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Methods

Inventory of metal complexes circulating in plant fluids: a reliable method based on HPLC coupled with dual elemental and high-resolution molecular mass spectrometric detection

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Summary

- Description of metal species in plant fluids such as xylem, phloem or related saps remains a complex challenge usually addressed either by liquid chromatography-mass spectrometry, X-ray analysis or computational prediction. To date, none of these techniques has achieved a complete and true picture of metal-containing species in plant fluids, especially for the least concentrated complexes. Here, we present a generic analytical methodology for a large-scale (> 10 metals, > 50 metal complexes) detection, identification and semiquantitative determination of metal complexes in the xylem and embryo sac liquid of the green pea, *Pisum sativum*.
- The procedure is based on direct injection using hydrophilic interaction chromatography with dual detection by elemental (inductively coupled plasma mass spectrometry) and molecular (high-resolution electrospray mass spectrometry) mass spectrometric detection.
- Numerous and novel complexes of iron(II), iron(III), copper(II), zinc, manganese, cobalt(II), cobalt(III), magnesium, calcium, nickel and molybdenum(IV) with several ligands including nicotianamine, citrate, malate, histidine, glutamine, aspartic acid, asparagine, phenylalanine and others are observed in pea fluids and discussed.
- This methodology provides a large inventory of various types of metal complexes, which is a significant asset for future biochemical and genetic studies into metal transport/homeostasis.

Introduction

In plants, metal species play an essential role in maintaining the structure and the enzymatic activity of cells. However, an excess of certain metals can lead to severe toxicity because of metal substitutions in enzymes and oxidative damage (Broadley *et al.*, 2012). As a consequence, it is crucial to determine the nature of metal species to better comprehend their fate and their role in organisms. Generally associated with organic molecules, metals are usually present in low concentrations (nM to μM ranges) and occur distributed in a plethora of chemical forms with various properties (covalent or noncovalent species and a wide range of molecular weights). These species are often unstable (labile and prone to oxidation), which makes the analysis cumbersome. Whereas covalent species can be approached by canonical metabolomic procedures, the identification of noncovalent metal complexes at basal concentrations remains a challenge.

X-ray absorption spectroscopy (XAS) and micro-X-ray fluorescence (μXRF) techniques are of particular interest to obtain information on the valence, coordination environment and spatial localization of elements within intact tissues (Sarret *et al.*, 2013) but they have a number of limitations. These include low sensitivity rendering the analysis of plant fluids at basal concentrations virtually impossible, and the critical dependence on *a priori* knowledge of the nature of the metal complexes present because their signals have to be compared with appropriate standards (Terzano *et al.*, 2013). *De novo* identification of previously unreported species and detection of minor species are therefore difficult to achieve.

An alternative is liquid chromatography with mass spectrometric (MS) detection which has been developed largely for metabolomics during the last decade (Dettmer *et al.*, 2007). However, successful applications to metal-speciation in plants have been rare and limited mostly to single elements with few ligands at relatively high concentrations, as recently reviewed by Álvarez-Fernández *et al.* (2014). The reasons are the difficult-to-control dissociation of metal complexes during sample preparation and during their exposure to the column stationary phase and electrospray source.

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Here, we report on the combination of a number of improvements at different levels of an analytical protocol, eventually enabling direct large-scale identification of metal–metabolite complexes, their semiquantitative determination and the elimination of analytical artifacts. The pivotal technique is hydrophilic interaction liquid chromatography (HILIC) coupled with mass spectrometry. This coupling was employed for the very first time to achieve the separation and identification of metal complexes for the speciation of nickel in the plant *Noccaea* (*Thlaspi) caerulescens* (Ouerdane *et al.*, 2006) and later, in different matrices including plants (Xuan *et al.*, 2006; Rellán-Álvarez *et al.*, 2010; Köster *et al.*, 2011; Pedrero *et al.*, 2012; Grillet *et al.*, 2014b). Here, this technique has been revisited with a focus on the optimization of the multispecies separation conditions with online control of metal recovery from the column, potential species transformations and separation efficiency by inductively coupled plasma mass spectrometry (ICP-MS). Subsequently, the online detection by high-resolution (> 100 000) Fourier-transform mass spectrometry (FTMS) allowed the elimination of isobaric interference which has plagued earlier studies. This permits the unambiguous observation of isotopic patterns, putative identification of the compounds by accurate (> 1 ppm) molecular mass and the validation of the identifications by tandem MS. The probability of analytical artifacts was limited by direct analysis, in contrast to multidimensional chromatography that requires intermediate fraction collection. These were eliminated by collating data from different chromatographic mechanisms/settings in positive and negative ionization conditions. In the xylem and the embryo sac liquid (ESL) of *Pisum sativum*, the combination of elemental and high-resolution molecular mass spectrometry allowed the identification, as well as the semiquantitative determination of species containing metals essential for plant growth. These two fluids were chosen for their biological relevance in the context of long-distance distribution of metals in plants.

Materials and Methods

Plant culture

Pea (*Pisum sativum* L., Dippes Gelbe Viktoria, DGV, cultivar) was used in this study. Plants were grown in a glasshouse at 23°C, in 3-l pots containing 0.5 l of quartz sand and 2.25 l of Humin substrate N2 Neuhaus (Klasmann-Deilmann, Bremen, Germany) irrigated with tap water. For xylem exudate collection, 15 plants were decapitated and a glass capillary was inserted in the central cylinder, after removal of the first 100 µl to avoid contamination by intracellular fluids. From this pool of samples, only *c.* 10 samples were kept which corresponded to the plants that produced comparable volumes of exudate, averaging *c.* 400 µl each. The exudates were then pooled. Embryo sac liquid (ESL), also called liquid endosperm, was obtained by inserting a glass capillary into the pea seed before complete seed maturation and pumping out the liquid surrounding the embryo as detailed elsewhere (Grillet *et al.*, 2014b). Samples were also collected from several plants and pooled until a volume of 200 µl was reached. Both types of samples were immediately stored at −20°C after collection. Pea plants were grown three times, which allowed the verification of sampling and analytical method reproducibility.

Analysis of total metal concentrations

Total metal contents were determined in xylem and LSE solutions after a 10- or 100- fold dilution with 2% nitric acid and were employed to check chromatographic recovery. Acidified sample solutions were then analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500cs) operating in hydrogen collision gas mode (H_2) to remove possible interferences. When possible, several isotopes were measured for each element to improve quantification accuracy.

Analysis of metal complexes using HILIC-ICP-MS and SEC-ICP-MS

Analytical reagent grade chemicals such as acetonitrile, acetic acid, formic acid, nitric acid and ammonia were purchased from Sigma-Aldrich. Ultrapure water (18 MΩ.cm) was obtained from a Milli-Q system (Millipore). Microbore HILIC HPLC separations were performed using a 1100 capillary HPLC system (Agilent, Tokyo, Japan) equipped with a 100 µl min^{-1} splitter module. ICP-MS detection was achieved using a model 7500cs instrument (Agilent) fitted with platinum cones, 1 mm i.d. injector torch and a T-connector allowing the introduction of 5% O_2. The HILIC-ICP-MS coupling was done via an Isomist interface (Glass Expansion, Melbourne, Vic, Australia) consisting of a 20-ml Cinnabar cyclonic spray chamber cooled to 2°C and fitted with a 50 µl min^{-1} Micromist U-series nebulizer. Size exclusion chromatography (SEC) separations were carried out with a 1100 HPLC system (Agilent). The metal elution was monitored by splitting part of the effluent to a 7500ce ICP-MS (Agilent).

The column used for HILIC separation was a TSK gel amide 80 (250 mm × 1 mm i.d., 5 µm) (Tosoh Biosciences, Stuttgart, Germany). Gradient elution, at a flow rate of 50 µl min^{-1}, was carried out using eluent A, acetonitrile, and eluent B, 5 mM ammonium formate (pH 5.5). The gradient program was: 0–5 min 10% B, 5–45 min up to 50% B, 45–50 min 50% B, 50–52 min up to 65% B, 52–55 min 65% B, 55–60 min down to 10% B, 60–65 min 10% B. Samples were diluted with acetonitrile and water to obtain a 1 : 2, sample to acetonitrile ratio, and centrifuged. A 7-µl aliquot of the supernatant was injected into the HILIC column each time. Addition of acetonitrile may create a small amount of precipitate containing < 5% of the metals of interest. To check the possible influence of the buffer ions on metal speciation, 5 mM ammonium acetate (pH 5.5) for eluent B was also tested. SEC separation was performed with a Superdex Peptide column. The elution was carried out with 5 mM ammonium acetate (pH 5.5) at 0.7 ml min^{-1} for 1 h. Both SEC and HILIC columns were cleaned regularly with 0.1% formic acid solution and 0.1% formic acid : acetonitrile (1 : 1) mixture, respectively, to avoid metal carryover between samples. For HILIC-ICP-MS and SEC-ICP-MS couplings, for elements for which it was possible (iron, copper, zinc, molybdenum, nickel, magnesium, manganese and calcium), at least two isotopes were

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monitored to confirm their presence and to discard possible interferences. Column recoveries for metals were determined by comparing the metal content in the eluting solution with that in the original sample, after sample injection and after blank injection.

Analysis for metal complexes using HILIC-ESI-MS and SEC-ESI-MS

For HILIC-ESI-MS and SEC-ESI-MS, the HPLC systems were connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For analysis with SEC, a splitter allowed the division by a factor of 10 of the chromatographic flow before injection into the electrospray ionization mass spectrometry (ESI-MS) instrument. In both cases, the coupling was achieved via a heated electrospray ionization source (H-ESI II) (Thermo Fisher Scientific). The chromatographic separation conditions were as given in the previous paragraph. For the post-SEC column acidification experiments, the solution added was 30% (v/v) formic acid delivered at 4 $\mu l\ min^{-1}$. The ion source was operated either in the positive or negative ion mode at 3.0 kV. The vaporizer temperature of the source was set to 120°C and the capillary temperature to 280°C. In full MS mode, the resolution was set at 100 000 (FWHM at m/z 400), whereas in MS/MS mode it was set at 30 000. MS data were processed using Xcalibur 2.1 software and MetWorks 1.2.1 software (Thermo Fisher Scientific) to screen spectra for metal containing molecules. To get accurate masses, MS and MS/MS spectra were recalibrated offline using precursor/fragment ions with known formulae, which resulted in the determination of a unique chemical formula for each metal complex. These formulae were checked against a natural compounds database (*Dictionary of Natural Products Online*, http://dnp.chemnetbase.com) to identify the organic ligands, knowing that ligands can be present several times in a metal complex. Putative metal species were fragmented during a subsequent chromatographic run with high energy collision dissociation (HCD) at 35% energy using an isolation width adapted to the number of isotopes present in the complex.

Results and Discussion

Method validation for the determination of metabolites involved in metal binding in xylem and embryo sac liquid from *Pisum sativum*

Hydrophilic interaction chromatography (HILIC) with dual inductively coupled plasma mass spectrometry (ICP-MS) and electrospray ionization–Fourier-transform mass spectrometry (ESI-FTMS) detection has been applied to the screening for covalent selenium metabolites in yeast (Gil Casal *et al.*, 2010; Arnaudguilhem *et al.*, 2012) and plants (Aureli *et al.*, 2012; Ouerdane *et al.*, 2013), and to the detection of stable and concentrated complexes in plants (Grevenstuk *et al.*, 2013; Grillet *et al.*, 2014b). However, metal–metabolite complexes are often prone to dissociation during sample preparation and concentration steps, which makes it difficult to develop this method for a global approach for metal complexes in plants. In order to avoid the risk of artifact formation, our method aimed at the direct (without any extraction or pre-concentration) analysis by single-dimension chromatography. The chromatographic mobile phase pH was set to mimic the pH of the sample (pH 5.5) and the mobile phase buffer was chosen to minimize competition with the endogenous metal complexes in the sample. Different chromatographic mechanisms (HILIC and size exclusion chromatography (SEC)), mobile phase conditions (ammonium acetate and ammonium formate) and electrospray modes (positive vs negative) were investigated to confirm the occurrence of a given metal species.

The chromatographic (HILIC and SEC) conditions were optimized to get high and reproducible metal recoveries. This optimization consisted in choosing the best compromise between conditions to allow for good compound separation and detection (lower concentrated buffer and longer chromatographic gradient) and opposite conditions to allow good metal recovery. Metal column recovery was checked regularly and good recoveries (60–90%, depending on the element) were obtained for the targeted elements in these two types of sample. Note that quantitative recoveries are important for quantification but not necessarily for the identification of endogenous metal species. To confirm that < 100% recovery was not affecting data interpretation, columns saturated with the metal ions of interest (resulting in a metal recovery of 150–500%) were tested. No significant changes were observed either in terms of the morphology of chromatograms with ICP-MS detection (only general intensity increased) or in terms of the species identification by ESI-MS in comparison with normal conditions. The absence, in these particular samples, of changes in metal speciation when metal recoveries stay within a reasonable range (60–200%) is likely to be due to the presence of higher than stoichiometric concentrations of the relevant ligands.

Two types of fluids, xylem sap and embryo sac liquid, were obtained from *P. sativum* and were analyzed by means of the different couplings and in the different modes described in the Materials and Methods section. Peaks corresponding to the metal-containing species were searched for in the ESI MS spectra for several elements by the recognition of specific isotopic patterns (exact inter-isotopic mass differences and isotopic ratios) when applicable (except for monoisotopic elements). Using the accurate masses of the precursor ions, as well as their corresponding fragments, empiric molecular formulae could be determined for all the metal species detected. Taking into account that metals can be bound to one or to several (identical or different) ligands, these formulae were checked against a natural products database to identify the organic ligands. The identification was validated by MS/MS. Only the identification of complexes resulting in an unique possibility were kept. Note that owing to the high mass measurement accuracy, the number of possible ligands was usually limited (< 5), which facilitated identification. More metal complexes were identified by HILIC-ESI-MS analysis than with SEC-ESI-MS analysis because of better compound separation in the former. The presence of the main metal complexes was always confirmed by SEC-ESI-MS coupling. To validate the occurrence of the less concentrated metal complexes, different HILIC chromatographic conditions were tested (ammonium acetate buffer instead of ammonium formate and a faster gradient). The

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different conditions led to variations in relative retention times of several species and, as a consequence, allowed the elimination of (minor) artifact compounds formed in the electrospray ion source because of the coelution of an endogenous metal complex and another free ligand. Finally, to avoid the possibility of metal complex formation that would be due to the reaction of sample free ligands with column metal pollution, free ligand standard solutions were also injected into the columns in the same conditions and the nonoccurrence of metal complexes was checked.

To conclude, the method developed in this study is based on the use of HILIC coupled to dual ICP-MS and ESI-FTMS detection for which the analysis pipeline was: to choose optimum chromatographic conditions (buffer pH, buffer salts, gradient. . .) in order to improve species detection and minimize degradations and artifacts; to check the significance of results by measuring chromatographic metal recovery, by verifying the nonformation of complexes when a free ligand alone is injected and by comparing results with other chromatographic conditions or type of chromatography (SEC); to use the high mass accuracy of the FTMS instrument to confirm species identification; and to evaluate relative amounts of metal complexes for each element by comparing peak areas in HILIC-ICP-MS chromatograms.

Identification and semiquantification of numerous metal complexes in *Pisum sativum* fluids

The identified and confirmed species for both fluids are presented in Table 1. More than 50 different metal complexes were identified. Numerous ligands were involved such as aspartic acid, asparagine, citric acid, glutamine, histidine, malic acid, nicotianamine, phenylalanine, tryptophan, pipecolic acid and aminoadipic acid. They were found to bind a great variety of metallic ions such as Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, K^{+}, Ni^{2+} and MoO_2^{2+}. The typical sets of chromatograms obtained for the analyses of xylem are illustrated in Figs 1 and 2 for HILIC and SEC, respectively. Matching retention times of metal complexes observed in ESI-MS extracted ion chromatograms with those observed in ICP-MS chromatograms enabled the semiquantitative evaluation of the contribution of each ligand in the binding of metal ions (as shown in Table 2). Despite differences in metal concentrations between xylem and embryo sac liquid (ESL), the nature and the proportion of metal complexes were similar between the two samples, which can be explained partially by the fact that xylem and ESL have a similar pH (*c.* 5.5). For this reason, detailed chromatograms are presented for xylem samples only. The results were reproducible during the three series of growth/analysis.

Several amino acids and carboxylic acids are involved in metal binding

Several metal complexes were identified for the first time, including complexes with elements for which no MS data about their speciation in plants have so far existed, as well as novel ternary metal complexes. One of the main (if not the main) binding molecule for zinc (Zn), copper (Cu(II)), cobalt (Co(II)) and nickel (Ni), as well as Fe(II) at trace levels, is nicotianamine (NA). This molecule is well known for its ability to complex metals, even if, to date, there has been no confirmation by MS demonstrating it as a key compound for trace metal homeostasis in plants and possibly for the transport of a large number of metal ions (at least by allowing them to be maintained in solution in order to access enzymes and membrane transport proteins). These results confirm the important role of NA in the studies of the fate of essential trace elements in plants. Its transport, driven by Yellow Stripe-Like (YSL) transporters, seems to closely control transient metal homeostasis and bioavailability within plants (Curie *et al.*, 2009; Schuler *et al.*, 2012). Our findings on the central role of NA in the metal speciation in the embryo sac liquid are thus in good agreement with previous reports on the genetic characterization of YSL transporters and their function in long-distance transport towards the seeds. In *Arabidopsis thaliana*, *AtYSL1* and *AtYSL3* were shown to regulate the simultaneous transport, in seeds, of NA with Fe (Le Jean *et al.*, 2005), Zn and copper (Cu) (Waters *et al.*, 2006). In rice, the mutation in *OsYSL2* also provoked a decrease in Fe and manganese (Mn) accumulation in grains (Ishimaru *et al.*, 2010).

Malic acid and citric acid were also observed to be involved in metal ion binding. Except for iron, the stoichiometry of the detected complexes was mostly 1 : 2 (metal : ligand). Such complexes have an expected low-to-medium stability, which contrasts with the more stable 3 : 4 (iron : organic acid) complexes. Organic acids were described as essential counter-ions for metal storage, especially in hyperaccumulating plants (Rascio & Navari-Izzo, 2011). Note that both ligands are α hydroxyl acids, which suggests that other organic acids belonging to this family could potentially play a role in other plants.

Several amino acids were also found to bind metal ions. For some of them, their roles in the complexation of metals in biological matrices have not yet been demonstrated by MS. Gln, Asn and Asp contain a $–CONH_2$ (amide) or a –COOH (acid) group in their side chain, which confers an increased capacity for metal binding. Therefore, one can also expect glutamate (Glu) to participate even if it was not shown to be complexing metal ions in this study. Some uncharged aromatic (phenylalanine, tryptophan) amino acids were also detected as minor ligands in overall metal speciation.

Although mostly identical, slight differences in speciation between xylem sap and ESL were noticed and, in particular, fewer amino acids were involved in metal binding in xylem than in ESL (see Table 1). This can be related to the fact that ESL is involved in seed feeding and should thus contain a wider variety and an increased concentration of nutrients (Patrick & Offler, 2001; Van Dongen *et al.*, 2003).

The fact that some metals have a higher affinity for some ligands than others is of considerable interest for obtaining clues about the roles of some ligands for element-specific homeostasis in the plant. For example, Ni was found to have a higher affinity towards histidine than all of the other metals. Interestingly, this particular affinity was actually suggested by earlier observations done for Ni-hyperaccumulating plants (Krämer *et al.*, 1996; Ingle *et al.*, 2005; Ouerdane *et al.*, 2006), which would indicate that

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Table 1 Metal complexes detected by hydrophilic interaction chromatography mass spectrometry (HILIC-MS) and size exclusion chromatography-elestrospray ionization mass spectrometry (SEC-ESI-MS) in xylem and embryo sac liquid (ESL) of pea (*Pisum sativum*) plants

No.	Compound[a]	Molecular formula (neutral form)	Exact mass[b] (*m/z*)	Experimental mass[b] (*m/z*)	Error value (ppm)	Retention time (min)	Ionization mode	Presence confirmed in xylem (X) and/or ESL (E)
1	Mg-(Asn)$_2$	$C_8H_{14}N_4O_6Mg$	287.08365	287.08363	−0.1	30.53	+	X
2	Mg-(Asp)$_2$	$C_8H_{12}N_2O_8Mg$	289.05168	289.05183	0.5	31.80	+/−	X
3	Mg-(Gln)$_2$	$C_{10}H_{18}N_4O_6Mg$	315.11495	315.11495	0.0	29.95	+/−	X/E
4	Mg-(Cit)$_2$	$C_{12}H_{14}O_{14}Mg$	407.03067	407.03063	−0.1	35.26	+/−	X/E
5	Ca-(Cit)	$C_6H_6O_7Ca$	230.98122	230.98101	−0.9	35.43	+	X/E
6	Ca-(PCA)$_2$	$C_{12}H_{20}N_2O_4Ca$	297.11217	297.11229	0.4	24.54	+	E
7	Ca-(Asn)$_2$	$C_8H_{14}N_4O_6Ca$	303.06120	303.06095	−0.8	30.64	+/−	X
8	Ca-(Asp)$_2$	$C_8H_{12}N_2O_8Ca$	305.02923	305.02906	−0.6	31.91	+/−	X
9	Ca-(Gln)$_2$	$C_{10}H_{18}N_4O_6Ca$	331.09250	331.09246	−0.1	29.89	+/−	X/E
10	Ca-(AAA)$_2$	$C_{12}H_{20}N_2O_8Ca$	361.09183	361.09210	0.7	22.33	+	E
11	Ca-(Cit)$_2$	$C_{12}H_{14}O_{14}Ca$	423.00822	423.00841	0.5	35.43	+/−	X/E
12	Fe(III)-(Asn)$_2$	$C_8H_{13}N_4O_6Fe$	318.02573	318.02584	0.3	30.64	+	X
13	Fe(III)-(Asp)$_2$	$C_8H_{11}N_2O_8Fe$	319.99376	319.99376	0.0	31.05	+/−	X/E
14	Fe(III)-(Mal)$_2$	$C_8H_9O_{10}Fe$	321.96179	321.96181	0.1	29.78	+/−	X/E
15	Fe(III)-(Gln)$_2$	$C_{10}H_{17}N_4O_6Fe$	346.05703	346.05707	0.1	29.95	+	X/E
16[c]	Fe(III)-(Cit)$_2$	$C_{12}H_{13}O_{14}Fe$	437.97275	437.97284	0.2	35.49 (18.83)	+/−	X/E
17[c]	Fe$_3$(III)-(Mal)$_3$-(Cit)$_1$	$C_{18}H_{17}O_{22}Fe_3$	753.83324	753.83379	0.7	37.29 (16.51)	+	E
18[c]	Fe$_3$(III)-(Mal)$_2$-(Cit)$_2$	$C_{20}H_{19}O_{24}Fe_3$	811.83873	811.83938	0.8	36.93 (15.65)	+/−	X/E
19[c]	Fe$_3$(III)-(Mal)$_1$-(Cit)$_3$	$C_{22}H_{21}O_{26}Fe_3$	869.84421	869.84526	1.2	39.19 (15.11)	+/−	X/E
20[b]	Fe(II)-(Asp)$_2$	$C_8H_{12}N_2O_8Fe$	318.98703	318.98667	−1.1	31.05	−	X/E
21[b]	Fe(II)-(Mal)$_2$	$C_8H_{10}O_{10}Fe$	320.95506	320.95482	−0.8	29.78	−	X
22[b,c]	Fe(II)-NA	$C_{12}H_{19}N_3O_6Fe$	356.05505	356.05517	0.4	32.17 (21.24)	−	E
23[b,c]	Fe(II)-(Cit)$_2$	$C_{12}H_{14}O_{14}Fe$	436.96602	436.96601	0.0	35.49 (18.83)	−	E
24	Mn-(Asn)$_2$	$C_8H_{14}N_4O_6Mn$	318.03666	318.03646	−0.6	30.64	+	X/E
25[b]	Mn-(Mal)$_2$	$C_8H_{10}O_{10}Mn$	319.95817	319.95747	−2.2	29.89	−	X
26	Mn-(Asp)$_2$	$C_8H_{12}N_2O_8Mn$	320.00469	320.00513	1.4	31.86	+/−	X
27	Mn-(Gln)$_2$	$C_{10}H_{18}N_4O_6Mn$	346.06796	346.06783	−0.4	29.78	+	X/E
28[b]	Mn-NA	$C_{12}H_{19}N_3O_6Mn$	355.05816	355.05813	−0.1	32.17	−	E
29	Mn-(Cit)$_2$	$C_{12}H_{14}O_{14}Mn$	437.98368	437.98313	−1.3	35.38	+	X/E
30	Co(III)-NA	$C_{12}H_{18}N_3O_6Co$	360.06004	360.05972	−0.9	32.78	+	X
31	Co(II)-NA	$C_{12}H_{19}N_3O_6Co$	361.06786	361.06824	1.1	31.16	+/−	X/E
32	Co(II)-(Cit)$_2$	$C_{12}H_{14}O_{14}Co$	441.97883	441.97903	0.5	35.49	+	X
33	Ni-(Asn)$_2$	$C_8H_{14}N_4O_6Ni$	321.03396	321.03380	−0.5	30.64	+/−	X
34	Ni-(Asp)$_2$	$C_8H_{12}N_2O_8Ni$	323.00199	323.00194	−0.2	31.16	+/−	X
35	Ni-(Mal)$_2$	$C_8H_{10}O_{10}Ni$	324.97002	324.96987	−0.5	28.33	+/−	X
36	Ni-(Gln)$_2$	$C_{10}H_{18}N_4O_6Ni$	349.06526	349.06528	0.1	29.72	+	X
37	Ni-NA	$C_{12}H_{19}N_3O_6Ni$	360.07001	360.07059	1.6	32.03	+/−	X/E
38[b]	Ni-(His)$_2$	$C_{12}H_{16}N_6O_4Ni$	365.05137	365.05153	0.5	32.55	−	X
39	Ni-(Phe)$_2$	$C_{18}H_{20}N_2O_4Ni$	387.08493	387.08509	0.4	18.32	+/−	E
40	Ni-(Cit)$_2$	$C_{12}H_{14}O_{14}Ni$	440.98098	440.98191	2.1	35.09	+	X
41	Ni-(Phe)$_3$	$C_{27}H_{31}N_3O_6Ni$	552.16391	552.16426	0.6	18.32	+/−	E
42	Ni-(Phe)$_2$-(Trp)$_1$	$C_{29}H_{32}N_4O_6Ni$	591.17481	591.17569	1.5	18.38	+	E
43	Cu(II)-(Asn)$_2$	$C_8H_{14}N_4O_6Cu$	326.02821	326.02834	0.4	31.34	+	X
44[c]	Cu(II)-NA	$C_{12}H_{19}N_3O_6Cu$	365.06426	365.06415	−0.3	31.80 (21.13)	+/−	X/E
45	Cu(II)-(Phe)$_2$	$C_{18}H_{20}N_2O_4Cu$	392.07918	392.07924	0.1	18.21	+	E
46	Cu(II)-(Phe)$_3$	$C_{27}H_{31}N_3O_6Cu$	557.15816	557.15852	0.7	18.27	+/−	E
47	Cu(II)-(Phe)$_2$-(Trp)$_1$	$C_{29}H_{32}N_4O_6Cu$	596.16906	596.17001	1.6	18.21	+	E
48	Zn-(Gln)$_2$	$C_{10}H_{18}N_4O_6Zn$	355.05906	355.05896	−0.3	30.59	+/−	X/E
49[c]	Zn-NA	$C_{12}H_{19}N_3O_6Zn$	366.06381	366.06387	0.2	31.34 (21.13)	+/−	X/E
50[c]	Zn-(His)$_2$	$C_{12}H_{16}N_6O_4Zn$	373.05973	373.05927	−1.3	31.98 (27.66)	+/−	E
51[c]	Zn-(Cit)$_2$	$C_{12}H_{14}O_{14}Zn$	446.97478	446.97505	0.6	35.78 (18.83)	+	X/E
52[d]	MoO$_2$-(Mal)$_2$	$C_8H_9O_{12}Mo$	394.91536 → 260.89383	260.89337	−1.8	17.43	+/−	X/E
53[b,d]	MoO$_2$-(Cit)$_2$	$C_{12}H_{14}O_{16}Mo$	510.92632 → 318.89931	318.89886	−1.4	18.63	−	E

[a]Asp, aspartic acid; Asn, asparagine; Cit, citric acid; Gln, glutamine; His, histidine; Mal, malic acid; NA, nicotianamine; Phe, phenylalanine; Trp, tryptophan; PCA, pipecolic acid; AAA, aminoadipic acid.
[b]Exact masses and experimental masses are always given in positive ion mode ($[M+H]^+$ ions) except for compounds that were observed only in negative ion mode ($[M-H]^-$ ions).
[c]Presence of these metal complexes in xylem and endosperm was confirmed by SEC-inductively coupled plasma (ICP)-MS and SEC-ESI-MS. The retention times corresponding to SEC are given in brackets.
[d]Compounds 52 and 53 were identified through their specific mass transition after MS/MS fragmentation.

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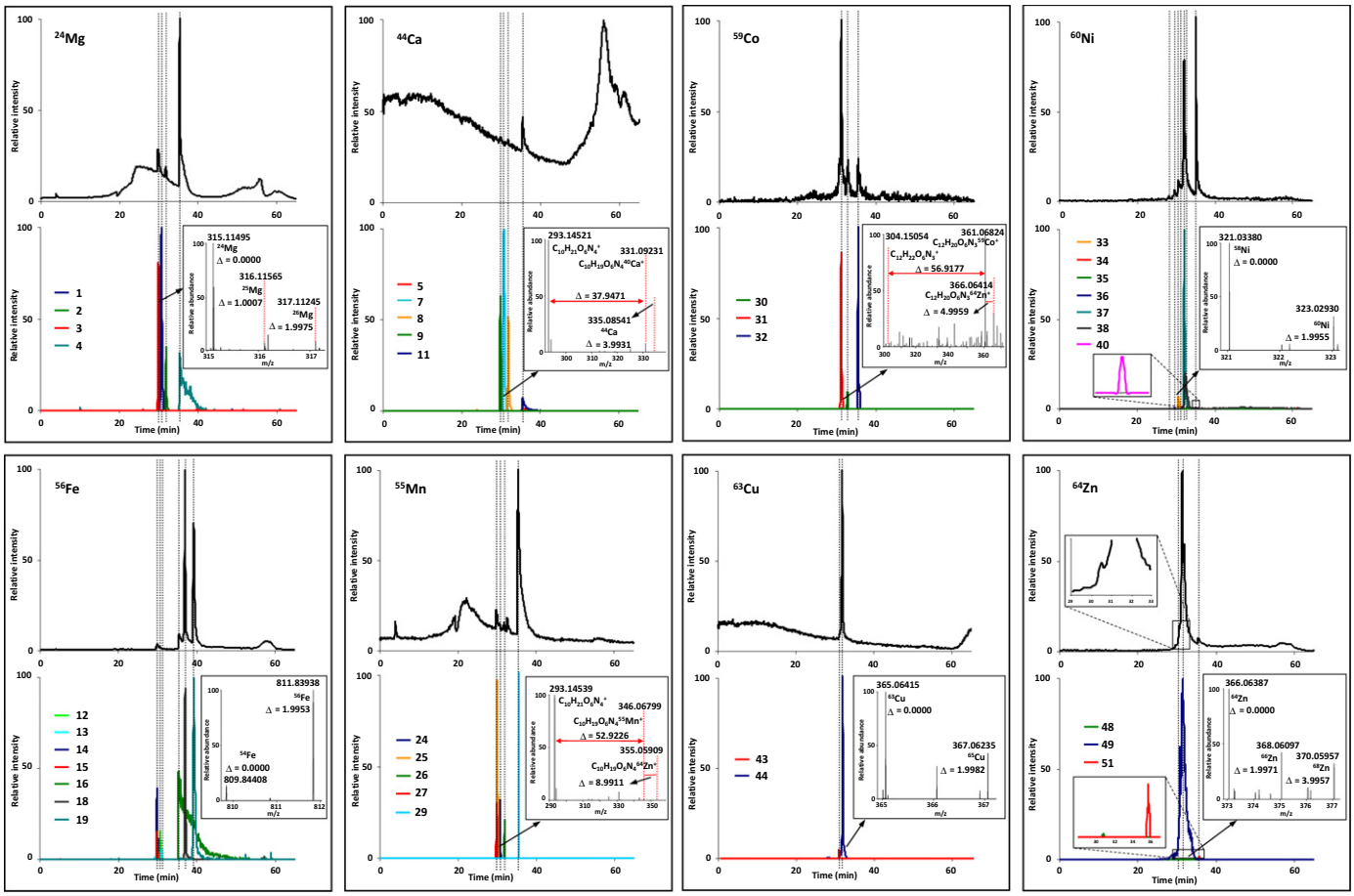

Fig. 1 HILIC-ICP-MS (upper part, chromatogram of mentioned isotope) and HILIC-ESI-MS (lower part, extracted ion chromatograms of *m/z* from compounds represented here by their associated numbers in Table 1) chromatograms of each main metal studied in pea (*Pisum sativum*) xylem exudates. The insets depict zooms of part of the mass spectra containing ions with characteristic isotopic signatures of the followed elements and/or metal-specific mass differences with free ligands.

this amino acid could definitively play a specific role in mobilizing this metal.

Iron complexes

Among the complexes found, special attention was paid to the mixed-ligand complexes $Fe(III)_3$-$(Cit)_{(4-x)}$-$(Mal)_x$ (with x ranging from 1 to 3) (see Fig. 3), which were identified on the basis of the consecutive elution in SEC. The three complexes containing different malate-to-citrate ratios differ by 58 Da. Their molecular formulae were established by tandem MS. The online post column acidification of the SEC eluate confirmed the presence of malic and citric acids at the elution volumes of these iron complexes, whereas the free citric and malic acids present in the sample elute much later. Finally, a 0.2 : 1 : 5 synthetic mixture of Fe (III) : citrate : malate at pH 5.5 was analyzed by infusion ESI-MS and showed the presence of the three iron complexes as main species. The biological implications of this finding for iron homeostasis in the pea have been discussed elsewhere (Grillet *et al.*, 2014a,b).

Concerning Fe(III), the involvement of citric acid in its binding confirms expectations from early works on the topic (Tiffin, 1966) and supports the recently discovered relationship between FRD3 (a citrate transporter) gene expression and Fe loading into the xylem (Durrett *et al.*, 2007; Roschzttardtz *et al.*, 2011). However, the role of malic acid in combination with citric acid was suggested only recently (Grillet *et al.*, 2014b). Also, the absence of the '$Fe(III)_3$-O-$(Cit)_3$' complex and $Fe(III)_3$-$(Cit)_3$ complex in the pea samples despite the presence of citric acid, would confirm a higher stability for the mixed iron complexes $Fe(III)_3$-$(Cit)_{(4-x)}$-$(Mal)_x$ (x = 1–3) compared to the '$Fe(III)_3$-O-$(Cit)_3$' complex or $Fe(III)_3$-$(Cit)_3$ complex as it was also observed with the standard mixture. Interestingly, one of the complexes in our study, Fe $(III)_3$-$(Cit)_1$-$(Mal)_3$, has exactly the same molecular formula (in neutral form) as the reported '$Fe(III)_3$-O-$(Cit)_3$' (Rellán-Álvarez *et al.*, 2010). In the latter study, the complex observed in tomato xylem sap is not in the same form as in their Fe citrate standard mixture (only '$Fe(III)_3$-O-$(Cit)_3$' is present in tomato xylem sap, but 60% $Fe(III)_3$-$(Cit)_3$ and only 40% '$Fe(III)_3$-O-$(Cit)_3$' are present in the standard mixture). As observed recently in *Plantago almogravensis* leaves (Grevenstuk *et al.*, 2013), the detection of the Fe(III): Cit mixture in plant samples should lead to the observation of the two forms: $Fe(III)_3$-$(Cit)_3$ and its hydrated form called '$Fe(III)_3$-O-$(Cit)_3$'. However, the latter is probably

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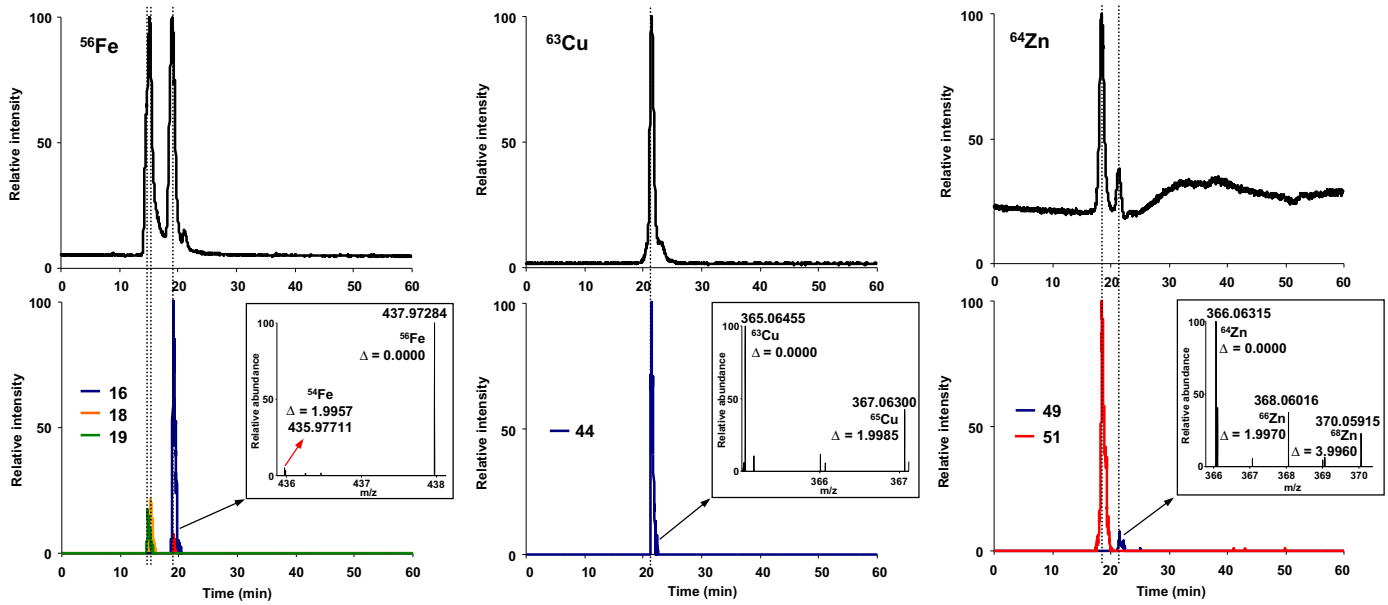

Fig. 2 SEC-ICP-MS (upper part, chromatogram of mentioned isotope) and SEC-ESI-MS (lower part, extracted ion chromatograms of *m/z* from compounds represented here by their associated numbers in Table 1) chromatograms of iron (Fe), copper (Cu) and zinc (Zn) (from left to right, respectively) from pea (*Pisum sativum*) xylem exudates. The insets depict zooms of part of the mass spectra containing ions with characteristic isotopic signatures of the followed elements.

just an adduct of a water molecule, giving $Fe(III)_3$-$(Cit)_3(H_2O)$, that stabilizes the complex structure without the insertion of an oxygen atom within the iron core, as discussed elsewhere (Fukushima *et al.*, 2012). Therefore, there is a possibility that the previously observed '$Fe(III)_3$-O-$(Cit)_3$' complex (Rellán-Álvarez *et al.*, 2010) was wrongly assigned and actually could have been the $Fe(III)_3$-$(Cit)_3(H_2O)$ or $Fe(III)_3$-$(Cit)_1$-$(Mal)_3$ complex in tomato xylem sap. Interestingly, malic acid is frequently found at high concentrations, sometimes in higher proportions than citrate in plant shoots, and its concentration can increase in the case of Fe deficiency (Abadía *et al.*, 2002; López-Millán *et al.*, 2009, 2012; Jelali *et al.*, 2010). The presence of malic acid could therefore favor (in combination with citric acid) the stabilization of Fe(III) in such mixed complex forms instead of Fe(II). Indeed, Fe(II) represents a potential threat for plant because its oxidation can lead to the Fenton reaction and production of reactive oxygen species (ROS).

Concerning Fe(II), it was found to bind citrate, malate and aspartate but only at trace levels compared to Fe(III). However, Fe(II) only was found to complex NA. To discriminate signal intensity due to Fe(II) compared to Fe(III) in HILIC-ICP-MS Fe chromatograms, the intensity ratios between *m/z* of Fe(II) complexes and Fe(III) complexes were checked after HILIC-ESI-MS chromatograms. Fe(II) was found to be present at a much lower concentration, which impaired its precise determination by HILIC-ICP-MS. Note that despite careful sample collection and soft electrospray conditions, a slight oxidation of a part of the iron is possible (Weber *et al.*, 2006). However, the remaining traces of Fe(II)-NA seem to confirm early observations that NA would contribute more than organic acids to stabilizing Fe(II) (Von Wirén *et al.*, 1999). It would participate stabilizing Fe(II) long enough to be available for metabolic/enzymatic reactions or transporters without producing oxidative damage through instantaneous re-oxidation. A role for YSL transporters for Fe (Le Jean *et al.*, 2005; Kakei *et al.*, 2012; Schuler *et al.*, 2012) or even for Fe(II)-NA (Ishimaru *et al.*, 2010) transport in plants has been demonstrated repeatedly, but this complex has only recently been observed (Grillet *et al.*, 2014b). Moreover, the formation of very stable complexes of Fe(III) with citrate or citrate/malate mixture favors the presence of Fe(III). It would have the effect of lowering the redox potential of the Fe(III)/Fe(II) redox couple to negative values, as observed for bacterial siderophores (Dhungana & Crumbliss, 2005). This favors and explains the presence of the majority of species-containing Fe(III) and not Fe(II), especially as the binding strength of NA towards Fe(II) is insufficient to balance this effect (Benes *et al.*, 1983).

Copper complexes

Because of its high affinity to NA (Benes *et al.*, 1983), Cu is bound mainly by this molecule in plant fluids. This non-proteogenic amino acid can therefore be considered as the main carrier of copper within pea plant vessels and ESL. Even though it is usually suggested that Cu is transported as Cu(I) via a Copper Transporter (COPT) family (Sancenón *et al.*, 2003; Eisses & Kaplan, 2005), it was also hypothesized recently that ZIP transporters and heavy metal ATPase (HMA) transporters could transport Cu(II) (Burkhead *et al.*, 2009; Pilon, 2011). More interestingly, a recent study demonstrated that YSL transporters (e.g. YSL16 in rice; Zheng *et al.*, 2012) were also involved in transport of the Cu(II)-NA complex, which meant that this complex could be immediately transported. Cu(II)-NA was putatively identified recently by MS in rice phloem but without confirmation by exact mass matching or MS/MS fragmentation

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Table 2 Semiquantitative determination of metal complex contributions to overall speciation of each metal determined in xylem of pea (*Pisum sativum*) plants by hydrophilic interaction chromatography mass spectrometry (HILIC-MS)

Metal	Ligand	Metal complex fraction relative to total peak area (%)
Ni	Asn	< 2
	Asp	< 2
	Gln	2–4
	NA	**30–35**
	His	**5–10**
	Cit	**15–20**
Cu	**NA**	**75–90**
Co(II)	**NA**	**30–40**
	Cit	**7–10**
Mg	Gln	5–6
	Asn	< 2
	Asp	2–3
	Cit	**10–25**
Ca	Asn	< 2
	Asp	< 2
	Gln	< 2
	Cit	**2–10**
Mn	Gln	2–5
	Asn	< 2
	Cit	**60–85**
Zn	Gln	< 2
	NA	**55–80**
	His	2–5
	Cit	**5–20**
Fe(III)	Mal	< 2
	Gln	< 2
	Asn	< 2
	NA	< 2
	Asp	< 2
	Cit	**30–40**
	Cit_2Mal_2	**20–30**
	Cit_3Mal	**20–30**
Mo	**Mal**	**25–55**
	Cit	**30–60**

Asp, aspartic acid; Asn, asparagine; Cit, citric acid; Gln, glutamine; His, histidine; Mal, malic acid; NA, nicotianamine.
Major ligands for each metal are indicated in bold. The metal complex fraction percentage of each ligand represents the proportion of metal that is bound to this ligand from the HILIC-ICP-MS peak areas compared to the sum of all of the peak areas above the baseline for this element. The missing percentage to reach 100% corresponds to unidentified complexes present as chromatographic peaks that are low intensity and/or have a broad shape (typical of unstable complexes and salts). A conservative cutoff value (< 2%) was set for minor species because their low peak areas are difficult to measure precisely below this threshold. Fe(II)- and Co(III)-complexes were in too low concentrations compared to Fe(III)- and Co(II)-complexes to be distinguished and quantified in HILIC-ICP-MS chromatograms.

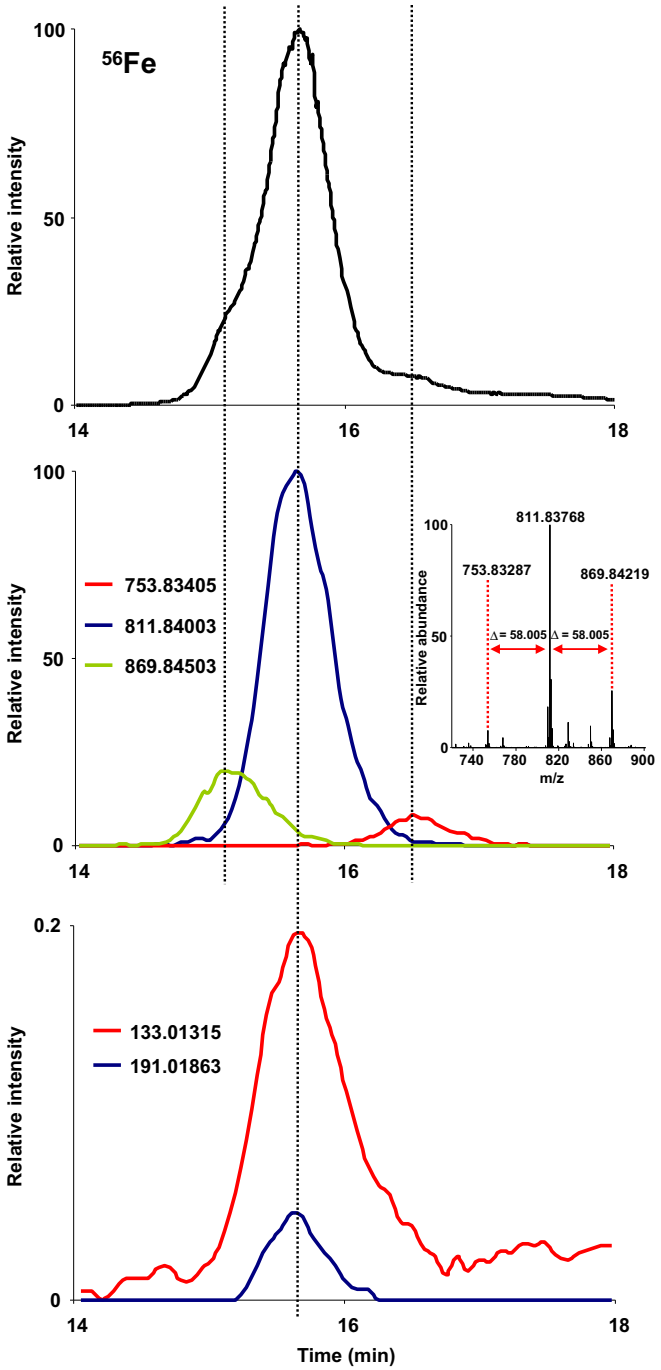

Fig. 3 SEC-ICP-MS (top), SEC-ESI-MS (middle) and SEC-ESI-MS after post column acidification (bottom) chromatograms that are zoomed around elution time of $Fe(III)_3$-$Cit_{(4-x)}$-Mal_x ($x = 1–3$) complexes (*m/z* 753.83, *m/z* 811.84 and *m/z* 869.85 corresponding, respectively, to compounds 17, 18 and 19 from Table 1) from pea (*Pisum sativum*) xylem exudates. Citric (*m/z* 191.02) and malic (*m/z* 133.01) acid are detectable after sample acidification at complex retention times whereas the free forms of these organic acids are observed to elute a few minutes later by SEC chromatography (data not shown).

(Ando *et al.*, 2013). The essential role of NA for Cu uptake in plants has been observed repeatedly (Pich & Scholz, 1996; Liao *et al.*, 2000; Irtelli *et al.*, 2009), but our results are the first showing the unambiguous detection of the Cu(II)-NA complex as the main form of this metal in xylem and ESL. As for Fe, the presence of Cu(II) instead of Cu(I) in xylem and ESL could be explained by the fact that the presence of the strong Cu(II) ligand, NA, without a counterpart for Cu(I), would lower the Cu (II)/Cu(I) couple redox potential. In this study, Cu was also found to have a specific affinity towards some aromatic (Trp and Phe) amino acids. The concentrations of the formed complexes

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are hard to evaluate (but low) because of the high background level for Cu during quantitative HILIC-ICP-MS analysis (see Fig. 1), especially at their retention times as given in Table 1. Therefore, despite the possibility of their creation in the electrospray ion source, the great specificity of the complexes for Cu with regard to other metallic ions is of a particular interest. An increase of Phe content in xylem sap was observed in a chicory plant exposed to high Cu concentrations (Liao *et al.*, 2000).

As Cu is observed mainly in a stable NA-Cu(II) complex, it can be asked if Cu(I) could be produced in the ESL by Cu(II) reduction and then transported into the seed embryo in a similar way to that described for Fe (Grillet *et al.*, 2014b). Cu reduction activity was then tested as described in Supporting Information Methods S1. As shown in Fig. S1, Cu(II) is reduced not only in the presence of the embryo plus its exudate, but also, to the same extent, in the presence of the exudate only. This observation suggests that the reducing activity is mainly due to the excreted species rather than to the reductases, such as FRO family members, present at the embryo surface (Mukherjee *et al.*, 2006). Among potential species involved in the Cu(II) reduction, ascorbic acid and glutathione are those expected to play a major role. An addition of ascorbate oxidase (AOX) indicates that at least half of the reducing activity is due to ascorbic acid (cf. Fig. S1), which confirms the implication of excreted low molecular weight reducing compounds for the reduction of a proportion of Cu(II) in Cu(I). As for Fe, it is interesting to note that among plant metabolites, not only metal ligands but also reducing/oxidizing compounds can remarkably affect metal speciation because they can locally influence metal redox status. More generally, it can be seen that the presence of ligands that maintain Fe and Cu in their initial oxidation states decreases the probability of Fenton or Fenton-like reactions that could occur during repeated redox cycles if these metal ions had been present in a free state (Barbusiński, 2009).

Zinc complexes

Zn(II) is bound primarily by NA. Citrate is also a major ligand of Zn. In some samples, other ligands such as His and, to a lesser extent, Gln were also observed. Proportions between NA, citrate and His varied between samples with NA dominating. Zn has less affinity for NA than Cu and the reason why this complex was observed is likely that the molar amount of NA is as high as, or higher than total Zn content. Note that this is not necessarily the case in all plants, especially those hyperaccumulating Zn (Cornu *et al.*, 2015), wherein the amount of Zn can greatly exceed the amount of NA that becomes a minor ligand compared to organic acids. Zn-NA was putatively observed by MS in rice phloem but with a distorted isotopic pattern, which made validation difficult (Nishiyama *et al.*, 2012). Our work is the first to detect the Zn-NA complex in plant xylem or ESL. In order to confirm the presence of Zn-NA, Cu-NA, other metal complexes with NA or deoxymugineic acid (a similar compound to NA present in graminaceous plants), the use of high-resolution MS and efficient chromatographic separation is indispensable to avoid any confusion due to isobaric interferences (Weber *et al.*, 2006). Our results confirm at the molecular level the strong link observed recently in several studies between NA and Zn transport in plants (Masuda *et al.*, 2009; Johnson *et al.*, 2011; Deinlein *et al.*, 2012; Haydon *et al.*, 2012; Clemens *et al.*, 2013; Schneider *et al.*, 2013).

The presence of Zn-$(Cit)_2$ was observed with both chromatographic set-ups; it gives a better defined peak and seems to be more stable in SEC. Citrate was found to be a commonly occurring counter-ion for Zn(II) in plants using X-ray fluorescence (XRF) (Sarret *et al.*, 2002; Terzano *et al.*, 2008). The Zn-$(His)_2$ complex can also be observed by XRF (Salt *et al.*, 1999; Küpper *et al.*, 2004). However, these two complexes have never been observed in plants using MS until now.

Manganese, cobalt and nickel complexes

Along with Fe and Zn, Mn is a major transition element in plants. It was found here bound mainly to citrate in a Mn-$(Cit)_2$ complex and, at a much lower level, in the Mn-$(Mal)_2$ complex, as predicted elsewhere by XRF in Mn hyperaccumulators (Fernando *et al.*, 2010). Small amounts of Mn-NA were also detected, which is consistent with a possible role of NA for Mn transport via YSL transporters in rice (Ishimaru *et al.*, 2010; Sasaki *et al.*, 2011).

Cobalt (Co) and Ni are two minor micronutrients and they are both observed to be bound mainly by NA and His. Ni-$(His)_2$ and Ni-NA have been observed by MS as minor ligands in hyperaccumulating plants (Ouerdane *et al.*, 2006). The Ni-NA complex could also be observed transported by YSL transporters (Mari *et al.*, 2006; Gendre *et al.*, 2007). With regard to Co, these are the first MS data concerning the speciation of this element in plants. The occurrence of Co-NA in plants implies that this complex may be recognized and transported by YSL proteins. There is an indication in this direction because, in the double *atysl1atysl3* Arabidopsis mutant, the remobilization of Co from senescing leaves is significantly reduced (Waters *et al.*, 2006). Thus, it seems that NA and His may participate in the transport of these two metals within the plant.

Magnesium and calcium complexes

Magnesium (Mg) and calcium (Ca) were mainly found to be complexed with Cit and Gln, but also with Asp and Asn. It is not surprising to detect Cit as a major ligand for these elements because Cit is one of the major organic acids in plants and contains several binding sites for metals (three carboxylic groups and one hydroxyl). Detection of such complexes is usually difficult because of their lability and their potential loss during chromatography. Some other chromatographic peaks (very broad shapes; Fig. 1) were also observed by ICP-MS, which is typical of weakly bound complexes. Their retention times and their number seem to indicate that they are not due to free metal ions. According to retention time, it is postulated that saccharides, polysaccharides or other organic acids could be involved in their binding. Some other molecules, aminoadipic acid and pipecolic acid, were detected as potential Ca chelators. However, the lack of similarity between the peak shapes of these complexes by

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HILIC-ICP-MS and HILIC-ESI-MS chromatographic profiles seems to indicate that the formation of these complexes may be just opportunistic and could occur in the ion source. Finally, chromatograms with ICP-MS detection were also recorded for K, Ti and Sr (data not shown) and they exhibited similar shapes/peak retention times as for Mg and Ca, which seems to indicate that they have the same binding targets.

Molybdenum complexes

Complexes of molybdenum (Mo) with Mal and Cit were observed to be the main forms of this element in xylem and ESL (two ligands for one MoO_2^{2+} ion). MS analyses of the Mo-containing species are detailed in Fig. 4. A zoom in MS/MS mass spectrum of the fragmented MoO_2-$(Mal)_2$ complex allows the observation of a fragment ion at *m/z* 260.89 with the characteristic isotopic pattern of Mo (Fig. 4c), which is consistent with MS/MS spectra of the MoO_2-$(Mal)_2$ ion of the standard mixture (Fig. 4e). Coelution of free malate and free citrate with their corresponding Mo complexes (Fig. 4d) confirms the identification of these organic acids in playing a role in Mo binding.

Contrary to other metals having affinity for organic acids, Mo seems to be preferentially bound by Mal over Cit. The coordinating environment of Mo(VI) that is surrounded by 2, 3 or 4 oxygen atoms (MoO_2^{2+}, MoO_3 and MoO_4^{2-}) makes the stability constants of the Mo(VI) complexes with Mal and Cit relatively similar (Cruywagen *et al.*, 1995, 1997), whereas for other metals Cit is usually a stronger binding ligand. The MoO_2-$(Mal)_2$ complex was recently observed to be the main Mo binding species in alfalfa plant leaves when accumulating high amounts of this element (Steinke *et al.*, 2008). Although Mo concentrations are three orders of magnitude lower in *P. sativum* xylem sap, the same characteristic affinity of malate for Mo(VI) was observed. Therefore, malate transport and production by plants could directly impact Mo(VI) uptake and translocation within the plant.

Summary on metal complexes potentially involved in metal transport in plant

The ligands involved in metal binding in plants observed in this study are summarized in Fig. 5. Several ligands reported in the literature to play a role in metal binding are also shown in this figure but were not observed in this study, mostly because they are either absent in *Pisum sativum* (e.g. phytosiderophores such as mugineic acid are present only in Gramineae), weakly produced in the case of absence of stress (e.g. phytochelatines and thiol-containing species) or in low concentrations in xylem/ESL samples (e.g. soluble phytate).

New information and a point of view on metal transport/homeostasis

In summary, the affinity for NA or for citrate is shared by several metals, which implies a competition for the binding. The metal speciation will then depend on the relative amounts of the different ligands and of the different metals (in addition to pH and ion

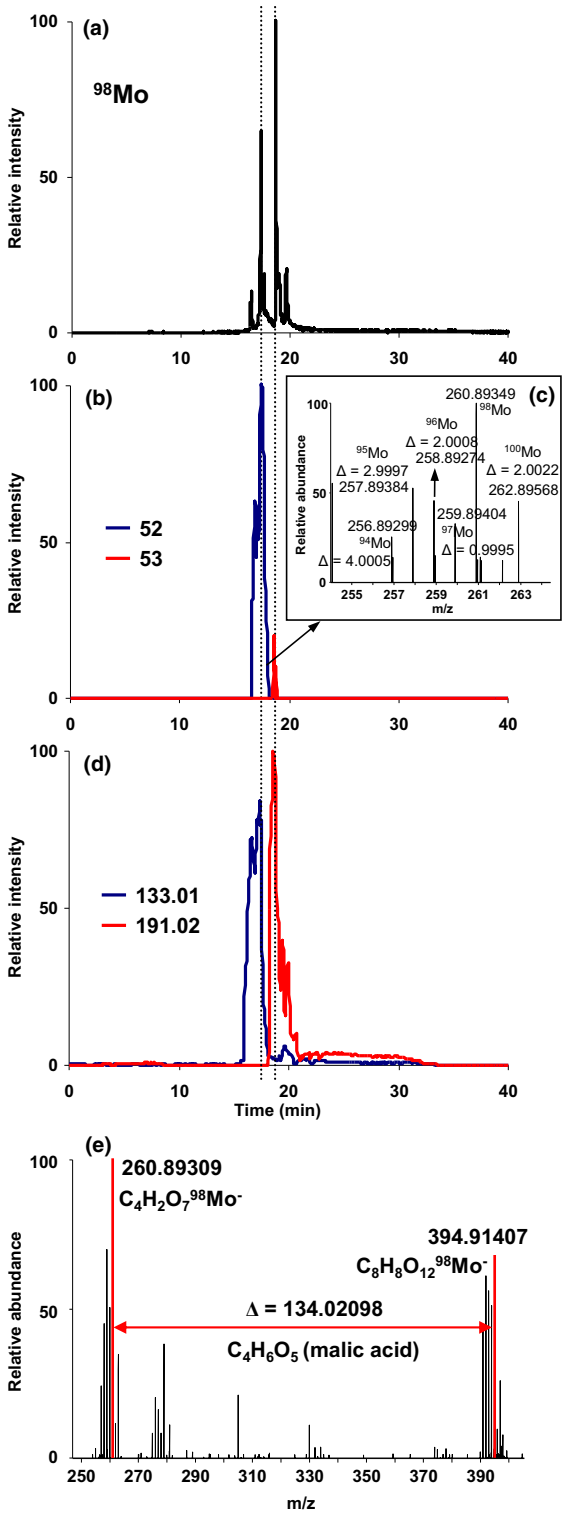

Fig. 4 Analysis of molybdenum (Mo) in embryo sac liquid (ESL) from pea (*Pisum sativum*) plants: (a) HILIC-ICP-MS chromatogram following ^{98}Mo, (b) HILIC-ESI-MS/MS (negative ionization mode) chromatogram following the specific mass transition for compound 52 (394.91 → 260.89) and compound 53 (510.93 → 318.89), (c) zoom of mass spectrum containing the ion at *m/z* 260.89 with characteristic isotopic pattern of Mo, (d) HILIC-ESI-MS (negative ionization mode) chromatograms following malic acid (*m/z* = 133.01) and citric acid (*m/z* = 191.02), (e) ESI-MS/MS spectrum obtained after analysis of Mo-malic acid (1 : 50) standard showing specific mass transition for compound 52.

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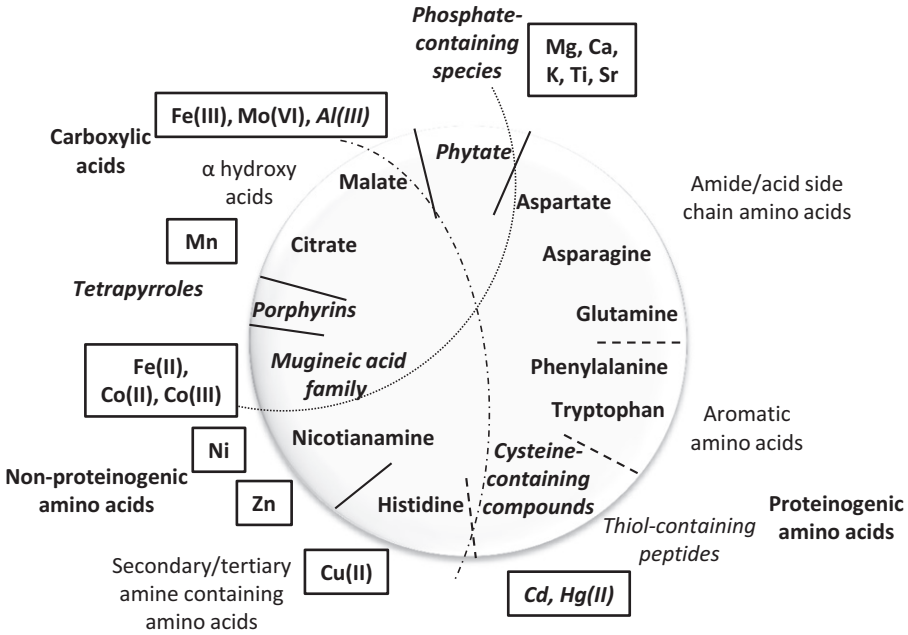

Fig. 5 Scheme representing the main low molecular weight ligands (in the central circle) involved in metal binding in plants according to present work and the literature. Metal ions (in boxes) are represented close to the ligands that are the most commonly found to play a key role in their binding. Key ligands are therefore the ones present in the closest vicinity for each metal. As examples, for Fe(III) (dotted semicircle) and for Ni (dashed semicircle), the most relevant ligands for each element are circled. Even if they were not observed in pea (*Pisum sativum*) xylem or embryo sac liquid (ESL), ligands and metals written in italics were described in the literature as being involved in metal complex formation in plants.

strength). All potential ligands and metals involved in metal chelation must be taken into account to evaluate the overall speciation. If some ligands, like NA, can bind several metals then the differentiation in the transport of the different complexes in plants will occur because of the specificity of dedicated transporters, such as YSL transporters for NA and its complexes (Curie *et al.*, 2009). However, if they are more concentrated, similar complexes can outcompete the metal complex that is expected to be transported, especially if the transporter is not very specific. To predict which metals and ligands have to be carefully monitored during an experiment dedicated to follow the transport of a particular metal ion, it is therefore obligatory to already know the potentially competing/involved metal species, which can be performed by the method developed here.

In addition, the nature of the involved ligands and their complexes such as multiple ligand for one type of metal ($Fe(III)_3$-$(Cit)_2$-$(Mal)_2$), different proportions (1 : 1, 1 : 2, 1 : 3, 1 : 1 : 2, 2 : 2, 3 : 3, 3 : 2 : 2,. . .) and mixtures of metals complexed by one type of ligand (Fe_2-Al_1-$(Cit)_3$ (Grevenstuk *et al.*, 2013)) are difficult to predict *a priori*, which can make computational extrapolation hazardous (Harris *et al.*, 2012). The recent observation in bacteria of transporters dedicated to $Fe(III)_3$-$(Cit)_3$ transport uniquely (Fukushima *et al.*, 2012) confirms the importance of having an appropriate tool to accurately determine the metal speciation, not only in terms of knowledge about the ligand potentially involved, but also in terms of the actual chemical form of the metal complex. The excess (hyperaccumulating plants) or absence (mutants, depleted media/soil) of one metal or one ligand can lead to a novel speciation for all elements. As an illustration, an experiment was also conducted with a pea DGV mutant, *dgl* (*degenerated leafs*), known to accumulate Fe due to a defective regulation of its uptake. Without addition of Fe, DGV and *dgl* plants did not show significant differences in growth and in Fe speciation (data not shown). However, in the presence of excess Fe (500 µM Fe added), both plant growth (Fig. S2a) and Fe speciation in xylem sap (Fig. S2b) were affected in *dgl* plants. Probably due to citrate overproduction as a response for Fe toxicity in *dgl* plant, proportion between the $Fe(III)_3$-$(Cit)_2$-$(Mal)_2$ complex (first peak) and $Fe(III)_3$-$(Cit)_3$-$(Mal)_1$ complex (second peak) changed in *dgl* mutant compared to DGV plant (Fig. S2b), which indicates that metal toxicity could also be revealed through changes in metal speciation.

Finally, this method also allows the detection of minor complexes for each element, which allows the identification of potentially important complexes even if they are at low concentrations. A transporter, an enzyme or a chemical reaction that specifically needs this complex to function properly, may need it in very minute amounts, which means that their detection is essential.

In conclusion, the proposed approach by HILIC-ICP-MS and HILIC-ESI-high-resolution FTMS is an important tool for determining metal speciation in biological matrices. The obtained qualitative and semiquantitative information of the nature of the potentially involved ligands as well as the probable metal-ligand, ligand-ligand and metal-metal interactions/competitions at the molecular level can contribute to a better understanding of metal homeostasis in molecular biology studies.

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Author contributions

L.O., P.F., R.L., S.M. and C.C. designed the research; P.F., L.O., L.G. and S.M. performed the research; L.O., P.F., L.G., R.L. and S.M. analysed the data; L.O., P.F., R.L. and S.M. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Copper reduction activity.

Fig. S2 DGV (WT) and *dgl* (mutant) plants grown in the presence of Fe in excess (500 μM).

Methods S1 Copper reduction assays and copper reduction activity measurement of exudates.

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