 with HCl (Merck). A reference protein mixture of aprotinin (~6.5 kDa), ribonuclease A (~13.7 kDa), carbonic anhydrase (~29 kDa), ovalbumin (~43 kDa) and conalbumin (~75 kDa) (GE Healthcare, Piscataway, USA) diluted in buffer solution was used to calibrate the column. A solution of 1.0 mg mL^{-1} of blue dextran (2000 kDa) was used to obtain the column void volume (V_o).

The gastrointestinal digestion was prepared with NaCl (Merck, Darmstadt, Germany), pepsin (Sigma-Aldrich, Saint Louis, USA), HCl