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Speciation of essential nutrient trace elements in coconut water

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ABSTRACT

Coconut water (*Cocos Nucifera*) is shown to be a source of essential elements present in the form of low-molecular weight stable complexes known for their bio-availability. The total element concentrations were in the range of 0.2–2.7, 0.3–1, 3–14 and 0.5–2 ppm for Fe, Cu, Mn, and Zn, respectively, and varied as a function of the origin of the nut and its maturity. Speciation was investigated by size-exclusion chromatography - inductively coupled plasma mass spectrometry (ICP MS), and hydrophilic interaction liquid chromatography (HILIC) - electrospray-Orbitrap MS. The metal species identified included: iron complexes with citrate and malate: $Fe^{III}(Cit)_3(Mal)$, $Fe^{III}(Cit)_2(Mal)_2$, $Fe^{III}(Mal)_2$, glutamine: $Fe^{III}(Glu)_2$ and nicotianamine: $Fe^{II}(NA)$; copper complexes with phenylanine: $Cu^{II}(Phe)_2$ and $Cu^{II}(Phe)_3$ and nicotianamine: $Cu^{II}(NA)$; zinc complexes with citrate: $Zn^{II}(Cit)_2$ and nicotianamine $Zn^{II}(NA)$ and manganese complex with asparagine $Mn^{II}(Asp)_2$. The contributions of the individual species to the total elements concentrations could be estimated by HILIC – ICP MS.

1. Introduction

Trace elements are required for normal growth, development and physiology of animals and man (Sigel, Sigel, & Sigel, 2013). Several metals, such as iron, copper, manganese and zinc, are vital for cellular functions, enzymatic activation, gene expression and metabolism of amino acids, lipids and carbohydrates (Sigel et al., 2013). One of the most widespread dietary problems in the world is mineral deficiency (Gregory et al., 2017). It is associated not only with the low concentration of an element in food but also depends on the chemical form in which the element is present. Indeed, some forms of an element can be more available than others (Gharibzahedi & Jafari, 2017).

The problem of trace element deficiency has been addressed by the production and marketing of feed and food supplements in the form of low-molecular weight metal chelates. These supplements are expected either to provide the metal as a highly available species or to increase its bioavailability by containing 'promoter' substances, such as ascorbate, β-carotene and cysteine-rich polypeptides which stimulate the absorption of essential mineral elements by the gut (White & Broadley, 2009). In these products, the mineral is bound to an organic (i.e., carbon-containing) molecule, typically a carboxylic or amino acid. The popular food supplements include glucinates of iron, zinc, copper and manganese, citrates of iron and zinc, glycinates of copper, iron, zinc and manganese, iron lactate and fumarate, zinc picolinate, acetate and orotate (Hurrell et al., 2004). Similarly, organic Cu sources have been shown to be more bioavailable than inorganic Cu sources in some animal studies (Spears, 2003). Zinc absorption from supplemental zinc citrate or gluconate was higher than from zinc oxide (Wegmüller, Tay, Zeder, Brnić, & Hurrell, 2014). The bioavailability of Mn-methionine for animals was found to be greater than that of manganese sulfate or manganese oxide (Henry, Ammerman, & Littell, 1992). A major inconvenience of the artificial supplementation is the effect of the increase of one element on the deterioration of the availability of the other; *e.g.*, it was shown that Cu and Zn inhibited Fe uptake, and while Fe inhibited Cu uptake, Zn did not (Rakhra, Masih, Vats, Vijay, Ashraf, & Singh, 2020). Also, some of the most efficient iron chelates cannot be used in many food vehicles because of sensory issues (Hurrell et al., 2004). Therefore, natural, equilibrated sources of trace elements present in low-molecular weight complexes have to be searched for.

A good example of such a source is goji beers (*Lycium Barbarum,* L.) which were demonstrated to contain a dozen of LMW complexes of Cu and Zn, with amino acids and anti-oxidant ligands (Ruzik & Kwiatkowski, 2018). Strategies to overcome the low iron and zinc bioavailability from cereals, attributed to their scavenging and complexation by phytates (EFSA, 2017), include a genetic modification aimed at the overproduction of an Fe-binding LMW ligand,

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nicotianamine (Clemens, 2014; Connorton & Balk, 2019; Lee et al., 2009). The tolerance to Fe and Zn deficiencies was observed in animals fed with genetically modified grains containing high contents of LMW metal complexes (Lee et al., 2009). The chemical speciation of manganese in foods has not been investigated so far.

Coconut water is the juice found inside a young coconut (fruit of the palm tree *Cocos nucifera*) which accounts for ca. 25% of fruit weight. It has become a trendy beverage, known for its nutritional properties and a good source of fiber, vitamin C and several important minerals, in particular potassium and manganese (Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996; Yong, Ge, Ng, & Tan, 2009). Data on the trace element concentration in coconut water are scarce. The concentrations obtained in a recent study of coconut water from Bangladesh reported the metal concentrations in the ranges of 0.3 to 1.5, 7.77 to 21.2, 0 to 0.71, 0 to 0.9, 0 to 0.2, 0.9 to 17.3, 0.1 to 0.9, 0 to 0.9 and 0 to 0.7 mg/l for Fe, Ni, Cu, Cd, Cr, Zn, Pb and Se respectively (Md Didarul, Rahaman, & Afrose, 2018). Another work, focused on Pb and Cd found that their concentrations in coconut water were below the maximum tolerated levels in Brazil (Paixao, Brandao, Araujo, & Korn, 2019). To our best knowledge, the speciation of trace elements in coconut water has never been studied.

The objective of this work was to verify the hypothesis that the essential trace metals present in coconut water occur as low molecular weight (LMW) complexes which could make concentrated coconut water a valuable natural food and feed supplement. As coconut water is, technically speaking, endosperm liquid, the methodology based on the coupling of different mechanisms of high performance liquid chromatography (HPLC) with element (ICP MS) and molecule specific (ESI MS) detection developed previously (Flis et al., 2016) for the endosperm liquid, and successfully used elsewhere, for the speciation of Zn in Goji berries (Ruzik & Kwiatkowski, 2018; Wojcieszek, Kwiatkowski, & Ruzik, 2017) was adapted. As sample preparation is known to highly influence species stability and thus, analytical results, sample preparation was limited to dilution with the chromatographic mobile phase and ultracentrifugation.

Size-exclusion chromatography using polymer stationary phase is as recognized technique for the fractionation of metal complexes in the biological cytosols (Szpunar, 2005). Although, the separation efficiency is poor and the elution volume is hardly a simple function of the molecular mass because of the presence of non-specific interactions (in addition to the molecular sieve effect) of the analytes with the stationary phase, the technique has the advantage of respecting the natural pH of the sample and measuring quantitatively the distribution between thermodynamically stable high and low molecular weight complexes and ionic or weakly bound species (Szpunar, 2005). Hydrophilic interaction liquid chromatography (HILIC) is the method of preference for separation of LMW polar species (Buszewski & Noga, 2012), and therefore has been chosen for studies of metal complexes present in the coconut water. When properly optimized, HILIC provides high resolution chromatograms with peaks corresponding to individual species (Montes-Bayón, Sharar, & Corte-Rodriguez, 2018).

In addition to the qualitative metal speciation, our second objective was to investigate the quantitative distribution of the metals amongst the identified metal species. Quantitative speciation of LMW metal complexes is hardly possible at the current state of the art of methodological developments. The principal reasons are (i) the difference in the behaviour of the synthetic species and those naturally present in the sample, and (ii) the risk of the modification of the equilibrium by the spike. Indeed, the stability and stoichiometry of mixed ligand complexes are dependent on the chemical environment and are difficult to control, especially in a natural sample (Dell'mour et al., 2012). The alternative is the use of another metal species for calibration (Boiteau, Shaw, Pasa-Tolic, Koppenaal, & Jansson, 2018), either in an independent HPLC run or by post-column addition of a known amount of metal, but this approach requires both the total recovery of the metal from the column and the baseline separation of the metal species of interest. Boiteau et al. proposed, for the quantification of iron species, a calibration curve constructed for the Fe-EDTA complex; in the case of co-elution, the peak area was deconvoluted on the basis of the relative intensities obtained byLC-ESI MS (Boiteau et al., 2018). Rellán-Álvarez et al. used post-column isotope dilution with ^{54}Fe to quantify the eluting Fe complexes but the recoveries of iron were very low (25%) (Rellán-Álvarez et al., 2010). Nischwitz et al proposed post-column Fe addition in size-exlusion LC – ICP MS but the chromatographic resolution was too low to distinguish amongst the individual species, and the metal recoveries could not be controlled (Nischwitz, Berthele, & Michalke, 2008). Finally, Latorre et al., proposed the metal-species fraction collection and the determination of the collected metal in *SEC* ICP MS but, again, the method quantified the metal fraction and not the individual species (Latorre et al., 2019). Our strategy has been based on the optimization of the HPLC conditions to allow the individual metal-species separation, good (close to quantitative) metal recovery, and correction of the effect of the change in the mobile phase composition on the sensitivity.

2. Experimental

2.1. Samples, materials, and reagents

Samples and materials. The coconut samples were acquired from different suppliers in France, Spain and Switzerland; two of the analysed coconuts were harvested directly from a coconut tree in Thailand. A standard reference material (CRM-MFD Mixed Food Diet, HPS, North Charleston, SC) was analysed to validate the results of the determination of the total Fe, Mn, Cu and Zn concentrations.

Reagents. The reagents used for digestions, dilutions and the preparation of HPLC mobile phases were obtained from Sigma-Aldrich, St. Louis, MO unless state otherwise. They were: ammonium acetate (≥98% for molecular biology), nitric acid (70%, Fisher Chemical, Loughborough, UK), acetonitrile (≥99.9%), hydrogen peroxide (30%), formic acid and hydrochloric acid (37%, Fluka, Steinheim, Germany). Standard solutions (1000 ppm) of Fe, Mn, Cu, Zn, Rh, Sc (SCP Science, Villebon-sur-Yvette, France) were used for the preparation of calibration curves and as internal standards (Sc and Rh). EDTA (ethylenediaminetetraacetic acid) was used for column cleaning. Milli-Q® Type 1 Ultrapure Water Systems (Millipore, Bedford, MA) deionized water was used throughout.

2.2. Instrumentation

A DigiPrep MS system (SCP Science, Quebec, Canada) was used for sample digestion. Samples were centrifuged using a MiniSpin centrifuge (Eppendorf, Hamburg, Germany). Two chromatographic systems were used for the separation of the analytes: Agilent 1200 Series (Agilent, Tokyo, Japan) fitted with a Superdex-75 10/300 GL *SEC* column (separation range 3000 and 70,000 Da, 10 × 300 mm) (GE Healthcare, www3.gehealthcare.com); and Dionex Ultimate 3000 RS (Thermo Scientific, Bremen, Germany) fitted with a Kinetex HILIC column (150 × 2.1 mm × 2.6 µm) (Phenomenex, www.phenomenex.com) and a Security Guard (2.1 mm × 3 µm) pre-column (ULTRA, www.phenomenex.com). The ICP MS spectrometers were ICP-MS 7500 (Agilent, Tokyo, Japan) equipped with an integrated autosampler (I-AS) used for the total analysis and Agilent 7700x (Agilent, Tokyo, Japan) used for coupling with HPLC pumps. Electrospray ionization mass spectrometer was Q Exactive Plus (Thermo Scientific, Bremen, Germany).

2.3. Procedures

Sample preparation. Coconut husks were opened by making an orifice with a drilling machine perforating the fruit mesocarp without getting to the albumen to avoid contamination or by removing the shell from one of three germination pores. Coconut water was manually extracted by pipette. The total volume of coconut water was centrifuged (80,000*g*, 30 min, 4 °C) and aliquoted in several Eppendorf tubes to be either analyzed immediately or to be frozen at −80 °C for further analysis. Due to the reported difficulties in sample preservation (De Sousa, Baccan, & Cadore, 2005; Obike, 2013), several approaches, described in the subsequent sections of this article, were investigated to guarantee the optimal storage conditions.

Total metal analyses. The method was based on that described earlier by De Sousa, Baccan et al. (2005), De Sousa, Silva, Baccan, & Cadore (2005). Briefly, coconut water samples were treated with a mixture of nitric acid and hydrogen peroxide (3:1, *v/v*) and digested until a clear solution was obtained (typically for 2.5 h). The maximum temperature did not exceed 60 °C. The digests were cooled down to room temperature. After an appropriate dilution and the addition of internal standards (Sc and Rh) the total concentrations of Fe, Mn, Cu, and Zn, was measured by ICP MS; ^{54}Fe, ^{56}Fe, ^{63}Cu, ^{65}Cu, ^{55}Mn, ^{64}Zn, ^{66}Zn and ^{68}Zn isotopes were monitored. The experimental parameters, such as plasma power, torch position, and voltage applied to extraction and focusing lenses, were optimized daily. Hydrogen was used as reaction gas to reduce spectral interferences. External calibration (5-points calibration curve) was used for quantification of the metals of interest. The calibration standards were: 0.5, 1, 2, 5 and 10 ppm for Cu, Fe, and Zn, and 1, 5, 10, 20, and 50 for Mn. The standard reference material and analytical blank were analyzed in parallel.

Speciation analyses. The HPLC separation conditions were summarized in Table 1. The *SEC* column was calibrated using a set of molecular weight standards: selenomethionine (198 Da) methylcobalamine (1.3 kDa), aprotinine (6.5 kDa), cytochrome *C* (12 kDa), ribonuclease A (13.7 kDa), myoglobine (17.1 kDa), carbonic anhydrase (29 kDa), bovine fetuine (48.4 kDa), ovalbumine (42.7 kDa), BSA (66.4 kDa), conalbumine (77.7 kDa), and ferritine (474 kDa). The calibration was performed by plotting the log of the molecular mass *vs.* log of the elution volume. The sole objective of the column calibration was to serve as reference of the reproducible column performance. As the separation is carried out according to the hydrodynamic volume and not molecular mass, the estimation of the molecular weight, on this basis, for metal complexes is highly approximate. For the ICP MS detection the exit of the HPLC column was simply connected to the entrance of the ICP MS nebulizer and the continuous signal (^{54}Fe, ^{56}Fe, ^{63}Cu, ^{65}Cu, ^{55}Mn, ^{64}Zn, ^{66}Zn and ^{68}Zn) was registered during the duration of the chromatographic run. Hydrogen was used as reaction gas. For electrospray MS detection, the ion source was operated in the positive mode. The following parameters were adjusted: capillary temperature, carrier gas temperature, shielding gas pressure and spray voltage; the typical values of these parameters are given in Table 1. Data processing was done using XCalibur software (Thermo Scientific, Bremen, Germany) used for the generation of theoretical masses and their comparison with the experimental ones.

Table 1
Experimental conditions used.

Chromatographic conditions		
separation mechanism	HILIC	*SEC*
column	Kinetex (150 × 2.1 mm × 2.6 µm)	Superdex 75 10/300 GL
guard column	Security Guard (XX × 2.1 mm × 3 µm)	none
mobile phase	A: 25 mM ammonium acetate, pH 5.5; B: ACN	100 mM ammonium acetate, pH 7.5
flow rate, mL/min	0.5	0.7
injection volume, µL	10	100
elution program	0–1 min 95% B; 1–10 min 80%; 10–13.5 min 80% B; 13.5–17 min 60% B; 17–21 min 60% B; 21.01–30 min 95% B.	isocratic
ESI MS operating conditions		
ionization mode		positive
resolution		140,000
AGC		$1e^{6}$
max injection time, ms		500
capillary temperature, °C		350
carrier gas temperature °C		400
ionization mode		40
spraying voltage, kV		3.2
shielding gas flow rate, arb		60
carrier gas flow, arb		20

Metal-species quantification. For the estimation of the quantitative distribution of metal of interest amongst the detected species, HILIC chromatography was repeated but with ICP MS detection using the above conditions. The detection sensitivity was normalized for each metal (Cu, Fe, Mn, Zn) and for each retention time using the curve of the metal signal intensity as a function of the mobile phase composition. The contribution of each species was calculated, on the peak area basis, as the percentage of the total metal amount eluted.

3. Results and discussion

3.1. Total analysis of metals in coconut water samples

Effect of centrifugation. Coconut water is a slightly turbid sample and requires to be centrifuged prior to chromatography. The distribution of trace elements between the solid phase and supernatant was investigated. The results shown in Fig. SI-1 indicate that no statistically significant differences in the total content of Mn, Fe, Cu and Zn in ultracentrifuged and non-ultracentrifuged samples were found which means that the totality of trace elements was present in the supernatant.

Total element concentrations. The results obtained for the analysis of the reference material: Cu 0.07 ± 0.01 (certified 0.06 ± 0.01) ppm, Fe 0.88 ± 0.11 (certified: 0.82 ± 0.11) ppm, Mn 0.20 ± 0.0 (certified 0.20 ± 0.00) ppm, and Zn 0.32 ± 0.03 ppm (certified 0.31 ± 0.03) fell within the certified ranges. The results obtained for the analysis of the coconut water samples are shown in Fig. 1. The concentrations ranges of the different elements are different and range: 0.3–1 ppm for Cu, 0.5 – 2 ppm for Zn, 0.5 – 6.5 ppm for Fe and 3 – 24 ppm for Mn. The concentrations depend strongly on the origin, those of Cu and Zn being correlated. The highest concentrations were found for manganese which makes coconut water a natural source of this element covering (for some samples and approximative volume of 250 mL) the quasi totality of the daily reference intake of Mn recommended by the European Food Safety Agency (3 mg/day) (EFSA Panel on Dietetic Products, (2013), 2013). The highest concentrations of Mn, Cu and Zn were recorded for the sample from Costa Rica. The values corresponding to Fe, Cu, and Zn are in the range as those reported by Midardul *et al.* who studied 15 coconut water samples from Bangladesh (Md Didarul et al., 2018). The total element concentrations in coconut water are affected by the environmental conditions. While comparing the data for different samples, a difference in volumes of the coconut water sampled which is dependent on the coconut size and maturity should be taken into consideration. All the fruits analyzed were considered as mature and ready for consumption.

3.2. Size fractionation of metal complexes present in coconut water

Representative size-exclusion chromatograms of the distribution of metal complexes in coconut water samples are shown in Fig. 2. The chromatograms for Cu, Zn and Mn show a single well shaped peak in the low-molecular weight zone of the chromatogram (Fig. 2a-c) In the case of iron (Fig. 2d), two peaks are observed. The morphology of *SEC*-UV/ICP MS chromatograms was identical for all the samples, only the intensities varied. Note that *SEC* does not allow the separation of individual species. Its role is to assure that metals elute quantitatively as LMW species which is the case except of iron".

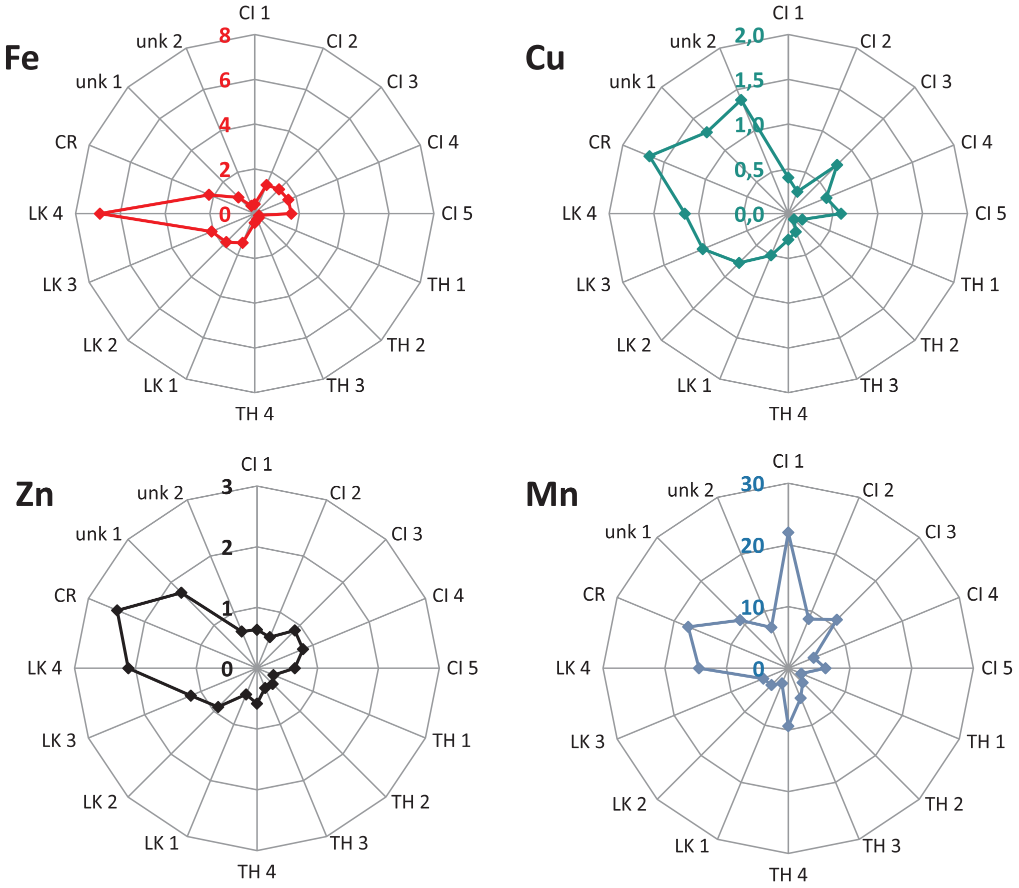

Fig. 1. Spider graphs of Fe, Mn, Zn and Cu contents in the studied coconut water samples (CI - Ivory Coast; TH – Thailand; LK – Sri Lanka; CR - Costa Rica; unk – unknown origin).

3.3. Effect of storage and pretreatment conditions on the coconut sample stability

Coconut water remains sterile inside the nut cavity but once the nut is opened, the biochemical composition and physical appearance of the liquid change (Prades, 2011). The effect of sample storage by freezing was examined but the chromatograms obtained before freezing and after one week freezing and thawing were identical. Also, no changes were observed for ultracentrifuged samples and those analyzed directly.

The possibility of the occurrence of oxidation-related changes in speciation was checked by studying the effect of an ascorbic acid addition on *SEC*-ICP MS chromatograms. The most pronounced effect was observed for iron. The appearance of new signals at elution times corresponding to lower masses than the original ones was observed. This effect was less pronounced for samples stored at 4 °C, while a number of new signals for Fe were recorded at a subzero temperature, with a decrease in the intensity of peak at 22 min. The effect is related to the reducing properties of ascorbic acid and its possible redox reaction with Fe(III), even in pH 5.5–7.0, which is typical for coconut water, and formation of ferrous-ascorbate complexes (Pervaiz, Farrukh, Adnan, & Qureshi, 2012). It was observed elsewhere that ascorbic acid was responsible for the reduction of Fe(III) in mixed citrate-malate complexes by embryos efflux of *Pisum sativum* (Grillet et al., 2014). Ascorbic acid did not influence the speciation of other metals. The effect of acetonitrile addition (42.5% corresponding to 50/50 dilution with the eluent used for HILIC) was investigated in view of the species preservation for the subsequent experiments with more resolutive HILIC chromatography. The chromatograms of coconut water and water-acetonitrile (42.5% *v*/*v*) solutions were practically identical for Cu, Zn, and Mn (not shown), with the exception of iron where the peak at a higher molecular mass observed in the aqueous solution disappeared (Fig. 2d). HILIC will therefore ignore this species accounting for up to 60% of iron depending on the sample. The identity of this peak is unknown; however its precipitation with acetonitrile accompanied by the disappearance of a peak recorded at 280 nm using UV detection (Fig. 2e) may suggest soluble Fe-binding proteins.

In summary, the samples for speciation analysis should be ultracentrifuged and – if not analyzed immediately - stored at low (at or below 4 °C) temperature prior to analysis. Such conditions do not affect the metals' speciation and the stability of coconut water is preserved for at least 4 months. The total concentrations, HPLC-ICP MS and *SEC*-ICP MS chromatograms measured for an example sample at $t = 0$ and $t = 4$ months were identical.

3.4. Speciation of LMW metal complexes in coconut water by HILIC-electrospray MS

Final conditions of sample preparation and storage were applied to compare the speciation of iron, zinc, manganese and copper. All coconuts were opened and the water analyzed on the same day, after ultracentrifugation. As samples were diluted with acetonitrile-containing eluent, the soluble proteins were removed prior to analysis.

ICP MS detection was used to complete the investigations of the effect of sample storage conditions. No significant changes in HILIC-ICP MS morphologies were recorded for ultracentrifuged and directly analysed samples; also the chromatograms obtained before freezing and after one week freezing and thawing were identical. Ultracentrifugation is however necessary to avoid the column filter clogging and pressure buildup.

The identification of the complexes of interest formed between

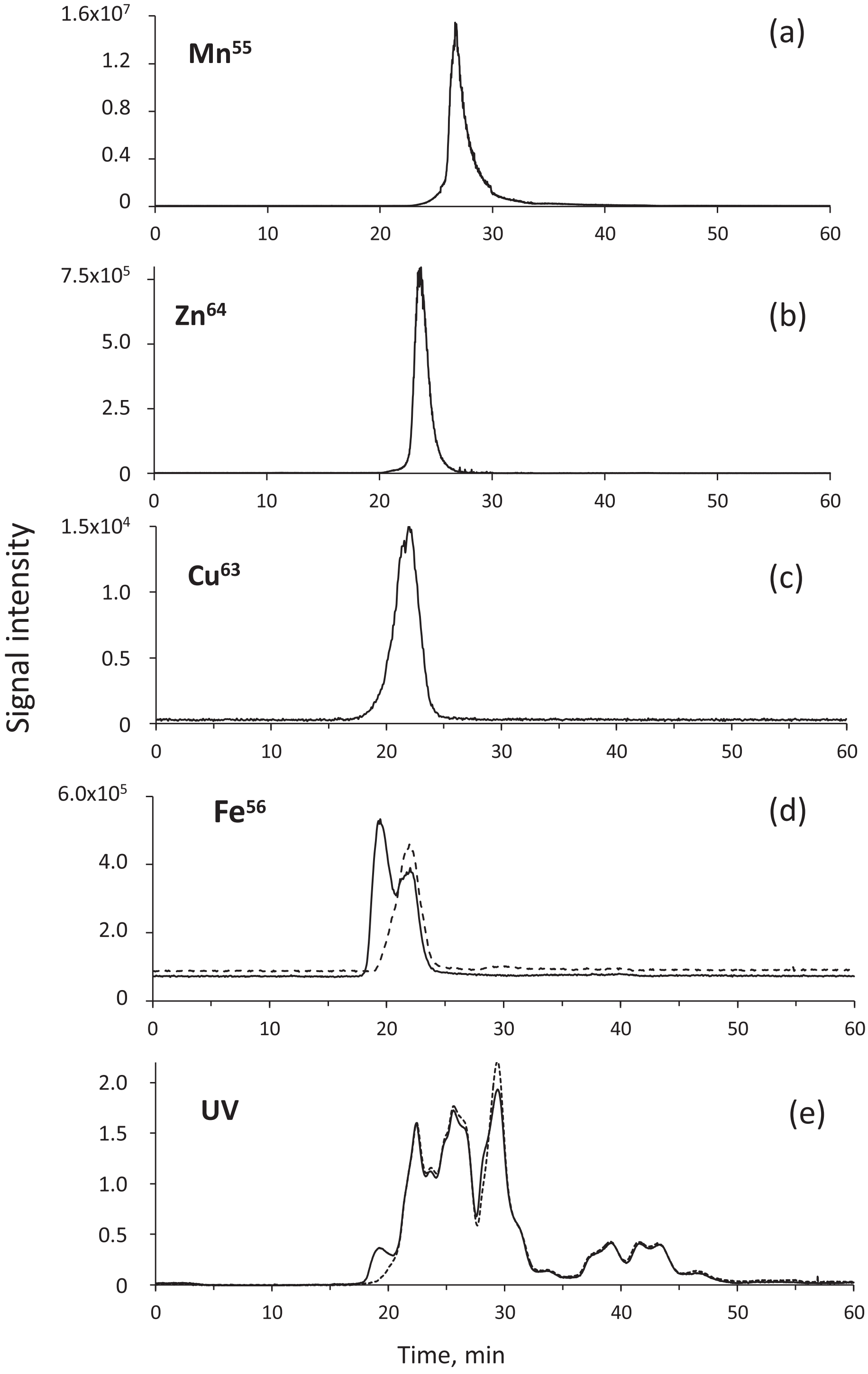

Fig. 2. Size-exclusion chromatograms (*SEC*) of coconut water (regular line) and of coconut water diluted 1:1 with 95% acetonitrile (dashed line). (a-d) ICP MS detection: (a) ^{63}Cu, (b) ^{56}Fe, (c) ^{55}Mn and (d) ^{64}Zn. (e) UV detection. Sample TH4.

metals and LMW ligands was carried out by coupling a HILIC column to an electrospray ionization mass spectrometer in the conditions adapted from the previous work (Flis et al., 2016). Data mining consisted of three strategies: (i) checking for the presence of metal-containing species at the retention times of peaks detected by ICP MS, (ii) targeted analysis based on the literature data and (iii) exploratory analysis based on automatic search for metal isotopic patterns (except for Mn which is monoisotopic). The molecular formulas, together with calculated and measured *m/z* for the identified complexes, are summarized in Table 2. The selected ion chromatograms are shown in Fig. 3. The comparison of the isotopic patterns of the molecular ions of the detected species with the theoretically expected patterns is shown in Fig. 2SI.

The most abundant iron containing peak was a complex of *m/z* 811.84, which consisted of three atoms of iron bound to two molecules of citrates and two malates (Fig. 3a). In addition, a complex of similar structure, but with three citrate and one malate residues (*m/z* 869.84) was observed. These two iron species, $(Fe_3^{III}(Cit)_2(Mal)_2$ and $Fe_3^{III}(Cit)_3(Mal)$, were reported by Grillet *et al.* in a liquid endosperm of pea (Grillet et al., 2014). In the coconut water, also smaller complexes formed with a single iron atom with two molecules of these ligands

Table 2
Metal complexes detected by hydrophilic interaction chromatography electrospray ionization mass spectrometry (HILIC-ESI-MS) in coconut water.

species	molecular formula	calculated *m/z*	measured *m/z*	error *m/z* [ppm]	estimated content (% of total)
copper species					
$Cu^{II}(NA)$	$C_{12}H_{20}O_6N_3Cu$	365.06426	365.06430	0.11	4 ± 2
$Cu^{II}(Phe)_2$	$C_{18}H_{21}O_4N_2Cu$	392.07918	392.07862	−1.43	92 ± 5
$Cu^{II}(Phe)_3$	$C_{27}H_{32}O_6N_3Cu$	557.15816	557.15863	0.84	5 ± 3
iron species					
$Fe_3^{III}(Mal)_2(Cit)_2$	$C_{20}H_{20}O_{24}Fe_3$	811.83873	811.83942	0.85	87 ± 5
$Fe^{III}(Mal)_2$	$C_8H_{10}O_{10}Fe$	321.96179	321.96205	0.81	
$Fe^{II}(NA)$	$C_{12}H_{20}O_6N_3Fe$	358.06960	358.07022	1.73	
$Fe^{III}(Gln)_2$	$C_{10}H_{18}O_6N_4Fe$	346.05703	346.05743	1.16	6 ± 2
$Fe_3^{III}Mal(Cit)_3$	$C_{22}H_{22}O_{26}Fe_3$	869.84421	869.84467	0.53	7 ± 3
manganese species					
$Mn^{II}(Asp)_2$	$C_8H_{13}O_8N_2$ Mn	320.00469	320.00461	−0.25	47 ± 6
zinc species					
$Zn^{II}(NA)$	$C_{12}H_{19}O_6N_3Zn$	366.06381	366.06256	−3.4	62 ± 3
$Zn^{II}(Cit)_2$	$C_{12}H_{14}O_{14}Zn$	446.97478	446.97574	2.1	

Mal - malate. Cit - citrate. Gln-glutamine. NA - nicotianamine. Asp-aspartate. Phe-phenylalanine. Sample : CH4

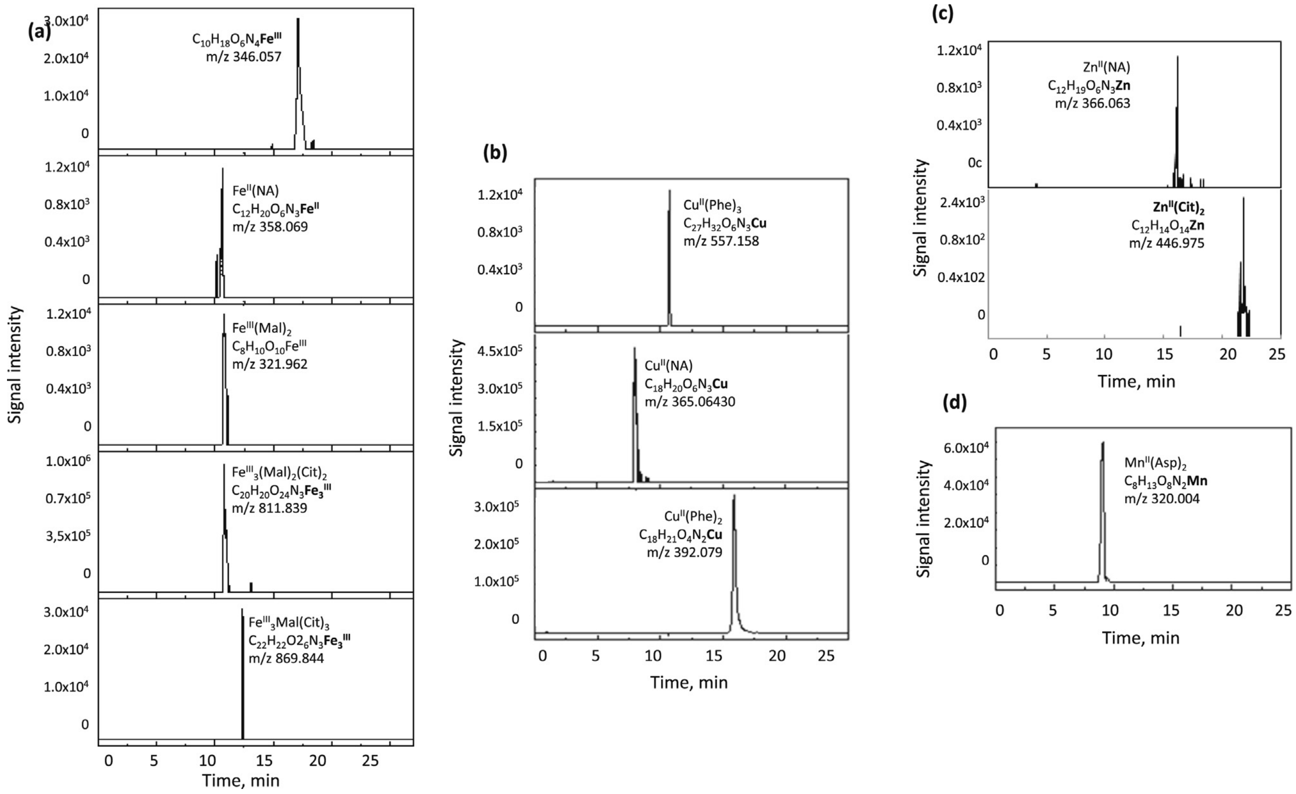

Fig. 3. Selected ion (XIC) HILIC - ESI MS chromatograms of (a) Cu-, (b) Fe-, (c) Mn- and (d) Zn-species detected in coconut water. Sample TH4.

were found. Additionally, iron complexes with glutamine (*m/z* 346.06) were identified; $Fe^{III}(Gln)_2$ was previously detected in xylem and ESL of pea plants (Flis et al., 2016). Additionally, significant differences ($P \leq 0.05$) between Fe-deficient and - sufficient values of glutamine in soybean plants were observed (Chu et al., 2019). Moreover, $Fe^{II}(NA)$ was identified in the endosperm of coconut; this complex was identified in the ESL of pea plants by Flis et al. (2016) and in wheat grains by Xue et al. (2014). Also, Tsednee *et al* reported the presence of several phytosiderophores-iron complexes, including $Fe^{II}(NA)$, in barley (Tsednee, Mak, Chen, & Yeh, 2012).

The most abundant copper complex was the Cu-phenylanine complex $Cu^{II}(Phe)_2$ (*m/z* 365.06) accompanied by a complex with nicotianamine and another complex with phenylalanine $Cu^{II}(Phe)_3$ (*m/z* 392.08 and 557.15) (Fig. 3b). These compounds are characteristic for xylem exudate or embryo sac liquid (known also as liquid endosperm) and were reported for *Pisum sativum* by Flis et al. (2016). Cu-nicotianamine was reported in chicory and tomato by Liao (2000), in xylem and phloem saps from rice (*Oryza sativa*) (Ando, Nagata, Yanagisawa, & Yoneyama, 2013) and in *Brassica carinata* by Irtelli, Petrucci, and Navari-Izzo (2009).

Two zinc complexes identified in coconut water included nicotianamine (*m/z* 366.06) and citrate (446.97) (Fig. 3c). Nicotianamine is the most common Zn binding ligand in plants and was shown to account for more than 70% of the total zinc content extracted from lettuce leaves (Wojcieszek et al., 2019). Zn-nicotianamine and Zn-citrate were accompanied by other LMW Zn-species with ligands including α-aminoadipic acid and sarcosine in lettuce (Wojcieszek et al., 2019) and malate, histidine and glutamine in *Pisum sativum* (Flis et al., 2016).

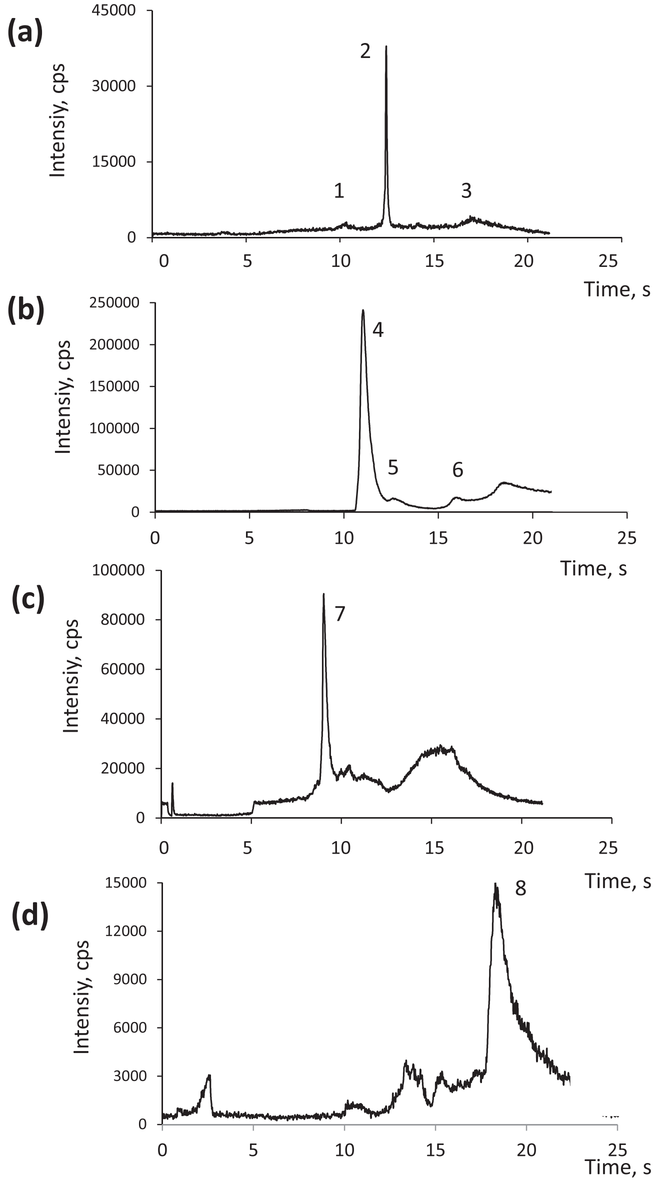

Fig. 4. HILIC - ICP MS chromatograms. (a) ^{63}Cu-, (b) ^{56}Fe-, (c) ^{55}Mn-, (d) ^{64}Zn-selective detection. Sample TH4.

The only manganese species identified in this work was its complex with asparagine (*m/z* 320.00) (Fig. 3d). To our best knowledge, no information about molecular forms of this element in foodstuffs has ever been reported. Manganese is, however, recognized as an essential element.

3.5. *Quantitative distribution of the individual metal species*

No valid and accurate quantification strategy exists for the individual metal-complexes for the reasons outlined in Introduction. The quantification strategy in this work was based on the optimization of the separation for baseline resolution (controlled by ESI MS), optimization of the recovery from the column, and the correction of the effect of the change in the mobile phase composition on the sensitivity (Fig. 3SI). Fig. 4 shows representative HILIC – ICP MS chromatograms obtained in the optimized conditions for Cu, Fe, Mn, and Zn. The morphology of the chromatograms obtained was similar for all the samples. The recoveries slightly exceeded 100% which suggests scavenging of the metals from the column and the chromatographic system. The recoveries do not affect the morphology of the chromatograms (qualitative speciation) as reported elsewhere (Flis et al., 2016). Therefore, it was considered acceptable to normalize them to 100% for the purpose of quantification. Table 2 shows an estimation of the average contribution of a given species (or a given set of species in the cases of insufficient resolution) in three coconut water samples. For iron, no sufficient resolution was achieved between $\mathrm{Fe^{III}(Mal)_2}$, $\mathrm{Fe^{III}(Mal)_2(Cit)_2}$ and $\mathrm{Fe^{II}(NA)}$ allowing one to quantify these species individually. Note that in contrast to electrospray MS detection, the ICP MS response is proportional to the amount of the eluted metal, the accuracy is improved by the normalization of the change in intensity as a function of the mobile phase composition. The accuracy of the measurement can be further improved by post-column isotope dilution as proposed elsewhere (Rellán-Álvarez et al., 2010) except for Mn which is monoisotopic at the expense of a more complex setup.

4. Conclusions

Coconut water is an attractive source of essential trace elements, such as Fe, Cu, Zn, and Mn which occur as low molecular weight complexes. The analytical approach based on coupling HILIC with ICP MS electrospray-Orbitrap MS turned out to be efficient for their identification. In particular, the concentration of manganese, present as its complex with asparagine, makes the daily consumption of a glass of coconut juice sufficient to match the daily intake level recommended by the European Food Safety Agency.

CRediT authorship contribution statement

Ghaya Alchoubassi: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Katarzyna Kińska:** Methodology, Formal analysis, Investigation, Validation, Writing - original draft, Writing - review & editing, Visualization. **Katarzyna Bierla:** Conceptualization, Validation, Writing - original draft, Supervision. **Ryszard Lobinski:** Writing - original draft, Writing - review & editing, Funding acquisition. **Joanna Szpunar:** Conceptualization, Validation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127680.

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