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1 Speciation of metals in indigenous plants growing in
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4 *Hypericum laricifolium*

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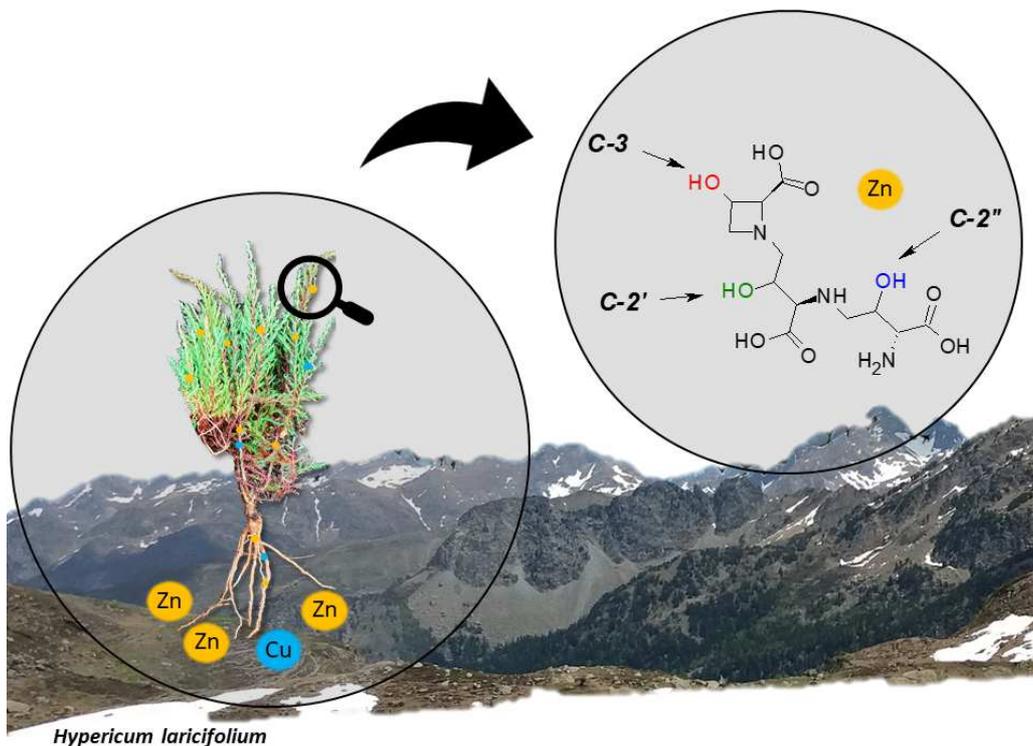
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14 ABSTRACT. Ag, As, Cu, Pb and Zn were found to be the principal metallic contaminants of a
15 post-mining area of Peru (Hualgayoc, Cajamarca). Study of metal distribution amongst roots,
16 stems, and leaves of four indigenous hypertolerant plant species, *Arenaria digyna*, *Puya* sp,
17 *Hypericum laricifolium*, *Nicotiana thyrsiflora* indicated significant translocation of Zn
18 ($0.6 < TF \leq 10.0$) and Cu ($0.4 < TF \leq 6.5$) into aerial plant organs and substantial water-extractable
19 fraction (20-60 %) of these metals, except for *A. digyna* (root and stems). A study of the metal
20 speciation by ultrahigh-performance size-exclusion (fast-SEC) and hydrophilic ion interaction
21 (HILIC) liquid chromatography with dual ICP (inductively coupled plasma) and electrospray
22 (ESI) Orbitrap MS detection revealed the presence of nicotianamine and deoxymugineic acid
23 copper and zinc complexes in roots, stem and leaves of *N. thyrsiflora* and *Puya* sp., and
24 nicotianamine alone in *A. digyna*. A previously unreported compound, dihydroxy-nicotianamine
25 was identified as the most abundant Cu and Zn ligand in *H. laricifolium*. The presence of
26 arsenobetaine and an arsenosugar was confirmed by ESI MS. Ag and Pb were hardly
27 translocated to leaves and were found as high molecular species; one of the Pb-containing
28 species co-eluted in fast-SEC- ICP MS with rhamnogalacturonan-II-Pb complex commonly
29 found in in the walls of plants.

30 KEYWORDS: post-mining areas, Andean flora, hyphenated techniques, metal speciation, low
31 molecular complexes, dihydroxynicotianamine.



33

34 INTRODUCTION

35 Peru is the second largest producer of copper (Cu), silver (Ag), and zinc (Zn) worldwide (1).
36 The contamination by mining environmental liabilities - including mine adits, prospecting pits,
37 waste dumps, shafts, open pits, trenches, old mine camps, old drill pads and non-mineralized pre-
38 strip material (2) - raises interest in native plants able to grow in such harsh conditions, with the
39 aim to use them for soil recultivation and phytoremediation. The molecular understanding of the
40 mechanisms allowing plants to adapt and grow in heavily contaminated post-mining areas is
41 critically dependent on the knowledge of the chemical species (metal-complexes) involved in the
42 uptake and transport of metals to the above-ground organs (stems and leaves) (3-5).

43 While there have been numerous studies of metal speciation in model plants grown in
44 hydroponics (6-13), studies of plants sampled in post-mining areas have been mostly limited to
45 the demonstration of the high total concentrations of accumulated metals. Information on metal
46 speciation has been scarce. These speciation studies have been carried out by synchrotron X-ray
47 absorption spectroscopy (XAS) and are thus restricted to the information about the coordination
48 environments of metal ions without revealing the molecular structure of the metal complexes
49 (14-18). In addition, these techniques are based on the comparison of the obtained spectra with
50 standards, and thus are incapable of untargeted (exploratory) analysis.

51 Molecular information on the metal species identity can be obtained by the combination of
52 their chromatographic fractionation with on-line mass spectrometric detection using: i) elemental
53 techniques (Inductively Coupled Plasma Mass Spectrometry, ICP-MS) in order to detect and
54 quantify metals, and ii) molecular techniques (Electrospray Ionization Mass Spectrometry, ESI-
55 MS), in order to identify the species (19). After a careful optimization, the approach was
56 demonstrated to be suitable to plant fluids (phloem (6) and xylem (19)) and water-soluble species
57 involved in transport and translocation. Although the majority of studies used hydroponically
58 grown plants, some studies led to a successful identification and quantification of metal-
59 speciation in wild-type plants. For example, a number of species of Ni (in hyperaccumulating
60 plants *Sebertia acuminata* (20) and *Thlaspi caerulescens* (21)), Al (22), and Fe (23) have been
61 reported. In comparison with XAS, hyphenated techniques require extraction and thus are
62 potentially vulnerable to species exchange between cells of different types and species-
63 transformations. However, for the few studies having used both approaches, data obtained from
64 XAS and hyphenated techniques were found in good agreement, which suggests that these
65 concerns may be exaggerated (24, 25). Indeed, the presence of As-S bond detected by EXAFS in

66 plant tissues was confirmed by the LC-ES-MS/ICP-MS identification of As-phytochelatin
67 complexes extracted from the fresh plant material without degradation or formation of artefacts
68 during the sample preparation step (24); a similar comparison was presented for selenium species
69 in yeast (25). While simple identification of metal species in wild-type plants has already been
70 challenging, data on the quantitative distribution of the metals amongst the identified complexes
71 have been even scarcer. The difficulties of quantification result from (i) the difference in the
72 behavior of the synthetic standards and species naturally present in the sample, and (ii) the risk
73 of modification of the equilibrium in the case an amount of standard is added to the sample.
74 Indeed, the stability and stoichiometry of metal complexes are dependent on the chemical
75 environment and are difficult to control, especially when using natural samples (26).

76 The goal of this study was to investigate, by means of the state-of-the-art hyphenated
77 techniques, the metal speciation in four native Andean plants growing in a post-mining metal
78 contaminated area, in order to identify the ligands responsible for the uptake and metal transport
79 to the aboveground organs.

80 EXPERIMENTAL

81 **Samples.** Four native plants growing in a post-mining metal contaminated area in the Andes
82 of northern Peru were studied. They included: *Arenaria digyna*, *Puya sp.*, *Hypericum*
83 *laricifolium* and *Nicotiana thyrsoflora*. The taxonomic identification was carried out by Dr. M.
84 Timaná (Pontificia Universidad Católica del Perú, Lima, Peru) and M.Sc. P. Gonzales Arce
85 (Laboratory of Floristics of the Herbarium of the Natural History Museum of the Universidad
86 Nacional Mayor de San Marcos, Peru). The plants were sampled in the Hualgayoc mining
87 district, that since Spanish colonial times has been known for Ag-rich polymetallic

88 mineralization, along with Zn, Pb, and Cu. Due to the long history of mining and, until recently,
89 the lack of appropriate legal regulations regarding mine closure, has nearly 1,000 catalogued
90 MEL sites. The sampling area is in the west of the province of Hualgayoc, department of
91 Cajamarca in the vicinity of a leach pad (a latitude 6°44'49" S, longitude 78°35'36" W and
92 altitude of 3412 m above sea level) is situated on top of a mine waste deposit which has no
93 drainage. The soil parameters are given in **Table SI-1** (27).

94 **Reagents.** The reagents used for digestions, dilutions, and the preparation of HPLC mobile
95 phases were obtained from Sigma-Aldrich (www.sigmaaldrich.com) unless specified otherwise.
96 The following were used: ammonium acetate ($\geq 98\%$ for molecular biology), nitric acid (70%,
97 www.fishersci.com), acetonitrile ($\geq 99.9\%$) and hydrogen peroxide (30%). Standard solutions
98 (1000 ppm, www.scpscience.com) of Pb, Ag, Cu, Zn, As, were used for the preparation of
99 calibration curves and Sc and Rh as internal standards. Deionized water (18.2 M Ω .cm) obtained
100 from a Milli-Q® Type 1 Ultrapure Water Systems (www.merckmillipore.com) was used
101 throughout unless stated otherwise.

102 **Instrumentation.** The ICP MS spectrometers were ICP-MS 7500 (www.agilent.com)
103 equipped with an integrated autosampler (I-AS) used for the total analysis and Agilent 7700x
104 (www.agilent.com) used for the coupling with HPLC. The electrospray ionization mass
105 spectrometer was Orbitrap Fusion™ Lumos™ (www.thermofisher.com). The chromatographic
106 systems used for the separation of the analytes were: Agilent 1200 Series (www.agilent.com) and
107 Dionex Ultimate 3000 RS (www.thermofisher.com). Samples were centrifuged using a MiniSpin
108 and 5415 R centrifuges (www.eppendorf.com).

109 **Sample preparation.** *Initial sample preparation.* The plants were washed with tap water (for
110 removing soil remains) and then with distilled water. Then, plants were separated into leaves,
111 stems, and roots, and dried at 30-40 °C before grinding to obtain a fine powder.

112 *Total analysis.* The samples were digested in DigiPrep heated-block digester
113 (www.scpscience.com) using the following two-step program: (1) 1 ml of 70 % nitric acid at 85
114 °C for 4 h 30 min, after leaving the sample with acid overnight, (2) 0.5 ml of 30 % hydrogen
115 peroxide at 85°C for 4.5 h. The digest was diluted to 5 ml with water prior to analysis by ICP-
116 MS.

117 *Extraction and mass balance.* 0.05 g of dried leaf, stem or root sample was shaken on a
118 horizontal shaker with 2.5 mL of 25 mM ammonium acetate pH 7 for 1 h at 400 rpm and
119 centrifuged for 5 min at 13 200 rpm. The supernatant was recovered and analyzed by HPLC. The
120 supernatants (250 µL) and the corresponding residues (after having been dried) were digested as
121 described above prior to the analysis by ICP-MS.

122 **Determination of the Total Metal Content.** Samples were analyzed by ICP mass
123 spectrometry (ICP-MS) using a reaction cell pressurized with He and H₂ gas. The isotopes
124 monitored were ¹⁰⁷Ag, ¹⁰⁹Ag; ⁷⁵As, ⁶³Cu, ⁶⁵Cu; ²⁰⁶Pb, ²⁰⁷Pb, ²⁰⁸Pb; ⁶⁴Zn, ⁶⁶Zn and ⁶⁸Zn.
125 Analytical blanks were analyzed in parallel. The Standard Reference Material 1573a Tomato
126 Leaves (NIST, Gaithersburg, MD 20899, USA) was used for quality control.

127 Quantification was performed in the calibration range of 0.2-100 ppb for Zn and 0.1-20 ppb for
128 the other elements; an 8-point calibration curve was used. Measurements were carried out in
129 triplicate; the results with relative standard deviation higher than 10% were discarded and the
130 measurements repeated. Values are reported as mean ± standard deviation (SD) of three
131 analytical replicates.

132 **Speciation of Metal Complexes. Chromatography.** The chromatographic conditions used
 133 are detailed in **Table 1**. For fast SEC separations, prior to injection, the supernatant from the
 134 extraction was mixed (1:1) with the eluent or ACN and centrifuged. Effluents from
 135 chromatographic column were analyzed directly or after post-column ACN addition (0.3
 136 mL/min); ACN was delivered with an Agilent 1200 Series system. For HILIC separations, the
 137 supernatant was mixed, prior to injection, with ACN (1:4). As the addition of ACN may create a
 138 small amount of precipitate, the sample was centrifuged for 5 min at 13 200 rpm.

139 **Table 1.** Chromatographic conditions used.

separation mechanism	fast size-exclusion chromatography (fast-SEC)	hydrophilic interactions liquid chromatography (HILIC)	
column	ACQUITY UPLC BEH 125 SEC (1.7 μ m 4.6 x 150 mm); 1-80 kDa	TSKgel Amide-80 (2.0 μ m, 2.0 x 150 mm)	
mobile phase	ammonium acetate 25 mM, pH=7	A: ammonium acetate 10 mM pH=7; B: ACN	
injection volume, μ L	5	10	
flow rate, mL/min	0.3	0.1	0.2
elution	isocratic	0 min -80 % B 2.5 min - 80 % B 22.5 min -50 % B 25 min -50 % B 26 min -80 % B 35 min -80 % B	0 min -80 % B 2.0 min - 80 % B 3.0 min - 65% B 4.5 min - 65% B 9.0 min - 60% B 12 min - 60% B 13.8 min -50 % B 16.8 min -50 % B 17.8 min -80 % B 25 min -80 % B

140 *ICP MS detection.* The exit of the column was directly connected to an ICP mass spectrometer
 141 equipped with a collision cell. O₂ was used as an optional gas (5%). Platinum cones were used.

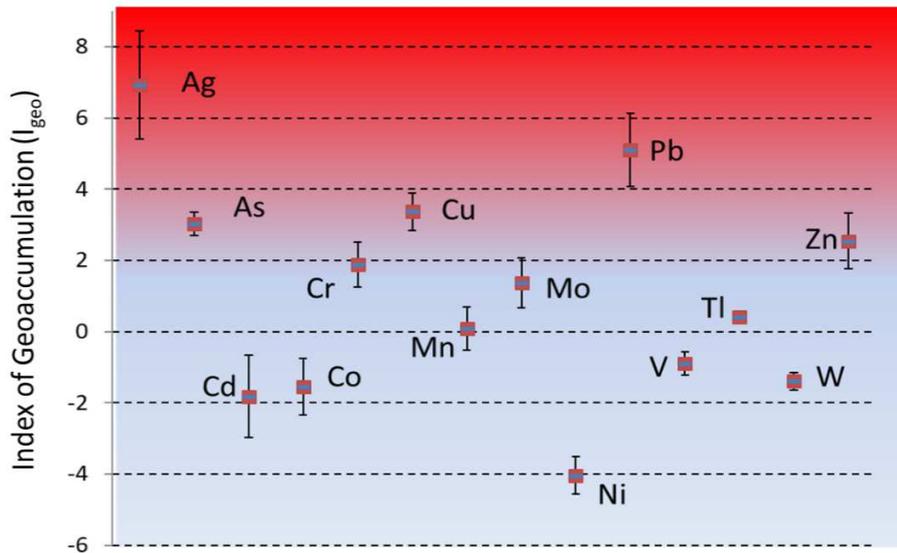
142 *Electrospray MS.* The analyses were performed in positive ionization mode with ion source
 143 conditions set as default, according to the current LC flow – 0.3 mL/min and 0.1-0.2 mL/min for
 144 Fast-SEC and HILIC, respectively, with post-column ACN addition at a flow rate of 0.3 mL/min

145 to enhance ionization for Fast-SEC. Data were acquired at resolution 240 000 in a scan range of
146 150-1000 m/z with a maximum injection time of 502 ms, $5e^5$ AGC target and 30% RF lens.
147 MS/MS was performed in a targeted analysis mode, based on the results from the FullScan mode
148 (Full MS/dd-MS²). The inclusion mass list was prepared on the basis of the already known
149 metal-containing compounds as well as compounds with metal characteristic isotopic patterns.
150 To confirm the presence those compounds, Thermo Scientific™ Compound Discoverer™ with
151 Pattern Scoring Node were used. Full MS/dd-MS² was performed as follows: 1) FullScan MS:
152 orbitrap resolution 240 K; scan range m/z 150-1000; maximum injection time 300 ms; AGC
153 target 80000; RF lens 30%; 2) MS² of targeted ions was performed in the HCD cell with a
154 collision energy of 30%; orbitrap resolution 60 K; maximum injection time 118ms; AGC target
155 75000 with the first mass of 100 m/z .

156 RESULTS AND DISCUSSION

157 **Choice of Elements of Interest.** Target elements were chosen on the basis of the soil
158 contamination assessed by using the Index of Geoaccumulation (I_{geo}) (28). The concentrations of
159 14 elements found in the soil where the plants were collected (**Table SI-2**) (27) were compared
160 with their abundances in the Earth's crust (29) and with the geochemical baseline values near the
161 sampling sites (**Table SI-3**) (30). The degree of contamination (31) depended on the element
162 (**Figure 1**). The soils were found to be extremely contaminated with Ag and Pb ($I_{geo} \geq 4$),
163 strongly contaminated with As, Cu, Zn ($2 \leq I_{geo} < 4$), moderately contaminated with Cr, Mo, Tl
164 ($1 \leq I_{geo} < 2$) and uncontaminated with other elements (Cd, Co, Mn, Ni, V, W, $I_{geo} < 1$). The five
165 elements for which the contamination was the highest, (Ag, As, Cu, Pb and Zn), were selected
166 for the study of their uptake and translocation in plants. As demonstrated in the previous study

167 concerning this area, these elements were mainly present in soil fractions with limited metal
168 mobility (mineral or bound to hydrated iron and manganese oxides) (32).



169
170 **Figure 1.** Index of Geoaccumulation (I_{geo}) for selected elements present in the soil in the
171 contaminated post-mining site.

172 **Choice of the Studied Plants.** All the plants growing in post-mining environmental
173 liabilities areas are indigenous to the Andes. *Nicotiana thyrsiflora* belongs to *Nicotiana genus*
174 which is important in traditional medicine in South America as a source of alkaloid nornicotine
175 (33). It is one of the most comprehensively studied flowering plant genus (34),(35) and raises
176 interest for environmental restoration activities (36).

177 *Hypericum laricifolium* (*Hypericaceae*) is distributed from western Venezuela along the
178 cordilleras of Colombia and Ecuador to central Peru (37). Its aerial parts are rich in phenolic
179 acids, flavonoids, triterpenoids (38), xanthones (39), and dimeric acylphloroglucinol derivatives
180 (40) and their infusion is used in traditional medicine (41). It is a source of essential oils
181 (acylphloroglucinol derivatives) (42) (43).

182 *Puya sp.* forms clumps of small 8-10 inch wide rosettes of very narrow powdery blue-gray
183 leaves and has no stem. The heart of the inflorescence of *Puya sp.*, known as "aguarongo", is
184 cooked and eaten in Ecuador (44) and is an important nutritious resource for hummingbirds, (45)
185 (46). *Puya sp.* tea infusions are known for antioxidant and aldose reductase inhibitory activity
186 (47).

187 *Arenaria digyna* belongs to family of *Caryophyllaceae* and is one of indicator species for high
188 altitude bofedales – type of wetlands found in Peruvian and Chilean Andes (48). Several species
189 of this family are used as traditional medicine (49).

190 **Bioaccumulation and Translocation Factors.** The total concentration of Ag, As, Cu, Pb
191 and Zn in individual plant organs are given in **Table 2**.

192 For As, Pb and Zn, the leaves of all the studied plants showed metal concentrations higher than
193 the maximum reference values generalized for mature leaf tissues from various plant species
194 (50). Moreover, Zn concentrations in leaves of *N. thyrsiflora* significantly exceeds the level of
195 excessive/toxic concentration (100-400 ppm). In the case of copper and silver, these values (50)
196 were exceeded in *N. thyrsiflora* and *A. digyna*.

197 The data on the total element concentration in plant organs allowed for the calculation of the
198 bioaccumulation (*BCF*) and translocation factors (*TF*) in order to assess the ability of plants to
199 mobilize elements from the soil (*BCF*) and translocate (*TF*) them from roots to aerial parts (51).
200 The bioaccumulation factors were lower than 1. The translocation factors were exceptionally
201 high for Zn (*N. thyrsiflora*, *H. laricifolium*, and *Puya sp.*) and Cu (*N. thyrsiflora*).

202

203 **Table 2.** Total metal concentrations (mg kg⁻¹) (mean ± SD) of As, As, Cu, Pb and Zn in different organs of the native plants the
 204 Hualgayoc district, Cajamarca region, Peru.

Native species	Organs	Ag		As		Cu		Pb		Zn	
<i>Puya sp</i>	L	0.52	± 0.01	8.33	± 0.14	13.3	± 0.82	28.5	± 5.31	145	± 2.57
	R	2.20	± 0.01	45.3	± 0.93	22.6	± 0.41	105	± 11.8	23.4	± 2.06
	<i>Bioaccumulation factor</i>	0.04		0.03		0.05		0.02		0.50	
	<i>Translocation factor</i>	0.23		0.18		0.59		0.27		6.20	
<i>Arenaria digyna</i>	L	3.24	± 0.13	8.29	± 0.14	77.6	± 0.55	286	± 46.0	390	± 8.6
	S	10.49	± 0.10	110.3	± 1.3	123	± 1.18	2114	± 46.5	447	± 8.8
	R	15.55	± 3.90	140.6	± 48.4	132	± 12.4	2205	± 103	590	± 12.4
	<i>Bioaccumulation factor</i>	0.14		0.02		0.15		0.10		1.97	
	<i>Translocation factor</i>	0.20		0.06		0.59		0.13		0.66	
<i>Hypericum laricifolium</i>	L	1.30	± 0.10	3.77	± 0.10	9.71	± 0.74	126	± 3.06	305	± 4.73
	S	0.81	± 0.02	1.07	± 0.05	9.12	± 0.95	147	± 5.39	164	± 3.46
	R	7.30	± 0.80	7.66	± 0.45	21.53	± 3.37	434	± 54.9	153	± 14.6
	<i>Bioaccumulation factor</i>	0.06		0.01		0.03		0.04		0.57	
	<i>Translocation factor</i>	0.18		0.49		0.45		0.29		1.99	
<i>Nicotiana thyrsoflora</i>	L	2.97	± 0.22	2.41	± 0.07	48.5	± 0.57	41.8	± 2.05	1051	± 20.6
	S	0.51	± 0.03	0.50	± 0.01	6.30	± 0.34	45.0	± 5.29	546	± 30.2
	R	3.46	± 0.15	3.37	± 0.16	7.47	± 0.09	66.1	± 9.11	105	± 3.78
	<i>Bioaccumulation factor</i>	0.16		0.01		0.24		0.02		0.73	
	<i>Translocation factor</i>	0.86		0.71		6.49		0.63		10.0	
leaf tissue generalized for various species*		0.5		1-1.7		5-30		5-10		27-150	

205 L: leaf, S: stem, R: root

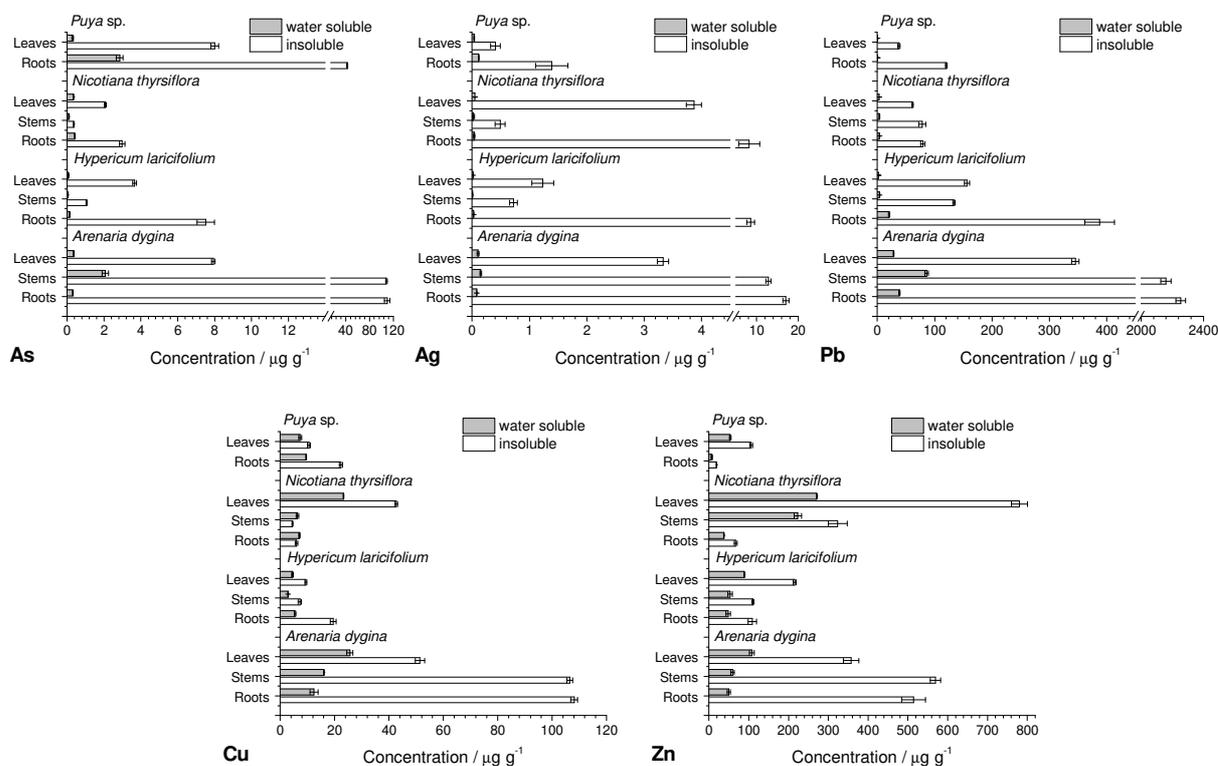
206 Bioaccumulation factor $BF = C_p/C_{so}$ (where C_p is the metal concentration in the plant leaves and C_{so} - in the soil)

207 Translocation factor $TF = C_s/C_r$ (where C_s is the metal concentration the leaves and C_r - in roots)

208 *according to: Kabata-Pendias, A., 2011. Trace Elements in Soils and Plants, 4th Ed. ed. CRC Press Taylor & Francis Group, Boca
 209 Raton London New York.

210

211 **Metal Water Soluble Fraction.** In order to get an insight into the ligands involved in metal
 212 translocation, the fraction of metals soluble in water was assessed. The data allowing for a
 213 comparison between the soluble and insoluble fraction are shown in **Figure 2**. These water-
 214 soluble fractions were highest for Cu and Zn, virtually regardless of the plant and similar for all
 215 the organs (root, stem and leaves) indicating the presence of a mobile pool of metal in the plant.
 216 For the other elements and plants, the mobile metal fraction was by far lower, and rarely
 217 exceeded 5%. In general, the water-soluble fraction percentage was negligible for Ag and Pb
 218 even if the total metal contents were relatively high.



219
 220 **Figure 2.** Ag, As, Cu, Pb and Zn fractionation into water soluble and insoluble fractions, in
 221 individual organs of the studied plants.

222 **Metal Speciation: the Approach.** Metal speciation was investigated coupling HILIC with
223 a parallel (not simultaneous) detection by ICP-MS and electrospray Orbitrap MS as described
224 elsewhere (19). In order to provide an independent set of chromatographic data, size-exclusion
225 chromatography was revisited. In particular, the regular size (10 x 300 mm, 13 μ m) column used
226 so far (19, 21, 52) was replaced by a ultrahigh performance (4.6 x 150 mm, 1.7 μ m) column.
227 This reduced considerably the analysis time (from 60 to 15 min), facilitated the online addition
228 of acetonitrile required to improve the species ionization, and increased sensitivity, as the peaks
229 were narrower than when using in the regular size SEC column.

230 Fast-SEC was inferior in terms of resolution to HILIC, but gave an estimation of the molecular
231 size distribution, including the high-molecular species which were precipitated (and thus could
232 not be analyzed) in the HILIC conditions. The metal recoveries were generally higher than 80%,
233 which is a satisfactory proof of good control of possible on-column dissociation effects and
234 carry-over. The results were considered as meaningful if similar data were obtained with both
235 fast-SEC and HILIC. Note that HILIC elutions are usually carried out with gradients, which
236 results in changing ionization conditions in ICP-MS and ESI-MS, and may affect the datasets
237 obtained (species more or less readily ionized).

238 In terms of MS, both targeted and exploratory approaches (based on the characteristic metal
239 isotopic patterns) were used for the search of metal species in the high-resolution high-accuracy
240 mass spectra of plant extracts. Elucidation of the structure of particular complexes was
241 completed by fragmentation studies of the complex (and free ligand, if present) using MSⁿ.

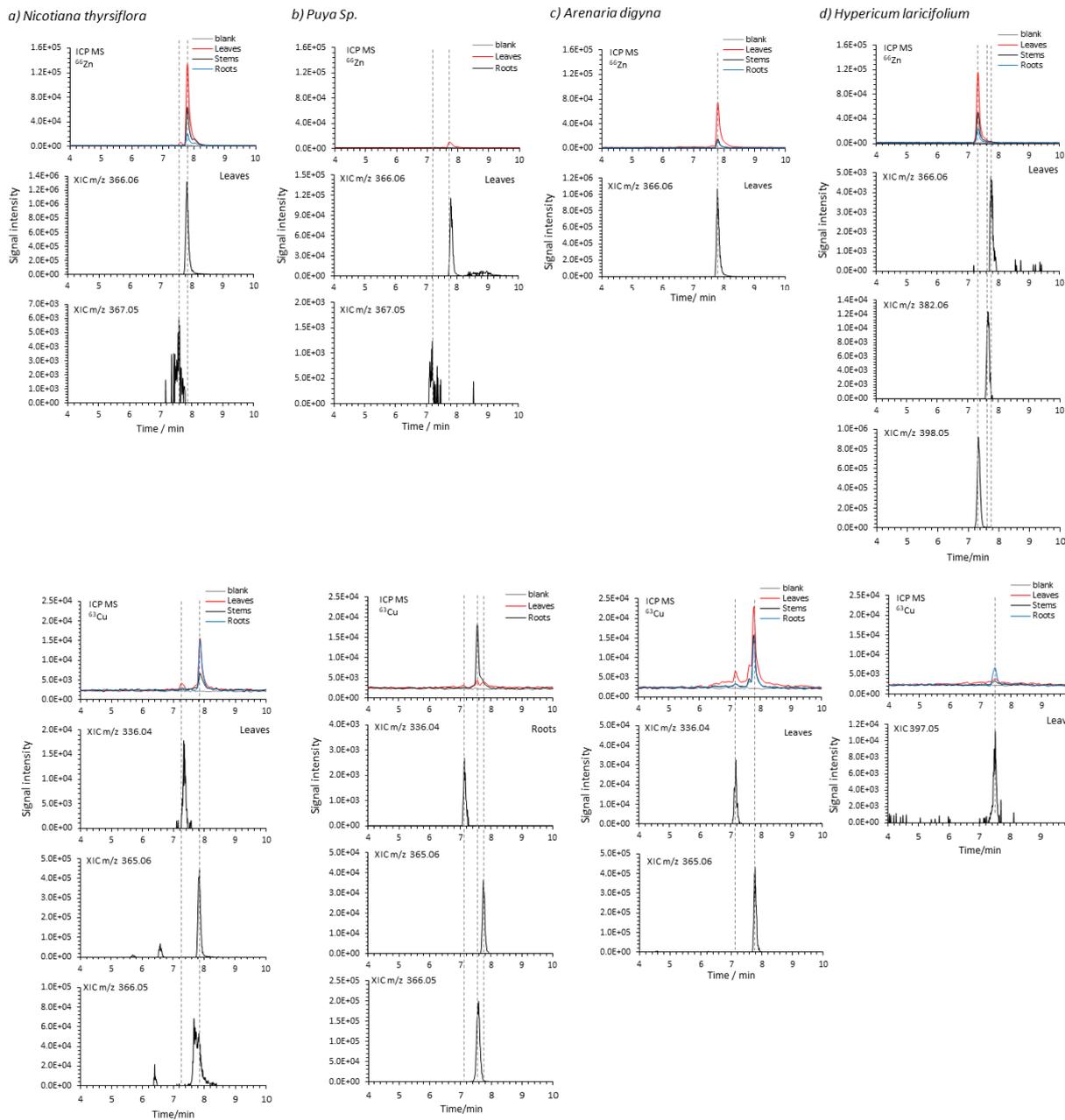
242 In terms of quantification, our strategy was based on the optimization of the HPLC conditions
243 to allow for individual metal species separation, good (close to quantitative) metal recovery, and
244 correction of the effect of the change in the mobile phase composition on the sensitivity. Indeed,

245 the intensity of signals obtained for Zn and Cu was the highest at ACN content of 80% and was
246 decreasing with the gradient changing to lower ACN percentage. Finally, to avoid this problem,
247 the FastSEC was applied to quantification, as the ACN concentration is constant throughout the
248 chromatographic run. The method employed here was based on the estimation of the ratio
249 between the signal intensity obtained for a specific compound (exact m/z) in different samples
250 based on LC ESI-MS results, and therefore can be considered as a semi-quantitative.

251 **Speciation of Zinc.** Figure 3 shows a set of HILIC – ICP MS/ESI MS chromatograms for
252 the speciation of Zn. The corresponding set of fast-SEC chromatograms is shown in **Figure SI-1**.
253 The compound with m/z 366.06 present in *Arenaria digyna*, *Nicotiana thyrsoflora*, and *Puya sp*,
254 and being only a trace in *Hypericum laricifolium*, corresponds to the Zn complex with
255 nicotianamine as demonstrated by MS/MS data (**Figure SI-2a**). Note that nicotianamine (NA) is
256 the most often reported LMW ligand complexing zinc in plants. Its stability constant ($\log K$) is
257 between 14.7 (53) and 15.4 (54). Zn-NA complex identification has been carried out both in
258 plant fluids (6),(19),(55),(52) and in extracts obtained upon plant tissue homogenization
259 (56),(13) (57). The Zn-NA complex was identified (as the only Zn species) in the phloem sap of
260 rice (6) and castor bean plants (*Ricinus communis*) (7), in the xylem of *Arabidopsis thaliana*
261 (55), in the xylem and embryo sac liquid of *Pisum sativum* (19), in coconut water (52), and in
262 hydroponically cultured lettuce leaves (13). Nicotianamine, glutathione and desGluPC₂ ligands
263 were identified in the Zn-containing SEC fractions of Tris-NaCl extracts of root and shoot tissues
264 of laboratory grown wild type *Arabidopsis halleri*, but no data for intact complexes were shown
265 in that study (56).

266 EXAFS spectra for *Thlaspi caerulescens* (Zn hyperaccumulator) indicate that nicotianamine
267 may be involved in Zn transport to aerial plant organs, but it is not a principal storage ligand

268 (58). In turn, XANES analysis of apple leaves exposed to Zn deficiency revealed the presence of
269 4 types of components: Zn-citrate, Zn-cell wall, Zn²⁺ and Zn-nicotianamine, wherein the largest
270 amount was associated with the cell wall (59). While studying Zn speciation in edible plants
271 grown in smelter contaminated soils by Zn K-edge XANES, the results were based on the use of
272 only three Zn complexes with histidine, malate, and cysteine so the results cannot be considered
273 as conclusive (60). In an XAS study of sweetcorn and maize, it was predicted that the large part
274 of Zn in the embryos was present as Zn-phytate, while in the endosperm the Zn was primarily
275 complexed with an N-containing ligand such as histidine and to a lesser extent with phytate (61).



276

277
 278 **Figure 3.** HILIC – ICP/ESI-MS chromatograms of water-soluble zinc and copper species in a)
 279 *Nicotiana thyrsoiflora*, b) *Puya sp.*, c) *Arenaria dygina* and d) *Hypericum laricifolium*.

280 In *N. thyrsiflora* and *Puya sp.*, Zn-nicotianamine was accompanied by the Zn-DMA complex
281 (m/z 367.05, confirmed by MS/MS data, **Figure SI-2a**). The contributions of Zn-DMA, which
282 has a lower complex formation constant (12.7) (62) than Zn-NA (ca. 15 (53, 54)), were much
283 lower, although free DMA was observed in the samples. The presence of DMA in *N. thyrsiflora*
284 is interesting because for a long time it has been thought that only graminaceous plants
285 biosynthesize MAs. However, at least two recent LC-TOF-MS studies reported the presence of
286 DMA in non-graminaceous plant species such as peanut (*Arachis hypogaea L.*) (63) and olive
287 trees (*Olea europaea L.*) (57). In the first one, the authors concluded that peanut's roots absorbed
288 Fe-DMA complex generated by DMA secreted from neighboring maize plants (63). Similarly,
289 the detected presence of Zn-DMA in lettuce was considered to be a contamination (13).
290 However, in the olive tree study of Suzuki *et al.*, possibility of intercropping was excluded and
291 DMA complexes were detected in both the xylem sap and leaves (57). The analysis of these two
292 samples - one analyzed directly (xylem sap) and the other one which required tissue
293 homogenization and extraction (leaves) - provided a mutual validation of these two approaches
294 (57). Marastoni *et al.* pointed out that more and more studies indicate difficulty in assigning only
295 one type of Fe acquisition strategy by different plant species. In their work, they showed that two
296 studied grapevine rootstock varieties exhibit different Fe acquisition strategies. Although they do
297 not belong to the graminaceous species, in their root's exudates they found mugineic acid
298 derivatives (64). Similar findings have been shown by Astolfi *et al.* (65). Their studies on tomato
299 plants support the hypothesis of double strategy of Fe acquisition by plants, previously discussed
300 for rice. In summary, more and more results indicate that clear distinction between Strategy I and
301 Strategy II plants (as graminaceous only), used so far, cannot be recognized as final, as the
302 overall picture is not so explicit.

303 In our case, although in field sampling intercropping could not be excluded, the presence of
304 free (apo) DMA in *N. thyrsoiflora* organs supports the detection of Zn-DMA (and also Cu-DMA)
305 complexes. The possibility of contamination during the analytical procedure (and, in particular,
306 chromatographic carryover) was carefully examined and discarded. The genes for DMA
307 biosynthesis were detected by transcriptome analysis of *Olea europaea L.* and it has been
308 suggested that NAAT and DMAS might have evolutionally loose substrate specificities in olive
309 plants in comparison with those in graminaceous plants (57). Indeed, recent studies stressed the
310 existence of several shared components of the metal complexing strategies among plants,
311 questioning the validity of the earlier concept of their mutual exclusivity (66).

312 Although nicotianamine and deoxymugineic acid are usually discussed in relation with metal
313 deficiency (67), studies have shown that NA may play a role in the rice tolerance to the Zn
314 excess (68). Indeed, in the case of the Zn excess, endogenous NA secretion in roots increased,
315 suggesting that NA is responsible for Zn tolerance, in addition to being the precursor of the
316 synthesis of DMA. Although the function of DMA in conditions of Zn excess is not known, the
317 synthesis of DMA in roots in such circumstances may also be involved in maintaining Zn
318 availability in rice plants (68).

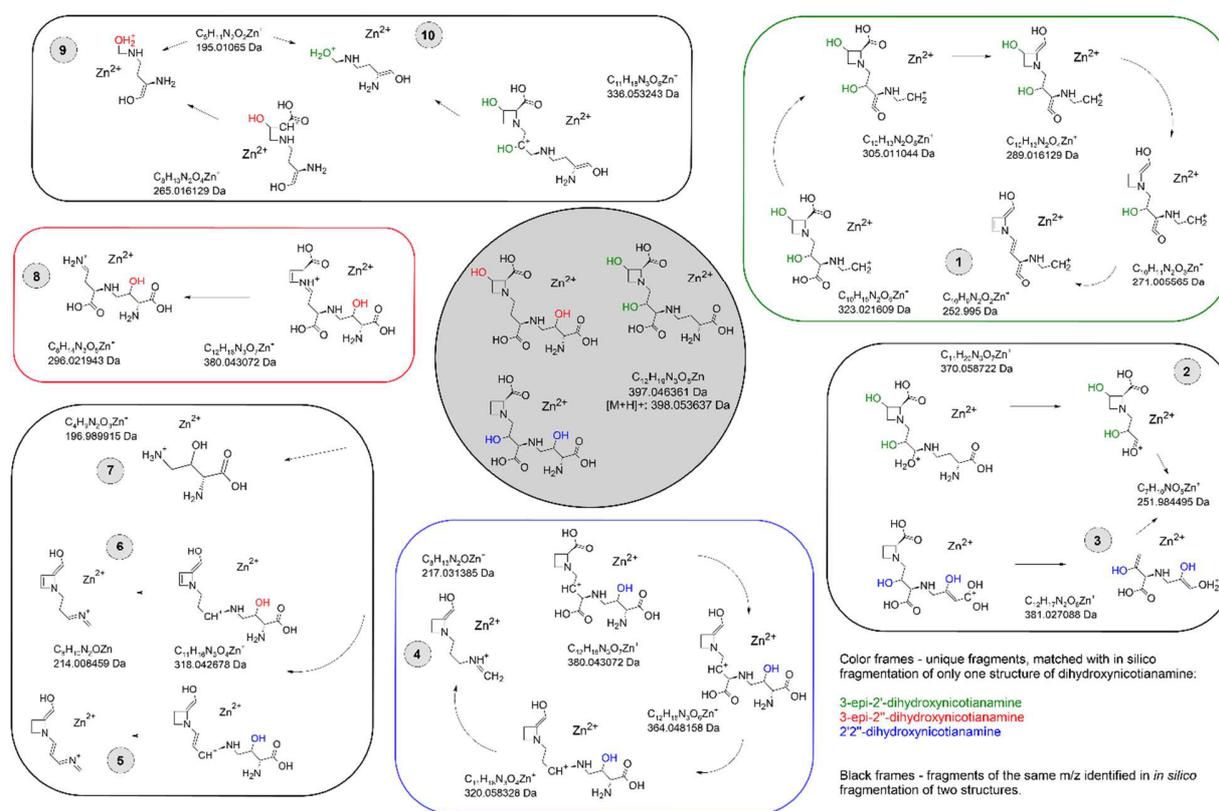
319 *Hypericum laricifolium* presented a distinctively different Zn speciation than the other three
320 plants studied. It showed the presence of two species of which the molecular mass suggested that
321 they were hydroxyl (mono- and di-) derivatives of nicotianamine. The hypothesis of
322 hydroxylation is corroborated by an observation that the introduction of hydroxyl groups into a
323 phytosiderophore skeleton increased metal-chelate stability in acid environments (48). An
324 indication of the hydroxylation positions may come from the observation that enzymes were

325 found to hydroxylate the C-2' positions of DMA and *epi*HDMA and the C-3 positions of MA
326 and DMA (69).

327 Monohydroxy-nicotianamine complexes of Cu and Zn were putatively identified in grassland
328 soil on the basis of their empirical formulas, the MS/MS fragmentation spectra, and the similarity
329 of metal binding to nicotianamine (70). However, the position of the hydroxyl group in the
330 molecule structure was not given (70). Free (apo) monohydroxy-nicotianamine was also isolated
331 from buckwheat powder, and shown to exist in some polygonaceous plants (71). A study of its
332 distribution within buckwheat plants revealed that it was predominantly localized in seeds (72).
333 Further studies have shown that the hydroxyl group is in the C-2" position (73). The same
334 compound was later identified and quantified in *Asparagus officinalis*, demonstrated for the first
335 time in non-polygonaceous plants (74). However, no information about possible metal
336 complexes was provided (71, 72).

337 The majority of Zn in *H. laricifolium* was present in the form of an unknown species with *m/z*
338 397.046361, which corresponds to the formula C₁₂H₁₉N₃O₈Zn. The structure of this species,
339 presenting a characteristic Zn isotopic pattern, was investigated by ESI-MSⁿ. The hypothesis of
340 the structure of a double hydroxylated nicotianamine derivative was confirmed by the
341 comparison with *in silico* fragmentation of three possible isomers: 2',2''-dihydroxy-
342 nicotianamine, 3-*epi*-2'-dihydroxy-nicotianamine and 3-*epi*-2''-dihydroxy-nicotianamine (**Table**
343 **SI-4**). However, the fragmentation of siderophores is characterized by multiple unspecific losses
344 of small fragments (hydroxyl, carboxyl, or amino groups) making the analysis of the
345 fragmentation spectra and the unambiguous confirmation of the species structures difficult. For
346 the detected Zn species, very similar coverages were obtained when comparing the experimental
347 data with three putative isomers of the dihydroxy-nicotianamine complex. The most probable

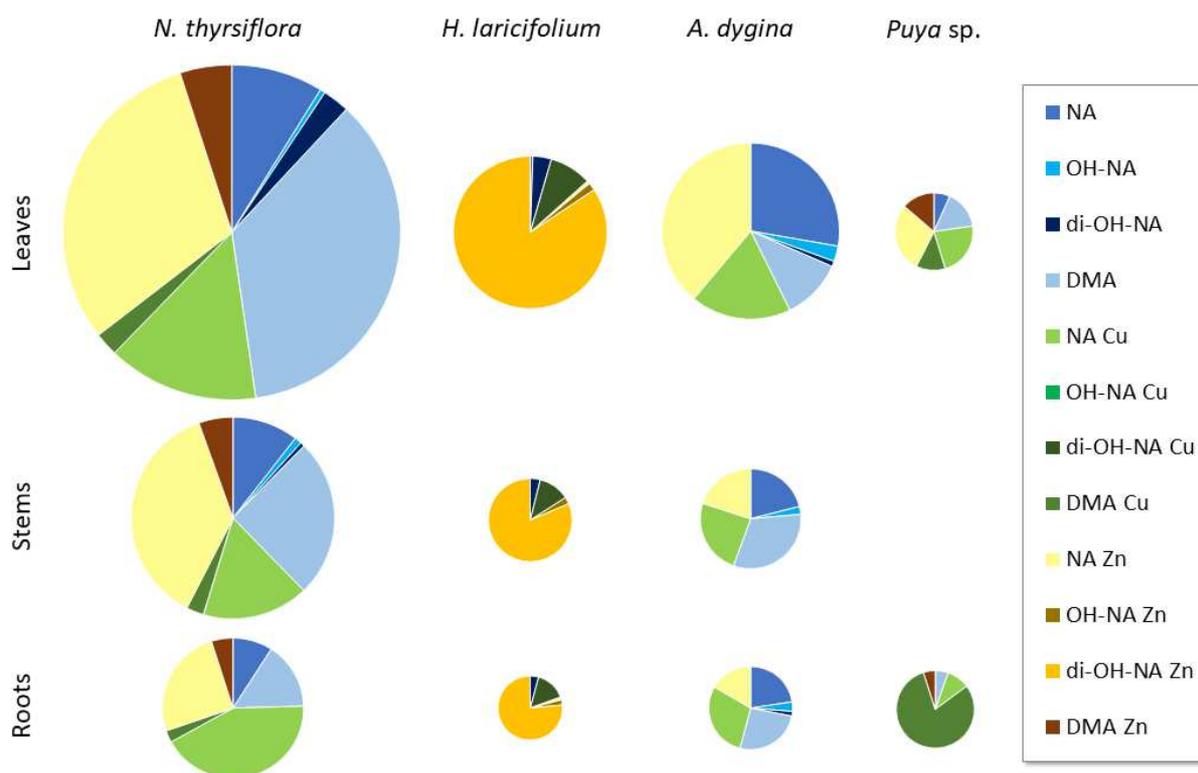
348 structure seems to be 3-*epi*-OH-2''-OH-NA having the hydroxyl groups in positions observed for
 349 other siderophores (69) and the highest agreement with the *in silico* fragmentation. In addition to
 350 the complex, in *Hypericum laricifolium* and *Nicotiana thyrsoiflora* free (non-complexed)
 351 dihydroxy-nicotianamine was detected. The set of the MSⁿ data supporting the proposed
 352 structures is shown in **Figure 4**.



353
 354 **Figure 4.** Fragmentation pathways of Zn-complex with di-hydroxy nicotianamine candidate
 355 species.

356 **Fig. 5** summarizes the estimated distribution of the species found in the different plant organs
 357 and accounts for the free ligands involved in the complexation of Zn and Cu, based on SEC-MS
 358 results (XIC signal intensity). It shows that in *A. digyna*, *N. thyrsoiflora* and *Puya sp.*, Zn-
 359 nicotianamine is the most abundant Zn species (and in *A. digyna* the only one). Also,

360 uncomplexed (free) NA was observed in these three plants. The contributions of Zn-DMA,
 361 which has a lower complex formation (12.7 for Zn(II)-DMA) (62) constants than Zn-NA (ca. 15
 362 (53, 54)) were much lower, although free DMA was also present in the samples.



363
 364 **Figure 5.** Distribution of free and complexed siderophore species in organs of the studied plants,
 365 based on XIC signals intensities recorded after separation on fast-SEC.

366 In *Puya sp.*, signals of Zn-DMA in roots were around twice lower than in leaves, while Zn-
 367 NA was present only in leaves (with signal intensity around twice higher than for the DMA
 368 complex). In *N. thyrsiflora*, the signals intensity for Zn-NA complex doubled when passing from
 369 roots to stems and then from stems to leaves. Relatively high signals for DMA-Zn were observed
 370 in all plant organs. In *A. digyna*, similar Zn-NA signals were present in roots and stems and

371 significantly higher (ca. 5 times) in leaves. No Zn-DMA complex could be detected. As it was
372 previously stated, the main compound in *H. laricifolium* was dihydroxy-nicotianamine-Zn,
373 which accumulated mainly in leaves (around 2.5x and 4x higher signals than in stems and roots
374 respectively). Moreover, a small part of Zn was associated with NA and monohydroxy-NA, and
375 localized mostly in leaves.

376 **Speciation of copper.** **Figure 3** (bottom part) shows a set of HILIC – ICP MS/ESI MS
377 chromatograms for the speciation of Cu. The corresponding set of fast-SEC chromatograms is
378 shown in **Figure SI-3**. The overall speciation pattern is very similar to that of Zn.

379 The presence of NA and DMA as the major ligands in *Puya sp.*, and *N. thyrsiflora* was
380 observed. Note that, in contrast to Zn, the binding constants (logK) of the Cu(II)-NA and Cu(II)-
381 DMA complexes are similar: 18.6 for Cu(II)-NA (53) 18.7 for Cu(II)- DMA (62). Indeed, NA
382 and DMA were reported in xylem and phloem of rice (*Oryza sativa*) (75) and castor bean plants
383 (*Ricinus communis*) (7). However, several other ligands such as histidine (18), proline (50),
384 citrate (76) and glutathione (17) were reported in the literature. Although our untargeted search
385 did not indicate any of such complexes, a targeted search for these complexes and free ligands
386 was attempted. No complex with Cu was detected with either of these ligands, despite the
387 presence of the free ligands, in particular histidine. XAS data obtained in a model hydroponic
388 experiment showed that the majority of Cu in the roots and leaves of tomato (*Solanum*
389 *lycopersicum*) and oat (*Avena sativa*) existed as sulfur-coordinated Cu(I) species resembling
390 glutathione/cysteine-rich proteins (77). Elemental distribution and chemical speciation of copper
391 in three metallophytes from the copper–cobalt belt in Northern Zambia was studied revealing a
392 complex picture of Cu handling across the studied plants; it was further complicated by the fact
393 that several of the XANES fits were poor, with significant fit residuals noted (78). The proposed

394 species included Cu(I) bound by S donor atom ligands (modelled as glutathione or cysteine) and
395 oxygen bound forms of Cu(I) and Cu(II); a significant contribution of Cu(II)-acetate complex
396 (78). Cu-N and Cu-O bonds were detected in wheat and tomato root apoplasts. Nitrogen/oxygen
397 ligands were identified in slightly higher proportions (40-70%) than single oxygen ligands. The
398 high-affinity N functional groups embedded in root apoplasts participated in copper binding in
399 the same magnitude than the low-affinity carboxylic groups (79). No siderophore metal
400 complexes have been included so far in the XAS standardization.

401 In leaves of *Puya sp.*, the intensity of the XIC signal corresponding to Cu-NA was almost
402 twice higher than the one corresponding to Cu-DMA, whereas in roots Cu-DMA dominated
403 (**Figure 5**). In *N. thyrsiflora*: Cu-NA was the main species in all the organs and was
404 accompanied by much smaller signals of Cu-DMA. The signals intensity of Cu-NA was the
405 highest in leaves and the lowest in stems. In *A. digyna* the intensity of the signals of Cu-NA was
406 similar in stems and roots and twice higher in leaves. As in the case of Zn, the speciation of Cu
407 in *H. laricifolium* was different from that in other plants. The only detected species was the Cu-
408 dihydroxy-NA complex which, was twice higher in leaves than in other parts.

409 The minor Cu species detected at m/z 335.0341 in all the plants except *H. laricifolium*, could
410 be attributed the empiric formula $C_{11}H_{16}N_2O_6Cu$. It corresponds to the ligand $C_{11}H_{18}N_2O_6$
411 previously reported (but not identified) in xylem sap of *Solanum habrochaites*, a native Andean
412 wild tomato (80). The abundance of this species was too low to provide meaningful
413 fragmentation data.

414 Relatively high signals corresponding to the free ligands NA and DMA were found in *N.*
415 *thyrsiflora*, *A. digyna* and *Puya* organs (only DMA in roots of *Puya*). Small signals of
416 hydroxylated nicotianamine forms were also observed in several organs of all plants, excluding

417 *Puya*. In addition to the already discussed phyto siderophores, ligands such as aminoadipic acid,
418 phenylalanine, tyrosine, and tryptophan, which could potentially bind metals, were observed.
419 However, no metal complexes with those ligands were detected.

420 **Speciation of arsenic.** Speciation of As in plants has been mainly studied in the context of
421 food safety, with the majority of works focused on rice (81-83). Indeed, rice is often harvested in
422 areas with high soil As content and the possible presence of toxic inorganic As(III) is of concern
423 in view of its popularity as staple food in many regions. The works on As speciation in plants
424 growing in post-mining sites have been scarce (84-88). All the studies were based on retention
425 time matching with standards in HPLC-ICP-MS. Our goal was to expand this approach to search
426 for As species by targeted and exploratory ESI-MSⁿ. For all the studied plants, the FastSEC
427 fractionation of As in water extracts of organs revealed the same profile consisting of just one
428 peak, eluting in the low (below 1 kDa) molecular weight range (**Figure 6a**). In contrast to the
429 metals discussed above, As forms covalent organic compounds, which allows using harsher
430 extraction conditions than those used for coordination metal complexes. In consequence, it was
431 attempted to increase the extraction recovery of As species in comparison with those obtained for
432 water extraction (**Figure SI-4**). However, the addition of methanol (or ethanol) (85-88) did not
433 significantly improve the extraction yield. A ten-fold pre-concentration by evaporation was used
434 to improve signal intensities in LC-MS/MS analysis.

435 A targeted analysis based on a Top3 MS² acquisition in positive mode permitted to confirm the
436 detection of two arsenocompounds on the basis of their fragmentation. Different fragmentation
437 collision energies and fragmentation modes - collision-induced dissociation (CID) and higher-
438 energy C-trap dissociation (HCD) - were applied to find the best conditions to fragment As
439 species and thus confirm their identification. The targeted fragmentation of arsenobetaine (m/z

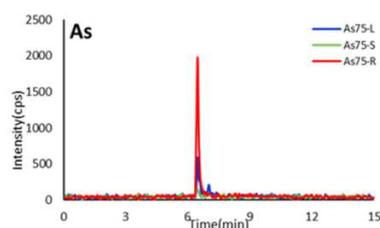
440 179.0048) and arsenosugar (m/z 329.0573) previously detected in a full MS scan permitted the
441 determination of five characteristic fragments of arsenobetaine and three fragments of
442 arsenosugar-glycerol (89). Both compounds were found in all organs of *A. dygina*. Furthermore,
443 arsenobetaine itself was found in leaves/roots of *Puya* and roots of *N. thyrsiflora*, for which the
444 signal intensities from LC ICP-MS were the highest (**Figure 6a**). The fragmentation data
445 obtained at HCD50 were compared with and matched those found elsewhere for algal extracts
446 (90) (89). The extracted ion chromatograms and the summary of the MS² fragments are
447 summarized in **Figure 6b**. Since As is known to occur in covalent complexes with glutathione
448 and phytochelatins (24), the presence of these putative species (**Table SI-5**) was screened for in
449 the target mode but without a positive result.

450 **Speciation of silver and lead.** SEC-ICP MS fractionation of (a) Ag and (b) Pb in individual
451 organs of the studied plants indicates the presence of compounds in the high-molecular weight
452 range (**Figure SI-5**). Neither Ag nor Pb species could be successfully separated on the HILIC
453 column which is suitable for the analysis of LMW species only. As it has been written earlier,
454 after diluting samples with HILIC mobile phase, containing a high percentage of acetonitrile,
455 precipitation of HMW complexes takes place. Hence, in the case of these two elements, the study
456 could only be based on the data obtained after the SEC separation. A SEC signal of Ag was very
457 weak and no hypothesis about its identity could be put forward. Regarding lead, two intense
458 peaks were usually present, one of the Pb-containing species co-eluting in fast-SEC-ICP-MS
459 (**Figure SI-6**) with the rhamnogalacturonan-II-Pb complex (MW of ca. 10 kDa) commonly
460 found in the walls of plants (91).

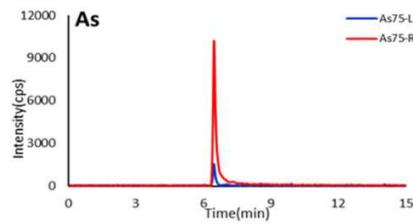
461 Unlike Zn or Cu, lead and silver are not the nutrient elements, and their bioavailability for
462 plants is reduced. Silver ions are preferentially bound by thiol groups and natural organic matter,

463 humic and fulvic acids play a significant role here. Consequently, the availability of Ag is
464 estimated at less than 5%, as it is strongly retained by the soil components (92). Whereas Pb
465 tends to form insoluble compounds, like sulfates and phosphates. Most of Pb is limited by the
466 roots, which usually accumulate the highest metal contents (93). The results of the study of lead
467 tolerance mechanisms of the plant species *Biscutella auriculata* L. from a mining area indicated
468 its high capacity to tolerate Pb and due mainly due to a very efficient mechanism to sequester the
469 metal in roots (94). Similar conclusions were drawn for the *Salix integra*, used for the
470 phytostabilization of Pb-Zn mine tailings. It was shown that the Pb taken up by the plant is
471 localized mainly in the cell walls, linked to the -OH or pectin groups. Furthermore, the
472 detoxification in the root cells may be related to metal precipitation in the phosphate or oxalate
473 forms (95).

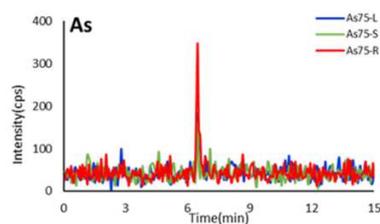
Nicotiana thyriflora



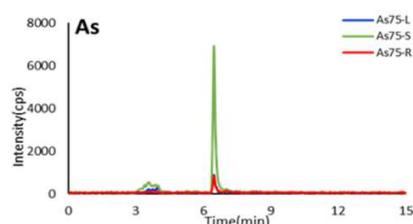
Puya sp



Hypericum laricifolium

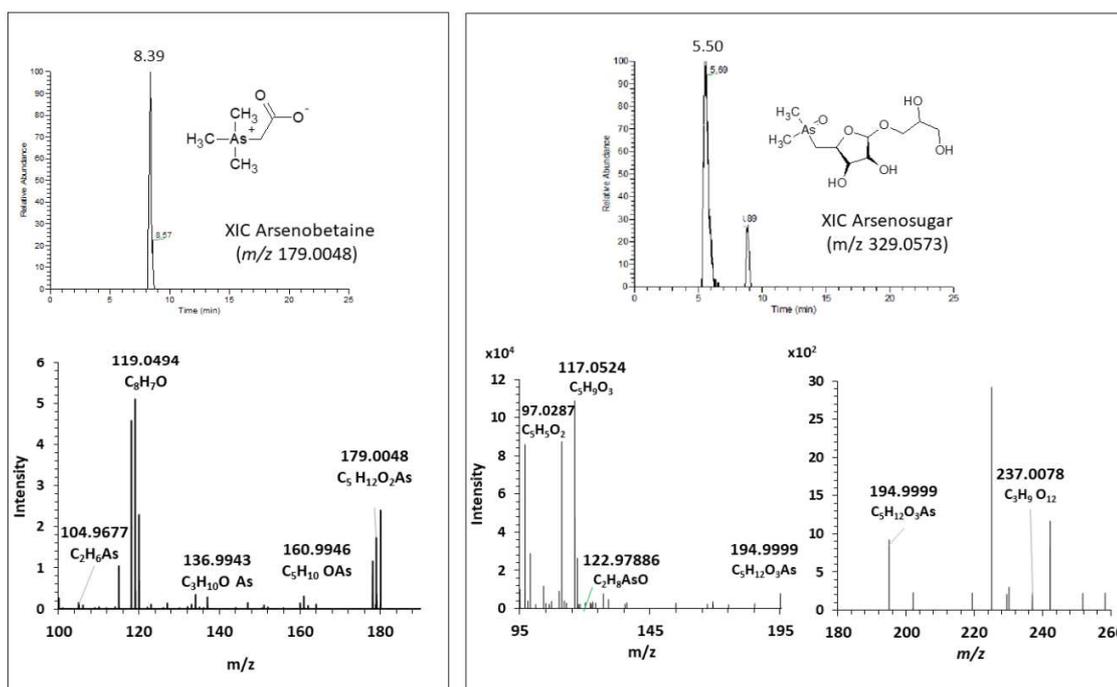


Arenaria dygina



474

a)



475

b)

476 **Figure 6.** Speciation of arsenic soluble species in organs of the studied plants; (a) SEC-ICP MS
477 fractionation, (b) XIC HILIC - ESI MS chromatograms (upper panels) and fragmentation spectra
478 (bottom panels) of water-soluble arsenic species found in organs of the studied plants.

479 CONCLUSIONS

480 The speciation of Cu and Zn in plants growing in post-mining areas in the Peruvian Andes
481 corroborate the hypothesis that, in contrast to hyperaccumulators which transport the metals
482 using weakly binding ligands, strong ligands such as NA and DMA are chosen in non-
483 accumulator plants (15, 96). The speciation does not differ much from that found in other plant
484 species reported in the literature, except for *H. laricifolium*. This species contained mono- and
485 dihydroxy-nicotianamine as the only water-soluble species complexing Zn and Cu, and the latter
486 is reported here for the first time. The observation of hydroxylation and double hydroxylation is
487 unique and interesting, since the process is likely to be genetically encoded and mediated
488 enzymatically (97). The study also showed the synthesis of organoarsenic compounds in
489 terrestrial plants. Ag and Pb are not transported to the aerial parts, except for Pb in *A. digyna*,
490 where the species involved in translocation is likely to be a complex with diRG -II.

491 ASSOCIATED CONTENT

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501 appreciated.

502 ABBREVIATIONS

503 NA, nicotianamine; DMA, 2'-deoxymugineic acid; epiHDMA, 3-epihydroxy-2'-
504 deoxymugineic acid NAAT, nicotianamine aminotransferase; DMAS, deoxymugineic acid
505 synthase.

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