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Speciation of essential nutrient trace elements in coconut water

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17 **Abstract**

18 Coconut water (*Cocos Nucifera*) is shown to be a source of essential elements present in the form of
19 low-molecular weight stable complexes known for their bio-availability. The total element
20 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,
21 respectively, and varied as a function of the origin of the nut and its maturity. Speciation was
22 investigated by size-exclusion chromatography - inductively coupled plasma mass spectrometry
23 (ICP MS), and hydrophilic interaction liquid chromatography (HILIC) - electrospray-Orbitrap MS.
24 The metal species identified included: iron complexes with citrate and malate: $\text{Fe}^{\text{III}}(\text{Cit})_3(\text{Mal})$,
25 $\text{Fe}^{\text{III}}(\text{Cit})_2(\text{Mal})_2$, $\text{Fe}^{\text{III}}(\text{Mal})_2$, glutamine: $\text{Fe}^{\text{III}}(\text{Glu})_2$ and nicotianamine: $\text{Fe}^{\text{II}}(\text{NA})$; copper complexes
26 with phenylalanine: $\text{Cu}^{\text{II}}(\text{Phe})_2$ and $\text{Cu}^{\text{II}}(\text{Phe})_3$ and nicotianamine: $\text{Cu}^{\text{II}}(\text{NA})$; zinc complexes with citrate:
27 $\text{Zn}^{\text{II}}(\text{Cit})_2$ and nicotianamine $\text{Zn}^{\text{II}}(\text{NA})$ and manganese complex with asparagine $\text{Mn}^{\text{II}}(\text{Asp})_2$. The
28 contributions of the individual species to the total elements concentrations could be estimated by
29 HILIC – ICP MS.

30 **Keywords**

31 Coconut water; trace elements; speciation; SEC-ICP MS; ESI MS; HILIC; low-molecular weight
32 complexes

33

34 **1. INTRODUCTION**

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

43 The problem of trace element deficiency has been addressed by the production and marketing of feed
44 and food supplements in the form of low-molecular weight metal chelates. These supplements are
45 expected either to provide the metal as a highly available species or to increase its bioavailability by
46 containing 'promoter' substances, such as ascorbate, β -carotene and cysteine-rich polypeptides which
47 stimulate the absorption of essential mineral elements by the gut (White & Broadley, 2009). In these
48 products, the mineral is bound to an organic (i.e., carbon-containing) molecule, typically a carboxylic
49 or amino acid. The popular food supplements include glucinates of iron, zinc, copper and manganese,
50 citrates of iron and zinc, glycinates of copper, iron, zinc and manganese, iron lactate and fumarate,
51 zinc picolinate, acetate and orotate (Hurrell, Lynch, Bothwell, Cori, Glahn, Hertrampf, et al., 2004).
52 Similarly, organic Cu sources have been shown to be more bioavailable than inorganic Cu sources in
53 some animal studies (Spears, 2003). Zinc absorption from supplemental zinc citrate or gluconate was
54 higher than from zinc oxide (Wegmüller, Tay, Zeder, Brnić, & Hurrell, 2014). The bioavailability of
55 Mn-methionine for animals was found to be greater than that of manganese sulfate or manganese
56 oxide (Henry, Ammerman, & Littell, 1992). A major inconvenience of the artificial supplementation is
57 the effect of the increase of one element on the deterioration of the availability of the other; e.g., it was
58 shown that Cu and Zn inhibited Fe uptake, and while Fe inhibited Cu uptake, Zn did not (Rakhra,

59 Masih, Vats, Vijay, Ashraf, & Singh, 2020). Also, some of the most efficient iron chelates cannot be
60 used in many food vehicles because of sensory issues (Hurrell, et al., 2004). Therefore, natural,
61 equilibrated sources of trace elements present in low-molecular weight complexes have to be searched
62 for.

63 A good example of such a source is goji berries (*Lycium Barbarum, L.*) which were demonstrated to
64 contain a dozen of LMW complexes of Cu and Zn, with amino acids and anti-oxidant ligands (Ruzik
65 & Kwiatkowski, 2018). Strategies to overcome the low iron and zinc bioavailability from cereals,
66 attributed to their scavenging and complexation by phytates (EFSA, 2017), include a genetic
67 modification aimed at the overproduction of an Fe-binding LMW ligand, nicotianamine (Clemens,
68 2014; Connorton & Balk, 2019; Lee, Jeon, Lee, Kim Y.K., Persson, Husted, et al., 2009). The
69 tolerance to Fe and Zn deficiencies was observed in animals fed with genetically modified grains
70 containing high contents of LMW metal complexes (Lee, et al., 2009). The chemical speciation of
71 manganese in foods has not been investigated so far.

72 Coconut water is the juice found inside a young coconut (fruit of the palm tree *Cocos nucifera*) which
73 accounts for ca. 25% of fruit weight. It has become a trendy beverage, known for its nutritional
74 properties and a good source of fiber, vitamin C and several important minerals, in particular
75 potassium and manganese (Santoso, Kubo, Ota, Tadokoro, & Maekawa, 1996; Yong, Ge, Ng, & Tan,
76 2009). Data on the trace element concentration in coconut water are scarce. The concentrations
77 obtained in a recent study of coconut water from Bangladesh reported the metal concentrations in the
78 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
79 0.7 mg/l for Fe, Ni, Cu, Cd, Cr, Zn, Pb and Se respectively (Md Didarul, Rahaman, & Afrose, 2018).
80 Another work, focused on Pb and Cd found that their concentrations in coconut water were below the
81 maximum tolerated levels in Brazil (Paixao, Brandao, Araujo, & Korn, 2019). To our best knowledge,
82 the speciation of trace elements in coconut water has never been studied.

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

93 Size-exclusion chromatography using polymer stationary phase is as recognized technique for the
94 fractionation of metal complexes in the biological cytosols (Szpunar, 2005). Although, the separation
95 efficiency is poor and the elution volume is hardly a simple function of the molecular mass because of
96 the presence of non-specific interactions (in addition to the molecular sieve effect) of the analytes with
97 the stationary phase, the technique has the advantage of respecting the natural pH of the sample and
98 measuring quantitatively the distribution between thermodynamically stable high and low molecular
99 weight complexes and ionic or weakly bound species (Szpunar, 2005). Hydrophilic interaction liquid
100 chromatography (HILIC) is the method of preference for separation of LMW polar species (Buszewski
101 & Noga, 2012), and therefore has been chosen for studies of metal complexes present in the coconut

102 water. When properly optimized, HILIC provides high resolution chromatograms with peaks
103 corresponding to individual species (Montes-Bayón, Sharar, & Corte-Rodriguez, 2018).

104 In addition to the qualitative metal speciation, our second objective was to investigate the quantitative
105 distribution of the metals amongst the identified metal species. Quantitative speciation of LMW metal
106 complexes is hardly possible at the current state of the art of methodological developments. The
107 principal reasons are (i) the difference in the behaviour of the synthetic species and those naturally
108 present in the sample, and (ii) the risk of the modification of the equilibrium by the spike. Indeed, the
109 stability and stoichiometry of mixed ligand complexes are dependent on the chemical environment and
110 are difficult to control, especially in a natural sample (Dell'mour, Schenkeveld, Oburger, Fischer,
111 Kraemer, Puschenreiter, et al., 2012). The alternative is the use of another metal species for calibration
112 (Boiteau, Shaw, Pasa-Tolic, Koppenaal, & Jansson, 2018), either in an independent HPLC run or by
113 post-column addition of a known amount of metal, but this approach requires both the total recovery
114 of the metal from the column and the baseline separation of the metal species of interest. Boiteau et al.
115 proposed, for the quantification of iron species, a calibration curve constructed for the Fe-EDTA
116 complex; in the case of co-elution, the peak area was deconvoluted on the basis of the relative
117 intensities obtained by LC-ESI MS (Boiteau, Shaw, Pasa-Tolic, Koppenaal, & Jansson, 2018). Rellán-
118 Álvarez et al. used post-column isotope dilution with ⁵⁴Fe to quantify the eluting Fe complexes but the
119 recoveries of iron were very low (25%) (Rellán-Álvarez, Giner-Martínez-Sierra, Orduna, Orera,
120 Rodríguez-Castrilln, García-Alonso, et al., 2010). Nischwitz et al proposed post-column Fe addition in
121 size-exclusion LC – ICP MS but the chromatographic resolution was too low to distinguish amongst the
122 individual species, and the metal recoveries could not be controlled (Nischwitz, Berthele, & Michalke,
123 2008). Finally, Latorre et al., proposed the metal-species fraction collection and the determination of
124 the collected metal in SEC ICP MS but, again, the method quantified the metal fraction and not the
125 individual species (Latorre, Herbello-Hermelo, Peña-Farfal, Neira, Bermejo-Barrera, & Moreda-
126 Piñeiro, 2019). Our strategy has been based on the optimization of the HPLC conditions to allow the
127 individual metal-species separation, good (close to quantitative) metal recovery, and correction of the
128 effect of the change in the mobile phase composition on the sensitivity.

129

130 **2. EXPERIMENTAL**

131 **2.1. Samples, materials, and reagents**

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

136 *Reagents.* The reagents used for digestions, dilutions and the preparation of HPLC mobile phases were
137 obtained from Sigma-Aldrich, St. Louis, MO unless state otherwise. They were: ammonium acetate
138 (≥98% for molecular biology), nitric acid (70%, Fisher Chemical, Loughborough, UK), acetonitrile
139 (≥99.9%), hydrogen peroxide (30%), formic acid and hydrochloric acid (37%, Fluka, Steinheim,
140 Germany). Standard solutions (1000 ppm) of Fe, Mn, Cu, Zn, Rh, Sc (SCP Science, Villebon-sur-
141 Yvette, France) were used for the preparation of calibration curves and as internal standards (Sc and

142 Rh). EDTA (ethylene-diaminetetraacetic acid) was used for column cleaning. Milli-Q® Type 1
143 Ultrapure Water Systems (Millipore, Bedford, MA) deionized water was used throughout.

144 **2.2. Instrumentation**

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

156 **2.3. Procedures**

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

165 *Total metal analyses.* The method was based on that described earlier by De Sousa *et al.* (De Sousa,
166 Baccan, & Cadore, 2005; De Sousa, Silva, N. Baccan, & Cadore, 2005). Briefly, coconut water
167 samples were treated with a mixture of nitric acid and hydrogen peroxide (3:1, *v/v*) and digested until a
168 clear solution was obtained (typically for 2.5 h). The maximum temperature did not exceed 60°C. The
169 digests were cooled down to room temperature. After an appropriate dilution and the addition of
170 internal standards (Sc and Rh) the total concentrations of Fe, Mn, Cu, and Zn, was measured by ICP
171 MS; ⁵⁴Fe, ⁵⁶Fe, ⁶³Cu, ⁶⁵Cu, ⁵⁵Mn, ⁶⁴Zn, ⁶⁶Zn and ⁶⁸Zn isotopes were monitored. The experimental
172 parameters, such as plasma power, torch position, and voltage applied to extraction and focusing
173 lenses, were optimized daily. Hydrogen was used as reaction gas to reduce spectral interferences.
174 External calibration (5-points calibration curve) was used for quantification of the metals of interest.
175 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
176 Mn. The standard reference material and analytical blank were analyzed in parallel.

177 *Speciation analyses.* The HPLC separation conditions were summarized in **Table 1**. The SEC column
178 was calibrated using a set of molecular weight standards: selenomethionine (198 Da)
179 methylcobalamine (1.3 kDa), aprotinine (6.5 kDa), cytochrome C (12 kDa), ribonuclease A (13.7
180 kDa), myoglobin (17.1 kDa), carbonic anhydrase (29 kDa), bovine fetuine (48.4 kDa), ovalbumine
181 (42.7 kDa), BSA (66.4 kDa), conalbumine (77.7 kDa), and ferritine (474 kDa). The calibration was
182 performed by plotting the log of the molecular mass vs. log of the elution volume. The sole objective
183 of the column calibration was to serve as reference of the reproducible column performance. As the
184 separation is carried out according to the hydrodynamic volume and not molecular mass, the
185 estimation of the molecular weight, on this basis, for metal complexes is highly approximate. For the
186 ICP MS detection the exit of the HPLC column was simply connected to the entrance of the ICP MS
187 nebulizer and the continuous signal (^{54}Fe , ^{56}Fe , ^{63}Cu , ^{65}Cu , ^{55}Mn , ^{64}Zn , ^{66}Zn and ^{68}Zn) was registered
188 during the duration of the chromatographic run. Hydrogen was used as reaction gas. For electrospray
189 MS detection, the ion source was operated in the positive mode. The following parameters were
190 adjusted: capillary temperature, carrier gas temperature, shielding gas pressure and spray voltage; the
191 typical values of these parameters are given in **Table 1**. Data processing was done using XCalibur
192 software (Thermo Scientific, Bremen, Germany) used for the generation of theoretical masses and
193 their comparison with the experimental ones.

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

200

201 **3. RESULTS AND DISCUSSION**

202 **3.1. Total analysis of metals in coconut water samples**

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

208 *Total element concentrations.* The results obtained for the analysis of the reference material : Cu 0.07
209 ± 0.01 (certified 0.06 ± 0.01) ppm, Fe 0.88 ± 0.11 (certified: 0.82 ± 0.11) ppm, Mn 0.20 ± 0.0
210 (certified 0.20 ± 0.00) ppm, and Zn 0.32 ± 0.03 ppm (certified 0.31 ± 0.03) fell within the certified
211 ranges. The results obtained for the analysis of the coconut water samples are shown in **Fig. 1**. The
212 concentrations ranges of the different elements are different and range: 0.3-1 ppm for Cu, 0.5 – 2 ppm
213 for Zn, 0.5 – 6.5 ppm for Fe and 3 – 24 ppm for Mn. The concentrations depend strongly on the origin,
214 those of Cu and Zn being correlated. The highest concentrations were found for manganese which
215 makes coconut water a natural source of this element covering (for some samples and approximative
216 volume of 250 ml) the quasi totality of the daily reference intake of Mn recommended by the
217 European Food Safety Agency (3 mg/day) (EFSA Panel on Dietetic Products, 2013). The highest
218 concentrations of Mn, Cu and Zn were recorded for the sample from Costa Rica. The values

219 corresponding to Fe, Cu, and Zn are in the range as those reported by Midardul *et al.* who studied 15
220 coconut water samples from Bangladesh (Md Didarul, Rahaman, & Afrose, 2018). The total element
221 concentrations in coconut water are affected by the environmental conditions. While comparing the
222 data for different samples, a difference in volumes of the coconut water sampled which is dependent
223 on the coconut size and maturity should be taken into consideration. All the fruits analyzed were
224 considered as mature and ready for consumption.

225 3.2. Size fractionation of metal complexes present in coconut water

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

232 3.3. Effect of storage and pretreatment conditions on the coconut sample stability

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

238 The possibility of the occurrence of oxidation-related changes in speciation was checked by studying
239 the effect of an ascorbic acid addition on SEC-ICP MS chromatograms. The most pronounced effect
240 was observed for iron. **The appearance of new signals at elution times corresponding to lower
241 masses than the original ones was observed. This effect was less pronounced for samples stored
242 at 4°C, while a number of new signals for Fe were recorded at a subzero temperature, with a
243 decrease in the intensity of peak at 22 min. The effect is related to the reducing properties of
244 ascorbic acid and its possible redox reaction with Fe(III), even in pH 5.5-7.0, which is typical for
245 coconut water, and formation of ferrous-ascorbate complexes (Pervaiz, Farrukh, Adnan, &
246 Qureshi, 2012).** It was observed elsewhere that ascorbic acid was responsible for the reduction of
247 Fe(III) in mixed citrate-malate complexes by embryos efflux of *Pisum sativum* (Grillet, Ouerdane,
248 Flis, Hoang, Isaure, Lobinski, et al., 2014). Ascorbic acid did not influence the speciation of other
249 metals. The effect of acetonitrile addition (42.5 % corresponding to 50/50 dilution with the eluent used
250 for HILIC) was investigated in view of the species preservation for the subsequent experiments with
251 more resolute HILIC chromatography. The chromatograms of coconut water and water-acetonitrile
252 (42.5 % v/v) solutions were practically identical for Cu, Zn, and Mn (not shown), with the exception of
253 iron where the peak at a higher molecular mass observed in the aqueous solution disappeared (**Fig.
254 2d**). HILIC will therefore ignore this species accounting for up to 60 % of iron depending on the
255 sample. The identity of this peak is unknown; however its precipitation with acetonitrile accompanied
256 by the disappearance of a peak recorded at 280 nm using UV detection (**Fig. 2e**) may suggest soluble
257 Fe-binding proteins.

258 In summary, the samples for speciation analysis should be ultra-centrifuged and – if not analyzed
259 immediately - stored at low (at or below 4°C) temperature prior to analysis. Such conditions do not

260 affect the metals' speciation and the stability of coconut water is preserved for at least 4 months. The
261 total concentrations, HPLC-ICP MS and SEC-ICP MS chromatograms measured for an example
262 sample at $t = 0$ and $t = 4$ months were identical.

263 3.4. Speciation of LMW metal complexes in coconut water by HILIC- electrospray MS

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

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

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

283 The most abundant iron containing peak was a complex of m/z 811.84, which consisted of three atoms
284 of iron bound to two molecules of citrates and two malates (**Fig. 3a**). In addition, a complex of similar
285 structure, but with three citrate and one malate residues (m/z 869.84) was observed. These two iron
286 species, $(\text{Fe}^{\text{III}}_3(\text{Cit})_2(\text{Mal})_2)$ and $\text{Fe}^{\text{III}}_3(\text{Cit})_3(\text{Mal})$, were reported by Grillet *et al.* in a liquid endosperm
287 of pea (Grillet, et al., 2014). In the coconut water, also smaller complexes formed with a single iron
288 atom with two molecules of these ligands were found. Additionally, iron complexes with glutamine
289 (m/z 346.06) were identified; $\text{Fe}^{\text{III}}(\text{Gln})_2$ was previously detected in xylem and ESL of pea plants (Flis,
290 Ouerdane, Grillet, Curie, Mari, & Lobinski, 2016). Additionally, significant differences ($P \leq 0.05$)
291 between Fe-deficient and -sufficient values of glutamine in soybean plants were observed (Chu, Sha,
292 Maruyama, Yang, Pan, Xue, et al., 2019). Moreover, $\text{Fe}^{\text{II}}(\text{NA})$ was identified in the endosperm of
293 coconut; this complex was identified in the ESL of pea plants by Flis *et al.* (Flis, Ouerdane, Grillet,
294 Curie, Mari, & Lobinski, 2016) and in wheat grains by Xue *et al.* (Xue, Eagling, He, Zou, McGrath,
295 Shewry, et al., 2014). Also, Tsednee *et al.* reported the presence of several phyto-siderophores-iron
296 complexes, including $\text{Fe}^{\text{II}}(\text{NA})$, in barley (Tsednee, Mak, Chen, & Yeh, 2012).

297 The most abundant copper complex was the Cu-phenylalanine complex $\text{Cu}^{\text{II}}(\text{Phe})_2$ (m/z 365.06)
298 accompanied by a complex with nicotianamine and another complex with phenylalanine $\text{Cu}^{\text{II}}(\text{Phe})_3$
299 (m/z 392.08 and 557.15) (**Fig. 3b**). These compounds are characteristic for xylem exudate or embryo
300 sac liquid (known also as liquid endosperm) and were reported for *Pisum sativum* by Flis *et al.* (Flis,
301 Ouerdane, Grillet, Curie, Mari, & Lobinski, 2016). Cu-nicotianamine was reported in chicory and
302 tomato by Liao *et al.* (Liao, 2000), in xylem and phloem saps from rice (*Oryza sativa*) (Ando, Nagata,

303 Yanagisawa, & Yoneyama, 2013) and in *Brassica carinata* by Irtelli *et al* (Irtelli, Petrucci, & Navari-
304 Izzo, 2009)

305 Two zinc complexes identified in coconut water included nicotianamine (m/z 366.06) and citrate
306 (446.97) (**Fig. 3c**). Nicotianamine is the most common Zn binding ligand in plants and was shown to
307 account for more than 70% of the total zinc content extracted from lettuce leaves (J. Wojcieszek,
308 Jiménez-Lamana, Bierla, Asztemborska, Ruzik, Jarosz, et al., 2019). Zn-nicotianamine and Zn-citrate
309 were accompanied by other LMW Zn-species with ligands including α -aminoadipic acid and sarcosine
310 in lettuce (J. Wojcieszek, et al., 2019) and malate, histidine and glutamine in *Pisum sativum* (Flis,
311 Ouerdane, Grillet, Curie, Mari, & Lobinski, 2016).

312 The only manganese species identified in this work was its complex with asparagine (m/z 320.00)
313 (**Fig. 3d**). To our best knowledge, no information about molecular forms of this element in foodstuffs
314 has ever been reported. Manganese is, however, recognized as an essential element .
315

316 **3.5. Quantitative distribution of the individual metal species**

317 No valid and accurate quantification strategy exists for the individual metal-complexes for the reasons
318 outlined in Introduction. The quantification strategy in this work was based on the optimization of the
319 separation for baseline resolution (controlled by ESI MS), optimization of the recovery from the
320 column, and the correction of the effect of the change in the mobile phase composition on the
321 sensitivity (**Fig. 3SI**). **Fig. 4** shows representative HILIC – ICP MS chromatograms obtained in the
322 optimized conditions for Cu, Fe, Mn, and Zn. The morphology of the chromatograms obtained was
323 similar for all the samples. The recoveries slightly exceeded 100% which suggests scavenging of the
324 metals from the column and the chromatographic system. The recoveries do not affect the morphology
325 of the chromatograms (qualitative speciation) as reported elsewhere (Flis, Ouerdane, Grillet, Curie,
326 Mari, & Lobinski, 2016). Therefore, it was considered acceptable to normalize them to 100% for the
327 purpose of quantification. **Table 3** shows an estimation of the average contribution of a given species
328 (or a given set of species in the cases of insufficient resolution) in three coconut water samples. For
329 iron, no sufficient resolution was achieved between $\text{Fe}^{\text{III}}(\text{Mal})_2$, $\text{Fe}^{\text{III}}(\text{Mal})_2(\text{Cit})_2$ and $\text{Fe}^{\text{II}}(\text{NA})$ allowing
330 one to quantify these species individually. Note that in contrast to electrospray MS detection, the ICP
331 MS response is proportional to the amount of the eluted metal, the accuracy is improved by the
332 normalization of the change in intensity as a function of the mobile phase composition. The accuracy
333 of the measurement can be further improved by post-column isotope dilution as proposed elsewhere
334 (Rellán-Álvarez, et al., 2010) except for Mn which is monoisotopic at the expense of a more complex
335 setup.
336

337 **4. CONCLUSIONS**

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

344 **Acknowledgements**

345 The funding from the French National Research Agency (ANR, EQUIPEX-MARSS ANR-11-EQPX-
346 0027) is acknowledged.

347 **Captions to Figures :**

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

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

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

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

358

359 **Supplementary information:**

360 **Figure SI-1.** Total concentrations of metals in centrifuged and non-centrifuged samples of coconut
361 water: CI – Ivory Coast; LK – Sri Lanka; TH -Thailand; CR - Costa Rica; unk –
362 unknown origin.

363 **Figure SI-2.** Zooms of mass spectra obtained for the species detected (left hand panel -theoretical,
364 right hand panel – experimental); (a) copper species: 1- $\text{Cu}^{\text{II}}(\text{NA})$, 2- $\text{Cu}^{\text{II}}(\text{Phe})_2$, 3-
365 $\text{Cu}^{\text{II}}(\text{Phe})_3$, (b) iron species: 4 - $\text{Fe}^{\text{III}}_3(\text{Mal})_2(\text{Cit})_2$, 5 - $\text{Fe}^{\text{III}}(\text{Gln})_2$, 6 - $\text{Fe}^{\text{III}}(\text{Mal})_2$, 7 -
366 $\text{Fe}^{\text{II}}(\text{NA})$, 8 - $\text{Fe}^{\text{III}}_3\text{Mal}(\text{Cit})_3$, (c) manganese species: 9 - $\text{Mn}^{\text{II}}(\text{Asp})_2$, (d) zinc species:
367 10 – $\text{Zn}^{\text{II}}(\text{NA})$, 11- $\text{Zn}^{\text{II}}(\text{Cit})_2$

368 **Figure SI-3.** Relatives changes of intensity of the ICP MS response during the elution gradient used
369 in HILIC separations

370

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- 494

Table 1. Experimental conditions used.

| Chromatographic conditions | | |
|--------------------------------------|--|------------------------------------|
| separation mechanism | HILIC | SEC |
| column | Kinetex (150 x 2.1 mm x 2.6 μ m) | Superdex 75 10/300 GL |
| guard column | Security Guard (XX x 2.1 mm x 3 μ m) | 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, μ L | 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 | | 140000 |
| AGC | | 1e ⁶ |
| max injection time, ms | | 500 |
| capillary temperature, $^{\circ}$ C | | 350 |
| carrier gas temperature $^{\circ}$ C | | 400 |
| ionization mode | | 40 |
| spraying voltage, kV | | 3.2 |
| shielding gas flow rate, arb | | 60 |
| carrier gas flow, arb | | 20 |

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

| species | molecular formula | calculated <i>m/z</i> | measured <i>m/z</i> | error <i>m/z</i> [ppm] | estimated content (% of total) |
|---|--|-----------------------|---------------------|------------------------|--------------------------------|
| copper species | | | | | |
| Cu^{II}(NA) | C ₁₂ H ₂₀ O ₆ N ₃ Cu | 365.06426 | 365.06430 | 0.11 | 4 _{±2} |
| Cu^{II}(Phe)₂ | C ₁₈ H ₂₁ O ₄ N ₂ Cu | 392.07918 | 392.07862 | -1.43 | 92 _{±5} |
| Cu^{II}(Phe)₃ | C ₂₇ H ₃₂ O ₆ N ₃ Cu | 557.15816 | 557.15863 | 0.84 | 5 _{±3} |
| iron species | | | | | |
| Fe^{III}₃(Mal)₂(Cit)₂ | C ₂₀ H ₂₀ O ₂₄ Fe ₃ | 811.83873 | 811.83942 | 0.85 | |
| Fe^{III}(Mal)₂ | C ₈ H ₁₀ O ₁₀ Fe | 321.96179 | 321.96205 | 0.81 | 87 _{±5} |
| Fe^{II}(NA) | C ₁₂ H ₂₀ O ₆ N ₃ Fe | 358.06960 | 358.07022 | 1.73 | |
| Fe^{III}(Gln)₂ | C ₁₀ H ₁₈ O ₆ N ₄ Fe | 346.05703 | 346.05743 | 1.16 | 6 _{±2} |
| Fe^{III}₃Mal(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 ₈ H ₁₃ O ₈ N ₂ Mn | 320.00469 | 320.00461 | -0.25 | 47 _{±6} |
| zinc species | | | | | |
| Zn^{II}(NA) | C ₁₂ H ₁₉ O ₆ N ₃ Zn | 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

Figure 1

[Click here to download Figure\(s\): Fig. 1 Total concentrations-1.pdf](#)

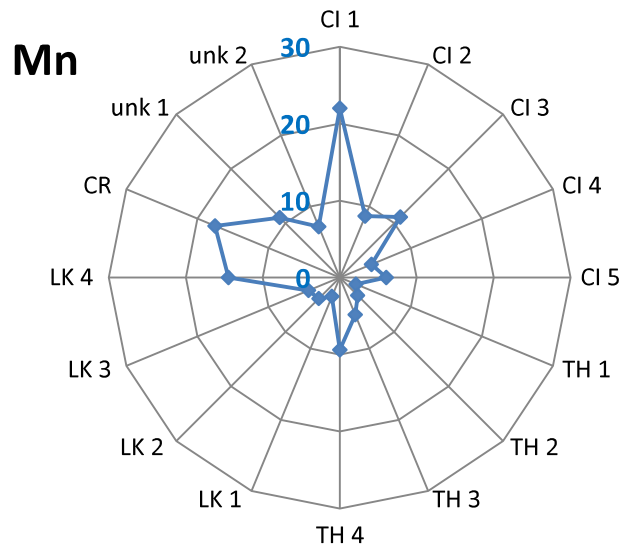
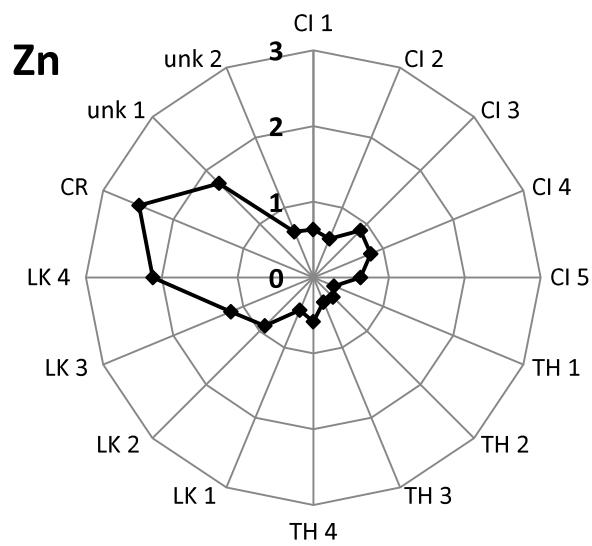
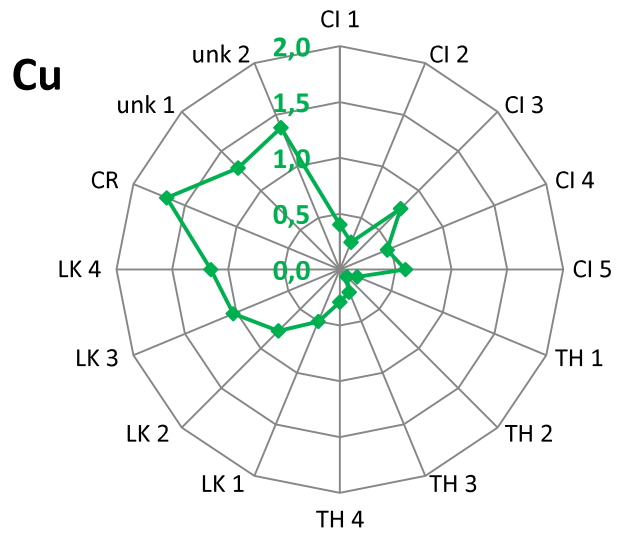
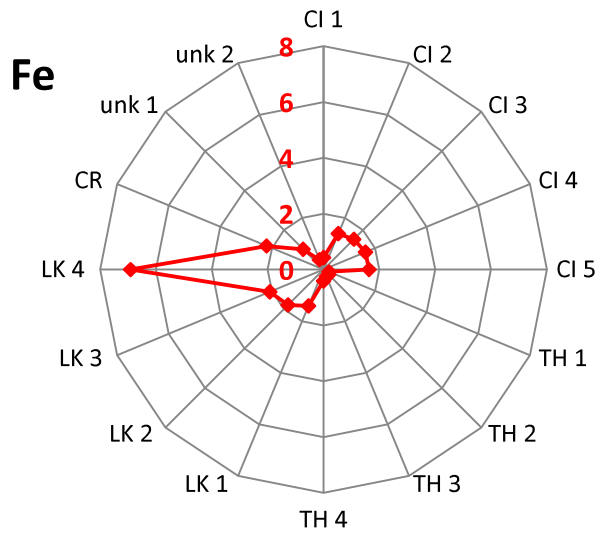


Figure 2 [Click here to download Figure\(s\): Fig. 2 SEC-ICP MS.pdf](#)

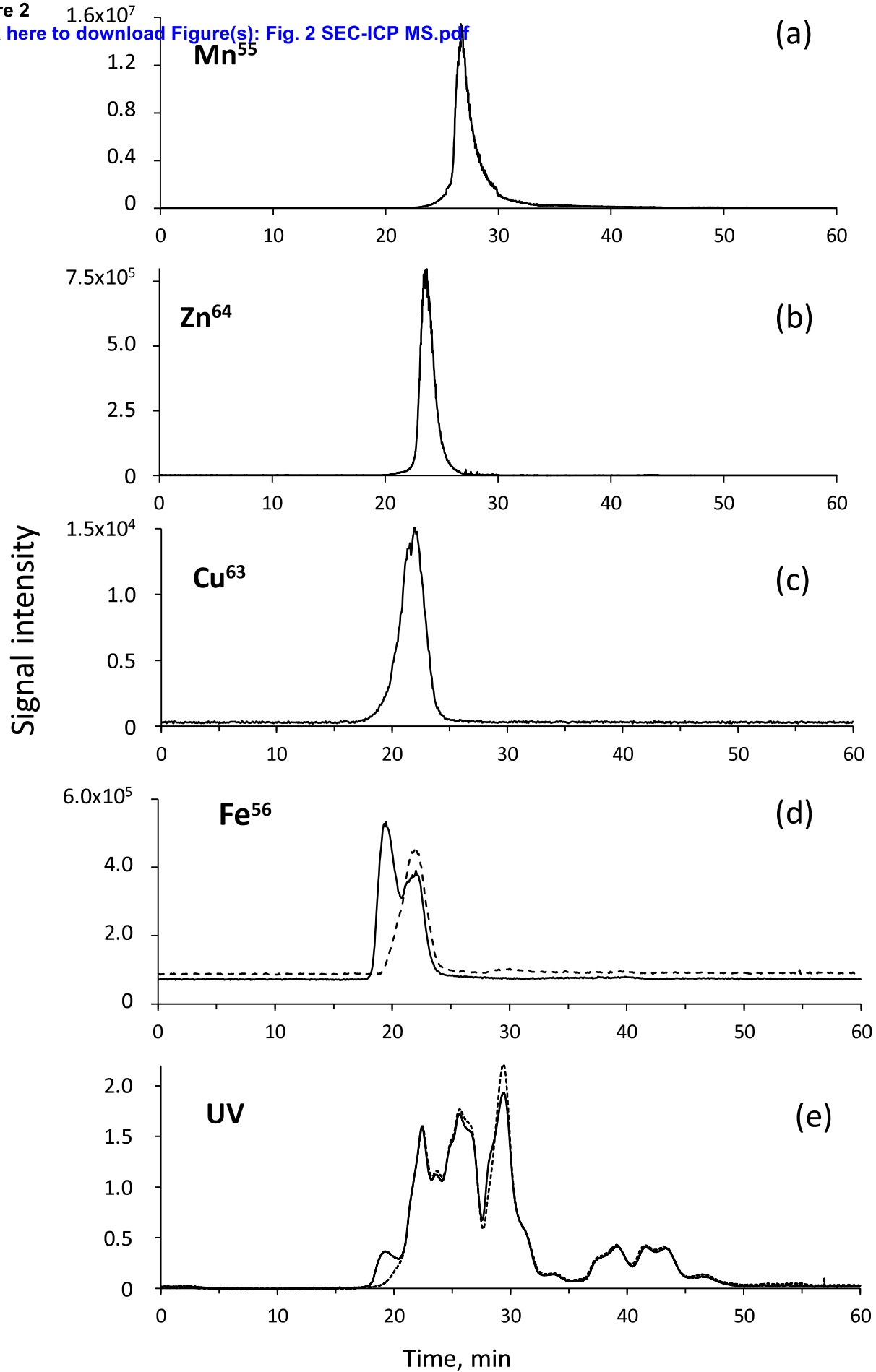


Figure 3
[Click here to download Figure\(s\): Fig. 3 HILIC-ESI MS.pdf](#)

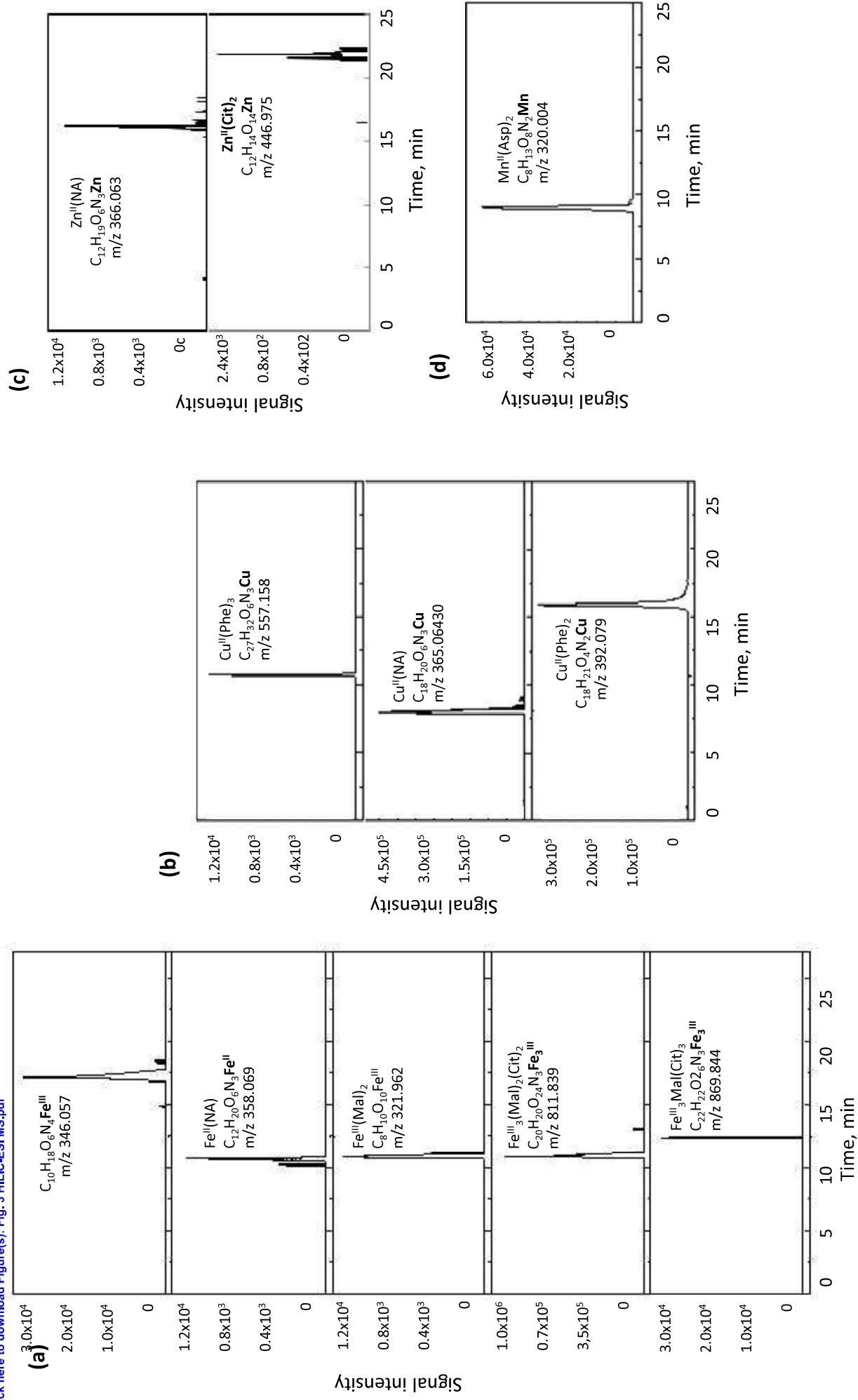
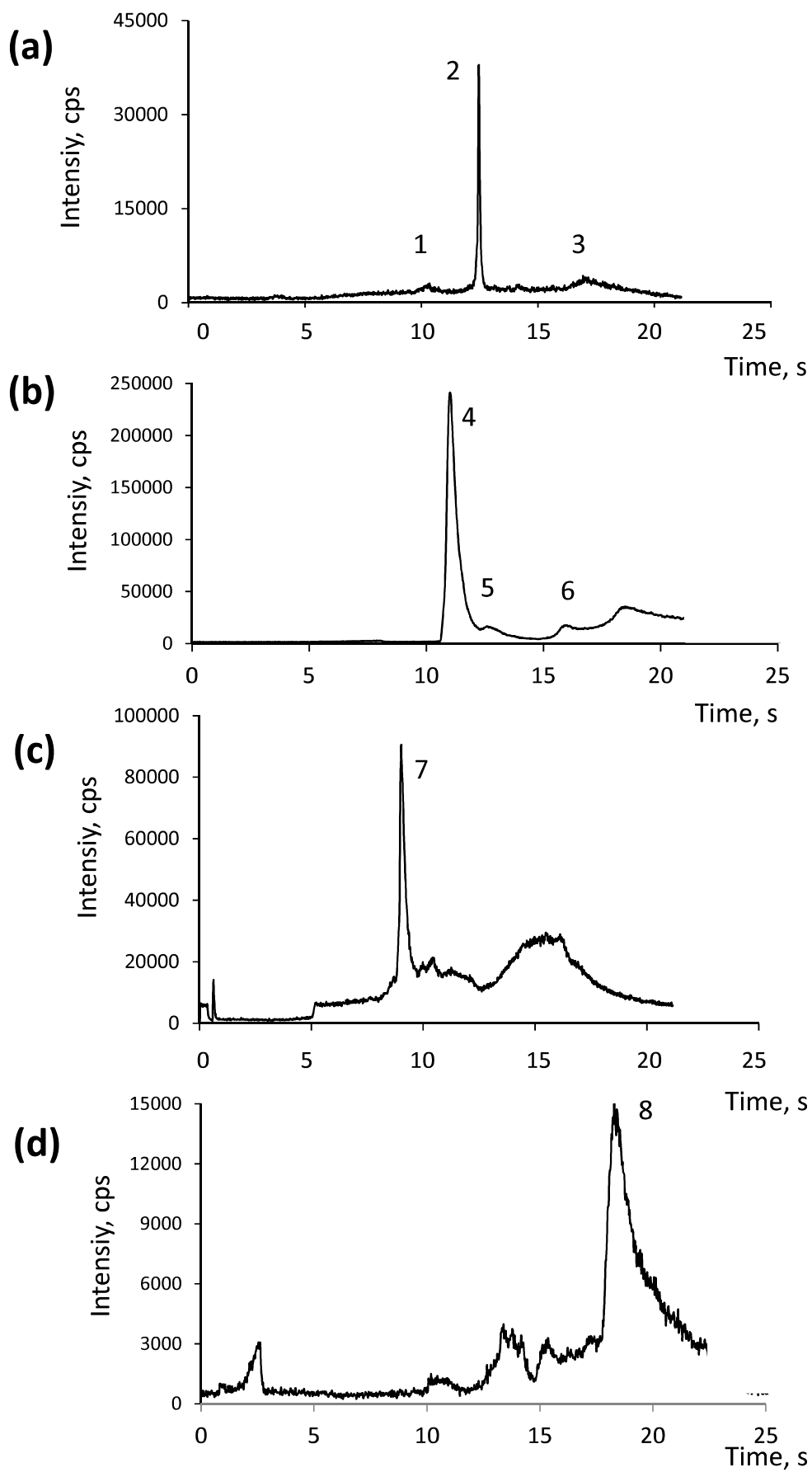


Figure 4
[Click here to download Figure\(s\): Fig. 4 HILIC-ICP MS.pdf](#)



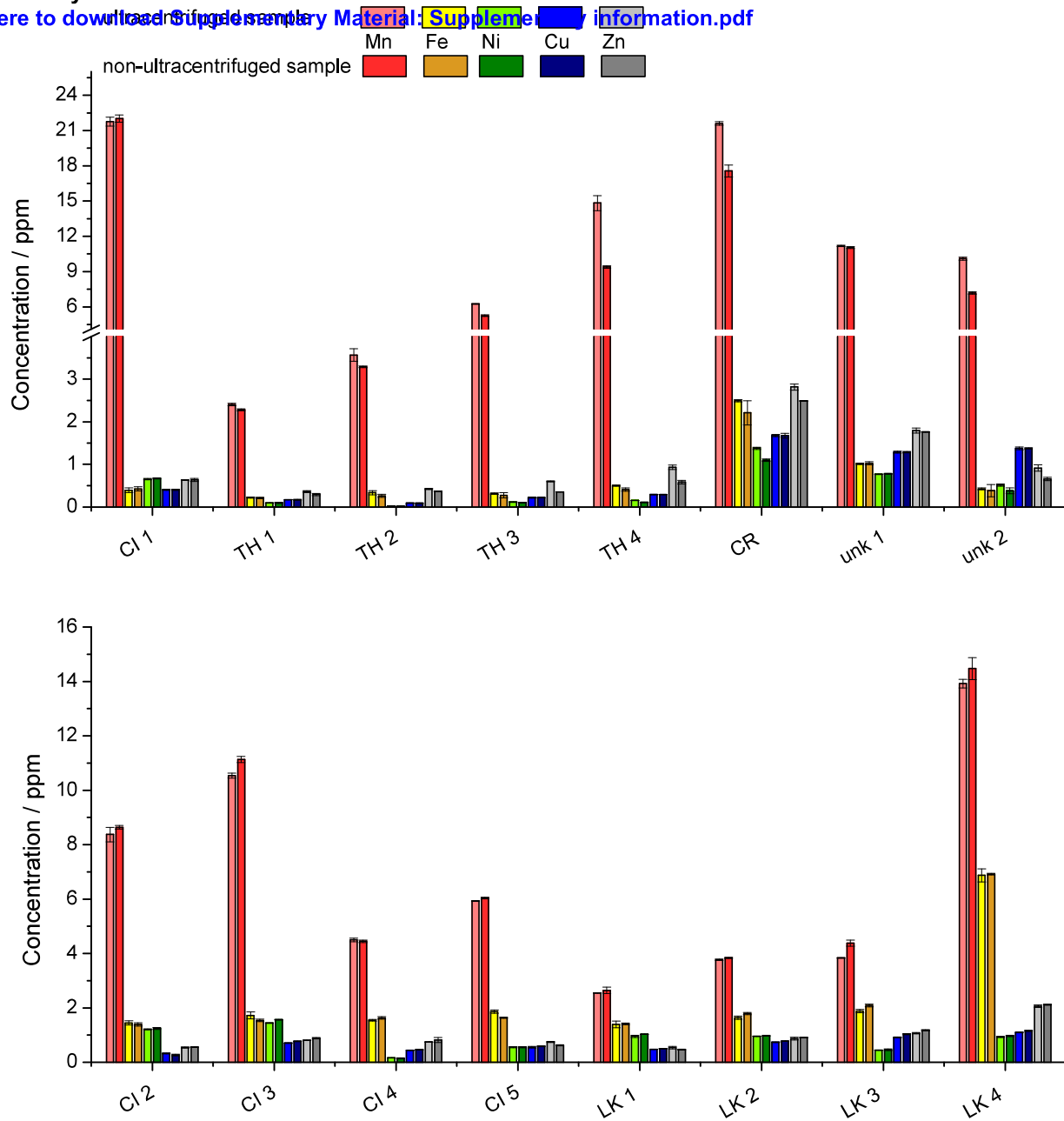


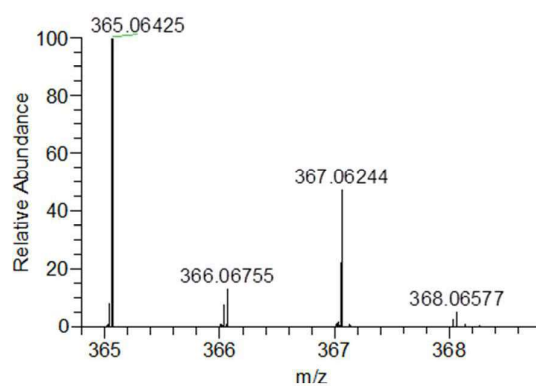
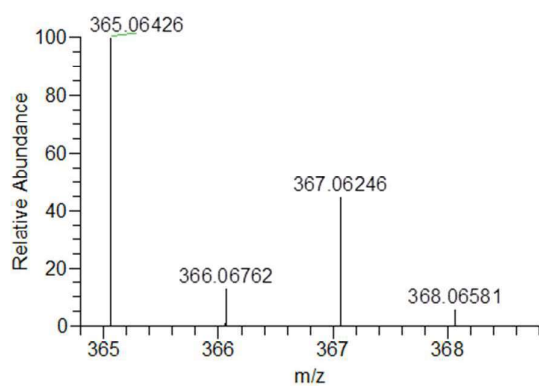
Figure 1 SI

Cu

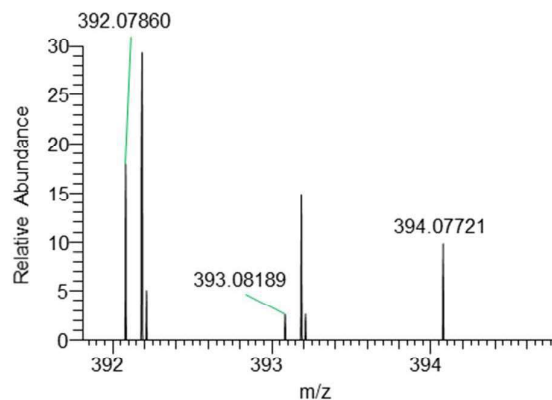
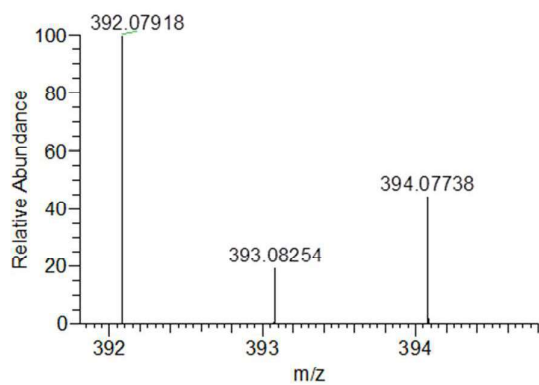
Theoretical m/z

Experimental m/z

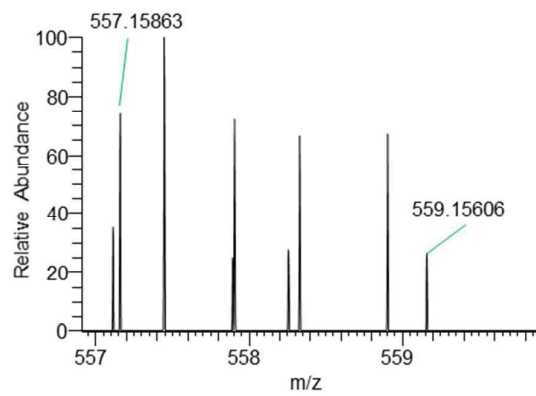
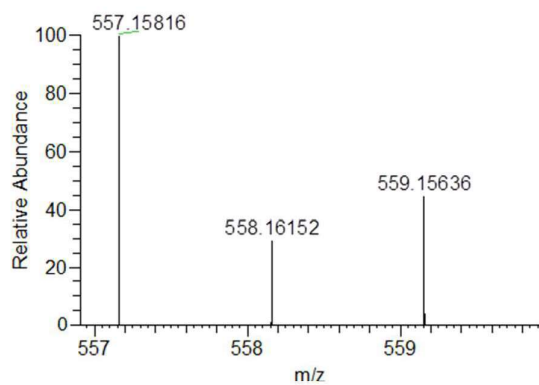
$\text{Cu}^{\text{II}}\text{NA} - \text{C}_{12}\text{H}_{19}\text{O}_6\text{N}_3\text{Cu}$



$\text{Cu}^{\text{II}}(\text{Phe})_2 - \text{C}_{18}\text{H}_{20}\text{O}_4\text{N}_2\text{Cu}$

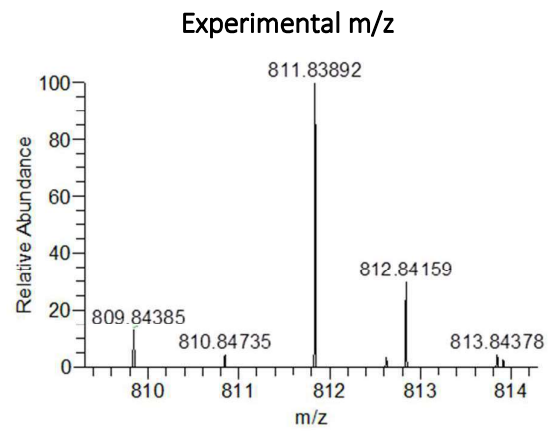
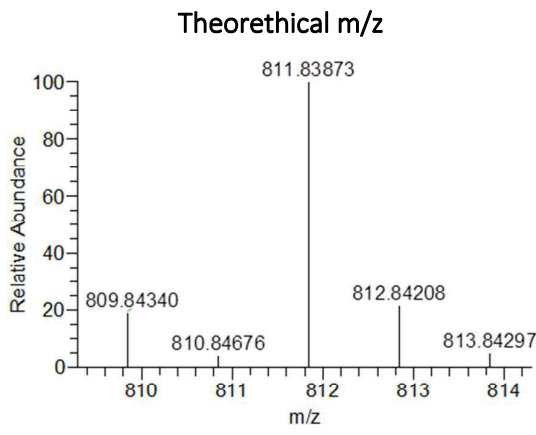


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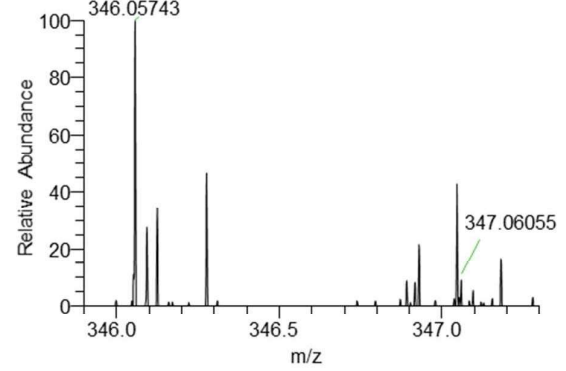
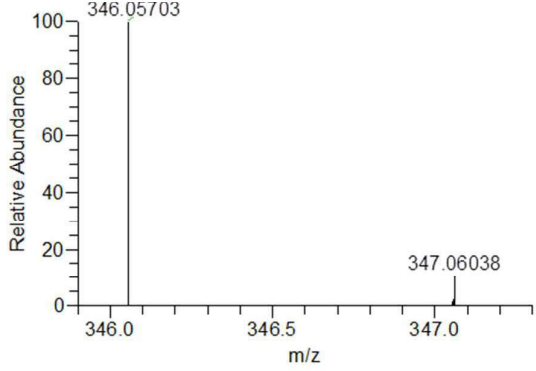


Fe

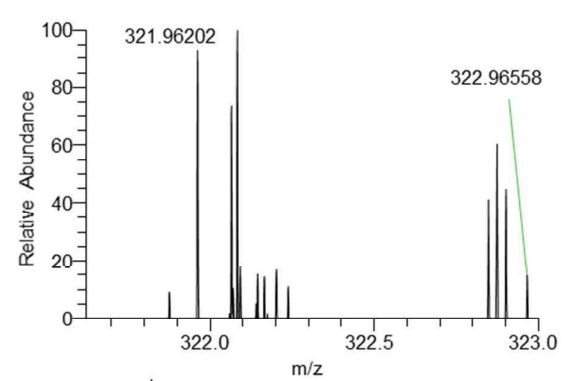
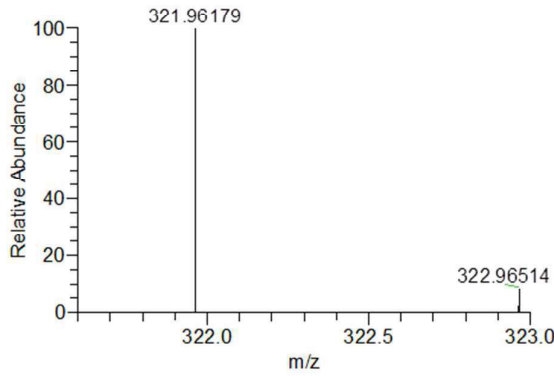
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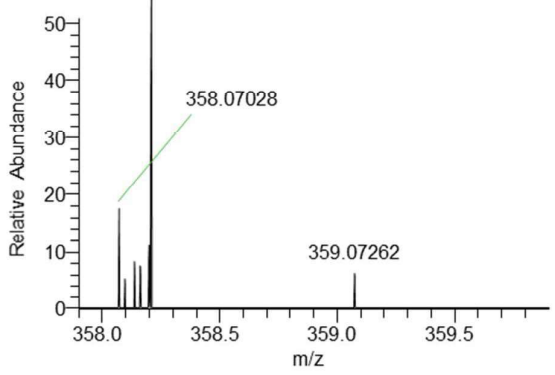
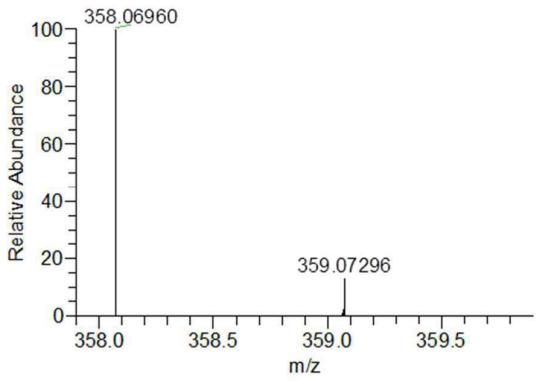
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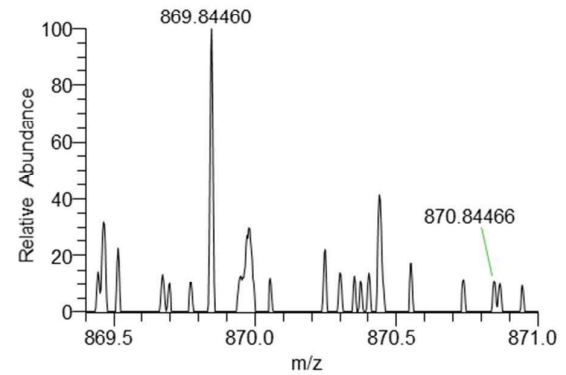
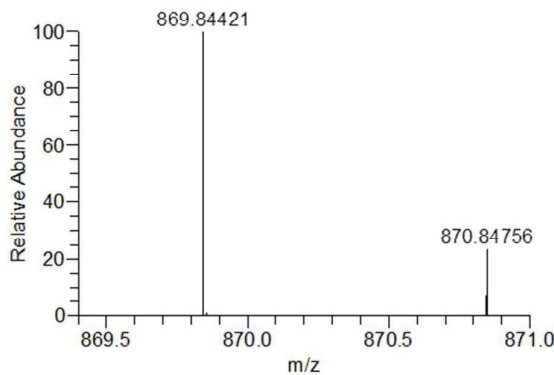
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$\text{Fe}^{\text{II}}(\text{NA}) - \text{C}_{12}\text{H}_{19}\text{O}_6\text{N}_3\text{Fe}$



$\text{Fe}^{\text{III}}_3\text{Mal}(\text{Cit})_3 - \text{C}_{22}\text{H}_{21}\text{O}_{26}\text{Fe}_3$

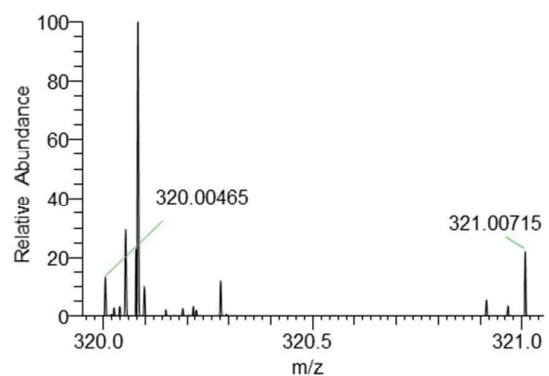
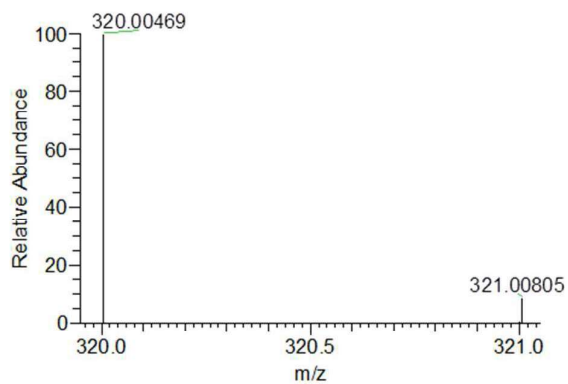


Mn

Theoretical m/z

Experimental m/z

$\text{Mn}^{II}(\text{Asp})_2 - \text{C}_8\text{H}_{13}\text{O}_8\text{N}_2\text{Mn}$

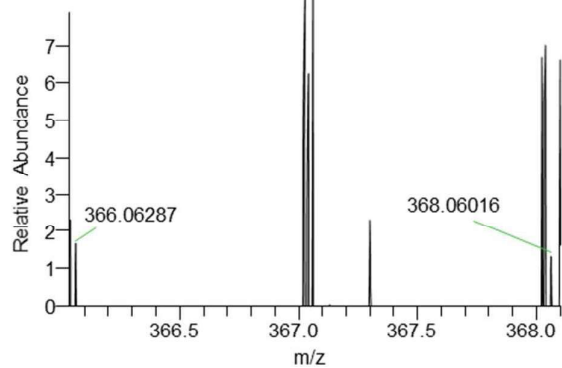
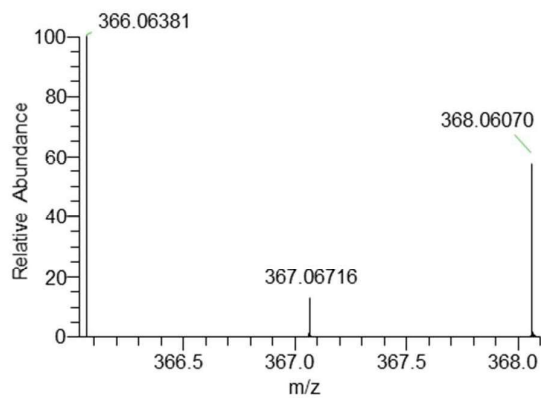


Zn

Theoretical m/z

Experimental m/z

$\text{Zn}^{II}(\text{NA}) - \text{C}_{12}\text{H}_{19}\text{O}_6\text{N}_3\text{Zn}$



$\text{Zn}^{II}(\text{Cit})_2 - \text{C}_{12}\text{H}_{14}\text{O}_{14}\text{Zn}$

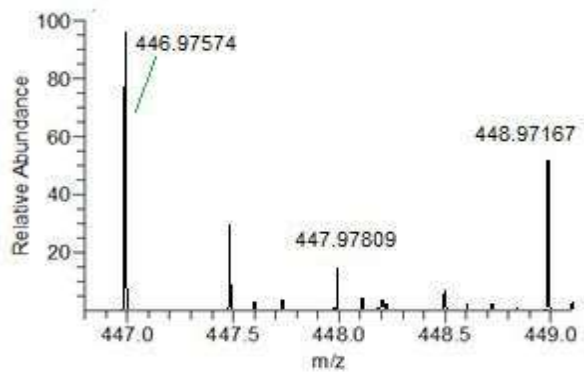
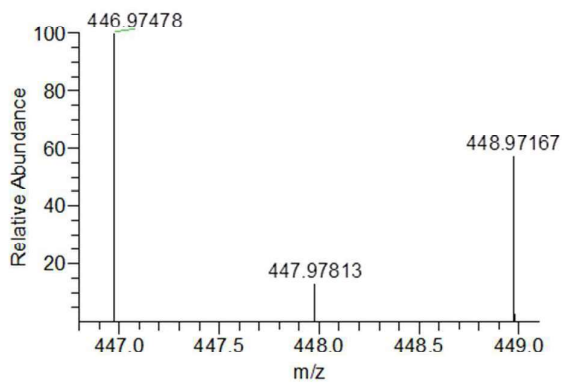


Figure 2 SI

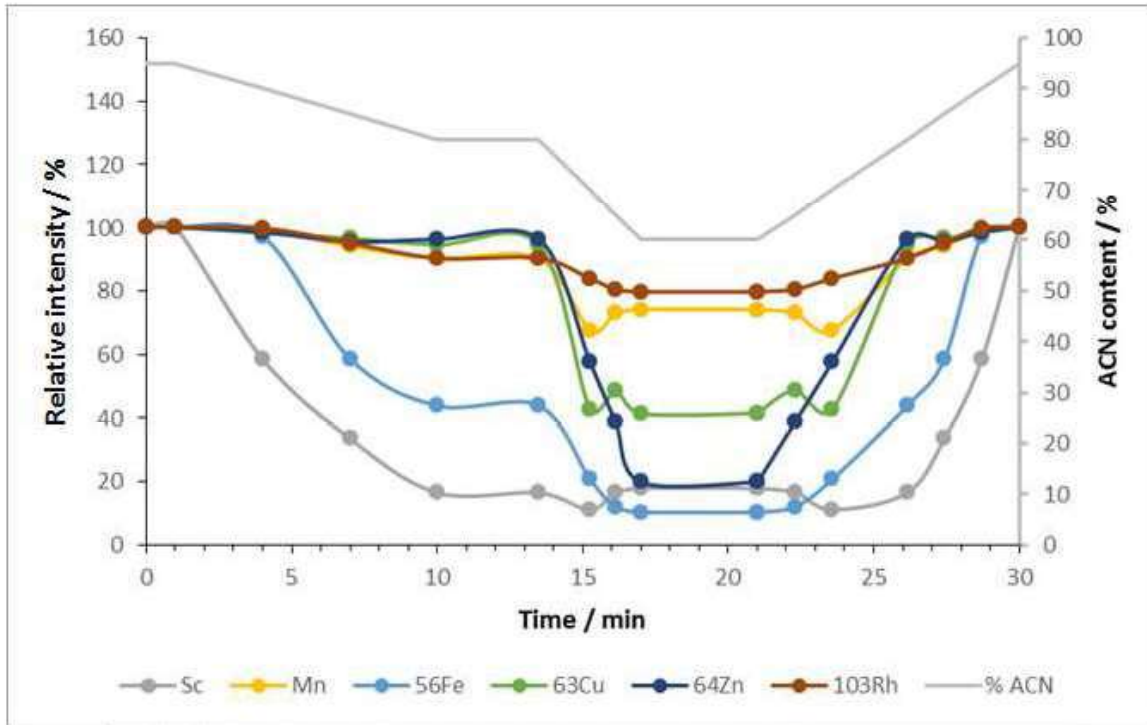


Figure 3 SI

Table 1-SI. Total metal concentrations in the studied coconut water samples.

| Sample name | Manganese, ppm | | Iron, ppm | | Copper, ppm | | Zinc, ppm | |
|-------------|----------------|------|-----------|------|-------------|------|-----------|------|
| | mean | SD | mean | SD | mean | SD | mean | SD |
| CI 1 | 21.7 | 0.38 | 0.39 | 0.06 | 0.40 | 0.01 | 0.63 | 0.01 |
| CI 2 | 8.37 | 0.26 | 1.45 | 0.08 | 0.33 | 0.02 | 0.54 | 0.02 |
| CI 3 | 10.54 | 0.10 | 1.72 | 0.12 | 0.71 | 0.01 | 0.81 | 0.01 |
| CI 4 | 4.50 | 0.07 | 1.55 | 0.03 | 0.43 | 0.00 | 0.74 | 0.01 |
| CI 5 | 5.93 | 0.02 | 1.86 | 0.06 | 0.55 | 0.03 | 0.74 | 0.02 |
| LK 1 | 2.54 | 0.01 | 1.39 | 0.12 | 0.47 | 0.01 | 0.54 | 0.05 |
| LK 2 | 3.77 | 0.03 | 1.64 | 0.06 | 0.73 | 0.01 | 0.87 | 0.05 |
| LK 3 | 3.84 | 0.01 | 1.88 | 0.06 | 0.92 | 0.02 | 1.07 | 0.02 |
| LK 4 | 13.9 | 0.16 | 6.87 | 0.24 | 1.10 | 0.01 | 2.06 | 0.05 |
| TH 1 | 2.40 | 0.03 | 0.22 | 0.01 | 0.17 | 0.00 | 0.36 | 0.02 |
| TH 2 | 3.56 | 0.15 | 0.33 | 0.05 | 0.09 | 0.00 | 0.42 | 0.01 |
| TH 3 | 6.24 | 0.03 | 0.31 | 0.01 | 0.22 | 0.01 | 0.60 | 0.01 |
| TH 4 | 14.8 | 0.63 | 0.50 | 0.01 | 0.29 | 0.01 | 0.93 | 0.05 |
| CR | 21.6 | 0.15 | 2.49 | 0.03 | 1.68 | 0.03 | 2.82 | 0.07 |
| unk 1 | 11.2 | 0.07 | 1.01 | 0.02 | 1.29 | 0.02 | 1.79 | 0.06 |
| unk 2 | 10.1 | 0.13 | 0.43 | 0.03 | 1.37 | 0.03 | 0.91 | 0.08 |