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Paulina Maria Flis

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Développement de méthodes analytiques pour une spéciation à grande échelle des composés métalliques dans les plantes

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Directeur de thèse : Ryszard Lobinski

Co-directeur : Laurent Ouerdane

Commission d'examen :

S. Clemens (Rapporteur)	Professeur, Université de Bayreuth, Bayreuth, Germany
D.E. Salt (Rapporteur)	Professeur, Université d'Aberdeen, Aberdeen, Scotland, United Kingdom
R. Lobinski	Directeur de recherche au CNRS, LCABIE, UMR 5254, CNRS-UPPA, Pau, France
L. Ouerdane	Maître de conférences, LCABIE, UMR 5254 CNRS-UPPA, Pau, France
M.-P. Isaure	Maître de conférences, LCABIE, UMR 5254 CNRS-UPPA, Pau, France
S. Mari	Chargé de recherche, INRA, BPMP, UMR INRA-CNRS-UM2-SupAgro, Montpellier, France

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Abstract. Numerous metals, such as, e.g. Zn, Fe, Cu or Ni play an essential role in normal plant growth and development as they are involved in different physiological processes that may be, however, disrupted by metal deficiency or excess. Therefore, to regulate metal uptake, translocation and accumulation plants have developed diverse mechanisms including the production of low molecular mass metal binding metabolites. The knowledge of these forms of metals and the processes they undergo in plants can be used in environmental, nutrition and toxicological studies. However, this knowledge was very limited as there was a lack of methodology that could be successfully applied to investigate trace metal speciation in plants. Therefore, the aim of this study was the development of the methodology for the analysis of metal species in complex plant samples. The novel approach is based on the use of combination of (i) HPLC – ICP MS coupling allowing the detection of numerous, often low concentrated, metal-containing species with (ii) a parallel identification using HPLC – electrospray Orbitrap MS/MS coupling. The developed approach allowed the identification of (i) ca. 60 different, mainly previously unreported metal species in saps of *Pisum Sativum* (green pea) and (ii) several mixed iron – aluminum – citrate complexes in *Plantago almogravensis*. This developed methodology was also applied to (iii) investigate selenium speciation in *Brassica nigra* allowing the identification of more than 30 selenium-containing low molecular mass compounds.

Résumé. De nombreux métaux tels que, par exemple le Zn, le Fe, le Cu ou le Ni jouent un rôle essentiel dans la croissance et le développement normal des plantes car ils sont impliqués dans différents processus physiologiques pouvant être cependant perturbés par une carence ou un excès en métaux. Par conséquent, afin de réguler l'absorption des métaux, leur translocation et leur accumulation, les plantes ont développé des mécanismes divers, comme la production de métabolites de faible poids moléculaire pouvant se lier aux métaux. La connaissance de ces complexes métalliques et des processus qu'ils subissent dans les plantes peut être utilisée dans des études environnementales, nutritionnelles et toxicologiques. Cependant, cette connaissance était très limitée à cause de l'absence de méthodologie appliquée avec succès pour étudier la spéciation des traces de métaux dans les végétaux. Par conséquent, le but de cette étude fut le développement d'une méthodologie d'analyse des espèces métalliques dans des échantillons de plantes. La nouvelle approche est basée sur l'utilisation conjointe du (i) couplage HPLC – ICP MS permettant la détection de nombreuses espèces, souvent peu concentrées, contenant des métaux avec (ii) une identification en parallèle par couplage HPLC – electrospray Orbitrap MS/MS. Elle a permis l'identification (i) d’environ 60 espèces métalliques, la plupart jamais observées précédemment dans différents fluides (xylème, endosperme liquide) de *Pisum sativum* (petit pois) et (ii) de plusieurs complexes mixtes fer - aluminium - citrate chez *Plantago almogravensis*. La méthodologie développée a également été appliquée à (iii) la spéciation du sélénium dans *Brassica nigra* permettant l'identification de plus de 30 composés de faible poids moléculaire contenant du sélénium.

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Introduction

Numerous metals, such as, e.g., iron, zinc, copper, nickel and cobalt, play vital role in life cycle of plants. They are essential for normal plant growth and development as they are involved in different physiological processes in plant. However, excess or deficiency even of an essential metal may adversely affect plant physiology and causes growth inhibition, metal substitution in enzymes, oxidative stress or even plant death. Therefore, plants have developed several mechanisms that allow them to control and regulate strictly the uptake, transport and storage of essential but also non-essential metals at the organismal as well as at the cellular level. These mechanisms include production of metal complexing proteins, peptides, organic acids and amino acids that help to maintain the fragile balance between essentiality and toxicity of metals. However, the analytical knowledge of molecular forms of the small metabolites produced by plants and responsible for detoxification, homeostasis and also uptake, translocation and accumulation of various metals to date is very limited. The characterization of the low molecular mass metal complexes would allow a better understanding of plant physiology and thus provide information (e.g. predict the bioavailability of different metals) that could be used in food, nutrient and toxicological studies, agriculture activities as well as in environmental research.

To fill the gap in knowledge of plant metallomics, a great interest is given to studies of metal speciation in plants. However, the identification of metal species in plants faces a few obstacles. The main issues are the lack of methodology that would work when applied to complex biological matrix and also low concentrations, instability and high diversity of metal complexes present in plants. Other issues include also various labilities of metal species during sample preparation and chromatographic separation. Part of these issues can be solved by using optimized extraction procedures, diverse techniques such as HPLC to purify, preconcentrate and separate compounds in unchanged form and MS for detection, quantification, identification of metal complexes and determination of their structure. Therefore, the best solution appears to be the combination of hyphenated techniques that are based on high-resolution separation by chromatography or electrophoresis with parallel element specific and molecule specific detection.

The aim of this doctoral project was:

- development of analytical methods that can be successfully applied for metals speciation in complex biological matrix;

- identification and determination of low molecular weight ligands responsible for metal transport in plants;
- identification of ligands responsible for detoxification of chosen metals;
- identification and characterization of metal complexes presents in plants saps;
- insight into the metal transport mechanisms in plant fluids;

The analytical approach present in this work is based on a coupling of size exclusion column (SEC) and hydrophilic interaction column (HILIC) to either a collision cell ICP MS for elemental mass spectrometry or an ESI-LTQ Orbitrap MS for high resolution molecular mass spectrometry. In the next step the obtained data are compared and combined to allow an unambiguous characterization of metal complexes. The PhD project aimed at further application of the developed methodology to achieve information about element speciation in different plant species and thus determination of the molecular forms of the low molecular weight metabolites produced by plants that are involved in metal transport and detoxification. The developed novel approach has been successfully used to achieve information about:

- ligands that are involved in metal transport in xylem and in to the seeds in the model organism – *Pisum Sativum* (green pea);
- ligands that are involved in aluminum detoxification process in aluminum accumulating plant *Plantago almogravensis* Franco;
- comprehensive selenium speciation in seeds of selenium accumulating plant *Brassica nigra* (black mustard).

Literature Part

List of the abbreviations

2D-LC – two dimensional liquid chromatography
AA – amino acid
ABC – ATP-biding cassette transporter
AC – alternating current
ACN – acetonitrile
AHA(1/2/7) – *Arabidopsis thaliana* H+ ATPase (1/2/7)
AtABCC1/2 – *A. thaliana* ABC Transporter C Family Member 1/2
AtITR1 – *A. thaliana* iron-regulated transporter 1
ATM(1/3) – mitochondrial inner membrane ATP-binding cassette (ABC) transporter (1/3)
AtOPT3 – *A. thaliana* oligopeptide transporter 3
ATP – adenosine-5'-triphosphate
AtPDR8 – *A. thaliana* pleiotropic drug resistance ABC transporter
ATX1 – Antioxidant Protein1 copper chaperone
AtYSL1/2/3 – *A. thaliana* Yellow Stripe 1/2/3-like proteins
AVA – avenic acid
BP – binding protein
CAD – charged aerosol (detection)
CCS1 – copper chaperone for superoxide dismutase 1
CEC – capillary electrochromatography
CID – collision induced dissociation
COPT – copper transporter family
COPT1 – copper transporter 1
COX17 – cytochrome c oxidase copper chaperone
CRC – collision/reaction cell
CTR1 – copper-regulated transporter 1
CW – cell wall
CZE/CE – capillary zone electrophoresis/capillary electrophoresis
DC – direct current
DMA – 2'-deoxy- mugineic acid
DMF – dimethylformamide
DNA – deoxyribonucleic acid
DW – dry weight
EDTA – ethylenediaminetetraacetic acid
ELSD – evaporative light scattering (detection)
Epi-HDMA – 3-epihydroxy 2'deoxy mugineic acid
Epi-HMA – 3-epihydroxy mugineic acid
ESI – electrospray ionization
ES MS – electrospray mass spectrometry
EXAFS – X-ray absorption fine structure
FER1/2/3/4 – ferritins 1/2/3/4
FL – fluorescence (detection)
FRD3 – ferric reductase defective3 protein
FT-ICR – Fourier Transform ion cyclotron resonance (analyzer)
FRO2/7 – ferric chelate reductases
GC – gas chromatography
HAVA – hydroxyavenic acid
HCD – higher- energy collisional dissociation
HILIC – hydrophilic interaction liquid chromatography

HIPPs – heavy metal-associated isoprenylated plant proteins
HPL – high pressure cell
HR – high resolution
HMA(1/2/3/4/5/6/8) – heavy metal ATPase (1/2/3/4/5/6/8)
HPLC – high performance liquid chromatography
HSP – heat shock protein
HSP 60/70/90/100 – 60/70/90/100 kilodalton heat shock proteins
HvIRT1 – *Hordeum vulgare* iron-regulated transporter 1
ICP MS – inductively coupled plasma mass spectrometry
IEC – ion-exchange chromatography
INAA – instrumental neutron activation (detection)
IRT(1/2) – iron-regulated transporter(1/2)
Isa1p/Isa2p – iron-sulfur proteins
IT – ion-trap (analyzer)
LA – laser ablation
LeIRT1/ LeIRT2 – *Lycopersicon esculentum* iron-regulated transporter ½
LIT – linear ion trap
LPC – low pressure cell
LTQ – linear trap quadrupole
MA – mugineic acid
MALDI – matrix-assisted laser desorption ionization
MATE – multidrug and toxic compound extrusion type transporters
MEKC – micellar electrokinetic chromatography
MC – multicollector
MS – mass spectrometer/spectrometry
MSn – tandem mass spectrometer/spectrometry
MS/MS – tandem mass spectrometer/spectrometry
MTP – metal transport protein family
MTP1/3 – metal transport protein 1/3
MTs – metallothioneins
MtZIP2 – Zn transporter from *Medicago truncatula*
NA – nicotianamine
NAAT – nicotianamine aminotransferase
NADH – reduced form of nicotinamide adenine dinucleotide
NADPH – reduced form of nicotinamide adenine dinucleotide phosphate
NP-LC – normal phase liquid chromatography
NRAMP(1/3/4) – natural resistance-associated macrophage protein (1/3/4)
NAS – nicotianamine synthase
OA – organic acid
OPT – oligopeptide transporters family
OsFRDL1 – *Orysa sativa* ferric reductase defective 3-like protein 1
OsIRT1 – *O. sativa* iron-regulated transporter 1
OsYSL2/15 – *O. sativa* Yellow Stripe 2/15-like proteins
OsZIP4 – *O. sativa* ZRT/IRT-like protein 4
PAA1/2 – P-type ATPases of *Arabidopsis*
PCs – phytochelatins
PC2/3/4/5/6 – phytochelatin 2/3/4/5/6
PEP – phosphoenolpyruvate
PIC1 – permease, membrane transport protein
PM – plasma membrane

ppm – parts per million
PS – phytosiderophores
Q – quadrupole (analyzer)
RF – radio frequency
RI – refractive index (detection)
RING – really interesting new gene
RNA – ribonucleic acid
ROS – reactive oxygen species
RP HPLC – reversed-phase high performance liquid chromatography
Ru-BP – ribulose-1,5-bisphosphate
SEC – size exclusion chromatography
SF – sector field
SCO1 – cytochrome c oxidase assembly protein
SOD – superoxide dismutase
sHSP – small heat shock protein familiy
STA1/AtATM3 – *A. thaliana* ABC transporter of the mitochondrion 3
TgMTP1 – metal transporter 1 from *Thlaspi goesingense*
THF – tetrahydrofuran
TOF – time of flight (analyzer)
TXRF – total-reflection X-ray fluorescence (detection)
UPLC – ultra performance liquid chromatography
UV – ultraviolet light absorbance (detection)
VIT1 – vacuolar iron transporter 1
XANES – X-ray absorption near-edge structure
XAS – X-ray absorption spectrometry
YS1 – Yellow Stripe 1
YSL – Yellow Stripe proteins family
YSL2/4/6 – Yellow Stripe like-2/4/6
ZAT1 – zinc transporter of *A. thaliana*
ZIC-HILIC – zwitterionic–hydrophilic interaction liquid chromatography
ZIP – zinc-regulated transporter, iron-regulated transporter -like protein
ZIP1/3/4 – Zrt- and Irt-like protein 1/3/4
ZmYS1 – *Zea mays* Yellow Stripe 1-like protein
ZNT1/2 – zinc transporters, members of ZIP family
ZRT – zinc-regulated transporter

1. Biological function of different elements in plants

Numerous elements play vital role in life cycle of plants. They can be present in huge amounts in plant tissues and thus they are regarded as essential macronutrients, such as, e.g. carbon, oxygen, hydrogen, nitrogen, magnesium or calcium. They can be also required by plants in much smaller concentration and thus they belong to the group of the essential micronutrients, such as, e.g. iron, manganese, zinc, copper, nickel or molybdenum. Some elements pose as beneficial elements that are not essential in plant physiological processes but they can promote plant growth. These elements include cobalt, silica, selenium, chromium or vanadium. There are also elements such as cadmium, lead or aluminum that are regarded as nonessential or even toxic for plants.[2] For this reason, the short characteristics of the most important elements concerning this study are presented in following paragraphs.

Magnesium. Magnesium is one of the most abundant mineral nutrients in plants. The majority of magnesium ions are bound by cellular components or incorporated into their structure. In the leaf cells magnesium is associated primarily with proteins while free ions are stored in vacuoles that are main storage compartments for this element in plants. In green leaves typically 15-20% of total magnesium is integrated with chlorophyll as the central atom in the molecule structure, albeit this percentage may change depending on the magnesium supply. Magnesium has very important function in protein synthesis. Approximately 75% of total amount of this element present in leaves is involved in this process as a bridging element, essential in the aggregation of ribosome subunits. There is a big variety of enzymes (e.g. RNA polymerases, glutathione and glutamine synthase, PEP carboxylase or fructose-l,6-bisphosphatase), substrates and reactions (e.g. the formation of RNA in the nucleus, the modulation RuBP carboxylase in the stroma of chloroplast, phosphorylation and dephosphorylation) that require or are promoted by magnesium (e.g. as a bridging cation). The deficiency of magnesium causes a decrease of photosynthesis rate and accumulation of starch and sugars in the source leaves.[1, 2]

Calcium. Calcium is a macronutrient that is essential for plants in form of divalent cation. It is required in the great deal of various processes concerning almost all aspect of plant growth and development. Calcium is a crucial element in process of crosslinking acidic pectin residue in the cell wall and thus is substantial for strengthening of the cell. Calcium is also essential for maintaining proper permeability of the plasma membrane and it acts as an intracellular messenger in the cytosol and a counter cation for organic anions (e.g. malate) or inorganic anions (e.g. nitrate, chloride) in the vacuoles. Plants also require calcium to maintain enzyme activity and nitrate uptake. If plants are growing on acidic, sandy, or coarse soils that contain a low amount of calcium, the calcium deficiency symptoms may occur, however, it does not happen in nature too often.[2, 3]

Iron. Iron is one of the micronutrients essential for many plant functions. It is taken up by plants as either Fe^{2+} (ferrous cation) or Fe^{3+} (ferric cation). Iron importance in various plant

processes is caused by its ability to accept and donate electrons. The facility of iron to form different complexes allows the modulation of its redox potential that may vary broadly depending on the ligand. This changeability is responsible for an important role played by iron in biological redox systems. Iron is a constituent of two major group of proteins – heme and Fe-S proteins. Among the heme proteins are cytochromes, catalase, peroxidase and leghemoglobin. Cytochromes are the components of redox system in the chloroplasts and the redox chain in nitrate reductase because of the characteristic iron-porphyrin complex which acts as a prosthetic group of the protein. Catalases and peroxidases as a heme enzymes facilitate different redox reactions and their activity decreases under iron deficiency condition. The second family of proteins is constituted of the iron-sulfur proteins like ferredoxin that is taking part in the transition of electrons. Concerning enzymes, iron can be either metal component, crucial to maintain their stability and activity or a bridging element between enzyme and substrate. For these reasons iron is an important element in the energy transfer, respiration, chlorophyll function and development of plants.[2]

Copper. The one of the essential micronutrients is also copper that is taken by plants as a cupric ion (Cu^{2+}) that can be easily reduced to monovalent copper. The copper ability to exist in different oxidation states is the main reason of importance of this element in many physiological processes in plants. The proteins that have Cu as a metal component can be divided in three groups. First group are blue proteins that are involved in one-electron transfer such as plastocyanin which bounds over 50% of copper localized in the chloroplast and is a part of the electron transport chain in photosystem I. The second group of Cu-proteins are non-blue proteins (e.g. peroxidases, oxidize monophenols) and the third one – multicopper proteins that contain in their structure at least four copper atoms (e.g. diphenol oxidase, scorbate oxidase). Copper as a constituent of different proteins and enzymes is implicated in the photosynthetic electron transport, lignin formation in the cell walls, respiration process in the mitochondria, hormone signaling and mechanism of detoxification of free radicals and thus in response to oxidative stress. In case of copper deficiency, the activity of enzymes and the formation of some proteins (e.g. plastocyanin) decrease rapidly that causes metabolic changes and at the end inhibition of plant growth.[2, 4]

Zinc. Zinc is a substantial micronutrient that plays different roles in the plant physiology. Plants are taking up zinc typically as a divalent cation which is redox-stable under physiological conditions. Zinc has strong tendency to form the covalent bonds with N-, O- and S- ligands that causes this element is an essential component of thousands of proteins and enzymes in plants. In enzymes zinc acts catalytic (e.g., carbonic anhydrase and carboxypeptidase), cocatalytic or structural (e.g., alcohol dehydrogenase) function. It is also responsible for gene regulation and stabilization of the protein structure including Zn fingers, Zn clusters and RING finger domains. Zinc is involved in ribosomes structure where is required for maintaining their structural integrity. It might be bound by phospholipid and sulfhydryl groups of membrane components and thereby maintain the integrity of biomembranes. Zinc is also involved in the processes such as photosynthesis and CO_2

fixation. Excess and deficiency of zinc in plants lead to high plant mortality, reduced and stunted growth, chlorosis, necrosis, small leaves and delay in flowering.[5, 6]

Manganese. In plant physiology manganese plays a role of an essential micronutrient. Manganese is taken up as a manganous ion (Mn^{2+}) because it is the most available form for plants. In plants a predominant manganese oxidation state is Mn(II) but it can also occur as a Mn(III) and Mn(IV). Therefore, this element is important in different redox processes. Manganese is component of the photosynthetic proteins and enzymes (manganese-protein in photosystem II and manganese-containing superoxide dismutase). It is also cofactor responsible for activation of nearly 35 various enzymes (e.g. superoxide dismutase, Mn-catalase and pyruvate carboxylase). For these reasons manganese is involved in photosynthesis, respiration, hormone activation, nitrogen metabolism, plants defense against oxidative stress, ATP synthesis and biosynthesis of chlorophyll, proteins, fatty acids, acyl lipids, aromatic amino acids and also secondary products such as lignin or flavonoids. In case of manganese deficiency the chlorophyll and lignin content decreases, the photosynthesis is disturbed and root and seed growth are inhibited.[2, 7]

Nickel. Nickel belongs to the group of micronutrients and in the biological systems its oxidation state is usually Ni(II) but other valences – Ni(I) and Ni(III) are also possible. In wide range of higher plants nickel is a constituent of the enzyme – urease. In the urease structure nickel is bound by N- and O-ligands and is substantial to maintain structure and catalytic function of the enzyme. Nickel together with urease is involved in nitrogen metabolism in some plant species. Nickel is essential for higher plants and its deficiency may have severe effects on plant growth, ageing, disease resistance, nitrogen metabolism, iron uptake and also the normal grain development.[2, 8]

Molybdenum. In comparison with other micronutrients, molybdenum is required by plants in lower amounts and is available for them just in the soluble forms of Mo(VI) wherefore is mainly taken up as molybdate ion (MoO_4^{2-}). Molybdenum can occur in different oxidation states such as Mo(IV), Mo(V) and Mo(VI) that results in that this metal is a constituent of different enzymes as cofactor. Molybdenum is important for the structural and catalytical function of the enzymes and is involved in the redox reactions. The molybdenum enzymes in plants are nitrate reductase, nitrogenase, xanthine oxidase/dehydrogenase, aldehyde oxidases and probably sulfite oxidase. Molybdenum as a component of these enzymes is involved in the nitrogen metabolism (nitrogen fixation and nitrification), purine catabolism, phytohormone biosynthesis of indoleacetic acid and abscisic acid and in formation of sulfate. Molybdenum deficiency may cause delay in flowering, inhibition of tasseling and anthesis, retarded development of sporogenous tissues, chlorosis in young leaves and stunted growth. However the visible symptoms of deficiency vary depending on plant species.[2, 9]

Selenium. Selenium is an essential element for animals, humans and microorganisms but there is no evidence that it is substantial for higher plants. However, selenium is regarded as

beneficial element for the growth of various plant species and it can be taken up by plants as selenate, selenite or organoselenium compounds (e.g. selenocysteine and selenomethionine). Chemical similarity of selenium to sulfur causes that selenium undergoes the same pathways as sulfur. The process of the non-specific selenium metabolism results in the replacement of sulfur by selenium in S-metabolites. Selenate that was taken up is reduced to selenite and selenide and then assimilated into the organoselenium compounds such as selenocysteine or selenomethionine. These compounds can replace cysteine and methionine in the protein structure by non-specific incorporation. The plant ability to accumulate and convert selenium into the different compounds and also the chemical nature of these compounds is of the great interest of scientists as it can have various implications for health and nutrition of animals and humans.[1, 10, 11]

Aluminum. Aluminum is not considered as an essential element even for plant species that accumulates this element. In acidic soils aluminum is solubilized and available for plants in the most toxic form – Al^{3+} ion. Therefore, aluminum toxic effects on plant growth and development are the main factor limiting crops efficiency on soils with low pH that pose approximately 40% of the earth's arable land. However, there are some plant species that tolerate high aluminum concentration in soil and are even able to accumulate this element in their tissues. For that reason, there is a big interest in studying mechanisms of aluminum tolerance and detoxification in the aluminum resistant plant species to understand the nature of these processes.[12, 13]

Cadmium and lead. Opposite to heavy metals, such as iron, zinc or copper that are essential micronutrients for normal plant growth, cadmium and lead are non-nutrient elements. They can be highly toxic for plants as they can inactivate many enzymes and disturb the metabolic processes such as transpiration, photosynthesis, chlorophyll synthesis or uptake of macro- and micronutrients. Plants growing on the soils polluted by heavy metals can take up cadmium and lead via the root system. However, the bioavailability of these elements depends on the soil pH and presence of other metals and chelating agents. In the case of most plant species (excluder group) cadmium and lead are accumulated mostly in the underground organs and the content of these metals in different plant parts decreases as follows: roots > leaves > stems > inflorescences > seeds. On the cellular level, up to 96% of absorbed cadmium and lead is accumulated in cell wall and vacuoles. The uptake of these heavy metals by plant activates, depending on the plant species, different detoxification mechanisms that are described in Chapter 4.[14]

2. Metabolic pathways of metals in plants – strategies of metal acquisition and homeostasis in plants

As described in previous chapter, there is a group of metals essential for normal plant growth and development while others may have diverse toxic effect on plants. However, even if the essential metal is present in inadequate amount compared to this required by plant, it may lead to deficiency or toxicity effects. Metals, such as zinc or copper, pose specific dilemma to plants because they are crucial for many metabolic processes but also potentially dangerous in the higher concentrations. Therefore, plants strictly control and regulate the accumulation of essential metals at the organismal as well as at the cellular level. The metal requirements depend on an organelle and they vary markedly. Hence, it is necessary to maintain the proper level of their concentrations in different tissues and compartments throughout tight coordination of processes like uptake, buffering, translocation and storage.[15]

The process of metal acquisition and maintaining homeostasis consists of a few stages that are shown in Fig. 1.

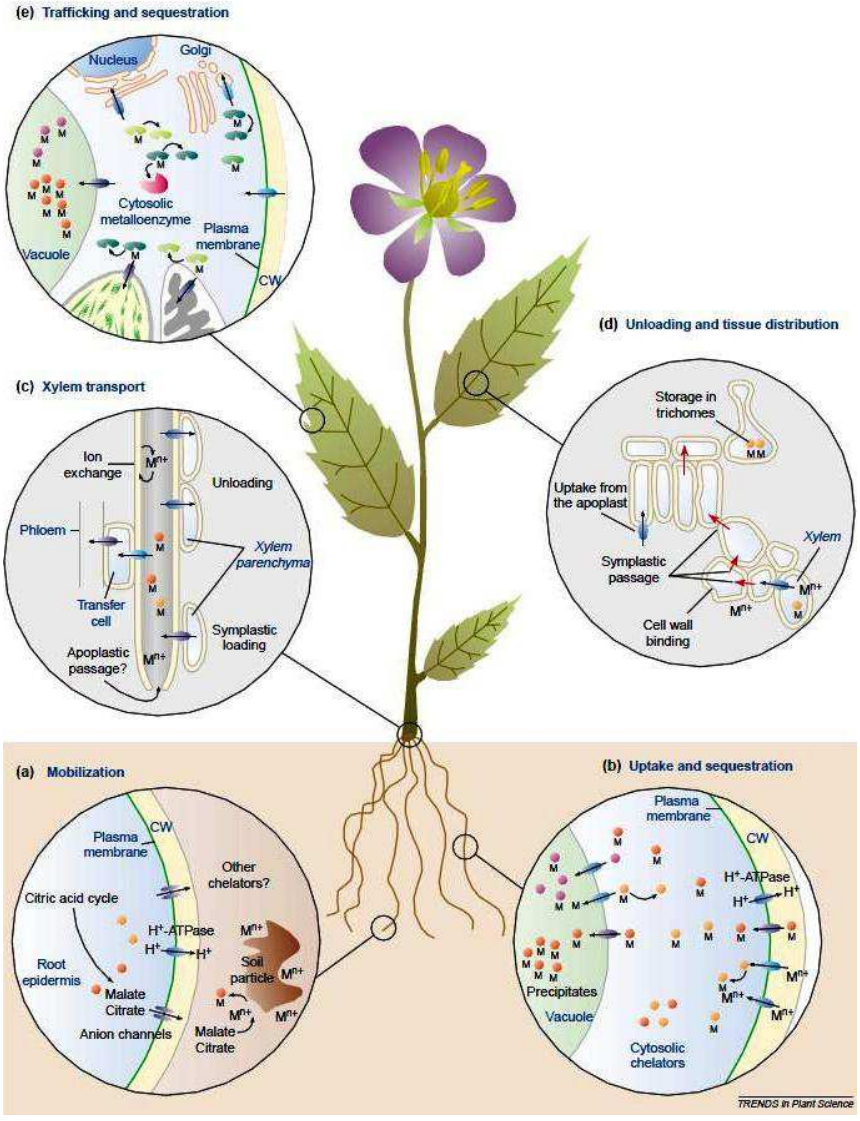

Figure 1. Molecular mechanisms involved in heavy metals uptake, transport, distribution and storage by plants.[15]

At the beginning metals are mobilized by acidification of the rhizosphere and secretion of chelators to be taken up from the soil through various systems placed in the plasma membrane in the next stage. Then, metals undergo the processes of compartmentation and sequestration within the root to be at the end loaded into the xylem and transported to the shoots. In leaves metals are unloaded to different leaf cell types and then moved through plasmodesmata form one cell to another. The last stage is metal distribution inside the cell and storage. During the passage through the plant just little amount of metals is transported as free hydrated ions. Presumably, they are bound to different ligands and the movement within the cell requires exchange reaction between ligands as it was recently reported for copper[16] and zinc[17] transport in yeast and bacteria, respectively. The efficiency of metal acquisition can be affected at every stage by the concentration and affinities of chelating molecules as well as by different selectivity of the transport systems in the plasma membrane.[15]

2.1. Strategies of metal mobilization and uptake

Soil is the main source of metals for plants. Despite the fact that an essential element is abundant in soil, its bioavailability and absorption by the roots can by limited by a few factors. These factors include soil type, aeration, pH, cation exchange capacity and redox potential as well as metal concentration, competing ions, the presence of microbial flora and fungi.[18, 19] Many of essential elements are present in soil in an insoluble form. Iron is usually found in soil as a component of insoluble oxyhydroxide polymers. These Fe(III) oxides are quite stable and insoluble at neutral pH and thus in an aerobic and aqueous environment the amount of free Fe(III) is very limited and not enough for optimal plant growth.[20] Also, zinc and copper are present mainly in an insoluble form because of the absorption to clay, $CaCO_3$ or organic matter. Therefore, an efficient mobilization and uptake is essential for normal plant development. There are different mechanisms developed by plants to enhance metal availability and uptake form soil and they include acidification of the rhizosphere, exudation of carboxylates, the reduction-based strategy commonly used by nongraminaceous plants or the chelation-based strategy developed by graminaceous plants. Sometimes the root-colonizing bacteria or the mycorrhiza may increase heavy metal availability for plants.[15, 21]

Rhizosphere acidification

The low solubility of different metals and thus their limited availability for plants causes that some plant species deal with this problem by releasing protons to the rhizosphere. This mechanism is used by nongraminaceous plants under the iron deficiency conditions. Protons extrude by roots lead to the decrease in soil pH and in consequence to the generation of free iron by releasing it from insoluble oxides. Acidification of the rhizosphere increases iron solubility thousand times for every one unit drop in soil pH making it available for plants. The production of protons can be quite fast and during a few hours the soil solution pH may drop to value of 3 or even lower. Presumably, the release of protons is caused by activation of ATPase proton pump expressed in the root epidermis in case of iron low supply but it has not been yet confirmed. However, there are assumptions that several proton ATPases which are

the members of AHA (*Arabidopsis* H+ ATPase) family may be responsible for this process. The most likely candidates from AHA family are AHA1, AHA2 and AHA7 as their expression in roots and upregulation are induced under iron deficiency. Among these three ATPases the AHA2 seems to be the most important because the AHA2 expression level is the highest one and the reduced rhizosphere acidification under iron deficiency was observed only during the loss of AHA2.[20, 22, 23] The rhizosphere acidification may also result in enhanced solubility of zinc and copper by encouraging cation exchange and leading, at the end, to the Zn and Cu releasing from the insoluble chelates with soil particles.[21] Apart from protons, roots can exude also organic acids that are responsible for acidification of rhizosphere, however, correlation between releasing organic acid and soil acidification is much more complex than in case of the proton exudation.[24]

Reduction

Some plant species, mainly dicots and nongraminaceous monocots, are using the reduction based strategy, also called Strategy I, for metal uptake because the transporters in plasma membrane of these plants have a specific affinity for a certain oxidation state of metal. In order to take up metals, such as iron or copper, these plants developed a mechanism consisting of the acidification of rhizosphere and exudation of organic acids and phenolic compounds that increase metals (e.g. Fe(III)) concentration in the soil solution. Then, possibly after reduction, metals are transported through different transporters placed in plasma membrane inside the root cells (Fig. 2).[21]

Although iron is present in soil in trivalent form, it is transported to cell in divalent form, after releasing from insoluble compounds. In plants using strategy I, Fe(III) is reduced by membrane-bound ferric chelate reductase (FRO2) to more soluble form – Fe(II) which is easily transported into the plant root cells via Fe(II) transporter – IRT1. The whole process takes place in plasma membrane of root epidermal cells. The mechanism of action of FRO2 is based on transmembrane redox system in which electrons are transferred from NADH across the membrane via four heme groups to the Fe(III) in the rhizosphere. These NADPH dependent Fe(III) chelate-reductase is expressed in the plasma membrane and its activity and accumulation is enhanced under condition of iron deficiency. It was shown that when expression of FRO2 is induced by iron low supply, it is also able to reduce copper – Cu(II). However, copper deficiency has no influence on upregulation of FRO2 expression.[21, 22]

Chelation

To acquire iron from the soil, grasses and cereals use Strategy II which is a chelation-based strategy. This strategy involves secretion of Fe(III) – chelating substances, known as phytosiderophores (PS), into rhizosphere. Then, intact Fe(III) – phytosiderophore complexes is taken up via a specific transport system (Fe – PS transporters) placed in the root cells as it is shown in Fig. 2. This mechanism of iron uptake is present in many nongraminaceous species but up till now the best characterization was obtained for *Arabidopsis thaliana*.[1, 21, 22]

Phytosiderophores are low molecular mass structural derivatives of mugineic acid (hydroxy – and amino – substituted iminocarboxylic acids). They have the ability to solubilize ferric compounds from the root environment for uptake by the plants.[24] The mugineic acid (MA) family phytosiderophores including mugineic acid, 2'-deoxy- mugineic acid (DMA), 3-epihydroxy mugineic acid (epi-HMA) and 3-epihydroxy 2'-deoxy- mugineic acid (epi-HDMA) are synthesized form L-methionine through nicotianamine (NA). The crucial enzyme in this process is NA aminotransferase (NAAT) that takes part in formation of 3" – oxo intermediate of deoxymugineic acid (DMA) by transferring the amino group from NA.[25]

Expression of the genes, implicated in MA biosynthesis, is upregulated in case of low iron supply. For instance, in barley the genes critical for sulfur uptake and synthesis of methionine and PS are highly upregulated during first 24 hours of iron deficiency. In consequence, secretion of MAs from the root epidermis is significantly increased. Presumably, the anionic channels or vesicles are involved in MAs secretion into the rhizosphere.[21, 22] The PS efficiency in mobilizing iron from soil may be impede by various factors, such as other metals ions that can be bound by PS, the soil pH, content of calcium in calcareous soils, PS adsorption on soil particles as well as microorganism in the rhizosphere that may cause degradation of PS. However, it was indicated that Cu has just mild inhibitory effect on iron mobilization by PS while Zn and Mn have no inhibitory abilities.[26]

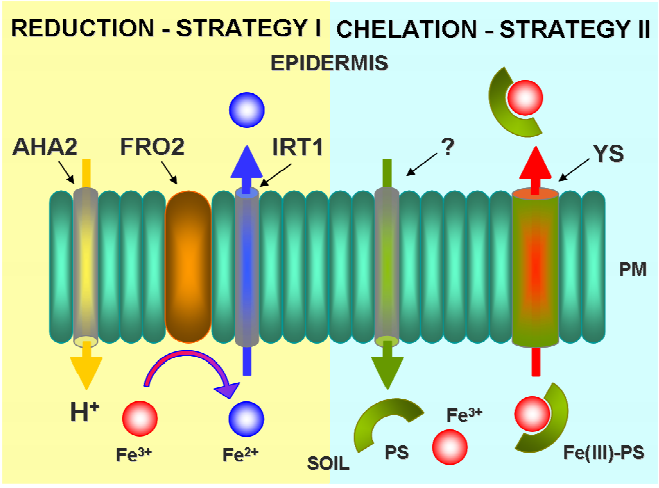

Figure 2. Reduction- and chelation-based strategies used by plants in order to mobilize and uptake metals from the soil. Modified after Guerinot and Yi, 1994.[20]

It was reported that the root exudates form Fe-deficient barley may solubilize metals, such as manganese, copper and zinc, and thus increase their concentration in soil. This process indicates that phytosiderophores, that exudation is induced by iron deficiency, can enhance mobilization of others micronutrients in the rhizosphere.[27] However, there are suggestion that the phytosiderophores are not involved in mobilization of Mn because of their low affinity to this metal compared to Fe, Zn and Cu. The experiments confirmed that Mn is not mobilized by PS either from calcareous or acidic soils.[26] Recent research pointed out that MA is also involved in mobilization of Zn in Zn-deficient barley. Zinc deficiency lead to enhanced expression of genes implicated in the biosynthesis of MAs and results in the increase of secretion of MAs from the Zn-deficient barley roots.[28] It was also demonstrated

that PS deoxymugineic acid can chelate zinc as well as other metals that can be easily taken up by maize.[15]

Mixed strategies

Some graminaceous species are able to combine elements of the reduction strategy that is common for nongraminaceous plants with the Fe-PS complexes uptake. The good example is rice which has the ability to transport Fe(III) – PS chelates as well as Fe(II) similarly to Strategy I plants. It is possible because rice has the orthologs of IRT1 – transporters of Fe(II) and also putative ferric chelate reductases that are responsible for reduction of Fe(III) to Fe(II). Rice ability to induce both responses seems to be quite logic as rice is grown in waterlogged soil that may be rich in Fe (II). However, there is no information to date about other graminaceous species that are able to induce the Strategy I as response for high content of Fe(II) in soil.[22, 23]

Other root exudates

Protons and phytosiderophores secreted by plants are just part of a huge group of diverse compounds that can be released to the rhizosphere to alter the amount of soluble ions and molecules. Their exudation is highly depended on the nutritional status of the plant. The root exudates have a complex composition that can even consist of over 200 various C-containing compounds. This huge variety includes low molecular mass compounds, such as, e.g. organic acids, amino acids, simple and complex sugars, phenols and different secondary metabolites. All these compounds are regarded to be the majority of root exudates. The second group is constituted by high molecular mass exudates, such as mucilage (polysaccharides) and proteins. Root exudates can also contain the root border cells, extracellular enzymes, vitamins, fatty acids, sterols, nitrogenous macromolecules, such as purines and nucleosides, as well as inorganic or gaseous molecules such as HCO_3^-, OH^-, H^+, CO_2 and H_2. Many of these compounds present in the root exudates is suggested, to be implicated in different functions, such as alteration of nutrient availabilities, increasing tolerance to heavy metals or attracting rhizobacteria (e.g. amino and organic acids, proteins or vitamins).[24, 29, 30]

One of the root exudates are phenols which secretion by plants is induced under iron deficiency conditions. These compounds increase the solubility of iron, phosphorus and other nutrients and are produced by different plants. The isoflavonoid phytoalexin[31] and caffeic acid[32] mobilize iron from insoluble iron sources and are produced by Fe-deficient *Medicago sativa* L. plants and Fe-deficient tomato, respectively.[24, 29, 30]

Among different root exudates are also organic acids (OA) including citric, malic, fumaric, succinic, aconitic, acetic, glycolic, malonic, oxalic, formic and piscidic acids. All of them were found in the root exudates of different plants and sometimes their presence were linked to the low nutrient supply in the soil. Organic acids are negatively charged anions that react strongly with various metals in soli solution. Therefore, they increase metals solubility

from highly insoluble soil mineral phases and thus they have important role in acquisition of nutrients, such as phosphorus, iron or manganese.[24, 29, 30]

It was observed that nutrient-deficient maize plants[33] and barley plants under Fe deficiency[34] exhibited higher exudation rates of citric and malic acids, respectively. These reports indicate that organic acids increase the iron mobility in soils by formation of Fe complexes that either are directly taken up by roots or they undergo subsequent ligand exchange.[29] Secretion of organic acid can also be a mechanism of detoxification and it is employed by some plant species. This subject is described more precisely in chapter Mechanisms of detoxification – Root exudates (4.3.).

There are also plant species that exhibit increased exudation of amino acids, for example barley roots produce glutamic acid under iron deficiency.[34] However, some experiments have strongly indicated that proteinaceous amino acids like lysine, methionine, glycine and glutamic acid secreted into the rhizosphere probably act minor role in mobilizing metals from the soil. It was observed that mobilization of nutrients, such as Mg, Ca, Na, K, Cu, Zn, Fe, Mn, was not significant, evidencing that ability of these amino acid to complex metals is very low.[30]

Interaction with microbial flora and mycorrhiza

Metal mobilization from the soil can also be affected by bacteria and fungi (mycorrhiza) that are living in the rhizosphere. It has been shown that the mycorrhiza may enhance the accumulation of selenium by garlic[35] and the microbial siderophores are capable to increase the mobility of iron in the rhizosphere and thus significantly improve the iron status in strategy I and II plants.[36] It has been also reported that iron from Fe-pyoverdine can be easily incorporated by *A. thaliana* Col[37]. The microorganisms living in the rhizosphere of hyperaccumulating plant *Thlaspi caerulescens* significantly enhanced the availability of water-soluble zinc in soil that resulted in increased Zn accumulation by hyperaccumulating plant.[38]

Transporters involved in metal uptake from soil

Once metal ions become available for plants, they are captured by the root cells. First, metals are bound by the cell walls which have low selectivity and then they enter the apoplast (space between the cell wall and the plasma membrane) by diffusion. However, further apoplastic transport is blocked by impermeable waxy layer called Casparian strip that stops water and solutes from entering the xylem. To cross the plasma membrane, metal must be transported actively by special transporters likely channel proteins and/or H^+-coupled carrier proteins.[15, 21]

Recently, a few compounds responsible for micronutrients transport to the symplast of the epidermis have been identified. Majority of transporter are presumably the members of the ZIP (ZRT – the zinc-regulated transporter, IRT - iron-regulated transporter -like Protein) and the Nramp (natural resistance-associated macrophage protein) family.

Once reduced, iron (II) is transported across plasma membrane of the root epidermis primarily by a high-affinity transporter IRT1 (Iron Regulated Transporter) that is member of ZIP (ZRT IRT-like Protein) metal transporter family.[15, 21] The IRT1 (AtITR1) is the main root iron transporter in *A. thaliana*, however, it has been reported that AtITR1 can also transport Zn, Ni, Mn, Cd and Co. The additional root epidermal transporters for these metals are still unknown but the studies on mutants indicated that under iron deficiency AtIRT1 is the main transporter for Mn, Zn and Co.[22] Another transporter – IRT2 was found in the root epidermal cells of Fe deficient plant and it has ability to transport namely iron and zinc.[23] Recently, an IRT1 ortholog – HvIRT1 was identified in barley (*Hordeum vulgare*) and heterologous expression in yeast showed that HvIRT1 is capable to transport Fe, Mn, Zn and Cd.[39] Other orthologs of IRT1 – LeIRT1 and LeIRT2 that were identified in tomato (*Lycopersicon esculentum*) plants have, similarly to HvIRT1, ability to transport wide range of metals, such as Fe, Zn, Mn and Cu.[40] These research shows that response to iron deficiency may lead to accumulation of other metals that can by potentially toxic (e.g. Cd).

Proteins that are responsible for Zn uptake form soil are yet unknown. There are some studies on zinc transport from the soil to root that show that *A. thaliana* ZIP1, ZIP3 and ZIP4 proteins can increase acquisition of zinc in yeast strain defective in Zn uptake. It suggests that these proteins may be involved in zinc transport. The expression of ZIP1 and ZIP3 takes place in root under Zn deficiency suggesting that these transporters can be involved in Zn transport across plasma membrane of the root epidermis. ZIP4 is expressed in both roots and shoots and probably it transports Zn intracellularly or between plant tissues.[41] Opposite to iron and zinc, copper is primarily taken up as Cu(I) and then transported via COPT1 transporters in plasma membrane. COPT (copper transporter family) proteins are the *A. thaliana* orthologs of the yeast transporter CTR1 and they were found to be upregulated in plants under copper deficiency.[23]

In plants using Strategy II for metal uptake from soil, the YSL (Yellow Stripe) proteins family is regarded as metal – phytosiderophores transporters. The well-known member of YSL family is YS1 (yellow stripe 1) from maize and is responsible for transporting Fe – PS complexes. The ZmYS1 proteins are expressed in roots and leaves of Fe-starved plants that suggest that YS1 may be involved in intercellular transport of iron. In rice the closest homolog of ZmYS1 is OsYSL15 (yellow stripe like) which is the primary Fe(III) – MA transporter.[21, 23] It was shown that ZmYS1 protein is capable to transport various phytosiderophore complexed metals including Zn, Cu, Ni and at the lower rate also Mn and Cd.[42] Thus, similarly to IRT1 in nongraminaceous species, ZmYS1 is able to transport broad range of metals even those that can be potentially toxic for plants.

2.2. Intercellular transport

In order to control the uptake of metal ions and different molecules from soil plants developed a cell layer, called endodermis, that surrounds the vasculature. The characteristic feature of endodermis cells is local thickening placed on their transverse cell walls. This structure is known as Casparian strip and contains the polymeric molecules: lignin and

suberin. The Casparian strip plays the role of a diffusion barrier that prevents water and nutrients from entering the water-conducting system through the apoplast. For this reason metal ions are forced to pass through plasma membrane and cytoplasm of the endodermal cells that act as selective diffusion barrier to be loaded to the vasculature.[43] An active transport allows metal ions to reach the symplastic space where they can be transported via plasmodesmata to the inner cell layers to be at the end loaded into the vasculature. Into the xylem, metals are loaded actively via different membrane transport proteins. The next stage is metal transport by the transpiration stream to the shoot tissues. However, seeds and young leaves cannot be fed by transpiration stream and the source of essential elements for them is phloem. Metals are transported in xylem and phloem sap in form of stable complexes that prevent metal precipitation from solution or adsorption to the cell walls. The processes of the vasculature loading and unloading are crucial for metal transport in the plant.[19, 21]

The transport from roots to shoots is taking place in xylem. Metals are effluxed into the xylem via various transporters. Zinc is loaded into the xylem by the heavy metal transporters HMA2 and HMA4 that are heavy metal ATPases and were found in the plasma membrane of root cells and in shoot vasculature.[44] Copper efflux to the vasculature seem to be also connected with HMA family transporters. It was showed that HMA5 is mainly expressed in the roots under condition of copper excess.[45] HMA5 is likely also involved in Cu translocation from roots to the shoots. The transporters responsible for loading iron to the xylem have not been yet identified.

Metals are probably transported to the xylem as different complexes. The proposed ligands are for example histidine, nicotianamine or citrate. Iron is most likely transported as citrate or NA complex to the xylem, however, concerning pH of xylem (pH 5.5) the most favorable form is Fe – citrate complex.[21]

In the xylem coexist a low molecular chelators, free hydrated metal cations and metal chelates which are in pH-dependent equilibrium with each other and also with stationary metal binding sites in the cell wall of xylem vessels.[15] It is known that inside the xylem Fe(III) is bound to citrate that prevent its precipitation on the apoplast walls. It was also reported that FRD3 (ferric reductase defective), a member of the multidrug and toxic compound extrusion (MATE) family, is expressed in the root vasculature and is responsible for citrate efflux and transport in the xylem. These processes are important for iron translocation to the shoots as iron is transported in the xylem in form of a ferric-citrate complex.[46] The FRD3 orthologue – OsFRDL1 was recently found in rice (*Oryza sativa* L.). The research indicates that in the xylem sap of rice iron is present as Fe(II) and Fe(III) that suggests partial oxidation of Fe(II) to Fe(III) in root cells before loading into the xylem. Additionally, the loss of OsFRDL1 leads to decrease in Fe(III) concentration, because of its precipitation in the central vascular part, but not in Fe(II) concentration. It suggest that there are additional chelators besides citrate for Fe(II) transport in xylem saps.[47]

The studies on copper transport in vasculature showed that amino acids are the most important long distance copper complexing compounds. Among them, histidine and nicotianamine are the most common as it was shown for *Brassica carinata*[48], chicory and tomato[49]. In the xylem of *Brassica carinata* the major amino acid was nicotianamine while

histidine and also proline played important role in copper chelation under conditions of Cu excess.[48] It was also shown that excess or deficiency of copper led to enhanced concentration of other amino acids, such as threonine, glutamine, methionine, asparagine, valine, glycine, leucine, phenylalanine, glutamic acid and γ-aminobutyric acid in the xylem sap of different plants.[48, 49] The primary ligands in xylem saps responsible for zinc and nickel transport are citrate and histidine.[19] In xylem saps of hyperaccumulating plants metal can be present as free hydrated ions or/and can be chelated by different ligands. The studies on Zn-hyperaccumulator *Thlaspi caerulescens* showed that in the xylem saps zinc was transported mainly as the free hydrated Zn^{2+} ion.[50] Similarly to zinc, cadmium was present primarily in the free ionic form in the xylem saps of the Cd-hyperaccumulator *Arabidopsis halleri.*[51] Additionally, both zinc and cadmium were found to be coordinated with organic acids in the xylem saps, however, the amount of Zn and Cd in form of metal-organic acid complexes was minuscule.[50, 51]

As it was mentioned before, the transport from roots to seeds and young leaves takes place in phloem. It is thought that in phloem metals are transported as chelates with nicotianamine that has been found in all higher plants. It has been shown that metal complexation by DMA dominates at acidic pH values. It indicates that this non-proteinogenic amino acid can be important xylem chelator, whereas complexation by NA is most favorable at alkaline pH values.[52] Thus, is believed that the metals complexation by NA prevent their precipitation in the alkaline phloem sap. However, recent research shown that in rice (*Oryza sativa* L.) phloem sap the main low molecular weight iron chelator is DMA, indicating that this compound can be also important in iron transport in phloem.[53] It was also suggested that DMA may bound mostly Fe (III)[53] whereas another research indicated that nicotianamine is an effective chelators for both Fe(II) and Fe(III). Additionally, it was reported that complexes Fe(II) – NA are kinetic stable and they do not autoxidize to Fe(III) – NA at physiological pH values.[52] The iron transport form xylem to phloem presumably requires the exchange of ligands as in xylem iron is present in form of Fe – citrate complexes and in phloem it was found to be complexed by NA and DMA. The exchange of Fe from citrate to NA occurs likely at pH 5.5 and after the ligand transposition, the complex Fe – NA is transported via specific NA – metal complex transporter.[22, 54] Studies on transgenic tobacco and tomato (*Solanum lycopersicum*) mutant (*chloronerva*) showed that nicotianamine plays an important role in transporting different metals, such as iron, zinc and copper, to young leaves, seeds and reproductive organs.[54, 55] Likewise in rice phloem saps, zinc was found to be complexed mainly by NA not by other possible chelates, such as amino acids.[53] It was also reported that in *A. halleri* depleted accumulation of NA in roots reduced significantly zinc hyperaccumulation. These results suggest that NA is an important Zn chelator that forms complexes with this metal in roots. In this way, NA facilitates zinc transport in xylem in *A. halleri.*[56] The results obtained in different research projects indicate that nicotianamine is the major ligand for zinc and is crucial in long-distance and intercellular transport of this metal in plants. Therefore, NA can be regarded as an important player in zinc homeostasis in plants.[57]

To date, the transporters implicated in loading and unloading phloem are not completely known. Presumably, they are members of the YSL (Yellow Stripe) group that is

subfamily of the oligopeptide transporters (OPT) family. YSLs are believed to take part in the iron translocation from the xylem into the phloem, the long-distance transport of Fe-complexes and also in iron loading from senescent leaves to be then transported to the flower and loaded into the developing seeds. YSL are hypothesized to transport metal – NA complexes in non-grasses while in *A. thaliana* were found eight members of YSL family. Three of them, AtYSL1, AtYSL2 and AtYSL3 were characterized, however, AtYSL1 (Yellow Stripe 1-like protein) seem to be the best described transporter.[22]

AtYSL1 is localized in the shoot vasculature, flowers, young siliques, pollen grains and developing seeds. Similarly to AtYSL1, the AtYSL3 (yellow stripe 3-like protein) transporter is also expressed in the shoot vasculature. The study on *A. thaliana* showed that YSL1, YSL2 and YSL3 have important role in Fe, Cu and Zn remobilization from leaf tissues as well as metal loading of the seeds. Additionally, it was shown that metals are provided to the leaves, pollen and developing seeds as metal – NA complexes.[58, 59] Recent studies revealed that AtYSL1 and AtYSL3 play important role in reproduction. In leaves, their expression is necessary for normal fertility and complete seed development while in inflorescences, the presence of AtYSL1 and AtYSL3 is crucial for proper metal loading into the seeds. [60] It was indicated that *A. thaliana* orthologs – ZmYS1 and OsYSL2 also play an important role in metal distribution via the phloem. ZmYS1 was found to mediate transport of phytosiderophore (DMA) bound Fe (III), Zn, Cu and Ni as well as transport of Ni, Fe(II) and Fe(III) complexed by nicotianamine.[42] OsYSL2 from rice (*Oryza sativa* L.) was found to transport Fe(II) – and Mn – NA complexes in phloem.[61] This research indicates that YSLs are presumably essential for metal – NA, especially Fe-NA, delivery to the developing seeds. This indicates also that seeds require not only the availability of NA to undergo normal development but also specific metal-NA transporters.[22] Recently, it was reported that *A. thaliana* AtOPT3 protein, the member of the family that includes ZmYS1 and the AtYSLs, is expressed in pollen, the siliques vasculature and in developing seeds. It may serve a role in loading iron of the seeds and thus having an essential role in embryo development.[62]

In rice, the OsIRT1 which is expressed in the phloem of roots and shots, is believed to transport Fe(II) into the phloem where it is complexed by NA.[22] In rice (*Oryza sativa* L.), the OsZIP4 protein is suggested to be involved in zinc translocation throughout the plant. It likely includes Zn loading in to the phloem where it is rapidly bound by nicotianamine, regulation of its supply in the developing leaves as well as the long-distance transport from old to young leaves.[53]

2.3. Intracellular transport – trafficking and sequestration

Via xylem and phloem metals are transported to different tissues where they have to be distributed properly to different cells and on the cell level to different organelles. The metal trafficking depends on organelle as each cell compartment has different nutrient requirements. The process of metal ions distribution within the plant cell aims at maintaining the proper concentration of metals in organelles. They ensure efficient delivery of metals to all compartments that require metal ions in different metabolic reactions as well as dispose the

excess of essential and non-essential metals to cell compartments, such as vacuoles, that are responsible for safe storing of metals. The processes of metal loading and unloading in different compartments are mediated by various transporters (Fig. 3). However, the information about the transport and storage forms as well as proteins involved in metal transportation are very limited to date.[15, 21, 23]

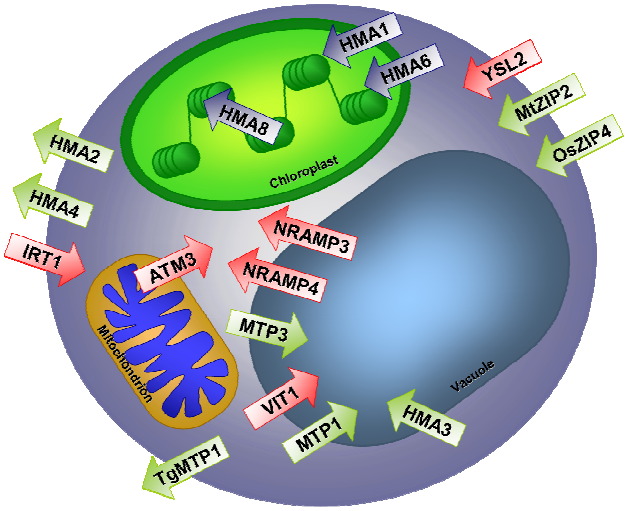

Figure 3. The membrane distribution of Cu, Fe and Zn transporters in different compartments of plant cells. The arrows show the direction of metals transport while the colour of arrow represents proposed transporter of: Cu – blue; Fe – red and Zn – green. Only the major metal substrates are shown for each transporter. Modified after Grotz and Guerinot, 2006.[23]

Vacuoles are important organelles in metal ions sequestration as they can store the excess of essential and toxic metals. In seeds, they play role as an initial store of metals that can be used by plants during the time when the metal uptake form the soil is not possible.[21] It was reported that vacuoles act as the most important iron storage compartments in seeds of *A. thaliana*[63] as well as in leaf and root cells of tomato (*Lycopersicon esculentum* Mill.)[64].

Apart from storing iron, vacuoles are also able to release iron as a response to changes in cytosolic iron concentration. In *A. thaliana*, Fe(II) was found to be transported to vacuoles via VIT1 (Vacuolar Iron Transporter 1) transporters. They are expressed in the vasculature of the roots and shoots especially during embryo and seed development.[22] It was shown that disrupted structure of these transporters impairs the proper iron distribution in the embryo and thus leads to disorders in seedling development.[65]

Unlike VIT1 that loads iron into vacuole, the *A. thaliana* NRAMP3 and NRAMP4, placed in vacuolar membrane, are responsible for mobilization of iron out of the vacuole. Their expression was found to take place in vasculature and is upregulated under iron deficiency. It was also reported that iron storage in vacuoles is strongly connected with nicotianamine. NA that is an important chelator in the intercellular transport, seems to be also required in the intracellular transport. NA was found in the vacuoles and presumably is chelating iron there, in order to retain it in a soluble form and probably to transport it in and out of the vacuole. There are even two transporters – YSL4 and YSL6 that could be implicated in Fe – NA complexes transport through vacuolar membrane.[15, 21, 22] It was also

proposed that NA may protect the cell from oxidative stress by chelating Fe(II) that can be easily oxidized to Fe(III) and thus leading to Fe(II) sequestration in the vacuole in form of Fe(II) – NA complexes.[65]

The studies on *A. thaliana* showed that in seed vacuoles iron seems to be complexed with phytate, not with ferritin as it was previously suggested and Fe – phytate is presumably the main storage form of iron in *A. thaliana* seeds.[22] Additionally, zinc was also found to be complexed with phytate in the vacuoles of the embryo and endosperm in *A. thaliana*. Zinc transport into the vacuoles is mediated by members of the MTP (Metal Transport Protein) family – MTP1 (called also ZAT1 – zinc transporter of *A. thaliana*) and MTP3 that are localized to the vacuolar membrane.[66, 67] The studies on *A. thaliana* showed that AtMTP1 is localized in the tonoplast (vacuolar membrane) and is involved in the zinc tolerance in this plant species. The Zn tolerance mechanism is based on vacuolar sequestration of excess Zn in the cytoplasm and the AtMTP1 is a key element in this process. For this reason AtMTP1 is essential to maintain Zn homeostasis and to protect plant tissues form high zinc concentrations.[66]

It was reported that MTP1 in hyperaccumulating plant *Thlaspi goesingense* presumably plays an important role in zinc hyperaccumulation. This protein is localized in vacuolar membrane in shoot tissues of *T. goesingense* plants and probably is involved in zinc transport into vacuole and thus is responsible for Zn tolerance and enhanced accumulation of this metal.[68] However, the transporters responsible for zinc transport out of the vacuole have not yet been identified.[21]

Vacuoles are also the storage compartments for metal – PC chelates. Recently, the studies on *A. thaliana* revealed that two ABC transporters – AtABCC1 and AtABCC2 that act as the vacuolar transporters that are implicated in the vacuolar sequestration of Cd(II)-PC and Hg(II)-PC. These transporters mediate the transport of the metal – PC chelates into the vacuole and for this reason they play an important role in Cd and Hg detoxification process in *A. thaliana*. Additionally, under the conditions of AtABCC2 absence the AtABCC1 transporter is able to support fully mercury and cadmium detoxification.[69]

Chloroplasts have an enormous requirement for iron and thus approximately 90% of iron in plants is localized in these organelles. Iron takes part in electron transport in the chloroplasts but also is required for synthesis of chlorophyll, heme and Fe-S clusters.[2] Zinc and copper are also important elements in the chloroplasts because they function as cofactors for superoxide dismutases (SODs). SODs are essential to prevent free radical produced in electron transport chain from damaging the cell. The transporters involved in metal transport into the chloroplast are not fully known but there are a few candidates that may act this function.[21] Recently, it was reported that the permease PIC1 that localizes in the inner envelope of the chloroplast is responsible for iron transport into chloroplast and hence is critical for iron homeostasis in plastid and normal chloroplast development. It was also suggested that PIC1 may be involved in other processes such as Fe export or Cu transport in and out of chloroplast.[70]

In vacuoles, two members of NRAMP family are important in iron mobilization out of vacuole. In chloroplast, another member of this family – NRAMP1 appears to have significant

role in transporting the excess of iron to the plastids to prevent its toxicity.[23] It was indicated that presumably part of cellular iron is trafficked to the chloroplast in Fe(III) form and then reduced to Fe(II) by ferric reductase FRO7 which was found to be expressed in the chloroplast membrane. Experiments on purified pea chloroplasts showed that Fe(II) is more favorable form to be transported across the inner envelope of the chloroplasts. It is suggested that inside the plastids ferritins (FER1, FER2, FER3 and probably FER4) are responsible for maintaining iron homeostasis. They likely sequester the excess of free metal ions that can cause oxidative stress. It can be especially important in leaf plastids where free iron bound to ferritins cannot move to flowers causing their damages.[22]

The molecules responsible for copper transport in chloroplasts are the P-type ATPases of *A. thaliana*: the PAA1 (HMA6) and PAA2 (HMA8) and HMA1. HMA6 is thought to transport Cu in to the chloroplast stroma whereas HMA8 is believed to be active on the thylakoid membrane. The HMA1 is localized in the chloroplast envelope and is also involved in maintaining copper homeostasis in chloroplasts.[23]

Similarly to the chloroplasts, mitochondria also require iron and copper for the respiration and synthesis of heme and Fe-S clusters. The experiments with *atm1* yeast mutants showed that STA1/AtATM3 in *A. thaliana* that is an ortholog of the yeast ATM1p may be involved in the export of Fe-S clusters from the mitochondria. Unfortunately, ATM3 is the only identified iron transporter in mitochondria of *A. thaliana* and the other transporters implicated in iron import still remain unknown.[21, 23] Iron homeostasis in the chloroplast must be strictly controlled to prevent oxidative damages in the cell. The important role in this process play iron sequestering proteins, such as ferritin and frataxin, that were found in the mitochondria of *A. thaliana*.[72, 73] Ferritin FER4 and frataxin were found to play an essential role in mitochondria of reproductive organs and presence of frataxin in embryo mitochondria is essential for its development. The importance of frataxin in mitochondria is based, first of all, on iron sequestering and, second of all, on mediating iron delivery to Fe-S cluster assembly scaffold.[74]

In maintaining zinc homeostasis in *A. thaliana* cells are implicated two members of the HMA family – the HMA2 and HMA4. It was reported that HMA2 and HMA4 function as membrane proteins and they may be involved not only in zinc homeostasis but also in Cd transport within the plant. It can be part of detoxification mechanism developed by plant and based on Cd transportation to specific tissues or organelles and its further sequestration.[23]

Concerning the intracellular transport, it is important to mention the soluble metal receptor proteins named metallochaperones that play an important role in metal ions trafficking inside the cells. The role of metallochaperones is to deliver the metal ions to certain location in the cell where they are then involved in different metabolic processes. For example, metal ions can be transported by metallochaperones to the enzymes that employ certain metals as co-factors and additionally are placed in different intracellular locations.[75]

The interaction between metallochaperones and metals were described very well for copper. It was observed that *in vitro*, majority of copper enzymes is able to acquire this metal without any help of metal transporting proteins. However, completely different situation was observed *in vivo* where the presence of auxiliary proteins was necessary for normal function

of copper enzymes. This is caused by very low concentration – 10^{-18} M of total cytoplasmic "free" copper (aquo Cu(I) or Cu(II) complexes) that is much less than one atom of "free" copper per cell. It means that all cytoplasmic copper is bound by different small molecules and specific copper proteins. For this reason, to be involved in metabolic processes, first copper has to be released from these chelates to be after transported by various proteins to the different enzymes.[75] There were also attempts to estimate the concentration of cytosolic "free" zinc. At the end, they brought the wide range of "free" zinc concentration, between 10^{-5} – 10^{-12} M, depending on the approach that was used. The studies on *Escherichia coli* revealed that metalloregulatory proteins are saturated at the "free" zinc concentration of around 10^{-15} M that is a few orders of magnitude lower than the concentration of single zinc atom in the cell. This result suggests that the pool of "free" zinc ions does not exist in the cell cytoplasm under normal growth conditions. It was also suggested that zinc, similarly to copper, is bound to metallochaperones during the trafficking within the cell.[17]

Concerning copper, to date, there are three distinct pathways of trafficking of this metal described in literature. The metallochaperones are implicated in copper delivery to the mitochondria, within this organelle and to the Golgi compartments and also in maintaining copper homeostasis in the chloroplasts (Ohalloran, 2000). It was proposed that two Cu chaperone COX17 and SCO1 may be responsible for Cu transport and incorporation into cytochrome oxidase in mitochondria. In yeast COX17 is localized to mitochondrial inner membrane space and cytosol while SCO1 is present just in inner membrane of the mitochondria. It was proposed that COX17 may play role of shuttle protein that delivers Cu to mitochondria while SCO1 probably directly transfers Cu to cytochrome oxidase.[71, 75]

In copper delivery to the Golgi compartments is involved protein ATX1 that pump Cu into the lumen of the Golgi. Thus, copper can be involved in different enzymes, however, the main targets are P-type copper transporters. In yeast, copper chaperone ATX1 binds one copper ion via two cysteine residue. The docking of Cu-ATX1 complex to copper transporter is based on physicals interaction of ATX1 with the ATX1-like domain of the Cu transporter and also on electrostatic interactions between ATX1 which "face" is positively charged and copper transporter which contains negatively charged residue as it is shown in Fig. 4.

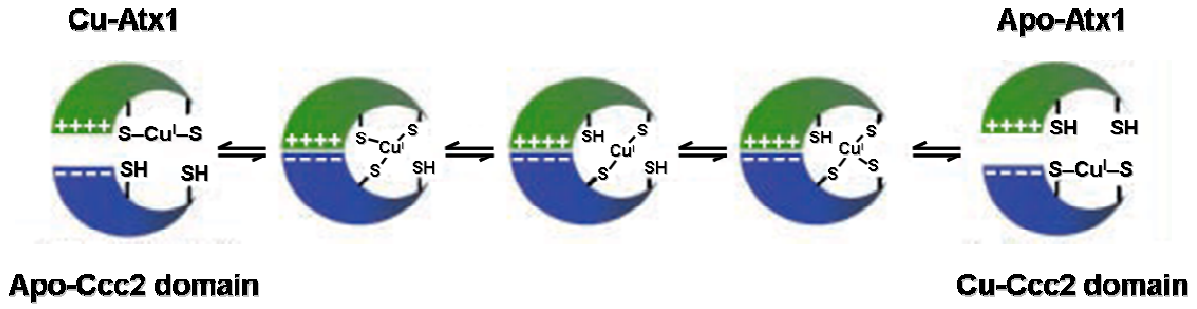

Figure 4. Proposed mechanism of copper transfer from ATX1 to Ccc2.[75]

Copper transfer from Cu-ATX1 complex to the P-type metal transporter is also shown in Fig. 4. The proposed mechanism involves attack of the cysteine of Cu transporter on Cu(I) that is in the center of ATX1. In the next stage, it takes place formation and decay of two or

three intermediates that contain Cu atom in the center and finally cysteines of ATX1 that coordinated Cu at the beginning are replaced with cysteines of the Cu transporter.[75]

The metallochaperones can be involved also in maintaining copper homeostasis in the chloroplasts, especially the copper chaperone for superoxide dismutase 1 (CCS1) that delivers copper to copper- and zinc-requiring enzyme SOD1. Their function in plants is not fully known but it was suggested that presumably CCS1 maintains the proper level of copper for plastocyanin and superoxide dismutases in chloroplasts.[23, 75]

The knowledge on intracellular copper trafficking is vast, however, presumably there are analogous proteins responsible for trafficking of other metals.[75] It was reported that in yeast *Saccharomyces cerevisiae* two proteins Isa1p and Isa2p may act as the iron chaperones. It was shown that Isa1p is present in mitochondrial matrix while Isa2p is localized to the mitochondrial intermembrane space and suggested that these proteins may be responsible for iron delivery to sites of the Fe-S cluster assembly.[76] Recently, it was reported that HIPPs (heavy metal-associated isoprenylated plant proteins) are metallochaperones that are involved in have metal homeostasis and/or detoxification. They show ability to bind wide range of metals including Cu, Ni, Zn, Cd, Hg and Pb as they contain metal binding domain. To date, HIPPs were found to be present in the cytosol, membranes and also in cell nucleus.[77]

2.4. Identification and modeling of low molecular weight metal complexes in plants

The studies described in previous chapters report that different low molecular ligands can be involved in metal transport within the plant. They involve nicotianamine that was shown to play an important role in transport of different metals such as iron, zinc and copper in xylem and also to young leaves, seeds and reproductive organs in different plant species.[48, 52, 53, 54, 55, 56] Other reported ligands are histidine, DMA and citrate that appear to be important chelators for different metals metal (e.g. Fe, Cu, Zn or Ni) in various plant species.[19, 22, 48, 49, 53]

Concerning the high metal tolerance mechanisms in hyperaccumulating plant species (described in details in chapter 4.5.), it was reported that histidine is one of the most important amino acids involved in hyperaccumulation. It is caused by its ability to form stable complexes with Ni, Zn and Cd. Histidine is responsible for nickel tolerance in Ni-hyperaccumulating plants such as *Alyssum lesbiacum, A. murale, A. bertolonii*[123], *Thlaspi goesingense*[124] and Zn homeostasis in Zn-hyperaccumulator *Thlaspi caerulescens*[50]. Nicotianamine seems to be also implicated in metals (e.g. Zn and Ni) tolerance in hyperaccumulating plants.[122, 125, 126] Organic acids such as oxalic, malic, citrate and malate were also found to be involved in metal detoxification and tolerance mechanism in different hyperaccumulating and non-accumulating plants. [50, 99, 124, 127]

Apart from these studies, there is much more works that are focused on identification of low molecular weight ligands that can be implicated in metal transport, storage and detoxification. Some of them concern also analysis of metal-ligand standards and modeling of low molecular weight metal complexes in plants.

The studies on Ni-hyperaccumulating and non-accumulating Iberian Alyssum species revealed that in hyperaccumulators nickel was mainly stored in vacuoles in form of water-soluble polar complexes. The analysis of purified Ni complexes using gas-liquid chromatography showed that nickel is mainly associated with malic and malonic acid suggesting that these organic acids (principally malic) are presumably involved in hyperaccumulating mechanisms allowing plants to tolerate high concentrations of nickel in soil.[78] The research on Ni accumulating plants *Psychotria douarrei* and *Phyllanthus serpentinus* revealed that 63% and 40% of nickel in these plants was also complexed by malic acid, respectively. Additionally, in *Phyllanthus serpentinus* species another organic acid responsible for complexation of 42% of nickel were identified as citric acid.[79] The analysis of purified leaf extracts of the Ni-hyperaccumulating plants *Dichapetalum gelonioides* subsp. *Tuberculatum* showed the presence of citric acid, malic acid but also traces of tartaric acid. The extract contained 18% of Ni, 24% of citric acid and 43% of malic acid that gave the mole ratio of Ni:citrate:malate as 1:0.4:1. The chromatographic analysis revealed the presence of two species containing nickel. The comparison with the chromatograms obtained for standards (aqua-Ni complex, Ni-citrate 1:1 complex, Ni-malate 1:1 complex and Ni-citrate-malate 1:0.4:1 mixture) showed that the first peak corresponds to nickel complexes with citrate and/or malate and the second is the aqua-Ni complexes. The results discussion in terms of the stabilities of Ni complexes with citrate and malate showed that Ni-citrate (1:1) complex is the most stable, however, the amount of citrate is insufficient to complex all nickel. The possible presence of mixed complexes Ni-malate-citrate was ignored as the stabilities of Ni-malate (1:1) and Ni-citrate (1:2) complexes are low so that this complex is unlikely to be the major one. The analysis of crystals obtained from the Ni-citrate-malate solution that mimicked the extract, using X-ray crystallography, revealed the presence of Ni-citrate complexes.[80]

The analysis of xylem saps of apple shoots showed that about 50% of total soluble calcium was present as a complexe with citrate and malate. The complexation of Ca by citric and malic acid presumably prevents or reduces adsorption of this metal at negatively charged sites and enhance its mobility in the xylem.[81] The citric acid was also found to complex iron in xylem exudates of tomato[82] and soybean[83]. It was suggested that this complex is the major form of iron in xylem fluids of soybean and in this form iron is feasibly translocated within the plant.[83] In case of studies on tamoto, the competetive experiments were performed. They showed that only iron was present in xylem saps as Fe-citrate complex while other metals – Zn, Co and Mn were present in uncomplexed form. However, it was suggested, taking in to account the stability constans of the citrate complexes of Co, Zn and Mn that these metals can migrate also in complexed form.[82] The studies on xylem exudates of soybean and tomato revealed the presence of wide range of different organic and amino acids. In xylem saps of soybean four organic acids – citric, maleic, malic and malonic had the highest concentration, however, citric acid was the major one. Concerning the tomato, there were only three organic acids – citric, maleic and malic found in xylem exudates, however, the maleic acid was present in the greatest amount. There were also twenty two amino acids identified in xylem saps of these two plants. In soybean xylem exudates asparagine was the most concentrated

amino acid while in tomato glutamine was the major one. In tomato xylems saps also two other amino acids – asparagine and γ-aminobutyric acid were present in great amount.[84]

Apart from attempts to identify metal complexes in plant fluids, also computer simulations were performed to predict presence of various metal species in plants. The computer program CHELATE was used in aim to calculate all equilibrium species, such as free metal ions, metal complexes or other metal species in xylem fluids. Six metal ions were taken in to account and their distribution in xylem saps of soybean and tomato was calculated. The obtained results indicated that majority of iron is complexed by citrate in both soybean (99.5%) and tomato (96.3%) xylem saps. Concerning copper, this metal is mainly bound to asparagine (82%) and histidine (15%) in xylem exudates of soybean while in tomato three amino acids – histidine, glutamine and asparagine are chelating 48%, 25% and 23% of copper, respectively. In this system neither copper nor iron exist as hydrated ions as approximately 100% of these metals is bound to different ligands in xylem saps of soybean and tomato. Zinc, manganese, calcium and magnesium are complexed mainly by citrate in stem exudates of soybean. However, malate and malonate are also involved in complexation of these metals but in less extent than citrate. In case of tomato xylem exudates, zinc and manganese are bound mainly by citrate and malate, however the amount of these metals complexed by citrate is smaller than in xylem saps of soybean. Calcium and magnesium are present mainly as hydrated ions in stem exudates of tomato. Only the small amount of these metals is complexed by citrate and malate in xylem of this plant.[85]

3. Toxicity of metals

Among 35 metals that are regarded as potentially dangerous for environmental health 23 are called heavy metals. The group of heavy elements includes antimony (Sb), arsenic (As), bismuth (Bi), cadmium (Cd), cerium (Ce), chromium (Cr), cobalt (Co), copper (Cu), gallium (Ga), gold (Au), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), platinum (Pt), silver (Ag), tellurium (Te), thallium (Tl), tin (Sn), uranium (U), vanadium (V) and zinc (Zn)[86]. Some of these metals, such as aluminium[12], arsenic[87], mercury[88], lead or cadmium[14], are nonessential for plants. However, as a result of industrial, mining and agricultural activities, deposition of sewage sludge, burning of coal and oil as well as use of the phosphatic fertilizers and pesticides, they became widespread and bioavailable for plants. Hence these elements (As, Al, Hg, Pb and Cd) are considered as the most common heavy elements that cause toxicity in plants. Other heavy metals such as iron, copper and zinc function as micronutrients and are required by plants in extremely low concentration. However, human activity contributed also to the elevated concentration of these elements in water and soil resulted in the potential toxic effects of these essential metals on plants.[89, 90]

The heavy metal content varies between different plant organs as well as organelles. Concerning various organs, the concentration of metals decreases in the following sequence: root > leaves > stems > inflorescence > seeds. However, the changes in metal concentrations in different plant parts may be observed and they depend on plant species and also on the age of plant organ. The metal content is the lowest in seeds because the seed coat is the first barrier for metals in germinating seeds. Decreasing concentration of metals can be also observed in cells and it follows the pattern: cell wall > vacuoles > golgi apparatus > endoplasmic reticulum > nucleus.[86]

Most of the metals can be readily taken up by plants[86] showing thus huge bioavailability referred to as availability for uptake into plants. However, there are metals that have very low solubility in soil and thus their bioavailability is very low as well. It causes that these metals can not be practically taken up by plants. Among these metals is lead that is one of the soil contaminants and is present in some areas in immense quantities. However, its low solubility and strong interactions with the soil particles cause that lead is hardly available for plants. Unlike lead, cadmium can be taken up by plants very easily. Therefore, this element is of particular concern as its huge bioavailability may lead to introduction of Cd to the food chain.[91] Besides the differences in bioavailability of various metals, there are also a few factors that can influence the metal fraction available for plants. These factor include the soil aeration, pH and cation-exchange capacity, presence of other metals, bacteria and fungi as well as plant species and chemical form of heavy metal.[18]

Once the metal is absorbed by plant, it undergoes different interactions within the plant. They may cause, for example, growth reduction which is the most common visible symptoms of metal toxicity. However, the symptoms like growth inhibition or changes in cell structure are just a side effects of direct mode of action of metals that is on plant metabolism.[92]

Metals can be bound to the cell wall or enter the cell. In both cases they interact with the functional groups of different molecules like polysaccharides, proteins and nucleic acids. Many heavy metals have high affinity for ligands containing sulfur, therefore, they easily form binding with molecules comprising SH-groups, such as proteins and enzymes. They may also substitute other metal ions that are already bound to functional groups of various molecules[86], such as copper, that can substitute magnesium in chlorophyll molecules and thus decreases photosynthesis efficiency.[92] Metal interactions with proteins lead to the changes in the native conformational structure of protein and thus to its impaired function. Metals can also block the SH-group in enzyme structure and mask its active site that leads to the enzyme inactivation and at the end to disruption of many metabolic processes. The examples are copper that inhibits ATPase activity in the plasma membrane in roots of *Zea mays*[93] and acid phosphatase activity in *Deschampsia cespitosa*[94] and also two other heavy metals – lead and cadmium that are well known as inhibitors of enzymes activity as they react with enzyme sulfhydril groups.[14, 89] Metals interactions with the cell wall polysaccharides decrease the cell wall plasticity causing metabolic disorders and ultimately growth inhibition.[86] Heavy metal may also cause genotoxicity when they interact with cell nucleus. Metal binding to the nucleus results in modification of DNA base, inter- and intramolecular crosslinking of DNA and proteins, breaks in DNA strand, rearrangements and depurination.[90]

Apart from interaction with different molecules, heavy metals are also involved in production of reactive oxygen species – ROS that induce the oxidative stress in plants. There are different mechanisms of generating ROS. Metals can be involved either directly in ROS production by taking part in Haber-Weiss reaction or indirectly by disturbing some metabolic processes that in consequence leads to overproduction of ROS and oxidative stress.[86, 89]

The activation of reduced forms of oxygen consists of a few stages that can be shown in simplified way. At the beginning of this process, molecular oxygen accepts electron from different molecules as well as metals such as iron and copper that have often unpaired electrons becoming oxygen free radical. In the next stage, oxygen free radical is reduced to superoxide ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2) during various intracellular reaction. These two molecules are not very reactive but undergoing Haber-Weiss reaction that can be catalyzed by metals (e.g. Cu and Fe) they form hydroxyl radicals (·OH). These radicals are highly reactive and are regarded as source of majority of the oxidative damages in biological systems.[90, 95]

Heavy metal may also impede some metabolic processes that results in the generation of free radicals. Mercury (Hg) toxicity in plants leads to the perturbation in biomembrane lipids and cellular metabolism. They are caused by reactive oxygen species generated as a side effect of mitochondrial activity disruptions induced by mercury.[89] Production of free radical is also a response to the photosynthesis inhibition caused by copper (Cu) and results in oxidative damages that accelerate leaf senescence.[92]

Generation of reactive oxygen species may also be induced by other heavy metals, such as lead, manganese or zinc.[92] However, it always leads to highly dangerous effects on plants, such as damages of macromolecules, metabolic alterations and at the end disruption of cell homeostatsis.[89] ROS can cause oxidative modifications of free amino acids and proteins

leading to denaturation of the latter and generation of protein hydroperoxides that through interactions with heavy metal ions may produce additional radicals.[86, 95] Reactive oxygen species, especially hydroxyl radicals, may be generated in the DNA vicinity with the participation of metal ions and lead to metal-induced DNA alterations. ROS may also induce production of the premutagenic adducts or add H atoms to DNA base or remove them from DNA backbone. All these interactions may impair DNA functions and increase its propensity for genetic mutations and alterations in transcription.[90, 95] Metals such as copper[2] and iron[90] together with oxygen are involved in lipid peroxidation that have crucial structural and functional role in the cell membranes. Changes in the cell membrane structure lead to increased membrane permeability and inhibition of membrane transport proteins and enzymes that disrupts ion homeostasis in cytoplasm. The membrane destruction may lead ultimately to the cell death.[86, 95]

Heavy metals can also decrease the uptake of macro- and microelements by being involved in one of two well-known mechanisms. The first mechanism – physical and chemical – depends on the size of metal ion radii. This mechanism can be observed for cadmium ions (Cd^{2+}) which have similar radii size to zinc (Zn^{2+}) and calcium (Ca^{2+}) and thus decrease the uptake of this two elements.[14] The same mechanism was observed for hydrated zinc ions that caused iron (Fe^{2+}) deficiency in some plant species.[2] The second mechanism is based on the metal-induced disorder in the cell membrane that lead to alteration in membrane enzymes activity and membrane structure.[86] The excess of copper impedes the activity of membrane enzymes results in enhanced potassium efflux that causes damages in the cell membrane and inhibits the root elongation.[2] Plants grown on soil contaminated with nickel, chromium and lead exhibited perturbed nutrient balance that resulted in disorder of the membrane functionality.[89] There are more examples of induced deficiency that can be caused by one of these mechanisms such as excess of zinc that may cause manganese and copper deficiency in plant shoots[89] or copper and manganese toxicity that may induce iron and magnesium deficiency[2], respectively.

Transpiration rate and water content in plants can be disturbed under conditions of heavy metals excess. Heavy metal induced stress in plants causes decreased loss of water, smaller size and number of leaves, their enhanced rolling and abscission, also smaller diameter and number of xylem vessels, decreased stomatal size and its increased resistance.[86] Dicot and monocot plants exposed to nickel excess display changes in water balance reflected in decline water content. Also, high level of lead and cadmium results in water uptake disorders and, in consequence, in water imbalance. Manganese toxicity probably causes loss of the metabolically controlled stomatal movements that result in increased transpiration rate. Another example is mercury that has ability to bind to water channels proteins. In that way it induces the closing of leaf stomata that leads to physical obstruction of water flow in plants.[89, 92]

Another target of heavy metal toxicity is the photosynthetic apparatus that is sensitive to the heavy metals toxicity. There are direct and indirect ways of metals to impede the process of the photosynthesis such as deactivation of enzymes of the Calvin cycle or causing CO_2 deficiency by closing stomata.[86] Chromium[86], cadmium, lead[14] and copper[4] disorganize

the ultrastructure of the chloroplasts. They affect the reaction center and electron transport of photosystem II by impairing protein structure and enzyme activity. All these processes lead to inactivation of photosystem II, reduction in synthesis of chlorophyll, carotenoids and plastoquinone and ultimately to decrease in the photosynthetic rate.[86, 89]

The metal-induced toxicity effects described above have influence on plants on cellular level. However, these various metabolic disorders caused by metals are reflected in visible toxicity symptoms that depend on metal ion, its concentration and plants species. Two most common symptoms of copper, cadmium, chromium, manganese, zinc and nickel toxicity are young leaves chlorosis and plant growth inhibition. If the severity of metal toxicity is not high just root growth reduction may appear. The chlorotic symptoms on young leaves, displayed by many plant species under copper and manganese toxicity, are probably caused by iron or zinc deficiency induced by excess of these two metals. Zinc can induce the phosphorus deficiency that is showed by plants as purplish-red colour in leaves. Other symptoms visible on leaves, petioles and stems are necrotic brown spotting that indicates zinc or manganese toxic effect on plant. With the time the number and size of spotting may increase leading to necrotic lesions, leaf browning and eventually entire leaf death. The roots of plants exposed to high metal concentration become yellow or brown (e.g. Zn, Cd and Mn toxicity) and in case of severe metal toxicity the death of root can be observed. Metals can also cause necrosis in different plants species (e.g. Ni) and accelerate the senescence (e.g. Zn).[92]

4. Mechanisms of detoxification

Many plant species are known to have ability to grow on soil contaminated with heavy metals without any toxicity symptoms. These plants possess a range of potential extracellular and intracellular mechanisms that allow them to avoid building-up the metal toxic concentrations at sensitive sites within the cell. All these strategies result in enhanced tolerance of plants to metal stress. Extracellularly they include roles for mycorrhizas, for binding metals to the cell wall and for secretion of different compounds to the soil. On the plasma membrane level, the tolerance mechanisms are based on either reducing the uptake of metals or, in case the metal have already entered the cytosol, on simulating the efflux pumping of metals.

There are also several detoxification mechanisms that are employed when the metal ions crossed the plasma membrane. First, metal ions are chelated in the cytosol by various ligands, such as phytochelatins, metallothioneins, metal-biding proteins as well as amino and organic acids. Afterwards, they are transported to the vacuole by tonoplast-located transporters where they are stored away from metabolic processes in non-toxic form. In case of the protein damages caused by heavy metal stress, there are also mechanisms for their repair that involve heat shock proteins.[86, 96] The possible mechanisms of heavy metal detoxification and tolerance used by plants are summarized in Fig. 5.

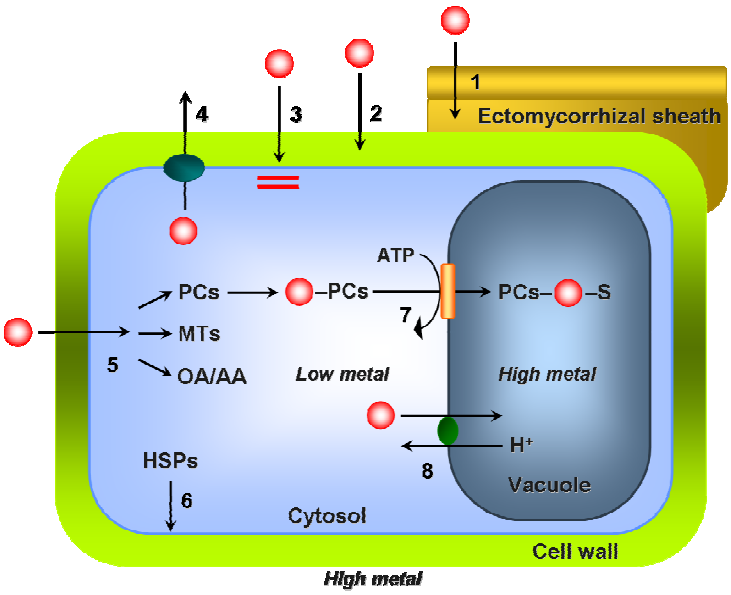

Figure 5. The possible mechanisms of heavy metal detoxification and tolerance used by plants. 1. Mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reduced metal uptake. 4. Active efflux into apoplast. 5. Chelation in cytosol by various ligands. 6. Activity of heat shock proteins. 7-8. Transport and storage of metals complexes in vacuole. Modified after Hall, 2002.[96]

4.1. Mycorrhizas

Under natural conditions, a lot of plants species is living in symbiotic association with different fungi. This phenomenon is called mycorrhizas and it appears to be very important in plant tolerance to enhanced metal concentrations in soil. The fungus hyphae are mostly

responsible for metal uptake. Presumably, in case of high metal concentration in soil they try to protect themselves against metal stress and thus they protect the host plant as well. The strategies developed by the fungi at the cellular level to tolerate metals are likely similar to some mechanisms developed by higher plants. They may involve metabolic processes, such as binding to extracellular materials, secretion of different metabolites that precipitate metals as well as intracellular metal accumulation in the vacuolar compartments. In majority of studies, concerning the role of ectomycorrhizas in metal tolerance by the host plants, the most mechanisms suggested for the protective effect of fungi is based on various exclusion processes. These processes, presumably, prevent heavy metal translocation to roots of host plant. These mechanisms include chelation by fungal exudates, absorption of metals by the hyphal sheath, reduced access to the apoplast due to the hydrophobicity of the fungal sheath, and adsorption onto the external mycelium.[96, 97]

The mycelium possesses physical barrier for heavy metals as it was shown for two mycorrhizal species *Thelephora terrestris* and *Suillus bovinus*. Under Cu excess condition, they protected *Pinus sylvestris* against copper toxicity by retaining large amount of this metal in the the extraradical mycelium.[98] However, these different exclusion mechanisms vary significantly between various plant-fungi interactions and they show also metal specificity.

4.2. Cell wall

The cell walls of monocot and dicots plants are made of water, hemicelluloses, quite large amount of pectin (roughly 35%), heteropolysaccharides and structural proteins. The content of different cell wall constituents may vary between plant species and tissues, which affects the cation binding capacity. Also, the binding strength between metals and certain components of cell wall differ highly.[86] Some studies revealed that cell wall has ability to bind different metals and thus it may act as one of diverse mechanisms of plant tolerance under heavy metal excess conditions.

The electron probe X-ray microanalysis was performed with the fresh leaves of the tea plants (*Thea sinensis* L.) in order to determine the manganese distribution pattern at the sub-cellular level. It showed that the majority of the Mn was accumulated in the cell walls of epidermis but as well as in collenchyma, bundle sheath cells and in vacuoles. The cell fractionation analysis confirmed the results of X-ray microprobe analysis showing that the cell wall is the main site of manganese accumulation.[99] The studies on heavy metal tolerant plant *Silene vulgaris* showed that this plant accumulate a range of heavy metals, such as, e.g. Fe, Ni, Cu, Al, Sn and Zn in the epidermal cell walls. Additionally, copper was found to be bound by cell wall proteins while zinc and tin were accumulated as silicate.[100] Similarly to *Silene vulgaris*, another plant growing on metalliferous areas, *Athyrium yokoscense*, is able to accumulate copper, zinc and cadmium in the cell walls. Approximately 70 – 90 % of the total copper, zinc and cadmium were located in the cell wall indicating that in *Athyrium yokoscense* cell wall is the main storage site for different metals. It may also act role in a heavy metal tolerance mechanisms in this plant.[101]

4.3. Root exudates

As it was described in previous chapters, different root exudates play an important role in metal mobilization from the soil. However, they can also be employed by plants to exclude or/and limit the uptake of heavy metal ions form the environment. The examples are organic acids that can chelate aluminum and thus transform it into the non-phytotoxic form. For instance, snapbean (*Phaseolus vulgaris* L.)[102] and Al-resistant maize (*Zea mays* L.)[103] release citric acid while Al-resistant wheat (*Triticum aestivum* L.) excretes malic acid[104] into the rhizosphere as a response for high concentration of Al in soil. Another study showed that citric acid together with histidine can be accumulated in the root exudates of non-hyperaccumulating plants resulting in significant reduction of nickel uptake. For this reason these compounds are regarded as important elements in a Ni-detoxification strategy.[96]

4.4. Plasma membrane

Plasma membrane is the first potential target for heavy metals that have affinity for both sulfhydryl groups of proteins and hydroxyl groups of phospholipids. Metal interactions with plasma membrane lead to disruption of plasma integrity and ionic homeostasis of cells. For these reason, the processes that aim in maintaining ionic balance and repairing the damages in plasma membrane are important issues under condition of heavy metal excess. The one of proposed mechanism is based on either preventing/reducing metals entry into the cell or efflux mechanisms.[96] For some metal ions, the limited entry to the cell can be achieved easily as some of them cannot readily cross the plasma membrane because of the size of atomic radius and valence. Concerning efflux of toxic metals through plasma membrane, the complete exclusion of them is not possible because many metals play an essential role in different metabolic processes. Thus, selective efflux appears to be more realistic. Selective membrane efflux transporter was recently found in *A. thaliana*. It was indicated that the ATP-biding cassette (ABC) transporter AtPDR8 is a cadmium-extrusion pump showing the resistance to the metal.[105, 106]

The heavy metal tolerance on plasma membrane level may involve also a more resistant structure of this cell component. There is some evidence that plasma membrane of heavy metal tolerant plants shows less damages induced by metal stress than the plasma membrane of sensitive plant species. However, the mechanism of this phenomenon still remain unclear.[106]

Another factor that can be important to maintain the plasma membrane integrity under heavy metal stress is the presence of molecules, such as heat shock proteins or metallothioneins, that can be involved in repairing damages in plasma membrane.[96]

4.5. Peptides, proteins and organic molecules

The production of ligands that exhibit high affinity to metals and can efficiently complex them in the cytosol seems to be one of the most important mechanisms of heavy

metals detoxification and tolerance. The variety of chelators produced by plants involves amino and organic acids, peptides – phytochelatins and metallothioneins – as well as different proteins.

Phytochelatins

Phytochelatins (PC) are low molecular mass, cysteine-rich polypeptides that exhibit high affinity to chelate various metals, such as, e.g. Cd, Cu, Zn or As. They are produced in the cytosol in cells of different organisms including plants but for the first time they were identified in yeast *Schizosaccharomyces pombe*.[107]

Phytochelatins consist of three types of amino acids: glutamic acid (Glu), cysteine (Cys) and glycine (Gly). The PCs form a group of structures consisted of different number of γ-Glu-Cys dipeptide units followed by a terminal Gly so that the general structure can be shown as $(\gamma\text{-Glu-Cys})_n$-Gly with n = 2 – 11. However the number of $(\gamma\text{-Glu-Cys})_n$ groups that is usually observed is between 2 and 5. Depending on the number of γ-Glu-Cys units, PCs were classified as PC2, PC3, PC4, PC5 and PC6 etc.[108] There are a few exceptions of the general structure of PCs that were found in different plants, such as γ-Glu-Cys-β-Ala in some legumes, $(\gamma\text{-Glu-Cys})_n$ and $(\gamma\text{-Glu-Cys})_n$-Glu in maize and $(\gamma\text{-Glu-Cys})_n$-Ser in some plants of the true grasses family.[90, 109]

The metal chelation by PCs is a primary cellular mechanism for heavy metal detoxification and the synthesis of PCs is rapidly induced in plants under heavy metal excess condition. Glutathione is the substrate in PCs synthesis that is controlled by an enzyme – PC synthase. Enzyme activation is induced by many heavy metals entering the cytoplasm.[109] It was reported that the catalysis is not activated by the direct interaction of metal ions with enzyme but by their interaction with the substrate. The main requirement for the reaction of dipeptdyl transpeptidation to be proceeded is the blockage of at least one of the thiol groups of the substrate molecule by the heavy metal.[110] The variety of metals activating PC-synthase include Cd, Hg, Cu, Ni, Ag, Au, Pb, As and Zn, however, cadmium is definitely the main PCs biosynthesis inducer.[109, 111] The recent research on PC-deficient *A. thaliana* mutant revealed that PCs play an important role in zinc tolerance as they act as important zinc chelators in case of Zn excess.[112]

The process of PCs synthesis is conducted till all metal ions are complexed as it was shown for *Brassica juncea*. The uptake of cadmium by *B. juncea* induced immediate biosynthesis of PCs and the amount of produced PCs was theoretically sufficient to chelate all accumulated cadmium.[113] Another research on *B. juncea* seedlings revealed that during first 6 to 12 hours of cadmium uptake the majority of intracellular Cd was coordinated by ligands containing oxygen atoms possibly organic acids. It suggests that organic acids can play important role in Cd detoxification process when the production of phytochelatins is reduced. It was also observed that after 36 hours of exposure to Cd, 60% of the intracellular Cd was chelated by PCs.[114]

The PCs interact with the metals through thiol (-SH) group of cysteine and the chelation of metals takes place in the cytosol. The small metal-PC chelates are transported then into the vacuoles via specialized transporters to be separated from metabolically active

compartments. Inside the vacuole heavy metal ions are stabilized by forming complexes with sulfides or organic acids.[111]

Recently, it was reported that PCs can be involved in long distance transport of metals (e.g. Cd) either from shoots to root or from root to shoots. It suggests that their detoxifying activity may be not the most important function in plants or it is the part of more complex mechanisms. Still, a lot of issues concerning PCs in plants, such as their exact role in the heavy metal homeostasis and tolerance mechanisms at the cellular level, remain unknown and require further research.[108, 111]

Metallothioneins

Metallothioneins (MTs) are low molecular weight (4 – 8 kDa) polypeptides containing large amount of cysteine a thus they have ability to bind heavy metals via the thiol group of the Cys residue. The MTs were found in some prokaryotes, eukaryotic microorganisms, higher plants and animals.[86] Despite of a huge diversity of MTs, there were divided into the Class I, Class II and Class III MTs depending on the arrangement of the cysteine residue. The Class I MTs are typical for vertebrates and they contain 20 highly conserved cysteine residues based on mammalian MTs. The Class II MTs do not have this strict arrangement of cysteine and are mainly found in plants and fungi as well as in nonvertebrate animals. In this MTs classification, phytochelatins are regarded as Class III MTs.[109]

Almost all of known plant MT genes were classified into four categories depending on amino acid sequence. These four types of MTs contain characteristic and invariant motifs in the peptide sequence that involve Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs in which X represent amino acid different than cysteine.[109] In general, the type 1 genes are mainly expressed in roots, type 2 in leaves, type 3 in ripening fruits while type 4 is expressed in seeds. The distribution and organization of cysteine residue in MTs sequence determines the MT isoforms and their capability to chelate and sequester various heavy metals. The biosynthesis of MTs is induced by various plant hormones, osmotic stress, drought, cold/heat shock, cytotoxic agents as well as by heavy metals ions, such as, e.g. Cd, Zn, Hg, Cu, Au, Ag, Co, Ni and Bi. The precise physiological function of these proteins in plants is not clear. However, there are a few proposition of the role that MTs may play. It includes detoxification and sequestration of toxic heavy metals, the cell protection against oxidative stress as well as transport, sequestration and control of concentrations of essential transition heavy metals.[86, 111]

Heat shock proteins

Heat shock proteins (HSPs) are a class of functionally related proteins that are involved in different processes including protein folding and unfolding, assembly, translocation and degradation in wide range of cellular processes. They also stabilize proteins and membranes and can be implicated in protein protection and repair under stress conditions. For all these reasons, HSPs are regarded as chaperones. There are five major HSP families

that include: the HSP70 (DnaK) family, the chaperonins (GroEL and HSP60), the HSP90 family, the HSP100 (Clp) family; and the small HSP (sHSP) family.[99, 115]

The HSPs were found in all groups of living organisms and their expression in these organisms is usually enhanced in response to increased temperature that is above the optimal growth temperature of certain organism. However, the HSPs expression can be increased also by other stress factors including heavy metals such as zinc, copper, cadmium and mercury. It appears that the mechanisms of proteins and membranes protection by HSPs, produced under heavy metal stress conditions, are similar to these used by HSPs expressed under heat stress.[99, 115]

The HSPs synthesis induced by heavy metals has been already observed in various plants species. It was reported that the HSP17 was synthesized in roots of *Armeria maritima* ssp. *halleri* growing on the copper rich soil.[116] Also, HSP17 is synthesized in cell cultures of a heavy metal tolerant *Silene vulgaris* and the sensitive *Lycopersicon peruvianum* exposed to the action of heavy metals, such as Hg, Cu, Cd and Zn[117]. Another study, on cell lines of sensitive plant species *Lycopersicon peruvianum*, revealed that another heat shock protein – HSP70 is expressed as response to Cd stress. Additionally, the localization of HSP70 was determined showing that except nucleus and cytoplasm this HSP was present also in plasma membrane. It suggests that HSP70 may be implicated in membrane protection against Cd damage.[118] The HSP70 expression was also enhanced in the seaweed *Enteromorpha intestinalis* after exposure to different stress conditions including copper.[119]

Other metal binding proteins and peptides

Apart from phytochelatins, metallothioneins and heat shock proteins, there are several proteins and peptides that are presumably involved in heavy metals detoxification and tolerance.

Recently, the new protein having ability to bind copper – Cu-binding protein (BP) – was isolated form the Asian periwinkle *Littorina brevicula* and characterized. The molecular weight of Cu-BP is between 11 – 38 kDa and under normal physiological condition it contains an equal amount of zinc. However, zinc can be replaced by copper either under the Cu excess conditions or after plant exposition to the metal ions for long time (60 days). In comparison with the metallothioneins, the copper-binding protein contains lower number of cysteine residues and is rich in aromatic amino acids such as tyrosine and phenylalanine. Additionally, histidine and methionine are present in the Cu-BP peptide sequence opposite to peptide sequence of the MT-like-Cd-BP of *Littorina brevicula*. The Cu-BP plays role in Zn and Cu homeostasis and presumably is involved in mechanism of copper detoxification under stress condition caused by excess of this element.[86, 120]

Two other proteins – ferritin and frataxin can also be involved in the oxidative stress response in plants and in protection of different organelles against oxidative damages. It is possible because they were found to sequester the excess of free iron ions in different cell compartments, such as mitochondria and chloroplasts.[74, 90]

In hyperaccumulating species glutathione is proposed to be involved in some mechanisms of high metal tolerance because it is known to play a role of a major cellular

antioxidant. The enhanced production of glutathione was observed in *Thlaspi* nickel hyperaccumulators indicating that glutathione may be responsible for cell protection against oxidative stress caused by high Ni concentrations.[121] Glutathione is as well a precursor in synthesis of phytochelatins, however, these polypeptides do not seem to act an important role in Cu, Cd, Co, Zn and Ni hypertolerance.[122]

Small molecules – organic acids and amino acids

Many plant species are producing organic and amino acid such as malic acid, citric acid or histidine. These small molecules are potential ligands for different heavy metals (e.g. Cd, Cu, Ni or Zn) and thus they can be involved in heavy metal detoxification and tolerance mechanisms. The role of amino and organic acid in the plant root exudates as the factors that exclude or/and limit the uptake of heavy metal ions form the soil was described in chapter 4.3.

However, organic and amino acids can be also produced intracellularly as a response for heavy metals excess. Histidine as a free amino acid is thought to be one of the most important amino acids involved in hyperaccumulation as it has ability to form stable complexes with Ni, Zn and Cd. The studies on nickel hyperaccumulator species *Alyssum lesbiacum*, *A. murale* and *A. bertolonii* showed that there is a linear correlation between concentration of nickel and histidine in xylem of these plants exposed to nickel. Additionally, it was observed that exposition of *A. lesbiacum* to elevated concentration of nickel caused 36-fold increase in the free histidine content in the xylem sap of this plant. These results suggest that histidine is likely to be responsible for nickel tolerance in *Alyssum* plants.[123] In another nickel hyperaccumulating plant – *Thlaspi goesingense* exposed to high concentration of this metal, the Ni that was not bound by cell wall, was associated with either citrate in vacuoles or histidine in the cytoplasm.[124] Histidine was also found to complex zinc in the root cells of Zn-hyperaccumulator *Thlaspi caerulescens* that suggests that this amino acid acts an important role in Zn homeostasis in the roots.[50]

Another amino acid – nonproteinogenic nicotianamine seems to be also implicated in metal tolerance in hyperaccumulating plants. It was observed that in hyperaccumulating species *A. halleri* the concentration of NA in roots was enhanced. It suggests that NA may play a role of Zn cytosolic buffer that is responsible for keeping zinc ions in nontoxic form but available for further transport to the leaves.[122] It was also shown that in *Thlaspi* hyperaccumulators, there was strong correlation between nickel and nicotianamine concentration in leaves[125] while in *T. caerulescens* NA complexes with nickel were identified in roots[126]. These results indicate that NA may be involved in Ni hyperaccumulation as well. Additionally, obtained data suggest that Ni and Fe may compete to be complexed by NA. There was also no evidence that NA is responsible for zinc hyperaccumulation in *Thlaspi* plant species.[125]

Other experiments on the tea plant (*Thea sinensis* L.) showed that the manganese accumulated in vacuoles was in form of Mn – oxalic acid complexes. The proposed mechanism of manganese detoxification was based on the Mn complexation by malate in the cytoplasm and further transportation of this complex through the tonoplast membrane into the

vacuole. Then, ligands are likely exchanged from malic to oxalic acid and the Mn-oxalic acid complex is stored inside the vacuoles.[99]

In hyperaccumulating plants, the role of organic acid is limited mainly to vacuolar sequestration. The low pH in the vacuole is favorable for formation of the metal-organic acid complexes.[122] The studies on Zn-hyperaccumulator *Thlaspi caerulescens*[50] and Ni-hyperaccumulator *Thlaspi goesingense*[124] showed that Zn and Ni were coordinated with citrate in vacuoles in shoot cells. Additionally, another research on *Thlaspi caerulescens* which is also known as Cd-hyperaccumulator indicated that in leaves cadmium was complexed by organic acids, mainly malate. However, there was no correlation between increasing Cd concentration in culture medium and malate concentration in leaves that suggests that synthesis of malate is not induced by cadmium.[127]

All these studies indicate that organic and amino acids can be involved in extracellular and intracellular response to heavy metal toxicity and thus enhanced plant tolerance for heavy metal stress.

4.6. Vacuolar compartmentalization

The transport of toxic metals into the vacuole is the way of reducing the level of these metals in the cytosol. Thus, it can be the mechanism of heavy metal tolerance and detoxification. As it was mentioned in previous chapters, vacuoles are the storage compartments for the metal – PC chelates[111] as well as for Mn – oxalic acid complex in *Thea sinensis*.[99] The studies on copper tolerant *Elsholtzia splendens Naki* and non-tolerant *Astragalus sinicus* plant species showed that in the tolerant plant the majority of copper was localized in the vacuoles and in the cell walls while the non-tolerant species accumulated more copper on the plasma membrane, in the chloroplast and cytoplasm under copper excess conditions.[128]

Comparative studies focused on the nickel hyperaccumulator *Thlaspi goesingense* and the nonaccumulator *Thlaspi arvense* showed that the hyperaccumulating plant was able to sequester nickel in the vacuole more efficiently than nontolerant plant. It indicates that the compartmentalization in leaf vacuoles play primary role in the intracellular mechanisms of nickel detoxification in this hyperaccumulator.[124] In general, the enhanced capacity of hyperaccumulating plants to store metals in the leaf vacuoles appears to be part of the mechanism responsible for the high metal tolerance in these plants.[122]

Earlier studies also showed that vacuoles are important compartments involved in storage of various heavy metals including zinc. The experiments on barley leaves using isotope ^{65}Zn showed that zinc taken up by leaves was immediately transported to the vacuoles. These results indicated that the transport and compartmentalization are an important homeostatic mechanism within the leaves that allow plant to deal with the toxic level of zinc in the shoots.[129] All these research point out that in various plant species vacuoles can be an important compartments responsible for the heavy metal storage and thus for increased plant tolerance for the heavy metals excess.

5. Sampling and sample preparation

Sampling and sample preparation are one of the most important, time-consuming and labor-intensive parts of the analytical procedure. The mistakes made during these steps may contribute even to important bias in the whole measurement process.

The way of sampling depends on the aim of the work and the main requirement is to collect the sample that is representative of the general population. If taken sample does not represent the original object, all the work put into the further analysis will be wasted because the obtained results will be invalid.[130]

The next step is the sample preservation that aims at the prevention the physical, chemical and biological processes to undergo in the sample, changing in the consequence its composition. The changes may arise during the delay between sample collection and analysis and include processes such as volatilization, diffusion, adsorption on the surface, photochemical reactions, oxidation, precipitation, biodegradation and enzymatic reactions.[130] The plant samples can be liquid or solid and the preservation method depends of the sample state of matter. Fresh liquid samples (plant juices) are usually filtrated or centrifuged and then they are stored by short-term at –4°C in the dark. Alternative methods are based on the shock-freezing and either storage as frozen samples or lyophilizing and storage as dried samples. Obviously, the latter technique can be applied just to the stable chemical species. The solid samples (e.g. leaves, roots, etc.) after harvesting should be gently rinsed with water to remove the surface contaminations and then shock-freezing of the sample in the gas phase above liquid nitrogen is recommended. If it is not possible, sample should be stored for short-time in –20 °C in dark place.[131]

The next step is sample preparation that is critical to obtain the accurate data and reliable interpretation of the results. The mistakes made on this stage of analytical procedure may distort information about the metal content in the sample as well as change the speciation. There are several rules that have to be taken in to account to avoid sample loss and contaminations, such as using the purified reagents and proper tools (e.g. containers, mortars etc.) as well as work in the clean laboratory. The way the sample is prepared to analysis depends on the analytical techniques that will be used and their capabilities. Majority of samples cannot be directly introduced into the instrument because of the sample concentration, its state of matter as well as the matrix complexity. If the chosen analytical technique requires the introduction of liquid sample, the solid samples have to be digested or extracted to obtain information about total metal concentrations and different forms of metals in these samples, respectively. Before these steps, samples have to be homogenized. Among different homogenization methods the most efficient appears to be cryogenic grinding. The use of liquid nitrogen at –196 °C causes that the solid sample becomes brittleness and thus the homogenization of plant material is better and less time consuming. Additionally, during this homogenization process, the plant tissues and the cell walls are effectively destroyed while the different chemical forms of metals stay unchanged. Hence, this homogenization technique can be used as a first step in extraction method.[132, 133]

The extraction step is needed during the sample preparation process to transfer species of interest from solid material into the soluble form. The extractions as well as other steps of sample preparation have to be performed in the way that guarantee maintaining the integrity of metal species and do not disrupt the equilibrium between different compounds present in sample. It can be difficult as in plant cells are present different species that can have differentiated kinetic and thermodynamic stabilities. During extraction some species may be degraded, dissociated or undergo ligand exchange that all results in loss of important species. Therefore, the selection of the extractant that ensure the good extraction efficiency together with unchanged speciation is the principal parameter in extraction procedure. In this choice different parameters must be taken into account such as buffer composition, ionic strength, temperature or extraction time. In practice, however, these requirements can be very difficult to fulfill. The use of the extractant which pH is adjusted to pH of the sample may not work in the case of biological samples, such as leaves and root because different cell compartments vary in pH considerably. The vacuoles are more acidic (pH ~ 5.5) while the cytosol pH is around 7.5. It was reported that pH of extractant have influence on extraction efficiency. The availability of low and high molecular weight species present in plant roots differed significantly depending on the extraction pH.[134, 135]

The pH has also influence on formation of metal-NA complexes. It was shown that, in general, the metal-NA complexes are hardly formed, partially formed and completely formed at acidic, neutral and basic pH values, respectively. However there is a few exceptions such as Fe(II)-NA and Zn(II)-NA complexes that are completely formed at neutral pH values and also Ni(II)-NA and Cu(II)-NA that complete formation is achieved at acidic pH values. Therefore, even small changes in pH can have a significant effect on metal-NA complexes speciation in plant samples. For this reason the extractant must be chosen very carefully to avoid the changes in speciation during extraction process.[136]

In addition, all chemical buffers that can be used as extraction agents have complexing properties for some metals and thus they may disturb the equilibria of species. The aqueous extraction procedures may results in low recovery from a solid matrix for some species and thus the use of more aggressive extractants is required. Sometimes, the sequential extraction is used to isolate different groups of compounds, such as the complexes situated in cytosol and vacuoles or bound to the cell wall, in each extraction step. However, only stable species can remain unchanged during that complex extraction procedure, such as high molecular weight metal species present in plants. These species are more resistant than small metal complexes which are often labile and unstable. Therefore, the most appropriate solution that can be used for extraction of low molecular weight metal species from plant material appears to be water. Water, as the natural constituent of each cell, does not affect the chemical form of metal species during extraction. However, a compromise is often needed between the extraction efficiency and preservation of the original metal forms.[134, 137]

The next step is analysis of prepared samples using the selective separation methods combined with sensitive detection techniques that allow the quantification and identification of various metal species present in plants.

6. Hyphenated techniques for metal speciation in plant samples

Many elements play an essentials or beneficial role in the physiology of living organisms. On the other hand, the same elements can be extremely dangerous because of either their high concentration in the organisms and surrounding environment or their chemical form. Hence the quantification of the total element concentration in biological material does not provide information about its toxicity or essentiality. It is due to the form of an element in which it is present in sample that critically implicates its chemical, biological and toxicological properties. Therefore, the identification, characterization and quantification of different chemical forms of elements, especially of metals, that are involved in various metabolic processes, are crucial to understand plant physiology. The main interest is in the mechanisms that are responsible for controlling essentiality and toxicity of metals including metal bio-availability, uptake, transport, detoxification and storage in plants. The speciation analysis that has become one of the fastest developing areas of the analytical chemistry is the tool that allow understanding the role of metals and processes that they undergo in plants.[138, 139]

Metals can interact with low-molecular-weight ligands (cellular metabolome) that are produced by plants to maintain the metal homeostasis within different tissues and cell compartments and also as a response to the metal toxicity. The cellular metabolome involves organic acids such as malate, citrate, oxalate or phytate, amino acids (e.g. histidine, nicotianamine), metallophores of mugineic acid family and metal-binding proteins. The metal chelation in plants and on the interface soil/root leads to formation of different metal complexes that possess the certain thermodynamic stability and kinetic inertness. To date, however, the majority of these metal species has not yet been identified or are relatively poorly characterized.[139]

The limited information about metal complexes present in plants is caused by their thermodynamic instability, high lability and complexity of biological matrix. Another issue is caused by the difficulties to convert metal complexes into volatile species that could be separated by gas chromatography (GC). Moreover, the lack of calibration standards for most of these metal species makes their identification difficult. Therefore, metal speciation in plants is a challenging task and requires suitable analytical techniques that should address several issues. First of all, the separation technique should exhibit the selectivity that allows the analyte species to reach the detector very well separated from potential matrix interference and from other species present in the sample. Additionally, the separation techniques should ensure that separated metal complexes present in plant samples quit the column in unchanged form. The separation technique should also not cause the generation of the artifact species on the column. Also, the element or molecular selective detection technique used should be very sensitive because the metals concentrations in environmental samples are usually very low, often at the picogram and lower levels. In addition, one metal can also be distributed among several species. Last but not least, the identification of metal species can be difficult to achieve as standards for majority of metal species present in plants are unavailable. Therefore, only the use of molecular-specific detection techniques can provide the information on the

identity of chemical forms of metal species in plant samples. These issues can be possibly solved by hyphenated techniques that are regarded as the fundamental tool to study metal speciation in plant samples. The variety of combined techniques is shown in Fig. 6.

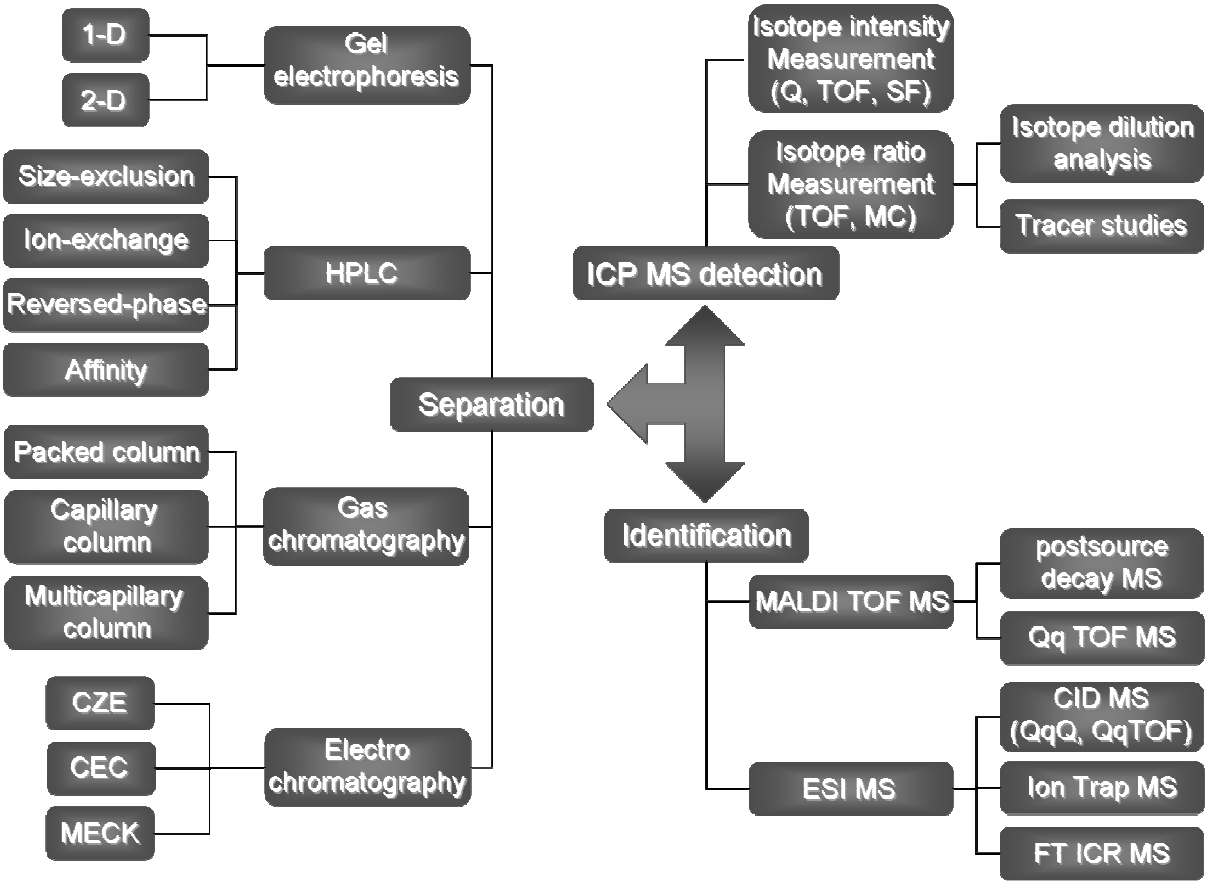

Figure 6. The hyphenated techniques usually used in speciation analysis.[137, 139]

The hyphenated techniques are based on high resolution separation techniques such as high performance liquid chromatography (HPLC), gas chromatography, capillary electrophoresis (CE) or gel electrophoresis. They are combined with sensitive element (e.g. ICP MS) or molecule specific (e.g. ESI MS) detection. The choice of the hyphenated setup depends on the aim of the work. The selection of a proper separation technique is critical if the species have similar physicochemical properties and high level of species- specificity is needed. The volatile compounds are separated using gas chromatography (GC) that can be directly coupled to the detection system.[137, 138, 140]

In case of non-volatile species the separation techniques, such as HPLC or CE, are recommended as the on-line coupling is usually easy to obtain. Also, the diversity of separation mechanisms and mobile phases allows the most suitable choice concerning the sample nature and thus preservation of the species identity. It is also possible to use the combination of two or more separation techniques for complex matrices to assure that the species of interest arrives at the certain time to the detector. The selection of detection technique depends on the concentration of species that are supposed to be quantified or identified. The choice becomes critical if the amount of compound of interest is relatively

small and low detection limits are required. It is challenging because the metal of interest is often a constituent of different species present is plant sample and the complexity of sample matrix poses usually an additional issue. Mass spectrometry with ionization in inductively coupled plasma copes with this issue and is one of the most widely used element specific detection techniques. Sometimes, the interface between HPLC and ICP MS may cause some problems as the separation conditions, such as the flow rate or mobile phase composition may be not compatible with the conditions required by the detector. The diversity of compounds in environmental samples and lack of analytical standards that could be used in order to identify various species has caused that the importance of the molecular specific detection increased significantly. Recently, ESI MS has become an increasingly popular detector for HPLC and CE separation techniques and gained a complementary role to ICP MS in the species identification in different biological samples.[137, 138, 140]

Development in speciation analysis including a decrease in detection limits of ICP MS, increase in sensitivity of electrospray MS and availability of efficient interface designs to HPLC and CE improved the effectiveness of the characterization of different metal species in biological samples. Information that can be provided by interpretation of data obtained using different hyphenated techniques, is important in many fields including environmental chemistry, medicine as well as health, nutrition and toxicity studies.[137]

6.1. Chromatographic techniques

The different chromatographic techniques are important in speciation analysis as they are used for sample purification and separation of diverse metal species in complex biological samples. However, the separation combine with identification using only chromatographic techniques is problematic and rare in metal speciation in plant samples because of the lack of analytical standards.[141]

The selection of a proper separation technique depends on physical and chemical properties, such as volatility, polarity and charge of analyte of interests. In case of separation of metal complexes that can be highly unstable the choice of proper mobile phase, its pH and ionic strength as well as gradient and time of analysis are critical. They are crucial not only to obtain the optimum separation but because they can affect the chemical form of the metal complex.[141]

The main techniques used for separation of various metal complexes in plant material are high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC is the robust, reliable and reproducible separation technique that provides high resolution, especially when used in gradient mode. It can be applied for different samples including plant saps and extracts.[140] The major HPLC separation mechanisms that found application in environmental speciation are size-exclusion (SEC), hydrophilic interaction (HILIC), reversed phase (RP) and ion-exchange (IEC) chromatography. Capillary electrophoresis is not used that widely in metal speciation in biological samples like HPLC. However, it offers also high separation efficiency, allows the use of small amount of sample (nanoliters) and additionally, has only capillary walls interacting with metals that may prevent

the disturbance of complexation equilibria. However, its coupling to MS detection techniques is not as good as with HPLC. Apart from HPLC and CE, also gas chromatography (GC) can be used for metal species separation in plant samples. However, its application is scarce because many metal species present in plant samples are non volatile and they cannot be converted in volatile species as well. Hence HPLC is the principal separation technique for metal species present in plants. Sometimes, however, it is necessary to use combination of two or more separation mechanisms, which is known as multidimensional separation, to cope with complexity of biological matrices. The multidimensional chromatography allows separation of great number of compounds even these that have similar physicochemical properties. In this approach, the SEC is often used as the first step that allows separation of various compound according to their sizes. In the next step, depending on the nature of the compounds present in different fractions collected from SEC column, other chromatographic techniques, such as ion-exchange, reversed-phase, or hydrophilic interaction LC have to be applied prior to electrospray MS. The multidimensional separation allows obtaining the high purity of separated compounds. Therefore, the perfectly isolated species of interest can arrive to the detector unaccompanied by other compounds. It is very important for the identification process as the presence of other species may interrupt the correct characterization of, for example, metal complexes by the molecular MS.[137, 139]

Recently, a new trend can be observed in analytical chemistry. It concerns the development of the miniaturized liquid separation techniques, such as capillary and nano high-performance capillary chromatography (nano HPLC). The miniaturization of chromatographic techniques brings several advantages, such as high separation efficiency, low solvent consumption, small sample volumes and shorter analysis time. These kinds of separation techniques are ideal for bioanalytical chemistry where the available amount of sample (e.g. biological tissues or a body fluids) is usually limited. Additionally, the reduction of the column inner diameter to less than 300 μm has led to the use of the nanoliter volumes of samples and the smaller flow rates compared to normal scale chromatographic techniques. It facilitated, therefore, the hyphenation to MS instruments.[142]

Size exclusion chromatography (SEC)

Size exclusion chromatography enables the separation of different compound mainly macromolecules including also different metal species. The primary separation mechanism is based on molecular sieve effect where molecules are separated depending on their size and, to a lesser extent, shape (Fig. 7). This mechanism works in most of the cases for the large proteins and polysaccharides. The average time they are spending in the pores of stationary phase can be related directly to their molecular weight. However, in case of small molecules (e.g. ions with high charge to mass ratio), there are other mechanisms, such as secondary adsorption and ion-exchange effects that can affect the separation process. The stationary phase must consist of the material that does not interact with the sample components. It must also have sufficient pore volume and the size of pore must be in the range that allows separation of compounds present in sample. The two major types of packing used in high performance SEC are polymeric gels or modified silica particles. The mobile phase should

highly eliminate the competition between buffer and cytosolic ligand as well as between these ligands and the packing material. In addition, mobile phase has to be compatible with chosen detection techniques. The water appears to be ideal eluent that does not cause structural changes, denaturation of proteins and destruction of metal-protein complexes. However, using water or diluted buffers as mobile phase lead to absorption of low molecular weight proteins to the stationary phase. Therefore, to avoid interaction with the column packing, the use of aqueous high ionic strength mobile phases is promoted.[141, 143]

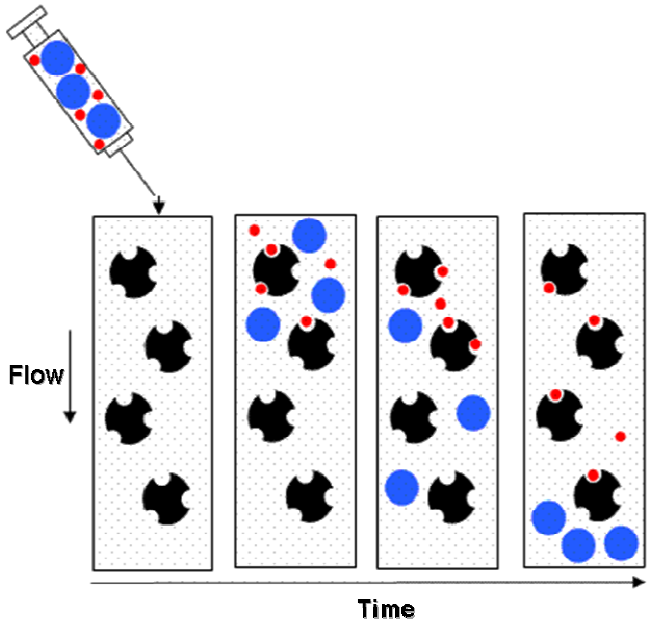

Figure 7. The primary separation mechanism of size exclusion chromatography.[185]

The advantages of using SEC in speciation analysis of biological samples are the flow rates between 0.7 – 1.0 mL min^{-1} that enable coupling SEC with ICP MS and the high tolerance of SEC to biological matrices. This robustness of SEC allows injection of complex samples without the prior extensive sample preparation steps. Moreover, SEC is also used in the multidimensional chromatographic approaches as the first chromatographic fractionation step. The disadvantages of SEC are the multifold dilution of sample during the chromatography and the small number of theoretical plates that cause the low separation efficiency. It leads to low purity of fraction and in consequence to problems with identification of metal binding species. Therefore, fractions collected form SEC require further analysis using other chromatographic techniques to obtain information about nature of species present in sample.[140, 141]

Size exclusion chromatography was used to investigate nickel speciation in latex of a hyperaccumulating tree *Sebertia acuminata*[144] and in the roots, xylem and shoots of the metal hyperaccumulator *Thlaspi caerulescens*[145].

Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction chromatography is an ideal chromatographic technique to effectively separate small polar highly hydrophilic and amphiphilic compounds. These compounds are usually too polar and have insufficient charge to be separated by reversed

phase and ion-exchange chromatography, respectively. Therefore, HILIC has solved some chromatographic problems, such as separation of small organic acids, basic drugs and several neutral and charged compounds. HILIC has similarities with normal phase chromatography (NP-LC) by employing the same polar stationary phases. The typical materials used for HILIC stationary phase are bare silica, silica gels modified with different polar functional groups (e.g. aminopropyl, amide, diol, cyanopropyl and other bonded phases) and also polymers that ensure that separation of polar compounds is selective and reproducible.[146] However, not all of the HILIC column can be used for separation of metal species. It was revealed that separation pattern for different metal complexes, such as Fe-citrate and Cu-histidine complexes varies depending on the stationary phase of HILIC column. The PFP columns exhibited the low retention of the metal species while the bare silica columns, the amino-type columns and the mixed-mode Trinity column showed strong retention and interactions with some metal complexes. The optimal separation of Fe-citrate and Cu-histidine complexes was achieved using three columns with the diol-, amide-, and zwitterionic (sulfobetaine) stationary phases.[147]

Zwitterionic sulfoalkylbetaine stationary phases are amongst the most widely used stationary phase in HILIC. They contain strongly basic quaternary ammonium groups and strongly acidic sulfonic acid groups that are separated by short alkyl group. This stationary phase is available under the tradename ZIC-HILIC.

HILIC is also similar to reversed phase chromatography (RP LC) as the mobile phases used in both techniques are the water-miscible polar organic solvents, such as methanol or acetonitrile with the small amount of water. However, the use of other solvents miscible with water, such as THF and alcohols, is also possible for HILIC. The increasing elution strength in HILIC is as follows: acetone < isopropanol ~ propanol < acetonitrile < ethanol < dioxane < DMF ~ methanol < water. There are also ionic additives, such as ammonium acetate and ammonium formate, used in HILIC that are supposed to control pH and ionic strength of mobile phase. They may also change the polarity of the compounds, which results in variations in retention times.[146, 148]

HILIC separation can be preformed in isocratic mode using as mobile phase high percentage organic solvents. Gradient elution, in contrast to RP LC, starts with a high content of organic solvent and polar compounds are eluted by continuous increase in proportion of aqueous solvent. It is believed that the surface of HILIC stationary phase is covered by water-rich layer formed by the mobile phase. The analyte retention is caused by its partitioning between a water-rich layer on the surface of the stationary phase and hydrophobic eluent as it is shown in Fig. 8.[146, 148]

Combining the properties of NP LC and RP LC, HILIC allowed many specific advantages over either of these two traditional chromatographic techniques. Compounds in HILIC are eluted in more or less opposite order than in RP. Consequently, HILIC enables separation of compounds eluting near the void in RP LC. HILIC also ensures a good solubility of polar compounds in the aqueous mobile phase employed by this technique opposite to NP LC where the use of nonpolar eluents causes poor solubility of polar sample. Additionally, the totally organic and nonpolar eluents characteristic for NP LC are not easily

ionized when LC is coupled to electrospray MS. Unlike NP LC, HILIC appears to be ideally suited to sensitive LC-MS analysis of water soluble polar compounds as the high organic content in the mobile phase leads to an immediate evaporation of solvent during electrospray ionization. The high volatility of organic solvents in HILIC comparing to RP LC results in tenfold increase in sensitivity. HILIC is also easy to combine with other detection techniques including fluorescence (FL), as ultraviolet light absorbance (UV), evaporative light scattering (ELSD), refractive index (RI) and charged aerosol (CAD).[146, 148]

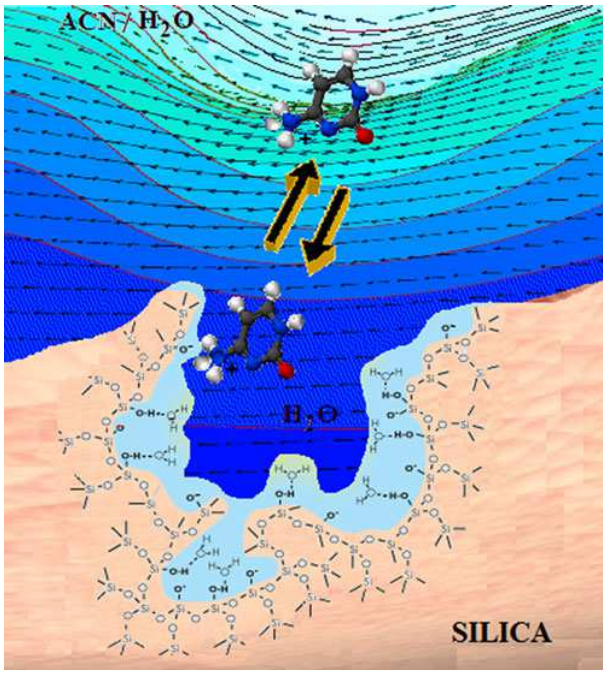

Figure 8. The separation mechanism in hydrophilic interaction chromatography.[146]

Hydrophilic interaction chromatography was used to separate small noncovalent nickel species, such as Ni complexes with malate, citrate, histidine and nicotianamine present in tissues of hyperaccumulating plant *Thlaspi caerulescens*.[145] It was reported that ZIC HILIC is also suitable for separation of small noncovalent metal-species, such as Ni-NA, Ni-aspartate, Ni-malate and Ni-citrate, present in xylem sap of *A. thaliana* grown with Ni or Cu-DMA and Zn-DMA present in the wheat root.[149]

Reversed phase liquid chromatography (RP)

Reversed phase liquid chromatography is a powerful technique for separation of peptides and proteins including metal complexes with different proteins present in plants. It was shown that RP HPLC can be applied for separation of polypeptides – phytochelatins produced by different plants.[150, 151] RP LC is ideal for peptide and protein separation because of excellent resolution, selectivity and reproducibility as well as high productivity and recoveries.

The separation mechanism of RP LC is based on the compounds partitioning between nonpolar stationary phase and a polar mobile phase. The separation is thus dependent on the hydrophobic binding of the compound present in the mobile phase to the immobilized

hydrophobic ligands that are constituents of stationary phase. Therefore, more hydrophobic compounds are retained on the column longer that hydrophilic molecules. The RP separation can be performed in isocratic mode with the constant concentration of organic solvent in mobile phase or using gradient elution where the content of organic solvent is continuously increasing during the analysis. The organic solvents used in RP HPLC are acetonitrile, methanol and 2-propanol that can contain ionic modifiers, such as trifluoroacetic acid. The ionic modifier, the organic solvent composition, the gradient slope or the operating temperature are the parameters that can be change or modified in order to manipulate the resolution and retention of different compounds. The stationary phases usually employed by RP HPLC are based on microparticulate porous silica that is modified commonly with alkyl group C18 and less often with n-butyl (C4) and n-octyl (C8). The C18 stationary phase exhibits higher selectivity than its homologs C4 and C8. The separation of proteins present in complex biological matrix using RP HPLC can be problematic and insufficient. Therefore, usually, sample pretreatment procedures are required in order to produce homogenous sample.[152]

Ion exchange chromatography (IEC)

Ion exchange chromatography allows the separation of ions and polar molecules depending on their charged. Therefore, IEC can be used for separation of wide variety of charged compound. They include proteins (e.g. metallothioneins, serum proteins), small nucleotides, amino acids as well as metal species, especially organoarsenic and organoselenium compounds. The IEC mechanism is based on coulombic (ionic) interactions where analyte ions in the mobile phase interact with the charged functional groups of stationary phase. The stationary phase, called ion exchanger, is insoluble solid material that has ions (cations or/and anions) covalently bound to it and ions that have opposite charge and are electrostatically bound to the surface. These ions possess exchangeable cation or/and anions of the stationary phase. When the mobile phase is eluted through the ion exchanger, the electrostatically bound ions can be exchanged for stoichiometrically equivalent amount of other ions of same sign. If the stationary phase has the negatively charged functional groups that interact with analyte cation in the mobile phase is called cation exchanger. The stationary phases with the positively charged groups, interacting with analyte anions in mobile phase, are known as anion exchangers. Certain materials are able to exchange both cations and anions and are called amphoteric exchangers. The cation and anion exchangers differ in acidic and base strength, respectively. The cation exchangers can be strong acidic with sulfonic groups or weak acidic if they contain carboxylic acid groups. Anion exchangers may contain weak base amino groups or strong base tertiary sulfonium and quaternary ammonium and phosphonium groups. [141, 153]

The separation using IEC requires drastic elution condition where acids, bases and high salt concentrations are used. This harsh condition must be used to allow elution of multicharged anions and cations in a reasonable time. However, the high concentrated buffers may cause problems during coupling ICE to ICP MS. The clogging of the nebulizer and sampler and skimmer cones are the main issues that can lead to variations of the detector

sensitivity. Depending on the compounds that are supposed to be separated, the ion exchangers with different acidic/base strength can be applied. To separate the metal complexes with metallothioneins the use of weak anion exchangers with diethylaminoethyl functional groups is sufficient while in the speciation analysis of arsenic and selenium more preferable are the strong anion exchangers.[141, 153]

6.2. ICP MS as an element specific detection in chromatography

Different techniques have been used as the detection systems (e.g. instrumental neutron activation – INAA or total-reflection X-ray fluorescence – TXRF) for HPLC. However, ICP MS equipped with quadrupole mass analyzer has become one of the most widely used detection techniques for this kind of applications. The mass spectrometry with ionization in inductively coupled plasma offers a lot of advantages. They include the detection limits at the femtogram level that do not depend on the chemical form and the presence of matrix components, quantification at the parts per million (ppm) level, multi-element and multi-isotopic measurement capacity as well as large dynamic range.[139, 154] Fig. 9 shows the elements that can be determined by ICP MS and the approximate detection capabilities of one of the ICP MS instrument models.

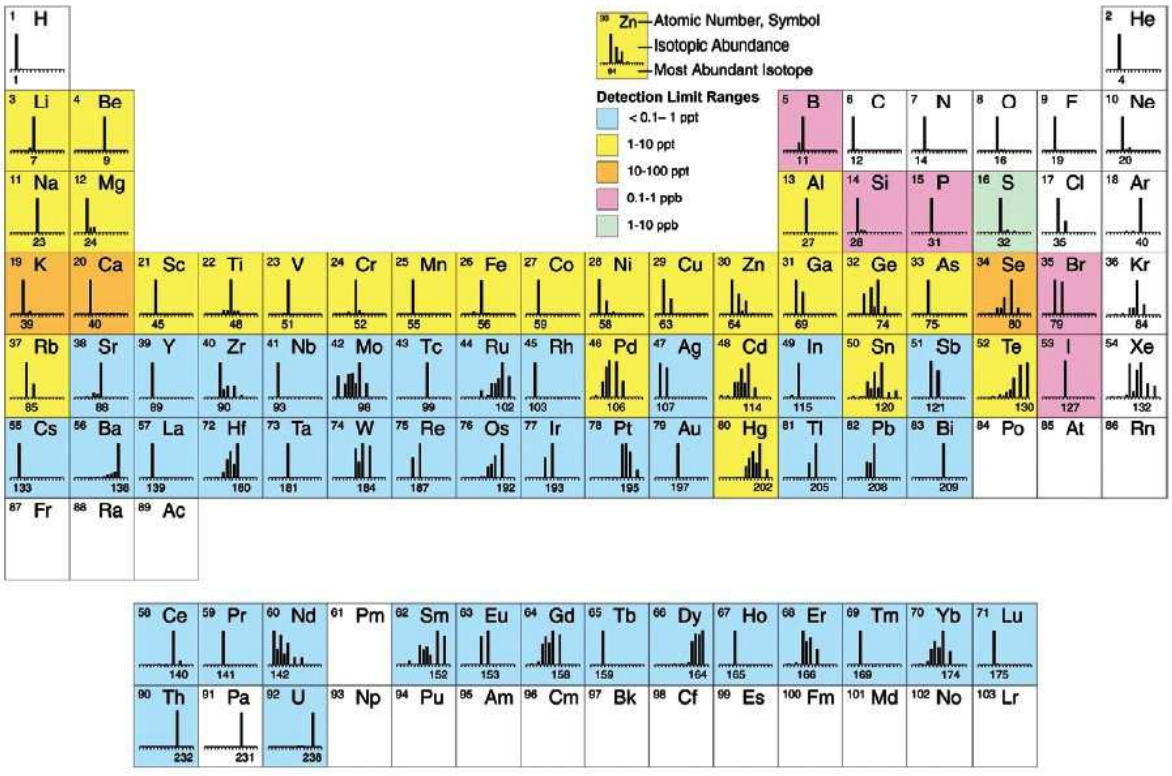

Figure 9. Elements that can be determined by ICP MS and approximate instrumental detection limits.[186]

Principles of ICP MS

An ICP MS system consists of a few elements that are similar in different available ICP MS designs. They include nebulizer, spray chamber, plasma torch and detector

(Fig. 10).[155] However, other components of ICP MS system, such as the interface, ion-focusing system, mass separation device and vacuum chamber, may vary significantly depending on the brand of ICP MS instrument.

Samples analyzed by ICP MS are usually liquid and are delivered to the nebulizer by a peristaltic pump. The role of the nebulizer is to convert liquid sample into a fine aerosol with presence of argon gas. In the spray chamber, the fine droplets of aerosol that represent only 1 – 2% of the sample are separated from large droplets which diameter is bigger than 10μm. In the next step, they are transported via a sample injector into the plasma torch which is positioned horizontally. The selection of small droplets that can reach the plasma torch aims at maintaining the stable temperature of plasma. The plasma torch consists of three concentric tubes: outer tube, middle tube and sample injector. The outer tube and the middle tube are responsible for plasma cooling and adjusting its position while the sample injector is responsible for transport of the aerosol into the plasma. The plasma formation starts when the RF power is applied to the load coil (usually copper) that surrounds the top end of the torch. The RF oscillation of the current in the coil creates the electromagnetic field at the top of the torch and the high-voltage spark is applied to argon gas flowing through the torch. This causes that some argon atoms lose their electrons that are caught up and accelerated in magnetic field. The collision of argon atoms with electrons causes the lose of next electrons leading, at the end, to the formation of the high-temperature plasma discharge (~10,000 K) at the open end of the torch.[156]

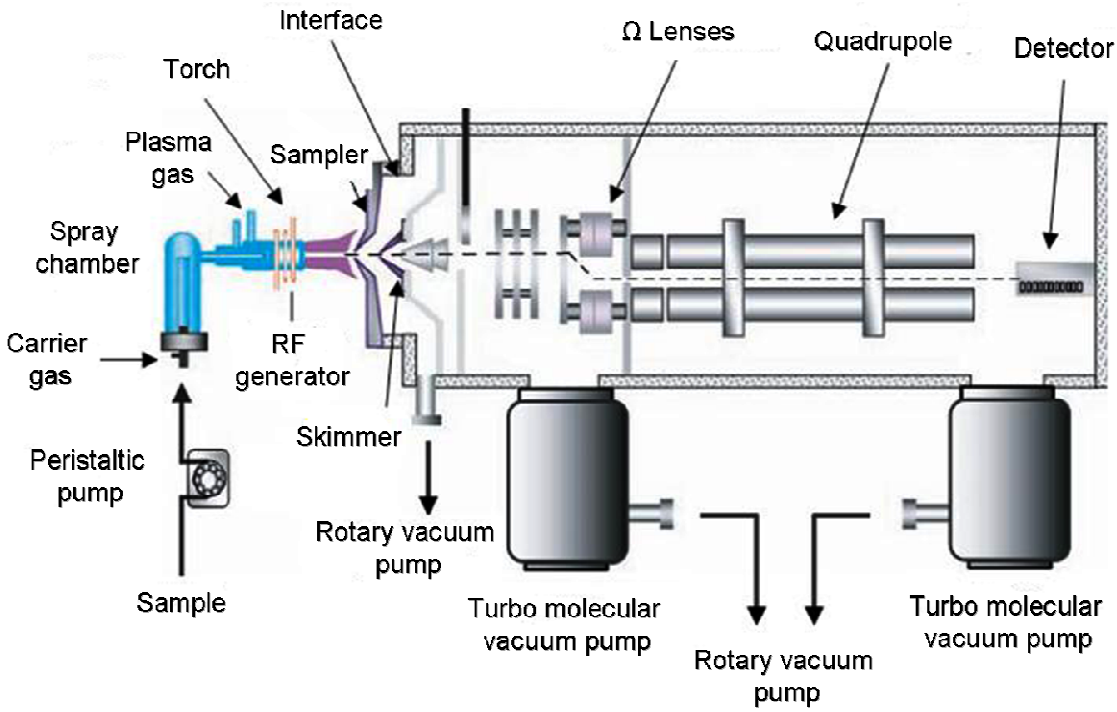

Figure 10. The scheme of the mass spectrometer with ionization in inductively coupled plasma.[155]

The aerosol reaching the plasma torch is passing then through different heating zones of the plasma that causes vaporization of solvent and formation of small particles. First, particles are changed into the gaseous form and then into the state-charged atoms. The atoms

due to the collision with energetic argon electrons are converted into the ions. Once the positively and singly charged ions are generated in the plasma, they are moved to the mass spectrometer via the interface region. The interface region is maintained at the vacuum (1 – 2 torr) and consists of two metallic cones – sampler and skimmer cone. The ions are passing through the cones and the ion optics to reach the mass separation device. The efficient transport of ions from the plasma which is at atmospheric pressure to the mass spectrometer analyzer (roughly 10^{-6} torr) through the interface region is one of the most critical parts of analysis. During this process ions must be transported efficiently, consistently and with electrical integrity into the mass analyzer region. There are different mass analyzers, such as quadrupole, magnetic sector and time-of-flight devices. The role of all of them is to allow the analyte ions of a particular mass-to charge ratio reach the detector. The mass analyzer is also responsible for filtering out all the nonanalyte, interfering and matrix ions.[156]

Quadrupole mass analyzer

The quadrupole (Q) mass filter is a constituent of roughly 85% of all ICP mass spectrometers that are used today. The advantages of this analyzer include simplicity, relatively low cost and absolute detection limits in subpicogram range. It caused that this kind of ICP MS system has found a lot of applications mainly in routine trace element analysis. The quadrupole mass analyzer consists of four cylindrical or hyperbolic rods made of metal (stainless steel or molybdenum) that have the same length and diameter. The rods are set in parallel to each other and arranged in the way that the direct current (DC) field and a time-dependent alternating current (AC) of radio frequency are placed on opposite pairs of rods as it is shown in Fig. 11. [154, 156, 187]

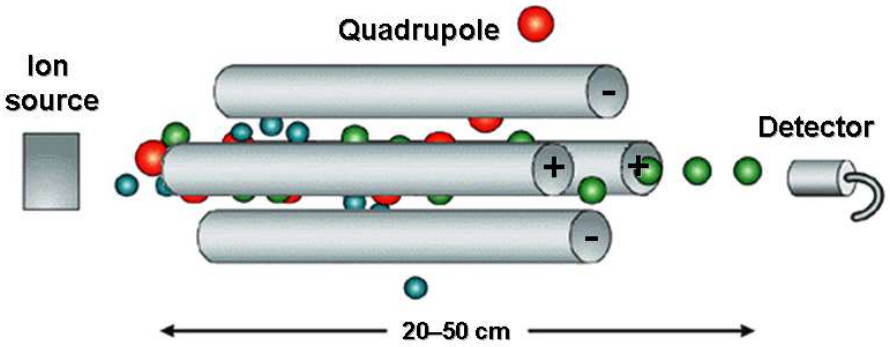

Figure 11. Scheme of quadrupole mass filter and principles of mass separation.[187]

Ions travel down the quadrupole between the rods and their trajectory is affected by applied voltages. The given AC/DC ratio on each pair of rods allows ions of only certain mass-to-charge ratio to reach the detector. Other ions are thrown out of their original trajectory and collide at the end with the rods. The ability of the quadrupole mass analyzer to separate different masses depends on a few factors, such as the shape, diameter and length of the rods, applied RF/DC voltages, frequency of quadrupole power supply and operating vacuum. Also, the movement and kinetic energy of the ions that enter and leave the quadrupole are important. [154, 156]

The quadrupole mass filter offers the resolution of 0.7 – 1.0 amu that is adequate for many routine applications. However, there are elements that suffer from different spectral interferences (e.g. argon-, solvent and/or sample based interferences) and their detection is not possible using quadrupole mass filter and requires use of high resolution mass spectrometry techniques.[154, 156]

Double focusing mass analyzer

The mass analyzer that offers resolving power much higher than quadrupole mass filter is the double focusing (SF) system that consists of the two analyzers: a traditional electromagnet and an electrostatic analyzer. The magnetic sector is responsible for mass focusing while the electrostatic sector acts as a kinetic energy selector. Therefore, the double focusing instrument is able to focus both the angular dispersions and energies of ions (Fig. 12).

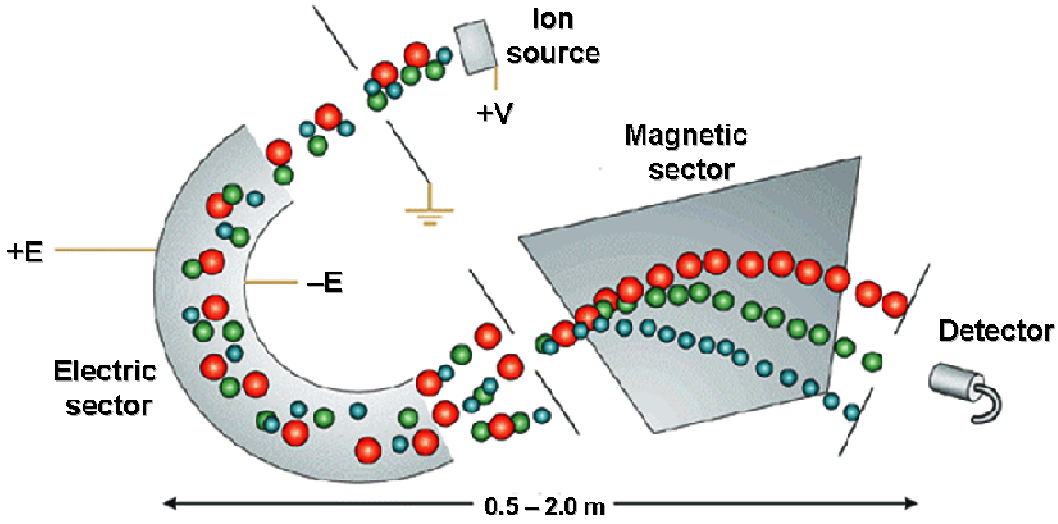

Figure 12. Scheme of double-focusing magnetic sector and principles of mass separation.[187]

The resolution offered by this kind of mass analyzer (up to 10 000) is high enough to allow the elimination of many polyatomic interferences that could not be achieved using a quadrupole mass analyzer. However, the improvement in resolution affects the sensitivity that decreases but is still high enough and makes possible detection of elements that are usually interfered. Additionally, use of this high resolution mass analyzer allows reduction of the background noise that led, in consequence, to the decrease in the detection limits. All advantages of use of double focusing system allow the detection of elements, such as sulfur. It is helpful in the determination of the stoichiometry of metal complexes with metallothioneins. The double focusing system offers also quantitative analysis with a great precision that caused the development of instruments that has found application in isotope ration analysis. Usually, these instruments, instead of the one detector, are equipped with multiple detectors referred to as multi-collector system. It allows a precise measurement of isotope compositions for a wide range of elements.[154, 156]

Despite many advantages offered by double-focusing magnetic sector systems, ICP quadrupole mass spectrometers are used more widely. They are ideal for rapid, high-throughput and multielement analysis and additionally, the cost of instrumentation and maintenances is lower compared to double-focusing magnetic sector ICP-MS systems.[154, 156]

Time of flight mass analyzer

The third type of mass analyzer that can pose as a "heart" of an ICP mass spectrometer is time of flight (TOF) mass analyzer. TOF mass spectrometers are based on a simple mass separation principle. At the beginning, all ions have the same position at the same time and are accelerated by the electric field so that all ions have the same kinetic energy that is proportional to their masses and velocities. As ions have different masses their velocities are also different. The correlation of ions velocities with their mass-to-charge ratio and arrival times at the detector allow the determination of their masses. Generally speaking, if there are three ions of different mass-to-charge ratios accelerated into a "flight tube", it is observed that the arrival time at the detector of each ion is different as it depends of the ion velocity. Therefore, the lightest ion that has the highest velocity is able to reach the detector first, followed by an ion of medium mass-to-charge ratio and, ultimately, the heaviest ion with the lowest velocity as it is shown in Fig. 13.

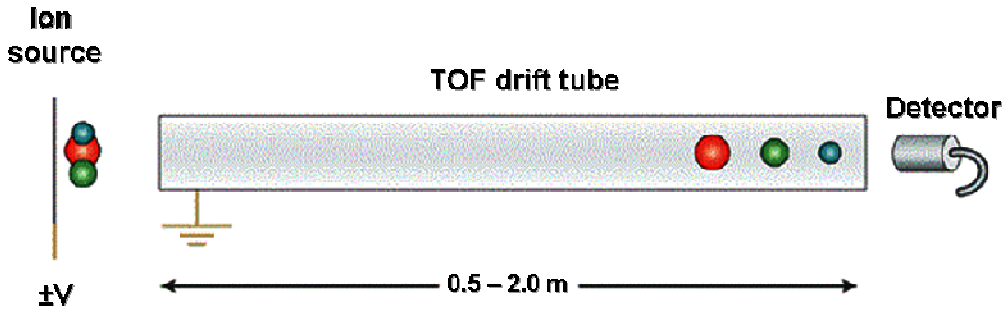

Figure 13. Scheme of time of flight mass analyzer and principles of mass separation.[187]

The main advantage of TOF mass analyzer over the scanning mass analyzers, such as the quadrupole, is that the latter are able to detect just one mass at the time. It results in the need of compromise between the number of monitored elements, detection limits, precision and time of analysis. Time of flight mass analyzer does not suffer from this kind of problems and allows the simultaneous extraction of all mass-to-charge ions and thus collection of multielement data sets without any significant losses in quality. The TOF analyzer permits to obtain roughly 20 000 complete mass spectra per second. It is thousand times faster than the sequential scanning mode of quadrupole mass analyzer. This abilities cause that TOF MS offers a lot of advantages including enhanced precision, determination of great number of elements in the fast transient peaks and rapid acquisition of data. It permits to carry out qualitative and semi-quantitative scans. Therefore, TOF MS is almost perfect to detect transient signals produced by high speed chromatographic techniques and is ideal for high-precision isotope ratio analysis.[154, 156]

Interferences in ICP MS

ICP MS is sensitive detection technique that allows acquisition of multielement concentration data in a small amount of samples. Additionally, the signal intensity in ICP MS is independent of the matrix composition. It gives possibility to quantify the proteins and others biomolecules using inorganic elemental standards. However, the one of the main issues that emerges during the analysis using ICP quadrupole mass spectrometer is formation of polyatomic spectral interferences that can disrupt the detection of some elements. The polyatomic interferences result from the combination of two or more isotopes from different elements including argon, solvent and matrix-derived ion. The different possible polyatomic interferences can be formed in the plasma torch and they may interfere with the detection of metals, such as zinc, copper or iron. As it is shown in Table 1 all isotopes of Cu, Zn and Fe can be interfered with different polyatomic ions and thus their detection can be disrupt

Table 1. The polyatomic interferences that can be formed in plasma torch.[157]

Isotope	Abundance	Interference
^{27}Al	100	$^{12}C^{15}N^+$, $^{13}C^{14}N^+$, $^{14}N^2$ spread, $^1H^{12}C^{14}N^+$
^{63}Cu	69.1	$^{31}P^{16}O_2{}^+$, $^{40}Ar^{23}Na^+$, $^{47}Ti^{16}O^+$, $^{23}Na^{40}Ca^+$, $^{46}Ca^{16}O^1H^+$, $^{36}Ar^{12}C^{14}N^1H^+$, $^{14}N^{12}C^{37}Cl^+$, $^{16}O^{12}C^{35}Cl^+$
^{65}Cu	30.9	$^{49}Ti^{16}O^+$, $^{32}S^{16}O_2{}^1H^+$, $^{40}Ar^{25}Mg^+$, $^{40}Ca^{16}O^1H^+$, $^{36}Ar^{14}N_2{}^1H^+$, $^{32}S^{33}S^+$, $^{32}S^{16}O^{17}O^+$, $^{33}S^{16}O_2{}^+$, $^{12}C^{16}O^{37}Cl^+$, $^{12}C^{18}O^{35}Cl^+$
^{54}Fe	5.82	$^{37}Cl^{16}O^1H^+$, $^{40}Ar^{14}N$, $^{38}Ar^{15}N^1H^+$, $^{36}Ar^{18}O^+$, $^{38}Ar^{16}O^+$, $^{36}Ar^{17}O^1H^+$, $^{36}S^{18}O^+$, $^{35}Cl^{18}O^1H^+$, $^{37}Cl^{17}O$
^{56}Fe	91.66	$^{40}Ar^{16}O^+$, $^{40}Ca^{16}O^+$, $^{40}Ar^{15}N^1H^+$, $^{38}Ar^{18}O^+$, $^{38}Ar^{17}O^1H^+$, $^{37}Cl^{18}O^1H^+$
^{57}Fe	2.19	$^{40}Ar^{16}O^1H^+$, $^{40}Ca^{16}O^1H^+$, $^{40}Ar^{17}O^+$, $^{38}Ar^{18}O^1H^+$, $^{38}Ar^{19}F^+$
^{64}Zn	48.89	$^{32}S^{16}O_2{}^+$, $^{48}Ti^{16}O^+$, $^{31}P^{16}O_2{}^1H^+$, $^{48}Ca^{16}O^+$, $^{32}S_2{}^+$, $^{31}P^{16}O^{17}O^+$, $^{34}S^{16}O_2{}^+$, $^{36}Ar^{14}N_2{}^+$
^{66}Zn	27.81	$^{50}Ti^{16}O^+$, $^{34}S^{16}O_2{}^+$, $^{33}S^{16}O_2{}^1H^+$, $^{32}S^{16}O^{18}O^+$, $^{32}S^{17}O_2{}^+$, $^{33}S^{16}O^{17}O^+$, $^{32}S^{34}S^+$, $^{33}S_2{}^+$
^{68}Zn	18.57	$^{36}S^{16}O_2{}^+$, $^{34}S^{16}O^{18}O^+$, $^{40}Ar^{14}N_2{}^+$, $^{35}Cl^{16}O^{17}O^+$, $^{34}S_2{}^+$, $^{36}Ar^{32}S^+$, $3^4S^{17}O_2{}^+$, $^{33}S^{17}O^{18}O^+$, $^{32}S^{18}O_2{}^+$, $^{32}S^{36}S^+$

However, the interferences can be minimized by using different approaches, such as correction equations, cool plasma technology, matrix separation and collision/reaction cells

(CRC). The latter appears to be the more efficient approach that permits to diminish production of many harmful species before they reach the mass analyzer.[156]

The collision/reaction cell is a chamber placed before the quadrupole mass analyzer that contains multipole, for example quadrupole, hexapole or octopole, that is usually operated in RF-only-mode. The collision/reaction cell is filled up with reaction/collision gases (one gas type at a time or a mixture of two of them), such as helium, ammonia, methane, hydrogen or oxygen. Inside the collision/reaction cell, the RF-only field is suppose to focus the sample ions which then collide and react with molecules of the collision gas or gasses. It results in the conversion of the harmful polyatomic interfering ions (a few examples are shown in Table 1) or analyte ions into harmless noninterfering species or other ions that are not interfered with, respectively.[156]

The principle of reducing the formation on polyatomic interferences in the collision/reaction cell is shown in Fig. 14. The polyatomic interferences that may occur during the analysis can be easily removed also by use of a sector field double focusing (high resolution) mass spectrometers.[156]

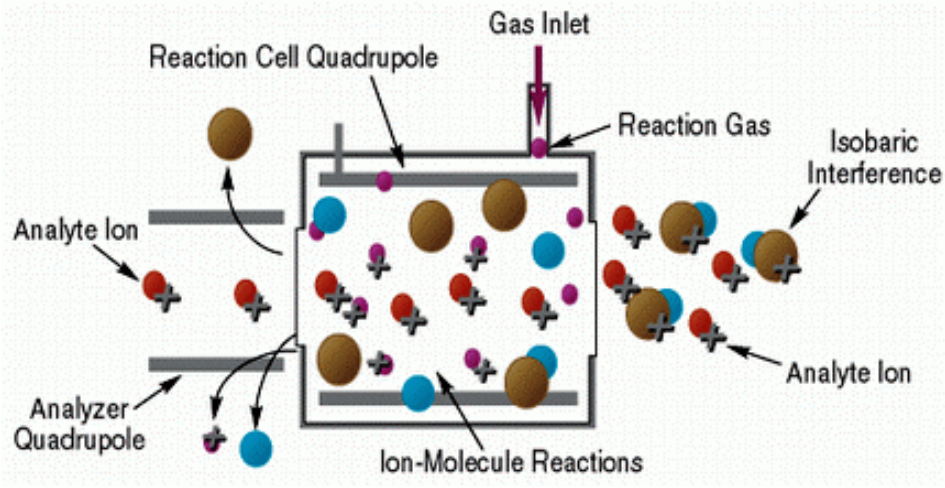

Figure 14. Principles of operation of a collision/reaction cell.[188]

Coupling HPLC to ICP MS

All advantages of ICP MS, such as possibility of multi-element analysis, low detection limits, element specificity and easy elimination of interferences, caused that ICP MS has become the main detector for HPLC and CE.[139]

ICP MS is practically the only technique able to detect, in on line mode, the trace concentration of different elements in LC and CE effluents. ICP MS also makes it possible to identify several elements in the same chromatographic or electrophoretic peak by scanning or hopping the mass analyzer. However, the coupling of HPLC and CE to ICP MS may encounter a few problems on the interface level, such as the lack of compatibility of the mobile phase composition and flow rate with ionization conditions of ICP MS. Therefore, the successful detection of various metal species depends on the proper choice of the interface that is one of the most important elements in HPLC or CE – ICP MS couplings.[137, 141]

The most common interface consists of a piece of narrow-bore tubing that connects the outlet of the HPLC column with the nebulizer. The HPLC column that diameter is between 4.6 – 10 mm can be connected to the pneumatic or cross-flow nebulizers operating in the flow rate that is optimal for the separation on these kind of columns. The use of the columns with smaller diameters, between 0.32 – 1.0 mm, requires the use of micronebulizers.[137, 141]

The mobile phases used in chromatographic separation should not contain elements that can cause polyatomic interferences in ICP MS. However, majority of eluents cause the production of polyatomic ions that interfere with the detection of different elements. The mobile phases containing volatile salts up to 100 mM are not generating any problems. However, higher salt concentration or non-volatile salts may cause the nebulizer and cones occlusion and thus leads to the either temporary depression or enhancement of signal.[140, 158, 159]

The chromatographic separation requires often the use of the mobile phases in which the organic solvent content is even up to 100%. The introduction of mobile phases rich in organic solvent into ICP MS affect negatively the stability of ICP MS and decrease signal intensity. Additionally, organic solvent present in mobile phase may cause deposition of carbon on the torch and cones. Therefore, the organic solvent can be introduced to ICP MS provided that cooled spray chamber or desolvation system are used accompanied by addition of oxygen to plasma gas, increased RF power and use of platinum cones. The coupling of HILIC and RP LC to ICP MS requires this kind of approach as both types of chromatography use high concentrations of organic solvents. Another issue that may appear during coupling HILIC and RP LC to ICP MS is the use of solvent gradients. The changing composition of mobile phase affects the plasma temperature and electron number density. These processes may make the ionization efficiencies vary during the analysis.[140, 158, 159]

Unlike HILIC and RP LC, SEC often exhibits good compatibility with ICP MS in terms of the flow rates between 0.7–1.0 mL min^{-1} and the mobile phases that do not contain organic solvents and can be limited in terms of concentration of salts. Concerning capillary and nano HPLC, where the flow rates are much smaller in comparison with conventional HPLC, the introduction of up to 100% of organic solvent into the plasma does not cause the problems mentioned before. These coupling can work without the use of a cooling spray chamber and addition of oxygen to the plasma gas. However, capillary and nano HPLC suffer from other issues that appear during coupling to ICP MS. The main one is incompatibility of capillary and nano HPLC with ICP MS because of the flow rates that are 100 – 1000 times smaller that those required by traditional nebulizers. The conventional spray chambers are also not suitable for these separation techniques as they have large dead volumes that cause long washout times and peak broadening. Therefore, the capillary and nano HPLC require the use of dedicated interfaces, such as small-dead-volume spray chambers and total consumption micro-nebulizers. They allow efficient nebulization and operate at the flow rates in the range 0.5 – 7.5 mL min^{-1}.[140, 158, 159]

The on-line couplings of HPLC to ICP MS have found applications in analysis of environmental samples. The SEC HPLC coupled on line to ICP MS was used for the determination of cadmium, copper, lead and zinc binding properties of phytochelatins in cell

cultures of *Silene vulgaris*[160] and for the separation and detection of different nickel species present in latex of nickel hyperaccumulating tree *Sebertia acuminate*[144]. These applications show that ICP MS is an excellent detector for on-line analysis of biological samples in terms of its high sensitivity, multielement detection and ability to accept liquid mobile phase at different flow rates.

6.3. Electrospray high resolution mass spectrometry in the metal species identification

The species selectivity in ICP MS depends on the time when the analyte molecule reaches the ionization source. Therefore, the metal species separated during the chromatographic step can be identified only if the information about possible compounds present in the sample is available and via matching retention times with those obtained for standards. However, the majority of metal species present in biological samples can not be identified and characterized using only HPLC ICP MS as the retention time standards are usually unavailable. Additionally, this approach assumes that compounds present in sample are completely separated and each peak corresponds to a single pure compound that in practice is difficult to achieve. Moreover, the progressively lower detection limits offer by ICP MS lead to detection of new and numerous compounds that need to be identified. Hence the use of techniques that allow molecular specific detection is mandatory to obtain information about accurate molar mass and structure of different metals species. They include previously unreported species and those for which standards are unavailable present in complex sample, often at the trace level. Two techniques, electrospray mass spectrometry (ESI MS) and matrix assisted laser desorption ionization (MALDI)-TOF MS, offer the possibility of determination of the accurate molecular weight and the structure of the diverse compounds in biological samples. However, ESI MS has become recently the one of the most widely used detection techniques that is complementary to ICP MS in HPLC and CE couplings. The ESI MS allows determination of precise molecular mass and characterization of the molecules that are present at the trace level in relatively complex matrices.[154]

ESI MS can mainly provide the identification of relatively stable metallospecies, such as cobalamins, porphyrins, metalloproteins or compounds containing metal-carbon bond (metalloids). The latter, usually can be easily ionized forming singly protonated ions that theoretically enables their identification concerning just the molecular mass. The labile complexes are likely to be destroyed as the metal-ligand bond is not resistant enough to survive the ionization process. ESI MS is widely used in functional proteomic analysis and to determine the noncovalent interaction. It is caused by the fact that the metal complexes with amino acids, peptides, proteins and carbohydrates can be readily ionized in ESI source and their gas-phase and solution properties are correlated. Additionally, there is a possibility to work in positive or negative ion mode that enables to find the ideal conditions to ionize different types of metal species. The identification of unstable metal complexes is also possible. The ligands can be identified and characterized by ESI MS/MS. However, the demonstration of the presence of the metal-ligand link requires the use of another complementary to ESI MS/MS technique.[137] The determination of precise molecular mass by

ESI MS is sufficient to determine the empiric formula and elucidate the metal-ligand stoichiometry. The information obtained after the fragmentation of protonated molecular ions by collision induced dissociation (CID) are very useful to establish ligand structure and confirm its identification as fragmentation gives deeper insight into the species identity.[139]

Electrospray ionization (ESI) is capable to produce ions of metal complexes in the gas phase that are then analyzed by MS. The electrospray ionization consists of a few stages that are shown in Fig. 15.

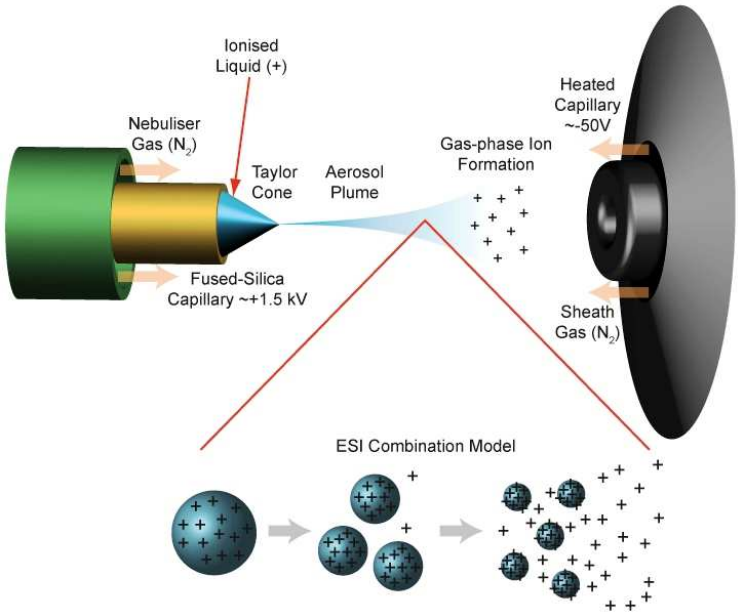

Figure 15. Principle of electrospray ionization.[189]

A solution containing analyte is introduced at the rate 1 – 10 μL min^{-1} to the source. Then the aqueous analytes are passing through the electrospray needle that is biased to high potential with respect to a ground planar electrode (counter electrode). This causes the generation of the spray of small, highly charged droplets. From the droplets, the neutral solvent molecules are evaporated leading to decrease of the droplet size and at the and to formation of molecular ions. The molecular ions are often multiple charged and in the next step they are transported from the atmospheric pressure to the high vacuum area. Then, in the mass analyzer, the ions are separated according to their mass-to-charge ratio. There are different types of mass analyzers including quadrupole (Q), ion-trap (IT), time-of-flight (TOF) and Fourier Transform ion cyclotron resonance (FT-ICR).[154]

The quadrupole mass analyzer seems to be ideal for routine application as it is robust and have modest vacuum requirements and compact size. However, other types of mass analyzers were gaining more and more attention for years as they offered better resolution and simultaneous detection or extraction of all ions. The use of these analyzers allowed also identification of species containing different metals and elucidation of metal-ligand stoichiometry in these compounds.[159] Recently, a great interest is given to mass spectrometers that provide accurate mass of analytes and they include TOF, FT ICR and Orbitrap detectors.

High resolution mass spectrometry

The great interest given to mass spectrometers, such as TOF, FT ICR and Orbitrap technology is caused by the high resolving power, mass and spectral accuracy, sensitivity and dynamic range exhibited by these MS techniques. It allows determination of the exact masses of different small molecules (up. to 400 Da), including metal complexes, and thus determination of their elemental composition. It is the first step for identification of unknown species. High resolution mass spectrometry allows the determination of elemental composition of compounds up to 1000 Da, the identification of proteins, lipids and other biological compounds as well as characterization of posttranslational modifications of intact proteins. Therefore, HR MS found application in fields such as proteomics, protein modifications analysis, metabolomics and petroleomics.[161]

The TOF mass analyzer (described in details in chapter 6.2.) gives possibility of fast and sensitive mass analysis as the measurement of all masses is simultaneous. TOF offers also better precision and mass measurement accuracy than other technologies, such as ion trap. Additionally, the lack of upper mass-to-charge limit makes this technique suitable for the detection of ions that are singly charged and heavier that 5000 Da. It can also be applied in analysis where more that 1 mass spectrum per second must be acquired. The resolution obtained using TOF can be above 10 000 with mass accuracy below 10 ppm.[154, 161] However, the FT MS technology offers greater resolution and accuracy than TOF. Additionally, the use of FT MS for identification of metal-bioligand complexes can bring just benefits as this detection system offers a large intrascan dynamic range and multistage fragmentation. It can make the identification of metal species much easier.[139]

One of the instruments that belong to FT MS family is FT ICR mass spectrometer (Fig. 16). In FT ICR MS ions are moved to the ICR cell that is sited in the middle of superconducting magnet. Thus, ions are trapped in a strong uniform magnetic field that causes that the ions motions are confined to circular orbits that have characteristic frequency which is called ion cyclotron frequency. The measurement of ion cyclotron frequency provides the information about ion masses. However, to measure it, the ions FT ICR motion must be excited by an RF pulse to larger ICR radius. Then, excited ions cyclotron motion is detected as an image current induced by the ions passing by the detection plates. The detected image current is digitized and the Fourier transform converts the signal to time domain and then to mass spectra. The stability and uniform magnetic field of the superconducting magnets combined with high accuracy and dynamic range of measured frequencies results that FT ICR MS offers the resolution that can be higher than 200 000 with the mass accuracy down to 0.2 ppm.[154, 162] This great potential of FT ICR MS allows determination of highly accurate mass and thus determination of molecular formulae. Also, elucidation of the structure is possible as the ions can be fragmented, usually by infrared multiphoton dissociation. The main disadvantages of FT ICR MS instruments are their high purchase and running costs.

The need to analyze more and more complex mixtures including biological matrices as well as faster HPLC resulted in searching for new solution to improve existing mass analyzers or create the new, more efficient ones. All attempts that have been made to enhance the

abilities of FT MS gave the groundwork for the appearance and development of a new member of FT MS family – the Orbitrap analyzer. Orbitrap analyzer was introduced to commercial use in 2005 and immediately became the part of mainstream mass spectrometry. The Orbitrap analyzer is a compact and robust mass analyzer that offers excellent quantification properties, high resolving power and accuracy (specified as 2 – 5 ppm, but peaks with signal-to-noise ratio>10 000 can reach 0.2 ppm), large space charge capacity, a minimum upper mass/charge limit of 6000 and great linear dynamic range up to four orders of magnitude. Other advantages include faster and more sensitive sample analysis, larger trapping capacity, more user-friendly interface and much lower purchase and running costs comparing to the FT ICR MS. Despite of the fact that the mass resolution and mass accuracy offers by the orbitrap are slightly lower than those offered by FT ICR, the other advantages given by orbitrap make it wide used mass analyzer.[161, 163, 164]

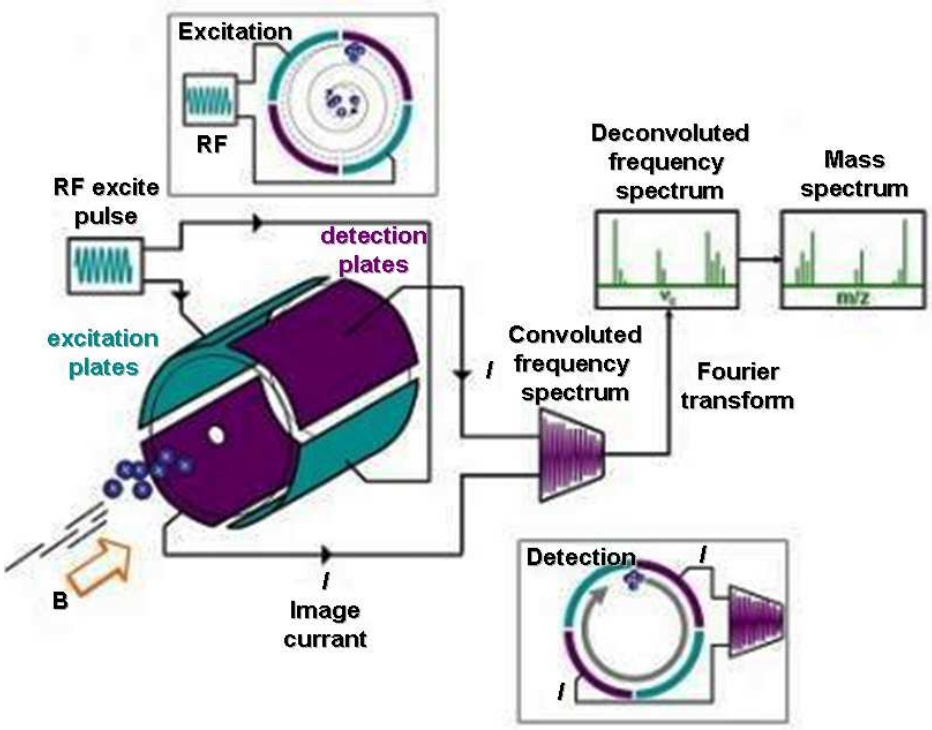

Figure 16. Principle of FT ICR MS.[190]

The first introduced analyzer was a hybrid instrument – LTQ Orbitrap. It consists of a linear ion trap and an orbitrap mass analyzer[163] as it is shown in Fig. 17. The orbitrap technology combines some features of FT ICR (trapping the ions in ultrahigh vacuum, principle of image current detection, FT data processing), radiofrequency ion trap (ions are trapped in precisely defined electrode structures) and TOF (electrostatic fields to constrain and to analyze the injected ions and pulsed injection) analyzers. It makes this mass analyzer very powerful tool. In addition, at the same time major limitation of the previous analyzers are avoided.[163] The different fields of analytical chemistry where the LTQ Orbitrap has been used include proteomics, metabolomics, environmental chemistry, drug analysis, lipidomics and identification of reaction products and small molecules.[164]

The complete Orbitrap based mass spectrometer (Fig. 17) is equipped with and electrospray ionization source (ESI), a dual cell linear ion trap, a curved linear storage trap, a collision cell and at the end the Orbitrap analyzer. The linear storage trap is required for storage and short pulse injection of ions into the orbitrap while a collision cell allows performing high energy CID experiments.[163]

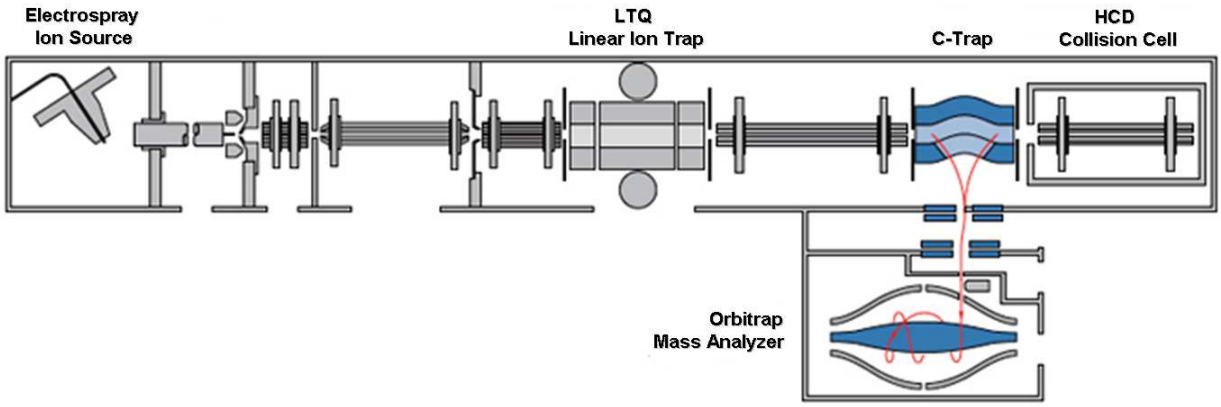

Figure 17. Scheme of LTQ Velos Orbitrap.[191]

The Orbitrap mass analyzer composed of three electrodes: two outer ones that are axially symmetric and together form barrel-like shaped trap that surface perfectly matches the shape of the central spindle-like third electrode. The outer electrodes are split in two halves because they act as receiver plates for image current detection. They are also electrically isolated from the central electrode by a hair-thin gap that is secured by made of dielectric central ring (Fig. 18).[163, 164]

The voltage applied between outer and central electrodes results in the electrostatic potential distribution that makes orbitrap mass analyzer – an electrostatic trap. Before entering the orbitrap, the significant ion populations are stored in C-trap (RF-only gas-filled curved quadrupole). Then, they are injected tangentially, in a short pulse, into the volume between the outer and central electrodes through a “deflector” placed in one of the outer electrodes. The short pulse injection results in the injection of ions population at different mass-to-charge ratio into the orbitrap analyzer at different times (sub-microsecond pulses). Once ions are injected to the orbitrap, they start rotate around the central electrode undergoing the process called “electrodynamic squeezing”. It means that when ions rotate around the central electrode they are confined in created between electrodes electric field. This process causes the contraction of the radius of the ion cloud and reduction of rotational radius. It prevents the ions to collide with the outer electrode once they start their axial oscillations.[163, 164, 165]

Unlike FT ICR, in the orbitrap, ions do not have to be additionally excited to start coherent axial oscillation because they are excited by injection. Apart from the orbiting motion around the central electrode, the stable ion trajectories involve also harmonic axial oscillations. As the ion packets of different mass-to-charge ratio are injected into the orbitrap analyzer in sub-second pulses, each ion packet is spread over the angular coordinate. Finally, each ion packet forms a thin rotating ring where the ions of larger mass-to-charge ratio have

larger orbital radius. The mass-to-charge ratio measurements are based on the image current detection of axial oscillations frequencies. These frequencies do not depend on the energy and amplitude of the ions just on the mass-to-charge ratio of the ion and the potential between the outer and central electrodes that is, however, keep constant during analysis. This independence causes that the orbitrap permits to obtain high resolution and mass accuracy measurements. The detected image current, similarly to FT ICR, is digitized in the time domain and the Fourier transform converts the signal to frequency domain which is then converted to mass spectrum.[163, 164, 165]

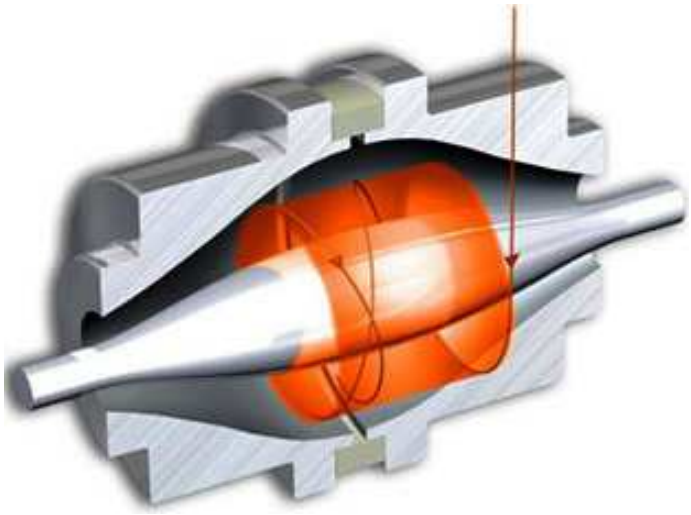

Figure 18. Orbitrap analyzer cell and example of stable ion trajectory.[192]

As it was mention before, the orbitrap mass analyzer offers high resolving power that is directly proportional to the acquisition time. For this reason enhancing the acquisition time higher resolving power can be obtained. The resolving power of orbitrap analyzers is up to 150 000 but usually it is lower (60 000 at m/z of 400 in a 750-ms detection period). However, the recent analyzers, such as Orbitrap Elite, can exhibit the resolving power of even 240 000 at m/z 400 for 768-ms detection period. It is four times higher that previous orbitrap analyzers could reach.[163, 164]

Comparing resolving power of orbitrap with FT ICR, the latter exhibits better resolving power which, however, decreases faster with mass-to-charge ratio unlike orbitrap. It is a consequence of the applied appropriate mathematic equations that describe the correlation between the detection time, period of the main oscillation and mass-to-charge ratio for FT ICR and orbitrap where ions move in magnetic and electrostatic field, respectively. Thus, for the fixed acquisition times, the resolving power of FT ICR inversely scales with m/z while in orbitrap mass analyzer resolving power is inversely proportional to the square root of m/z. The consequence of this correlation is that for any Orbitrap and any FT ICR analyzer exists a critical mass-to-charge ratio. Below this critical mass-to-charge ratio FT ICR exhibits higher resolving power than orbitrap analyzer but once this critical value is crossed the orbitrap fronts in resolving power as it is shown in Fig. 19.[163, 164]

Apart form high resolving power, the orbitrap analyzer offers also a high mass accuracy measurement. It is the one of the main advantages of the orbitrap as an excellent

mass accuracy is highly desired attribute of mass analyzers. The ability to measure the compound masses with high accuracy allows direct determination of elemental composition of these compounds as the majority of other possibilities can be easily eliminated. The determination of elemental composition of different compounds is the first step for elucidation of structure of unknown molecules. It is possible to achieve using accurate mass of the compound as each isotope of every chemical element has different mass defect.[166]

The mass defect is defined as the mass difference between the calculated exact mass of compound (atom, molecule, ion or radical) and its nominal integer mass (number of protons and neutrons in the atom nucleus) based on convention defining carbon ^{12}C = 12.0000 u with zero mass defect. All other isotopes of every element have exact masses above or under the nominal mass as the amount of energy released during formation and stabilization of nucleus is slightly different for each of them. Thus, each isotope of every element has either positive or negative mass defect. It causes that each molecule with different elemental composition has a unique exact mass. Therefore, it is possible to elucidate the elemental formulae of the molecules knowing their exact masses.[166]

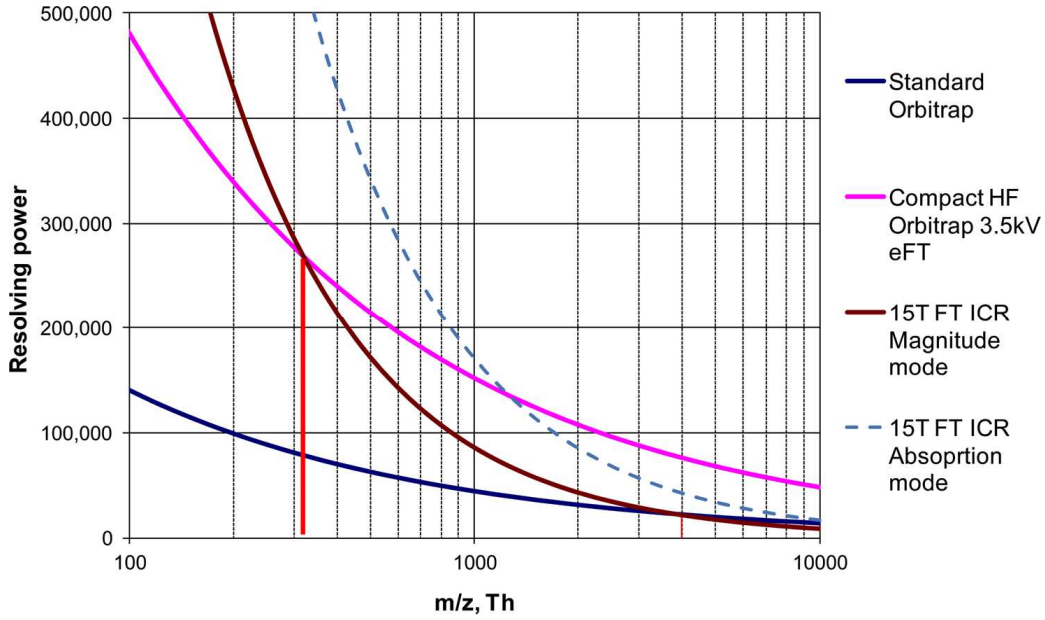

Figure 19. Correlation between the resolving power and mass-to-charge ratio for different types of orbitrap and FT ICR mass analyzers.[163]

However, sometime the information about accurate mass of the molecule is not enough to elucidate the correct elemental composition. Then, additional information is required, such as recognition of isotopic pattern or detection or relative isotopic mass defect. Among all naturally occurring stable elements, 20 of them is called monoisotopic elements because they are present as the only one stable isotope. The rest of elements occur in two or more isotopes and they are termed as polyisotopic elements. Each of these elements has different isotopic composition that results in characteristic distribution that can appear in the mass spectrum and is called isotopic pattern. The exact mass of the most abundant isotope of an element is defined as monoisotopic mass. Concerning molecules, the sum of the

monoisotopic masses of the isotopes of each element forming the molecule is called monoisotopic mass of the molecule. However, molecules of the one compound are not only composed of the most abundant isotopes of the elements given in the formula but also other isotopes have their contribution in the composition of the molecules. For this reason, each compound is the mixture of different isotopic compositions. It results in characteristic for the analyte isotopic distribution that appears in the spectrum as a superimposition of the mass spectrum into the mass spectra of all isotopic species of the compound. These isotopic species are called isotopologues. The isotopologues can be observed in spectrum as A+1, A+2…A+n isotopic clusters of the molecule. The mass difference between the monoisotopic mass of the molecule and the mass of its isotopologue is termed as inter-isotopic mass defect. Because of the superimposition of mass spectrum and the presence of inter-isotopic mass defect, analytes have the characteristic isotopic distribution of the elements they contain. It can be considered as an elemental fingerprint that allows identification of different compounds by comparison of the experimental isotopic pattern with the calculated one.[167, 168]

The elucidation of the molecule structure and confirmation of its identity can be provided by tandem mass spectrometry (MS/MS). The dissociation of the precursor ions in LTQ-Orbitrap can be performed in linear ion trap by collision induced dissociation (CID), in the C-trap and also in the octopole collision cell.[164] One of the most important features offered by orbitrap is the possibility to obtained tandem mass spectra with high mass accuracy.[169] Linear ion trap (LIT) mass spectrometer offers the multiple stage MS/MS or MS^n fragmentation of precursor ion but also of its product ions. In the LIT, ions are collected in high pressure cell (HPC) (Fig. 20. A) where applied voltages allow isolation of a single ion (Fig. 20. B). In the next step, the chosen precursor ion is excited by the applied voltages. Then, it collides with neutral molecules (e.g. helium) that causes the bond breakage and results in the fragmentation of precursor ion into the product ions (Fig. 20. C). The product ions are transferred to the low pressure cell (LPC) and then ejected (Fig.20. D). All product ions can be scanned out but there is also possibility to isolate one of the product ions, excite it and then scan out all of the secondary products to obtain the MS^3 scan.[170, 171]

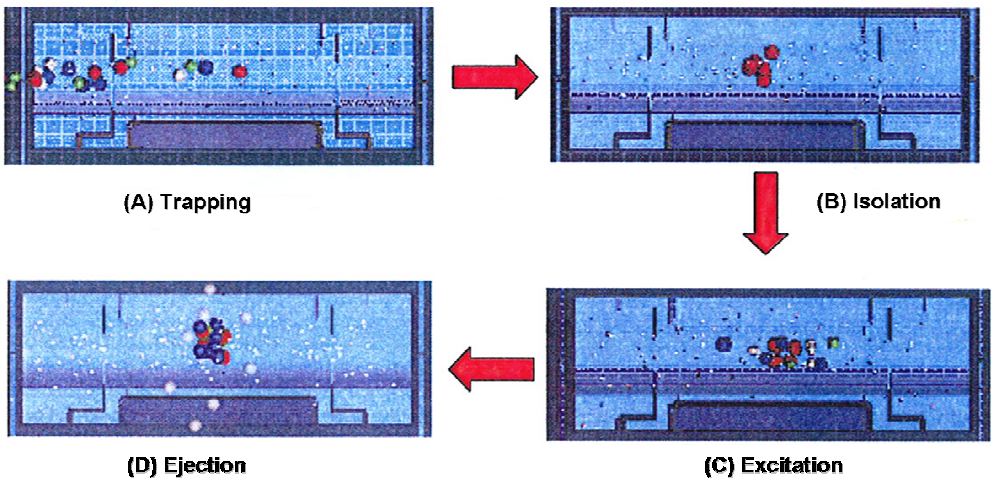

Figure 20. The operation of linear ion trap during MS/MS (or MS^n) mode.[171]

The repetition of isolation (B) and excitation (C) steps on chosen product ions permits to obtain the MS^n spectra. In consequence, LIT yields the structural information of fragmented ion that is regarded as mass spectral fingerprint.[171]

Two models of LTQ Orbitrap – LTQ Orbitrap Discovery and LTQ Orbitrap XL give the alternative to CID dissociation method – high-energy collisional dissociation (HCD). It can be performed in octopole that is integrated with C-trap. In this fragmentation technique, the precursor ions are isolated in the linear ion trap to be in the next step transferred to the C-trap. In the C-trap, precursor ions are accelerated into the octopole collision cell where their kinetic energies increase and they ultimately collide with the neutral gas molecules (e.g. nitrogen). However, the number of collisions that precursor ions undergo with gas molecules is relatively small. Fragmented ions, produced in octopole collision cell, are then returned to the C-trap using potential gradient. Then, they are injected to the orbitrap mass analyzer. The high energy collisions permit to obtain the MS/MS spectra with no low mass cut-off and can be used to elucidate the structure of both small molecules and peptides or proteins.[170, 171] It was reported that sensitivities of both, the CID and HCD spectra, are similar. Additionally, in collision induced dissociation the normalized energy of 35% is efficient to perform fragmentation of majority of small molecules while for HCD the optimal collision energy depends on molecular weight and structural features of the compounds. As both dissociation techniques provide different fragmentation pathways to generate fragmentation fingerprint, they complement each other. It permits to obtain the comprehensive information about fragmented ions. Therefore, the combination of the CID and HCD methods enables confident and accurate small molecule structure elucidation.[170]

The orbitrap technology combines different features of previously available mass analyzers. It caused that orbitrap gained far better sensitivity, resolution and mass accuracy[164], however, as every mass analyzer, orbitrap technology has also its physical limitation that luckily are still far from being reached. Therefore, orbitrap technology continuously attains the novel expectations in different fields of analytical science.[163]

The attributes of the orbitrap mass spectrometer caused that orbitrap mass spectrometry has become a powerful tool for the comprehensive identification of low molecular weight molecules including metal complexes with small metabolites. The high diversity and the wide range of concentrations of small molecules make their analysis very tedious using only the conventional mass spectrometry approaches. The high resolving power offered by Orbitrap allows minimization of the chromatographic separation steps. It is important in case of analysis of the low molecular weight metal complexes as they are usually unstable and can be degraded if the multidimensional separation is applied. Additionally, high accuracy allows significant reduction of the number of possible molecular formulae that facilitates identification of a previously unreported species.[164] The advantages given by orbitrap mass analyzer caused that this technology was used in this study to identify metal complexes with low molecular weight metabolites produced by plants. Presented comprehensive metal speciation in plants shows clearly the abilities of orbitrap technology for plant science applications.

Coupling HPLC to ESI MS

Many advantages of ESI MS has led that this technique is regarded, similarly like ICP MS, as an excellent detector for HPLC and CE techniques. The one of issues that can appear in these kinds of hyphenated techniques is the lack of compatibility of the chromatographic mobile phase flow rate and composition with the ionization conditions required by ESI. The availability of different sources, such as nanospray, micro ionspray or turbo ionspray sources gives the possibility to cope with incompatibility of flow rate because different ion sources can be operated at different flow rates, in range from nL min^{-1} up to mL min^{-1}.[171]

However, the choice of the mobile phase composition that is compatible with ESI still remains a problem. The ESI MS is vulnerable to presence of high concentration of salts (more than 10 mM) and some compounds, such as ion pairing reagents or solubilizing agents that can negatively affect the ionization process. Therefore, the use of nonvolatile salt buffers as mobile phases and different additives to mobile phase should be eliminated. The non-volatile buffers, such as phosphate, borate, sulphate or citrate buffers, may deposit on lens elements while the surfactants (e.g. Triton-X 100) may suppress ionization and coat the ion optics. All those processes can result in significant sensitivity reduction. The inorganic acids and alkali metal bases should be also avoided as they may cause the damages of the source components. The addition of ion-pairing reagents, such as trifluoroacetic acid (TFA) or trichloroacetic acid (TCA), to the mobile phase can lead to ion suppression in negative and positive ionization mode. For this reason, the use of these additives in HPLC ESI MS experiments should be limited. The mobile phases that are compatible with ESI contain volatile salts, such as ammonium formate, ammonium acetate or ammonium carbonate. It is also possible to use some additives in the mobile phase that can enhance the ionization efficiency. Therefore, the addition of proton donors, such as acetic acid or formic acid, can be used for more efficient ionization of basic compounds in positive ion mode. Proton acceptors, such as ammonium hydroxide or ammonia solution, can enhance ionization of acidic compounds in negative ion mode. In Fig. 21 is shown the response given by peptide that was ionized in positive ion mode in presence of different solvent system. The graph is not representative for all compounds but it gives the view how the use of different organic solvents and mixture of organic-aqueous solvents as well as different additives to mobile phase may affect the ionization process.[171]

The mobile phases employed by HILIC that are highly volatile organic solvent, such as acetonitrile or methanol, are compatible with ESI. The separation condition of RP LC are nearly ideal for ESI MS detection, however, the use of the ion-pairing reagents causes a drastic decrease in sensitivity. However, not only the components of mobile phase may suppress the ionization of analytes but also the contaminants that are easy-to-ionize matrix components present at high levels. Their presence in ion source may negatively affect the ionization of compounds of interest, especially metal species that are difficult to ionize and present at trace level. Therefore, the additional purification steps of samples using multidimensional chromatography are usually required. The species purification prevent the analyte and contaminant compounds to arrive to the source at the same time.[140, 141, 171]

It was reported that reserved phase liquid chromatography was used in the purification step, prior to ESI MS/MS analysis, to separate the bulk of phytochelatins from matrix salts that could cause the suppression of ionization.[151] Apart from using multidimensional chromatography to purify samples, it is also recommended to use high-purity additives and solvents. Solvents should not be degassed, filtered or transferred just stored in original containers and replaced weekly to maintain low background level. Both solvents and additives should not have any contact with plastics and detergents that may cause ion suppression or increase of background noise.[171]

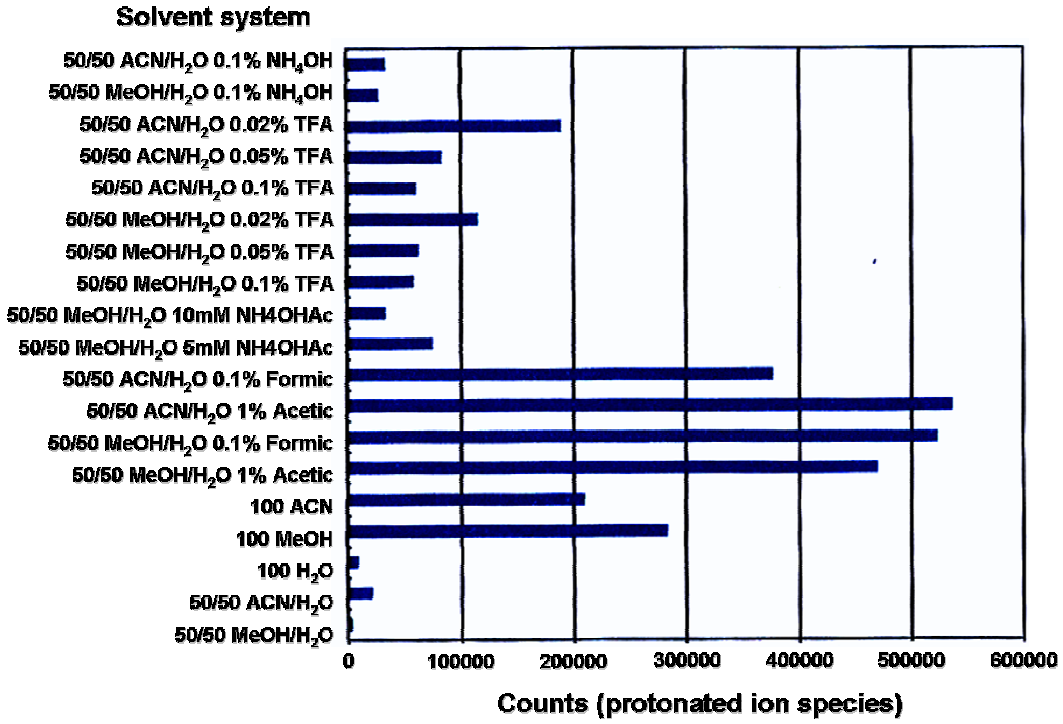

Figure 21. The electrospray response of peptide Tyr-Gly-Gly-Phe-Leu depending on the solvent composition.[171]

Coupling of HPLC to ESI HR MS is very powerful combination and for this reason it has found application in different fields of analytical chemistry. There are several reports about combining Orbitrap MS with HPLC. The most frequently employed LC technique in these kind of couplings is RP HPLC that found application in proteomics and analysis of small molecules. Orbitrap is also ideal for coupling to U-HPLC. It was reported that Orbitrap MS is able to collect a great number of data points across the chromatographic peaks in case of very narrow peaks (peak width of 5 – 10 s) characteristic for U-HPLC and still maintain good sensitivity and mass accuracy. The combination of U-HPLC with LTQ Orbitrap MS was used for metabolic profiling of serum samples and comprehensive residue analysis in food samples. Apart from RP LC and U-HPLC, also HILIC has been coupled to LTQ Orbitrap MS to identify different Se-species. Use of this hyphenated technique allowed identification of nine Se-compounds that was not possible to obtain using HILIC coupled to TOF MS where just two of nine species were identified. The study shows a superiority of LTQ Orbitrap MS over TOF MS concerning intra-scan dynamic range, high mass accuracy especially in MS^n

mode and large fragmentation window. HILIC LTQ Orbitrap MS couplings were employed also in peptide analysis, bioanalysis as well as in metabolomics applications.[169]

However, there are no reports regarding use of HPLC combined with LTQ Orbitrap MS for analysis of metal complexes with different metabolites produced by plants. Other detection techniques, such as TOF MS or FT ICR MS were only used for metal speciation analysis in plants samples (Table 2).

The on-line coupling of ZIC HILIC to ESI MS in the negative ionization mode allowed detection and identification of DMA complexes with Fe(III), Zn and Cu in wheat roots as well as Ni complexes with nicotianamine in *A. thaliana*.[172] Information of nickel speciation in the latex of Ni-hyperaccumulating tree *Sebertia acuminata* was achieved by the use of SEC with parallel ICP MS and ESI MS/MS detection.[144] The nickel speciation was also studied in *Thlaspi caerulescens* using 2D LC for species isolation and Q-TOF MS/MS for their identification. The use of hydrophilic interaction liquid chromatography in combination with size-exclusion chromatography allowed purification of noncovalent nickel species from plant aqueous extracts and their further identification by electrospray TOF MS. This approach allowed identification of five nickel complexes with nicotianamine, citrate, malate, histidine and EDTA.[145] The RP HPLC-tandem mass spectroscopy found also application for metal species separation and identification in plant samples. The reserved phase chromatography allowed separation of different Cd-PC and Pb-PC complexes present in Cd-hyperaccumulator *Sedum alfredii*, while the ESI MS/MS operated in positive ion mode gave possibility of their characterization.[173]

The other cases, when ESI MS were used for identification of different metal complexes in plants samples, are shown in Table 2. The garnered data show that, to date, the available information about metal species present in plant is very limited. The main interest was given to phytochelatins complexes with different metals and also to nickel speciation mainly in hyperaccumulating plants. The knowledge about speciation of less studied elements in plants including iron, zinc and copper is highly incomplete while the forms in which other metal such as cobalt, manganese or aluminum are present in plants still remain unknown. Therefore, there is a need to investigate the metal speciation in plants. The extended knowledge about the processes that metals undergo in plants and thus better understanding of plant physiology will provide the great amount of data that can be used in environmental, nutritional or toxicity studies. For these reasons, the novel analytical approach was developed in this study to obtain information about the different metal species present in plants. The HPLC combined with ICP MS and LTQ Orbitrap MS was successfully used for comprehensive speciation of low molecular weight metal complexes present in various plant species.

Table 2. The couplings of HPLC to ES MS that have found application in metal speciation analysis in plants samples.

Separation technique	Detection technique	Plant species	Metal	Metal species	Reference
RP HPLC	ESI–MS	*Silene cucubalus* *Agrostis tenuis* *Rauvolfia serpentina*	Cd	Cd-PCs	151
SE HPLC	ESI–MS	*Sebertia acuminata*	Ni	Ni-NA, Ni-$(citrate)_2$	144
SE HPLC CZE	ESI–MS/MS	*Thlaspi caerulescens*	Ni	Ni-NA	126
2D-LC : SEC-RPLC	ESI–MS	*Arabidopsis thaliana*	Cd	Cd-PCs	150
ZIC–HILIC	ESI–MS	*Arabidopsis thaliana*	Ni	Ni-NA	172
ZIC–HILIC	ESI–MS	Wheat (cv.,Bezostaya)	Fe, Zn, Cu	Fe(III)-DMA Zn-DMA Cu-DMA	172
SEC–RPC	ESI–TOF–MS	*Hordeum vulgare* L.	Cd	Cd-PCs	174
RP HPLC	ESI–MS	*Brassica napus*	Hg	Hg-PCs	175

Separation technique	Detection technique	Plant species	Metal	Metal species	Reference
2D LC: SEC–HILIC	Q–TOF–MS/MS	*Thlaspi caerulescens*	Ni	Ni-EDTA, Ni-(malate)$_2$ Ni-(citrate)$_2$ Ni$_2$-(citrate)$_2$ Ni-NA Ni-histidine Ni-(histidine)$_2$	145
SE HPLC	ESI–MS/MS	*Brassica chinensis*	Cd	Cd-PCs	176
RP HPLC	ESI–MS/MS	*Sedum alfredii*	Cd, Pb	Cd-PCs Pb-PCs	173
SE HPLC	ESI – LTQ FT ICR MS	*Sebertia acuminata*	Ni	Ni-(citrate)$_2$ Ni-(malate)$_3$ Ni-(erythronic acid)$_3$ Ni-(galacturonic acid)$_3$ Ni-tartaric acid Ni-(aconitic acid)$_3$ Ni-(saccharic acid)$_2$ Ni-(malonic acid)$_4$	177

Separation technique	Detection technique	Plant species	Metal	Metal species	Reference
ZIC–HILIC ZIC–cHILIC	ESI–MS	*Arabidopsis thaliana*	Ni	Ni-NA Ni-$(Asp)_2$ Ni-$(malate)_2$ Ni-citrate Ni-$(citrate)_2$ Ni-$(histidine)_2$	149
SE HPLC RP HPLC	ESI–MS^n	*Pisum Sativum*	Cd	Cd-PCs	178
ZIC–pHILIC	ESI–TOF–MS	*Solanum lycopersicum*	Fe	$Fe_3(citrate)_3$	179
UPLC	ESI–Q–TOF–MS	*Hordeum vulgare* L.	Fe	Fe(III)-DMA Fe(III)-Epi-HMA Fe(III)-MA Fe(III)-AVA Fe(III)-HAVA	180
SE HPLC	ESI–TOF–MS	*Arabidopsis halleri*	Zn	NA GSH	56
SE HPLC CE HPLC	ESI–TOF–MS	*Oryza sativa* L.	Zn, Fe	Zn-NA Fe(III)-DMA	53

6.4. Other techniques for metal speciation

Apart from HPLC as separation technique and ICP MS and ESI HR MS as detection techniques, also other approaches such as X-ray absorption spectroscopy (XAS) or laser ablation (LA) have been used to obtain information about metals present in various samples.

There are three regions in the spectrum generated by XAS data (Fig. 22). The one region referred to as X-ray absorption near-edge structure (XANES) allows the determination of the oxidation state of the central atom (e.g. metal) and provides the information on the first coordination layer. The extended X-ray absorption fine structure region (EXAFS) provides local structural information including the coordination number, radial distances and types of ligands complexing metal. Apart from the information listed above that can be attained using XAS, this technique can be also used in lateral-resolved analysis that permits to obtain the distribution pattern of metal species. XAS allows also analysis of solid and liquid samples that can be prepared for the analysis as a powder, a solution or as frozen solution. The latter preparation method is usually applied to biological samples. Information that can be obtained using XAS caused that this technique is used to study element speciation in hyperaccumulating plants. However, the meaningful information can be obtained only if the metal is high concentrated and bound by a single ligand. The low concentration of metal, its complex speciation and potential unknown species reduce the possibility to provide significant results.[139, 181, 182]

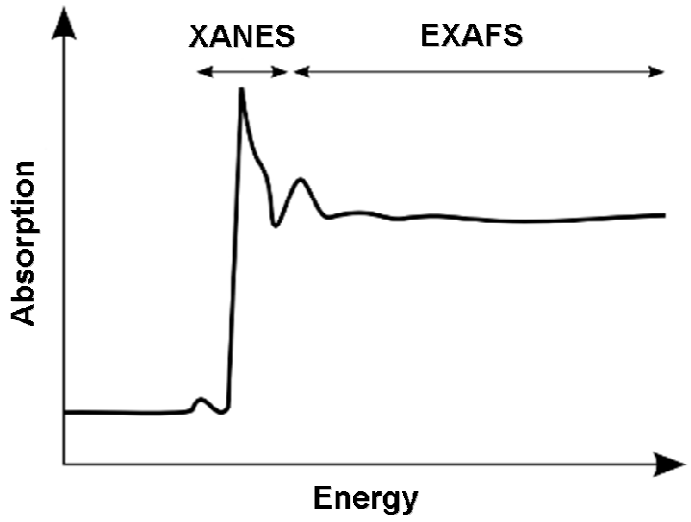

Figure 22. Regions in the spectrum generated by XAS data.[193]

Another technique that found application in the analysis of environmental samples is laser ablation coupled to ICP MS. ICP MS offers high sensitivity and thus allows acquisition of multielement concentration data, in addition, in samples that amount is very small. For this reason, ICP MS is perfect detector for laser ablation sampling technique that permits to obtain multielement images of e.g. brain or leaf tissues with micrometer resolution.[139] LA-ICP MS together with gel electrophoretic separation techniques was used to study the distribution of humic acid – metal (Zn, Cu and Mn) complexes in environmental samples[183] as well as the

distribution of metals, such as Mg, Mn, Fe, Cu and Zn in leaves, shoots and roots of tobacco. The same hyphenated technique found application in studies on metal and non-metal accumulation and distribution in Cu-tolerant plant *Elsholtzia splendens* and permitted to obtain the distribution profiles of elements, such as Cu, K, Mn, P and B that were quantified using calibration curves.[184]

However, the techniques shortly described in this chapter are not as powerful as couplings of HPLC to either ICP MS or ESI HR MS in studies on metal speciation in plant samples but they can be highly useful in elucidation of complex structure (XAS) and determination of metal distribution (XAS and LA-ICP MS).in different intact plant tissues and organelles

6.5. Summary

The hyphenated techniques are a convenient tool to investigate metal speciation in different environmental samples. The proper sample preparation including optimization of the extraction step and selection of suitable separation and detection techniques allow the obtaining the quantitative and qualitative information about metal species present in plant sample. Additionally, the development of new techniques (e.g. LTQ Orbitrap MS) that offer high sensitivity, resolving power and mass accuracy gives the more efficient tools and approaches to study metal speciation.

Results and discussion

7. Research projects

Three different projects were carried out during this PhD research. All of them were focused on identification of low molecular weight metal/metalloid complexes present in plants saps and organs. To obtain information about metal speciation in plant samples novel analytical approaches based on combination of HPLC or GC with the parallel element and molecule specific detention were used.

7.1. *Pisum Sativum*

The first project aimed at the identification of metal complexes present in *Pisum Sativum* (green pea) saps and thus determination of ligands that are responsible for metal transport in xylem and into the seeds in this plant. This project was carried out in collaboration with Stéphane Mari from UMR Biochimie et Physiologie Moléculaire des Plantes AGRO-M/INRA in Montpellier (France).

The plant chosen to study processes of metal transport in xylem and into the embryo – *Pisum sativum* belongs to the *Leguminosae* family and dicotyledonous plants. Green pea is regarded as a model organism since the work of Gregor Mendel concerning the laws of inheritance. It is perfectly suited to study the metal transport into the embryo as the seeds produced by this plant are large and thus contain a huge amount of endosperm (embryo sac liquid) that is the main source of nutrients for a developing embryo. Therefore, the embryo sac liquid can be collected in relatively large amount and then analyzed to obtain information about its composition.[194, 195]

This research project was focused on multielement speciation analysis including iron, zinc, copper, nickel, manganese, cobalt, magnesium and calcium. The interest was given to these metals as they are micro-(Fe, Zn, Cu, Ni, Mn and Co) and macro- (Ca and Mg) elements that play essential role in plant physiology. The deficiency or excess of these metals may have negative effects on plant physiological processes and induce, in consequence, inhibition of plant growth or even plant death. Therefore, it is important to determine the low molecular weight metabolites that are produced by plants and involved in metal uptake, transport, accumulation, storage and detoxification. The better understanding of plant physiology can provide a lot of useful information for food and nutritional studies and also agricultural activities because legumes are the second most important crop plant family. Additionally, green pea is also second most widely grown plant that belongs to grain legumes.[194]

7.2. *Plantago Almogravensis*

The second project was carried out in collaboration with Tomás Grevenstuk from University of Algarve, Faculty of Sciences and Technology, IBB/CGB in Faro (Portugal) and was focused on determination of ligands that are involved in aluminum detoxification processes in *Plantago almogravensis* Franco.

Plantago almogravensis belongs to the *Plantaginaceae* family that is endemic to the southwest coast of Portugal. This plant is on the IUCN Red List of Threatened Species assessed as a critically endangered because the total population of *Plantago almogravensis* is between 3 000 and 4 000 individuals and is still decreasing.[196] *Plantago almogravensis* has ability to thrive on acidic soils that are rich in iron and aluminum and additionally, it was reported that is able to hyperaccumulate aluminum in its shoots.[197]

The plant species, such as *Plantago almogravensis* that are resistant to high concentrations of aluminum in soil, are ideal models to study mechanism of aluminum tolerance and detoxification. Understanding the aluminum pathways in plants is interesting because aluminum is considered as non-essential element. Additionally, in acidic soils, aluminum is available for plants as a trivalent cation which is the most toxic form Al. Aluminum toxicity symptoms include mainly the inhibition of plant growth. For this reason, this metal is one of the main factors that limit the crop efficiency in acidic soils that pose approximately 40% of the earth's arable land. Therefore, the knowledge about processes that aluminum undergoes in Al-tolerant plant species is likely to be useful to enhance the tolerance of commercial crops for this element.

7.3. *Brassica nigra*

The third project aimed at the identification and characterization of low molecular weight selenium compounds present in *Brassica nigra* seeds. The project was carried out in collaboration with Federica Aureli from Istituto Superiore di Sanità, Department of Food Safety and Veterinary Public Health in Rome (Italy).

Brassica nigra, also called black mustard, belongs to the *Brassicaceae* family. Black mustard is an annual herbaceous that is cultivated in different parts of the world and it poses the primary source of the mustard seeds. The seeds are used as a medicine and for production of condiment sauce and table mustard.[198] The oil-free protein fraction of the seed is used also in the animal feed concentrates.

The plants from family *Brassicaceae* are known as a primary and secondary selenium accumulators. *Brassica nigra*, if cultivated on soils rich in selenium, has also ability to accumulate selenium in relatively large amounts (several hundred g Se g^{-1} DW) in different organs. However, the seeds of this Se-accumulating plant are the best source of this element.[2]

Selenium is regarded as an essential micronutrient for human and animals mainly because of its antioxidant and catalyst properties as well as the role in successful functioning of the immune system. However, the beneficial effect of selenium on human and animal health is strongly dependent on its concentration and speciation. The nature of the Se-compounds can have various implications for health and nutrition of animals and humans. Hence recently, there is a big interest in studying selenium speciation especially in plants as they are the main source of selenium in diet.[199] As the seeds of *Brassica nigra* can provide large amounts of selenium in human and animal diet, it is important to obtain information about the chemical nature of Se species present in black mustard seeds. This information can be use later in food, nutrient and agronomy studies, especially in the field concerning human and animal health.

8. Techniques of choice

The novel systematic analytical approach that was developed as a part of this doctoral project to study metal/metalloid speciation in plant material is based on chromatographic separation using size exclusion chromatography (SEC) and hydrophilic interaction chromatography (HILIC) with parallel element specific detection by inductively coupled plasma mass spectrometry (ICP MS) and molecule specific detection by electrospray LTQ Orbitrap mass spectrometry (ESI LTQ Orbitrap MS). The analytical techniques that are presented in this work were chosen because they offer a lot of advantages that will be explained in following chapters, which allows a new insight into trace element speciation in complex biological samples.

8.1. Chromatographic techniques

The samples that were analyzed during research projects include plant saps – xylem and embryo sac liquid and also leaves, roots and seeds extracts. Gas chromatography was usually not suitable for this kind of application as metal complexes present in plant samples are usually not volatile and their conversion in volatile species is also not possible.

Liquid chromatography seemed to be perfectly suited for analysis of complex liquid plant samples because it allows the separation of different metal species and sample purification. The latter is important, especially in HPLC-ESI HR MS couplings, where the presence of non-analyte molecules, eluted at the same time as analyte, may disturb the ionization process of molecules of interest.

The chromatographic techniques were chosen taking into account the nature of the species, their possible high diversity and the fact that low molecular mass metal complexes may be usually unstable. The selected stationary phase had to have low metal binding properties. The chromatographic conditions (buffer, gradient and time of analysis) were optimized to ensure good separation of different metal species and the stability of separating compounds. One of the critical parameters of the mobile phase is pH that was adjusted to the pH value of analyzed samples to avoid degradation of metal species. To avoid any changes in form of analyzed compounds, due to their instability, monodimensional chromatography was used and simple sample preparation methods were applied. The samples were analyzed undiluted and the off-line preconcentration steps were avoided. If extraction step was needed (the case of solid samples such as leaves, roots or seeds), simple one-step extraction procedures using mainly water as extractant were used.

Size exclusion chromatography

Size exclusion chromatography was chosen for analysis of plant samples because of its high tolerance to biological matrices that allows injection of complex samples without prior extensive sample preparation steps. Therefore, SEC appeared to be perfectly suited for analysis of undiluted plant saps and extracts.

To detect metal complexes, especially the low concentrated ones, it was necessary to connect SEC column to sensitive detector. For this reason, SEC was on-line coupled to the ICP MS instrument. It was possible as the flow rates in range between 0.7 – 1.0 mL min^{-1} and used mobile phases, employed by this chromatographic technique, are compatible with ICP MS. This coupling did not allow the identification of metal complexes as retention standards are unavailable for many compounds and also there is still plenty of unknown species. Therefore, SEC column was coupled also to ESI LTQ Orbitrap mass spectrometer.

SEC separations were carried out with either an Dionex UltiMate 3000 system (Thermo Fisher Scientific, Bremen, Germany) or a model 1100 HPLC pump (Agilent, Wilmington, DE) used as a delivery systems. The SEC column, used in all performed SEC analysis, was Superdex Peptide HR 10/30 (300 x 10mm i.d.) purchased from GE Healthcare Bio-Science AB (Sweden). This kind of column was chosen in this study because it is dedicated for high performance gel filtration of peptides and other small biomolecules and it offers the separation range between 100 – 7 000 Da. Therefore, Superdex Peptide was suitable to obtain a good separation of low molecular weight metal species in plant tissues. The samples, undiluted plant saps and extracts (10 μL), were analyzed using as a mobile phase – 5mM ammonium acetate buffer at pH either 5.5 or 6.2 depending on the sample pH. The separation was obtained using an isocratic mobile phase flow of 0.7 ml min^{-1}.

The used flow rate and low concentrated mobile phase allowed the connection of the outlet of SEC column to the ICP MS instrument. The used interface between the pump and ICP MS instrument is described in chapter 8.2. The coupling of SEC to ESI MS instrument was more complex than in case of ICP MS because of the incompatibility of the mobile phase flow rate with the ionization conditions required by electrospray ionization source. The reduction of the mobile phase flow rate prior to the ionization source was, therefore, necessary. It was obtained by the use of the post column flow splitter. The splitting setting allowed reduction of flow rate to approximately 100 μL min^{-1} and also introduction of 30% formic acid to the eluent to perform the post-column acidification experiments. These experiments aimed at confirmation that demetallated ligands were eluted at the identical retention time as their metal complexes.

Hydrophilic interaction liquid chromatography

Hydrophilic interaction chromatography is an ideal chromatographic technique to effectively separate small polar highly hydrophilic and amphiphilic compounds. Additionally, this technique has been reported elsewhere to separate metal complexes with low molecular weight metabolites, such as organic and amino acids, produced by plants. For these reasons, HILIC was employed in this study to separate metal complexes with different polar compounds including organic acids, amino acids, glucosinolates and sugars.

The microbore HILIC separations were performed using a model Agilent 1100 capillary pump (Agilent, Tokyo, Japan) as a delivery system equipped with a 100 μL.min^{-1} splitter module. The HILIC column, used in all performed HILIC analysis, was a TSK gel amide 80 (250 x 1 mm id) (Tosoh Bioscience, Germany). The employed mobile phase was (A) 10 mmol.L^{-1} ammonium formate buffer at pH 5.5 and (B) acetonitrile. The ammonium

formate buffer was chosen because it is the one of the recommended buffers for HILIC as it is highly soluble even in concentrated organic solvents. The HILIC gradients were optimized to obtain the best separation of diverse metal/ metalloid compounds present in plant tissues. In the case of the analysis of *Pisum Sativum* and *Plantago Almogravensis* samples, the optimized HILIC gradient started with 10% of A and was increasing to 50% of A during 45 min. The separations of selenium compounds present in seeds of *Brassica nigra* were performed using the gradient that started with 2% of A and was increasing to 40% of A during 50 min. The sample preparation was reduced to simple dilution with acetonitrile to obtain a 1 to 2 ratio (aqueous sample: acetonitrile) and aimed at precipitation of salts present in samples. After dilution samples were centrifuged and either 5 µL or 7 µL of the supernatant was analyzed at the flow rate of 50 $\mu L.min^{-1}$.

The outlet of HILIC column was coupled to either ICP MS or ESI MS/MS instrument. The coupling of HILIC to ICP MS required the use of special interface (chapter 8.2) that allowed introduction of mobile phase rich in organic solvent into the plasma torch. Concerning coupling HILIC to ESI Orbitrap MS, HILIC appears to be ideally suited to sensitive LC-MS analysis of water soluble polar compounds as the high organic content in the mobile phase leads to immediate evaporation of solvent during electrospray ionization. Therefore, composition of used mobile phase – up to 90% or 98% of acetonitrile and low concentrated (10 mM) volatile buffer and additionally flow rate were compatible with the ionization conditions required by electrospray ionization source. The HILIC column was connected with ESI MS instrument just via PEEK tubing.

8.2. Detection techniques

The metal concentrations in plant samples are usually very low. In addition, the metals of the interest can be distributed among several species. Another issue is high complexity of plant samples. Therefore, the element or molecule specific detection techniques have to be sensitive enough to cope with these issues. The detection techniques that fulfill this requirement are ICP MS and ESI Orbitrap MS that were chosen in this study for element and molecule specific detection, respectively.

Inductively coupled plasma mass spectrometry

Mass spectrometry with ionization in inductively coupled plasma (ICP MS) was chosen in this study because it offers low detection limits, quantification on parts per million level, multi-element and multi-isotopic measurement capacity and large dynamic range. All these advantages of ICP MS were necessary in trace metal speciation analysis presented in this work and allowed detection and quantification of different metallated species

ICP MS detection was achieved using a model 7500cs instrument (Agilent) equipped with a collision cell with hydrogen as collision gas. The use of the collision cell was necessary to remove the polyatomic spectral interferences that could disrupt the detection of metals of interest.

As it was mentioned in the previous chapter, the SEC and HILIC columns were connected to the ICP MS instrument. In case of the coupling SEC to ICP MS, a standard interface between pump and ICP mass spectrometer was used. It consisted of a quartz double pass Scott style spray chamber, a MicroMist EazyFit® nebulizer (Glass Expansion, Australia), a 2.5 mm i.d. quartz torch and a set of nickel cones (Agilent Technologies, USA).

The composition of the mobile phase and flow rate employed by HILIC were not compatible with the conditions required by ICP MS. Therefore, in order to connect a HILIC column to the ICP MS instrument, it was necessary to use the interface allowing the introduction of mobile phase containing high concentration of organic solvent. For this reason, the interface between HILIC column and the Agilent 7500cs consisted of a glass Cinnabar cyclonic spray chamber (Glass Expansion, Australia), a 50 $\mu L.min^{-1}$ Micromist U-series nebulizer (Glass Expansion, Australia), a 1 mm i.d injector torch (Agilent Technologies, Japan), a T-connector allowing the introduction of oxygen and a set of platinum cones (Agilent Technologies). The Cinnabar cyclonic spray chamber was used because of the smaller dead volume compared to the double pass Scott style spray chamber. The smaller dead volume of the spray chamber was required for HILIC analysis as the flow rate employed by this chromatographic technique (50 $\mu L\ min^{-1}$) was much smaller than the one employed by SEC (700 $\mu L\ min^{-1}$). In consequence, the use of this model of spray chamber permitted to avoid long washout times and peak broadening. The addition of oxygen to plasma gas and the use of platinum cones were necessary. Otherwise, the high content of organic solvent in the mobile phase could cause carbon deposition on the torch and cones, and thus affect negatively the stability of ICP MS and decrease signal intensity.

The use of the ICP MS instrument required also the optimization of conditions. The procedure was always done daily for highest intensities and lowest interferences with 1 ppb solution of Li, Y and Tl in 2% nitric acid using a standard built-in software procedure.

Electrospray LTQ Orbitrap mass spectrometry

ICP MS allowed detection of different metal species in plant material. However, it did not provide any information about the structure of metal complexes present in plant tissues. The lack of available standards that would allow the identification of metal complexes according to the retention times matching of peaks on chromatograms obtained for sample and standard as well as the presence of unknown species caused that the use of molecular-specific detection techniques were required. To identify and characterize the metal/metalloid compounds present in plants, the electrospray LTQ Orbitrap mass analyzer was employed. This instrument was used in this work because of its high sensitivity, resolving power and mass accuracy. These advantages of Orbitrap mass spectrometer allowed determination of exact masses of molecules and their structures.

The MS/MS instrument, use in this work, was an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with heated electrospray ionization source (H-ESI II) (Thermo Fisher Scientific). The instrument was operated either in the positive ion mode with the electrospray voltage set at 3 kV or in negative mode at –3 kV. The analysis were performed in both ionization modes to ensure that

observed metal/metalloid species were not just adducts formed in electrospray source. The vaporizer and ion transfer tube temperatures as well as sheath and auxiliary gas flow rates were set up according to the parameters recommended by manufacturer for different mobile phase flow rates. As the all HILIC experiments were performed at the mobile phase flow rate of 50 μL min^{-1}, the source and capillary temperature were usually 120 °C and 280 °C, respectively. For this flow rate of mobile phase the sheath and auxiliary gas flow rates were set at 15 arb and 5 arb, respectively. The ESI MS spectra were obtained in different mass ranges but as the aim of the work was identification of low molecular mass metal/metalloid complexes the chosen mass ranges were always between 100 and 1200 u with the resolution set at 100 000 (full width half height, FWHM, at m/z = 400).

Obtained MS data were processed using *Xcalibur 2.1* software and *MetWorks* software (Thermo Fischer Scientific). The high resolution mass spectra were searched for metal specific isotopic pattern, for mass defects and for mass differences with chemical analogues or demetallated ligands to identify metal/metalloid containing molecules. Once all the potential metal/metalloid species were found, the second chromatographic analysis of the same sample was performed. The species of interest were fragmented by either collision – induced dissociation (CID) or high energy collision fragmentation (HCD) at various energy levels. In the MS/MS mode, the resolution of the instrument was set at 30 000.

9. Principles of identification of metal complexes

ESI LTQ Orbitrap mass spectrometer was used to obtain information about metal/metalloid species present in tissues of three different plant species. The unambiguous identification of metal/metalloid compounds required comprehensive approach that was based on searching the high resolution mass spectra for metal/metalloid specific isotopic pattern, mass defects, inter-isotopic mass defect/ratio and mass differences with chemical analogues or demetallated ligands. In order to discard false positive identifications additional analysis, different separation mechanisms and different ionization modes were investigated. This approach allowed identification and characterization of diverse metal/metalloid species, even the low concentrated ones.

First of all, the coupling HPLC-ICP MS permitted to obtain the information on the retention times, the number and the amounts of potential metal/metalloid species present in analyzed samples. It facilitated searching for these compounds in complex mass spectra. Knowing the retention times of possible heavy element containing species, the high resolution mass spectra obtained using Orbitrap mass spectrometer were searched for these compounds at retention times corresponding to retention times of peaks observed on HPLC ICP MS chromatograms. As the elements of interest, including selenium, iron, copper, zinc and nickel, have at least two stable isotopes, the characteristic isotopic pattern could be observed in the spectrum. Therefore, at the beginning mass spectra were searched for complete metal/metalloid specific isotopic profile using the isotopic pattern screening tool of *MetWorks* software. In order to find the low abundant species the spectra were searched for partial isotopic pattern and the searching parameters of *MetWorks*, such as tolerance on inter-isotopic mass defect/ratio were increased. The ideal isotopic signatures of elements of interest obtained with *Xcalibur* software are shown in Fig. 1.

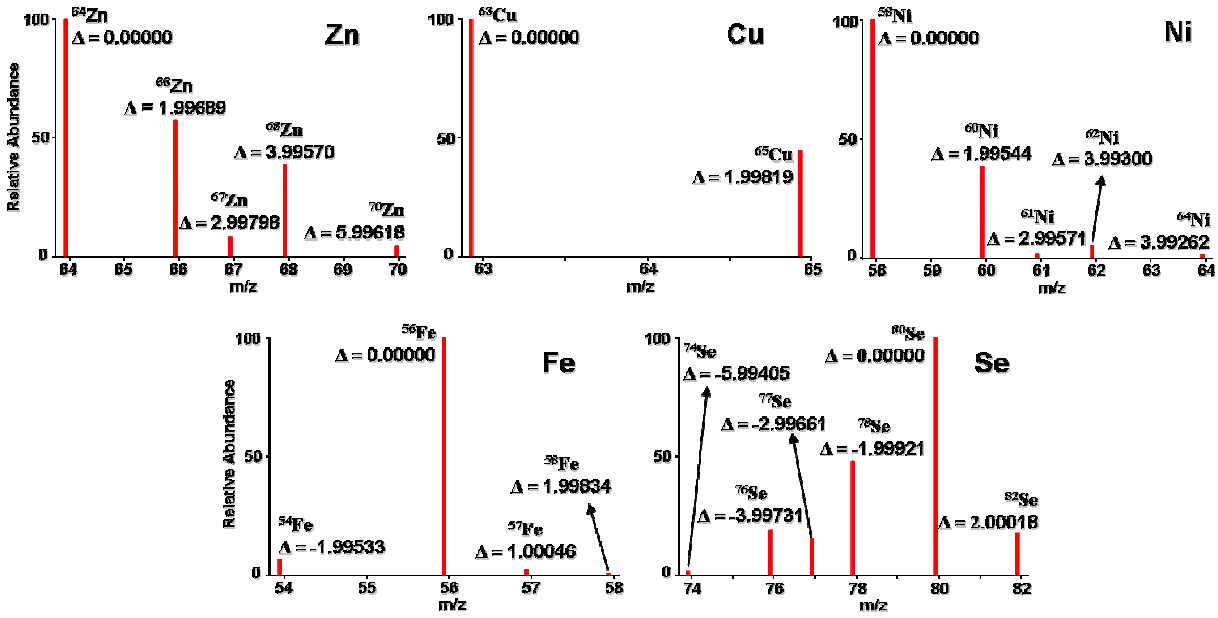

Figure 1. The ideal isotopic signatures of elements of interest: zinc, copper, nickel, iron and selenium.

However, not only multi-isotopic but also mono-isotopic elements, such as aluminum, cobalt or manganese were investigated in this study. In order to find species containing a mono-isotopic element, it was useful to find correlation with multi-isotopic element. The HILIC ICP MS chromatograms were essential to reveal the co-elution of different metal species, such as multi-isotopic zinc was co-eluted with mono-isotopic cobalt. Then, the mass spectra were searched for mass difference between the co-eluted complexes consisted of the same ligand and different elements (chemical analogues). For example, if the metals were complexed by the same molecule the mass difference between these molecules is 4.9959 Da, 6.0038 Da, 8.9911 Da and 28.9534 Da if they contain "$^{64}Zn^{2+}$ and $^{59}Co^{2+}$", "$^{64}Zn^{2+}$ and $^{59}Co^{3+}$", "$^{64}Zn^{2+}$ and $^{55}Mn^{2+}$" and "$^{56}Fe^{3+}$ and $^{27}Al^{3+}$", respectively. An example of using mass difference between two similar species containing just different elements, in order to identify the complexes with mono-isotopic elements, is shown in Fig. 2.

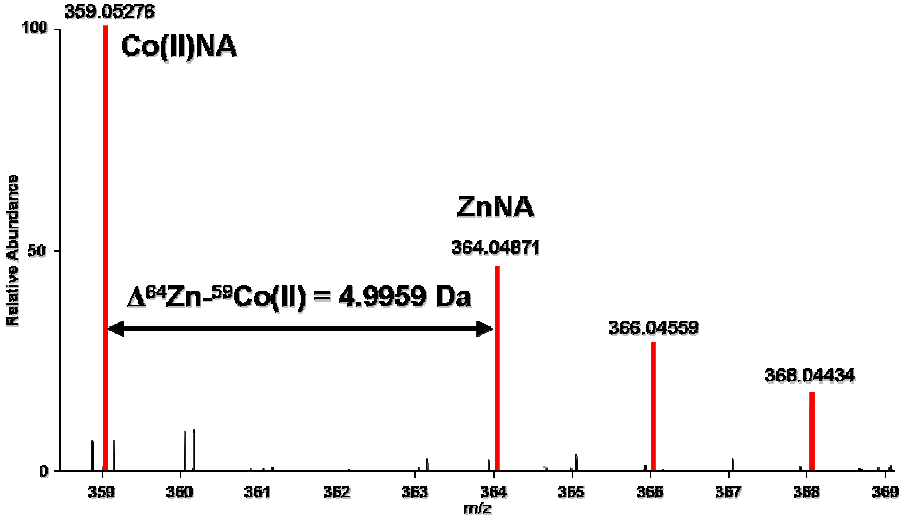

Figure 2. Principles of searching mass spectra for mono-isotopic containing species.

Once the ions containing metal/metalloid were found, the *Xcalibur* software allowed the determination of several possible formulae using ions exact masses. From all formulae generated by the software the one corresponding to metal/metalloid complexes with possible metabolites were chosen. These metabolites involved the compounds that have been already reported to be produced by plants or belonged to the same family of compounds. The mass error was also taken into account and it could not be higher than 5 ppm. In this way the amount of possible formulae was limited to one or two.

In order to confirm the identity of the species several additional steps were performed. Concerning the metal complexes, it was possible to obtain information that is more specific to heavy elements and is based on searching the mass spectra for mass difference with demetallated ligands. For this reason, the mass spectra were searched for the mass difference between the ion containing the metal of the interest and its corresponding free ligand. In this case, the mass difference should be the same as mass of the metal ion that is the part of the complex diminished by the mass of the substituted protons. Therefore, in order to confirm the identity of some metal complexes the mass spectra were checked for mass differences of 61.9135 Da, 55.9197 Da, 52.9115 Da, 60.9139 Da, 52.9224 Da and 56.9176 Da for ^{64}Zn,

^{60}Ni, ^{56}Fe(III), ^{65}Cu, ^{55}Mn and ^{59}Co(II), respectively. The example of using mass difference between the metal complex and its demetallated ligand in order to confirm that a determined formula is correct, is shown in Fig. 3.

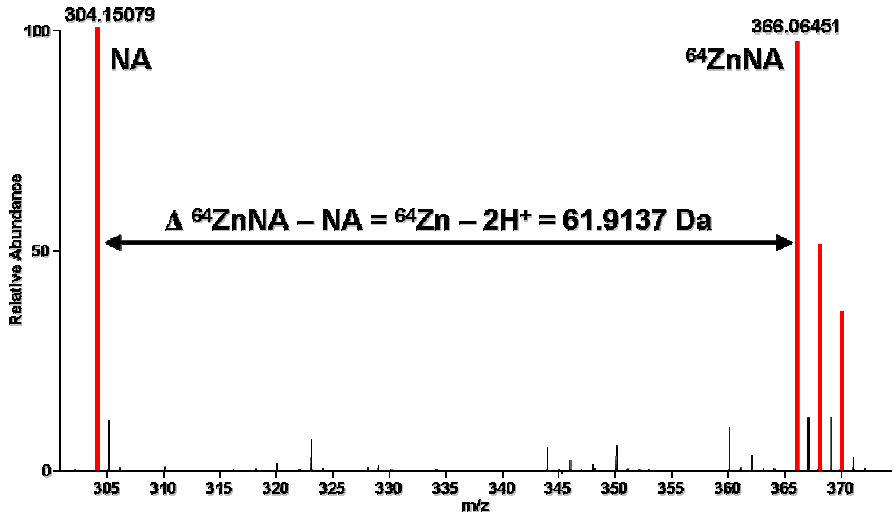

Figure 3. The use of mass difference between the metal complex and its demetallated ligand in order to confirm the identity of metal species.

In order to discard false positive results the analyses were performed in positive and negative ionization mode and using two different kinds of chromatographic separation – SEC and HILIC. First of all, the use of both ionization modes (only for HILIC) permitted to confirm the presence of the most intense and representative complexes in mass spectra and to prove that the observed species were not adducts formed in electrospray source but only stable species produced by plants. Then, performing analysis using HILIC and SEC allowed the observation of some species in both separation modes. It was a confirmation that observed metal/metalloid compounds were not formed or destroyed during chromatographic separation as an effect of interaction with stationary phase or accidental co-elution of ligand and slats containing metal. Finally, several performed analyses of the same sample permitted to validate the presence of different species, especially low concentrated. It confirmed that any misinterpretation of mass spectra because of possible spectral artifacts or ligand exchange between partially co-eluting species containing different metals did not occur.

Once the all potential formulae for all metal/metalloid species were determined the next chromatographic analysis of the same sample was performed and the species of interest were fragmented. In order to confirm that observed species contained a metal in the structure, the isolation width was set up to preserve the isotopic signature of the metal complex of interest. The analysis in MS/MS mode allowed the characterization of metal/metalloid compound structure.

In case of compounds whose identity was uncertain, the identification was confirmed by comparing mass spectra obtained for the sample with that obtained for standard.

All these steps combined together offer a systematic approach that in this study allowed unambiguous identification and structure determination of metal/metalloid compounds present in plant tissues.

Article I

Exhaustive characterization of metal complexes in xylem and in liquid endosperm of pea (to be submitted)

Exhaustive characterization of metal complexes in xylem and in liquid endosperm of pea

Paulina Fils, Laurent Ouerdane*, Louis Grillet, Catherine Curie, Stéphane Mari and Ryszard Lobinski

Manuscript in preparation (to be submitted)

(abstract)

A direct and exhaustive description of metal species in plant fluids such as xylem, liquid endosperm or phloem remains a complex task usually achieved by liquid chromatography mass spectrometry couplings, x-ray analysis or computational simulation according to supposed values for metal concentrations, ligand concentrations and equilibrium constants. However, up to now, none of them could achieve a complete and undisputable overview of metal species in plant fluids, especially in the least concentrated such as xylem, for example. The association of hydrophilic interaction chromatography (HILIC) with inductively coupled plasma mass spectrometry (ICP MS), high resolution electrospray mass spectrometry (HR ESI MS) and systematic computed metal species searching in ESI MS mass spectra allowed the detection and quantification of more than 50 metal complexes in xylem and liquid endosperm of pea, *Pisum sativum*. Metal ligands such as nicotianamine, citrate, malate, histidine, glutamine, aspartic acid, asparagine, phenylalanine and others were identified for ten different metals. Species observed by this methodology convey considerable information for metal transport/homeostasis understanding and will be a very valuable tool to use in parallel to molecular biology experiments.

(Introduction)

In plants, metallic species play an essential role in maintaining structure and enzymatic activity of cells. On the other hand, excess of metal can lead to severe toxicity because of metal substitutions in enzymes and oxidative damages (Broadley et al., 2012). As a consequence, it is crucial to determine the nature of these species to better comprehend their fate and their role in organisms. Generally associated with organic molecules, these elements have a huge variety of chemical forms and properties (covalent or non-covalent binding, low to high molecular weight ligands), are often unstable (metal complexes lability, oxidation-sensitive species) and are usually low concentrated, which makes their analysis being tedious to achieve. Moreover, even if several techniques can achieve a good screening of covalently binding elements, the characterization of non-covalent metal complexes present at basal concentration in biological matrices is still to date a challenging issue.

X-ray absorption spectroscopy (XAS) and micro-X-ray fluorescence (μXRF) techniques are of particular interest to get a spatial localization of elements within intact tissues (Sarret et al., 2013) but, even if these techniques keep improving, they have two main drawbacks, their limited sensitivity and the necessity to know *a priori* the nature of all

potential ligands (to record signals of metal complex standards to allow proper deconvolution of X-ray signals later), which impairs identification of novel species. On the other hand, mass spectrometry instruments, especially when there are coupled to liquid chromatography, are usually one of the most recommended tools to achieve the simultaneous characterization of several unknown low concentrated species in complex biological matrices. However, LC MS techniques imply sample collection, sometimes extractions and, finally, interaction of compounds of interest with a chromatographic support, which can contribute to modify the nature of the original species. Among chromatographic techniques, some of them gave promising results and seems the most appropriate to avoid degradation of metal complexes during analysis, size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC) and reverse phase chromatography (RP). SEC columns have been used for several applications. Usually coupled to ICP-MS, their lack of resolution would generally only permit to discriminate between high (> 10kDa), medium (0.5 to 10 kDa) and small (< 0.5kDa) molecular weight compounds. It has been of a particular interest to distinguish metal complexes with metallothioneins (MTs) (Lobinski et al., 1998) and phytochelatins (PCs) (Vacchina et al., 1999) from metal complexes binding organic acid, amino acids or proteins. On the other side, HILIC technique is based on the use of chromatographic material with polar groups allowing the retention of hydrophilic compounds, which results only in minor degradation of metal complexes. The coupling of HILIC was used for the very first time to achieve the separation and the identification of metal complexes for the screening of nickel speciation in the hyperaccumulating plant *Thlaspi caerulescens*, also called *Noccaea caerulescens* (Ouerdane et al., 2006). It was directly coupled to ICP MS and metal complexes were identified By ESI MS after fraction collection. Several other works using HILIC columns were then performed successfully to characterize metal complexes in different matrices (Xuan et al., 2006; Rellán-Álvarez et al., 2010; Köster et al., 2011). Finally, RP chromatography could also be successfully used to separate and identify small protein binding metals like MTs (Chassaigne and Lobinski, 1998; Mounicou et al., 2010). Multidimensional chromatography was also proposed as a tool to improve separation of metallated species although it can be more difficult when it is applied to low concentrated complexes because of the increased risk of degradation during sample purification. Regarding MS techniques, ICP-MS and ESI-MS are instruments of choice to get respectively quantitative and qualitative information on metal species present in aqueous media. However, if ICP-MS can easily achieve unambiguous detection of metal species at low concentrations and, this, for most of the metallic elements (especially when using equipment with collision

cells or with high resolution sector field), ESI-MS instruments are more prone to face problem of detection because of sample matrices. Indeed, presence of numerous adducts in spectra or a lack of interscan dynamic range were usually the reason why quadrupole or time-of-flight instruments were mostly unable to identify metal complexes at trace levels (ng/g to µg/g), especially that coelution of concentrated free ligands causes ion suppression. In the recent years, the apparition of high resolution ESI-MS instruments, such as ESI LTQ Orbitrap MS, allowed real advance in terms of detection limit and of spectral resolution (R > 100 000 for compounds at m/z < 400 Da). Therefore, for LC MS analysis of metallated metabolites, it became possible to employ in parallel ICP-MS and ESI-MS instruments and achieve similar detection limits. LC methods (HILIC and SEC) were optimized to achieve a good recovery of metal complexes for the analysis of xylem and embryo sac liquid (ESL) from *Pisum sativum*, which allowed then their analysis by the above mentioned instruments.

RESULTS

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

Parallel use of HPLC ICP MS and HPLC high resolution ESI MS couplings, especially when using HILIC chromatography, could be applied recently for the exhaustive screening of covalent species in natural samples, for instance selenocompounds in yeast (Arnaudguilhem et al., 2012) or in plants (Aureli et al., 2012; Ouerdane et al., 2013), and of stable metal complexes with proteins in dolphin liver (Pedrero et al., 2012; Pedrero Zayas et al., 2013). However, low molecular weight metal complexes are sometimes unstable and can be degraded during sample preparation and preconcentration steps before analysis or between chromatographic steps. To avoid these issues, sample analyzed in this work did not go through any kind of extraction procedure and monodimensional chromatography was chosen for direct analysis. Finally, chromatographic mobile phase pH and nature of buffer were chosen to mimic pH found in sample and to minimize competition with endogenous metal complexes, respectively, which insures minimum degradations of metal species initially present. Two different chromatographic conditions were also tested for HILIC MS analysis to sort out which metal complexes were definitively present in the sample and which of them could be due to a simple coelution.

Chromatograms of both samples were recorded coupled to ICP MS or ESI MS with SEC or HILIC column. Moreover, ESI MS spectra were obtained in positive and negative mode. To obtain meaningful data about metal complexes present in xylem and LSE samples, metal recovery was checked after analysis for both HILIC and SEC columns. Most elements had an almost quantitative recovery, which allow to get significant data on metal speciation in those fluids. To confirm that a recovery not exactly at 100% was not affecting much data interpretation on metallic species characterization and quantitation, saturated columns (resulting in metal recovery from 150% to 500%) were tested for sample analysis and no significant change were observed in ICP MS profiles and species identification from ESI MS. It indicates that no important changes seem to occur in metal speciation of these particular samples when column recovery for metals stays within a reasonable range (50% to 200%), probably because of a much higher concentration of ligands than metallic ions.

To test analytical methodology, plant transport (xylem) and feeding (ESL) fluids from *Pisum sativum* were analyzed through the different couplings and modes described above. Metallic species were searched in ESI MS spectra for several elements and the resulting species are presented in Table 2. Using the accurate masses (recalibrated offline with known background ions) of the precursor ions as well as their corresponding fragments, exact molecular formula could be determined for all of them. Taking into account that metals can be bound to one to several identical (or different) ligands, these formulas were compared to natural compounds database to find the most probably involved organic ligands. Then, to discard any doubts considering species nature, fragment ions in MS/MS spectra was checked and had to be consistent with expected molecules. Finally, only identifications of complexes resulting in a unique possibility were kept. It is worth notice that once the exact molecular mass with high resolution MS instrument is known, the range of compounds possibly associated to the metal is quite limited, which facilitates easier identification work.

HILIC ESI MS analysis is more sensitive than the one performed with SEC and allows the identification of numerous metal complexes. However, the presence of the main metal complexes was confirmed by SEC ESI MS coupling. To confirm the occurrence of the less concentrated metal complexes different chromatographic conditions was tested for HILIC chromatography (ammonium acetate buffer instead of ammonium formate and a faster gradient). The different salt compositions of the two buffers and chromatographic gradients lead to a variation in relative retention times of several species (chromatograms are only shown for ammonium formate buffer) and, as a consequence, allowed to distinguish the very few low concentrated metal complexes only formed in electrospray source because of the

coelution of a metal ion and a ligand from metal complexes stable in both HILIC chromatographic conditions. Despite differences in metal concentrations between xylem and ESL, the nature and the proportion of metal complexes were similar between the two types of sample and were reproducible during the three campaigns of growth/analysis. This could be partially explained by the fact that xylem and ESL samples have similar pH (around 5.5). Finally, more than 50 different metal complexes were identified by HILIC ESI MS. Numerous ligands could be characterized such as aspartic acid, asparagine, citric acid, glutamine, histidine, malic acid, nicotianamine, phenylalanine, tryptophan, pipecolic acid and aminoadipic acid. There 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^{+} and Ni^{2+}. Matching retention times of ESI MS extracted ions of metal complexes with ICP MS chromatograms enable to evaluate the quantitative contribution of each ligand in the binding of metallic ions in these plant samples for both HILIC (Fig. 1) and SEC (Fig. 2) couplings.

Mixed citrate-malate-iron complexes

Among those complexes, the most intriguing was the mixed complexes $Fe(III)_3Cit_{(4-x)}Mal_x$ (with x ranging from 1 to 3), which were identified because of the consecutive elution in SEC of the three complexes (two were detected in HILIC mode) containing different malate to citrate ratios characterized by their mass difference of 58 Da. The determination of their exact molecular formula was established by MS/MS fragmentations. However, to confirm this result, post-column acidification was set during a SEC chromatographic run and confirmed the coelution of malic acid and citric acid at the retention time of this iron complex as shown in Fig. 3, whereas the corresponding free form of these organic acids are eluting much later,. Finally, a 0.2 : 1 : 5 mix of Fe(III) : citrate : malate at pH 5.5 was analyzed by infusion with ESI MS instrument and these three iron complexes were actually found to be the major metal complexes forms for m/z > 500 (data not shown) indicating the preferential formation of these mixed citrate-malate complexes against those containing a single type of organic acid. The biological implications of such discovery for iron homeostasis in pea were further developed in an iron-dedicated study (Grillet L, Ouerdane L, Flis P, Isaure MP, Lobinski R, Curie C and Mari S, unpublished work).

DISCUSSION

Several amino acids and carboxylic acids are involved in metal binding

Several metal complexes were characterized for the first time, including complexes with elements for which no MS data existed yet about their speciation in plants as well as complexes containing ligands of different nature. One of the main (if not the main) binding molecule for Zn, Cu(II), Co(II) and 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, no confirmation by mass spectrometry could clearly demonstrate that it is a key compound for trace metal homeostasis in plants and possibly for transport of large number of metal ions (at least by allowing them to be maintained in solution to access enzymes and membrane transport proteins). Regards to these results, NA is obviously of a great interest to study the fate of essential trace elements in plants and its transport, driven by YSL transporters, is probably of a tremendous importance to control sharply transient metal homeostasis and bioavailability within plant cells (Curie et al., 2009).

Malic acid and citric acid are also observed to be involved in metal ion binding, even if the form of the detected complexes for elements other than iron (Zn, Ni, Ca…) were mostly 1:2 metal:ligand species that have an expected low to medium stability, which contrasts with the more stable 3:4 iron/organic acids complexes. Organic acid are usually described as essential counter-ions for metal storage of these elements, especially in hyperaccumulating plants (Rascio and Navari-Izzo, 2011). Several amino acids were also found to bind metal ions among which a few were never described by mass spectrometry to be involved in metal complexes in biological matrices. Gln, Asn and Asp are three amino acids containing -NH_2 or -COOH group in their side chain, which confers them an improved capacity for metal binding. Actually, some of them also present uncharged aromatic (phenylalanine, tryptophan), which is clearly unexpected. However, these amino acids are only minor ligands in overall metal speciation.

Differences in speciation between xylem sap and ESL sample could be noticed quantitatively but also qualitatively as less variety of amino acids were involved in metal binding in xylem than in ESL. It can be related to the fact that ESL are involved in seed feeding and should then contain a wider variety and an increased concentration of nutrients.

More generally, it is worth noticing that both samples shared the same major ligands for several elements.

It could then be noticed that some metals had a higher affinity for some ligands relatively to other metal ions, which is of a considerable interest to obtain clues about the role of some ligand for element-specific homeostasis in the plant. For example, Ni was found to have a higher affinity towards histidine than all other metal species. Interestingly, this particular affinity was actually suggested by former observations done for Ni hyperaccumulating plants (Krämer et al., 1996; Ingle et al., 2005; Ouerdane et al., 2006), which would mean that this amino acid could definitively play a specific role in mobilizing this metal.

Metal species present in xylem and in ESL

For Fe(III), the implication of citric acid confirms early works done by (Tiffin, 1966) as well as more recent characterization of "$Fe(III)_3$-O-Cit_3" metal complex in plant (Rellán-Álvarez et al., 2010). However, the role of malic acid in combination with citric acid was never suggested and the absence of "$Fe(III)_3$-O-Cit_3" complex or $Fe(III)_3$-Cit_3 complex in the pea samples despite the presence of citric acid would confirm a higher stability for the mixed iron complexes $Fe(III)_3Cit_{(4-x)}Mal_x$ (x = 1 to 3) compared the "$Fe(III)_3$-O-Cit_3" complex or $Fe(III)_3$-Cit_3 complex (as observed also with the standard mixture). Interestingly, one of the complex in our study, $Fe(III)_3Cit_1Mal_3$, has exactly the same molecular formula (in neutral form) than the "$Fe(III)_3$-O-Cit_3" complex predicted by (Rellán-Álvarez et al., 2010). In this previous study, the complex observed in tomato xylem sap is not in the same form than in the Fe citrate standard mixture (only "$Fe(III)_3$-O-Cit_3" in tomato xylem sap but 60% $Fe(III)_3$-Cit_3 and only 40% "$Fe(III)_3$-O-Cit_3" in standard mixture). As it was observed recently in *Plantago almogravensis* leaves (Grevenstuk et al., 2013), the detection of Fe(III): Cit mixture in plant sample 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" even if it is probably just an adduct of a water molecule, $Fe(III)_3$-$cit_3(H_2O)$, that stabilizes complex structure without the insertion of an oxygen atom within iron core (Fukushima et al., 2012). Therefore, there is a possibility that the formerly observed "$Fe(III)_3$-O-Cit_3" complex (Rellán-Álvarez et al., 2010) was wrongly assigned to $Fe(III)_3Cit_3$ complex and could have been actually $Fe(III)_3Cit_1Mal_3$ complex in tomato xylem sap. Remarkably, malic acid is frequently found in high concentration, sometimes in higher proportion than citrate in plant shoots and, commonly, its concentration increases in case of

Fe deficiency (Abadía et al., 2002; López-Millán et al., 2009; Jelali et al., 2010; López-Millán et al., 2012). Its presence could then favor (in combination with citric acid) the stabilization of Fe(III) in those mixed complexed forms instead of Fe(II) that represents a potential threat for plant as its oxidation can lead to Fenton reaction and production of ROS. Concerning Fe(II), it was found to bind at trace level citrate, malate and aspartate as well as Fe(III) but only Fe(II)-NA complex could be observed without its oxidized counter-part. It is worth noticing that despite careful sample collection, a slight oxidation in contact with air of a part of iron during sampling is possible. However, the remaining traces of Fe(II)-NA as a specific complex for Fe(II) seems to confirm early observations that this complex more than those with organic acids would contribute to stabilize Fe(II) (Von Wirén et al., 1999). It would participate in making Fe(II) stable long enough to be available for metabolic/enzymatic reactions or transporters without producing oxidative damage through instantaneous re-oxidation, as commented above. A role for YSL transporters for Fe (Jean et al., 2005; Kakei et al., 2012; Schuler et al., 2012) or even for Fe(II)-NA (Ishimaru et al., 2010) transport in plant was repeatedly demonstrated but this complex could never be observed. Moreover, the formation of very stable complexes for Fe(III) with citrate or citrate/malate mixture as described above favors the presence of Fe(III) and would have the effect of lowering the redox potential of Fe(III)/Fe(II) redox couple to negative values as observed with bacterial siderophores (Dhungana and Crumbliss, 2005), which favors and explains the presence of a majority of species-containing Fe(III) and not Fe(II). It is especially true as binding strength of NA with Fe(II) is not sufficient to balance this effect (Benes et al., 1983). These observations could be confirmed in a parallel work dedicated to iron transfer from ESL to embryos in peas through the contribution of ascorbate exudation leading to Fe(III) reduction to Fe(II) before transport (Grillet L, Ouerdane L, Flis P, Isaure MP, Lobinski R, Curie C and Mari S, unpublished data).

Concerning other metals, Cu(II), as expected because of its high affinity for NA (Benes et al., 1983), is mainly bound to this molecule in plant fluids. This non-proteogenic amino acid can then be considered as the main carrier of copper within pea vessels and to seeds. Even if it is usually proposed that Cu is transported as Cu(I) via Copper Transporter (COPT) family (Sancenón et al., 2003; Eisses and Kaplan, 2005), it was also observed that ZIP transporters and heavy metal ATPase (HMA) transporters could transport Cu(II) and, more interestingly, recent study could finally demonstrate that YSL transporters (*e.g.*, YSL16 in rice; (Zheng et al., 2012)) are also involved in transport of Cu(II)-NA complex, which means that this complex could be immediately transported. Cu(II)-NA was putatively identified recently by mass spectrometry in rice phloem but without confirmation by exact

mass matching with high resolution MS instrument or MS/MS fragmentation (Ando et al., 2013). The essential role of NA for Cu uptake in plants was repeatedly observed (Pich and Scholz, 1996; Liao et al., 2000; Irtelli et al., 2009) and these results constitute the first unambiguous detection of Cu(II)-NA complex as the main form of this metal in xylem and ESL of a plant. As for Fe, the presence of Cu(II) instead of Cu(I) in xylem and ESL could be explain by the fact that the presence of the strong Cu(II) ligand, NA, without counterpart for Cu(I), would lower 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. Despite the very low concentrations of these complexes (hard to evaluate because of high background level for Cu during quantitative HILIC ICP MS analysis, Fig.1) and therefore the possibility of their artifactual creation in electrospray source, the great specificity of the complexes for Cu regards to other metallic ions was found of a particular interest. Increase of Phe content in xylem sap was observed once in chicory plants exposed to high Cu concentration (Liao et al., 2000). Additional work could probably elucidate the potential minor participation of these ligands in plant Cu homeostasis. As Cu is mainly observed as a stable NA-Cu(II) complex, it can be wandered if Cu(I) could be produced in ESL to be then transported into seed embryo. As shown in Fig.4, Cu(II) is reduced in presence of the embryo plus its exudate but, also, and in the same extent, in presence of the exudate only, which means that reducing activity is mainly due to excreted species more than by reductases present at embryo surface such as FRO family members (Mukherjee et al., 2006). Among potential species involved in Cu(II) reduction, ascorbic acid and glutathione are the one that are expected to play a major role. Addition of ascorbate oxidase (AOX) seems to indicate that almost half of this reducing activity is due to ascorbic acid, which confirms the implication of excreted low molecular weight reducing compounds for the reduction of a part of Cu(II) in Cu(I) that can then also be transported by COPT transporters. It is very important to realize then that, at a low molecular weight metabolites level, not only metal ligands are involved in metal speciation but also reducing/oxidizing transported compounds that can control locally metal species redox status and so their chemical form. More generally, it can be noticed that the presence of ligands that maintain Fe and Cu in their initial oxidative state contributes also in reducing the occurrence of Fenton or Fenton-like reaction that could occur during repeated redox cycles if these metals would have in a free state (Barbusiński, 2009).

In a similar way than Cu, Zn(II) is also binding to NA almost quantitatively with citrate as a secondary ligand. In some samples, other ligands such as His and, in a lesser extent, Gln were also observed. Proportions between NA, citrate and His could vary between

samples with NA dominating. Zn has less affinity for NA than Cu and the reason why this complex can be observed is obviously that molar amount of NA is as high as or higher than total Zn content, which is not obligatory the case in plants, especially the one accumulating Zn. Zn-NA was already observed by mass spectrometry in rice phloem but with a distorted isotopic pattern, which makes authentication difficult (Nishiyama et al., 2012). However, it was never detected in plant xylem or ESL. To confirm the presence of Zn-NA, Cu-NA, other metal-NA complexes or metal-deoxymugineic acid (a similar compound to NA present in graminaceous plants) complexes, the use of high resolution MS and sharp chromatographic separation, as performed in this study, is indispensable to get clear identification and to discard any confusions with isobaric interferences as previously stated (Weber et al., 2006). These results are confirming 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). Presence of Zn-Cit_2 is observed with both chromatographic set-up even if this complex seems more stable and give a more define peak with SEC column. Even if the formation of this complex was deduced after x-ray analysis (Sarret et al., 2002; Terzano et al., 2008) showing that citrate can be a useful counter-ion for Zn(II) in plants, it is for the first time identified by MS in plant. Similarly, Zn-His_2 complex signal could also be deconvoluted by x-ray analysis (Salt et al., 1999; Küpper et al., 2004) but never confirmed by MS.

With Fe and Zn, Mn is usually one of the major transition element in plants. In this study, it is found mainly bound to citrate in a Mn-Cit_2 complex and much lower level in a Mn-Mal_2 complex, as predicted by x-ray in Mn hyperaccumulators (Fernando et al., 2010). Same small amounts of Mn-NA could also be detected for the first time, 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).

Co and Ni are two minor micronutrients and there are both observed to mainly bind NA and His. Ni-His_2 and Ni-NA were already observed by MS as minor ligands in hyperaccumulating plants (Ouerdane et al., 2006) and the later one could also be observed as being transported by YSL transporters (Mari et al., 2006; Gendre et al., 2007). Concerning Co, it is simply the first MS data concerning the speciation of this element in plant. It seems so that NA and His plays an important role in transport of these two metals within the plant.

Concerning K, Mg and Ca there were mainly found to be complexed with Cit and Gln but also Asp and Asn. It is not surprising to detect Cit as a major ligand for these weakly binding elements because Cit is usually one of the major organic acids in plants and it

contains several binding sites for metals (three carboxylic and one hydroxyl groups). Concerning the amino acids, there were probably also among the most concentrated in plant xylem. 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 witnessed by ICP MS, which is typical of weakly bound complexes. Their retention times and their number seem to indicate that there are mainly not metal in free form. According to retention time, it is postulated that saccharides and polysaccharides could be involved in this binding. Some other molecules, aminoadipic acid and pipecolic acid were detected as potential Ca chelators but the lack of similarity between ICP MS profile and the peak shapes of these complexes by HILIC ESI MS seems to indicate that the formation of these complexes is maybe just opportunistic and only occur in ion source.

On a general point of view, it can be noticed that the affinity for NA or for citrate, for example, is shared by several metals, which involves a competition for its binding. The metal speciation will then depend on ligand and metal concentrations. All potential ligands and chelated 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 their differentiation in plant will occur because of the specificity of dedicated transporter, like YSL transporter for NA (Curie et al., 2009). To achieve a correct simulation, it is therefore obligatory to know already metal species, which can be performed by this HPLC MS based method. Indeed, the nature of involved ligands and their complexed forms such as multiple ligand for one type of metal ($Fe(III)_3Cit_2Mal_2$), different proportions (1:1, 1:2, 1:3, 1:1:2, 2:2, 3:3, 3:2:2 etc…) and mixture of metals around one type of ligand ($Fe_2Al_1Cit_3$ (Grevenstuk et al., 2013)) are difficult to predict a priori, which makes this kind of simulation sometimes hazardous (Harris et al., 2012). It can be easily understood that the excess or absence of one metal (hyperaccumulating plants) or one ligand can lead to novel speciation for all elements. Concerning x-ray analysis, most of works published to date were conducted on hyperaccumulating plants because of the drawback of this technique for low concentrated elements and the necessity to know in advance the potential involved complexes (Terzano et al., 2013). To complement the lacks of the above mentioned methods, the proposed approach by HILIC ICP MS and ESI high resolution MS is therefore an important tool to develop metal speciation in biological matrices. The obtained information at a molecular level could then be of a great significance for the further understanding of metal homeostasis at a proteomic or a genomic level.

MATERIAL AND METHODS

Plant culture

Pea (Pisum sativum L., Dippes Gelbe Viktoria, DGV, cultivar) was used in this study. Plants were grown in a greenhouse 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) irrigated with tap water. Xylem sap sample was collected after insertion of a glass capillary in the stem of the decapitated plants. Embryo sac liquid, also called liquid endosperm, was obtained by inserting a capillary glass in pea seed before complete seed maturation. Both type of samples were immediately stored after collection at -20°C. Samples were collected from several plants and pooled until reaching a volume of 200 µL. Peas were grown three times to get replicates and check sample content and analytical method reproducibility.

Analysis of total metal content in plant samples

Total metal contents in xylem and LSE solutions were determined after a 10 or 100 fold dilution with 2% nitric acid solution. Acidified sample and standard solution of metallic elements were then analyzed by ICP MS (Agilent 7500ce) operating in hydrogen gas mode to remove possible interferences. When possible, several isotopes were screened for each element to confirm quantification results.

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 ammoniac were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and ultrapure water (18 MΩ.cm) was obtained from a Milli-Q system (Millipore, Bedford, MA). Microbore HILIC HPLC separations were performed using an Agilent 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 μHILIC HPLC-ICP MS coupling was done via an Isomist interface (Glass Expansion, Melbourne, Vic, Australia) consisting of a 20 mL model Cinnabar cyclonic spray chamber cooled at 2 °C and fitted with a 50 µL.min^{-1} Micromist U-series nebulizer. Size exclusion chromatography

separations were carried out with an Agilent 1100 HPLC system (Agilent, Wilmington, DE). The metal elution was monitored by splitting part of the effluent to an Agilent 7500ce ICP MS (Agilent, Tokyo, Japan). The column used for HILIC separation was a TSK gel amide 80 (250 mm × 1 mm i.d., 5 µm) from Tosoh Biosciences (Stuttgart, Germany). Gradient elution, at a flow rate of 50 $\mu L.min^{-1}$, 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 to 2 ratio (sample : acetonitrile), then centrifuged and 7 µL of the supernatant were injected on the HILIC column each time. Addition of acetonitrile creates a small precipitate that contains only trace amount (< 5%) of metallic element of interest. 5 mM Ammonium acetate (pH 5.5) for eluent B was also tested to check if buffer ions influence metal speciation. Size exclusion chromatography was performed with a Superdex Peptide and 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 regularly cleaned respectively with 0.1% formic acid solution and 0.1% formic acid/acetonitrile (1:1) mixture to avoid metal carryover between samples. For µHILIC HPLC-ICP MS and SEC-ICP MS couplings, at least two isotopes (when possible) of iron, copper, zinc, molybdenum, nickel, cobalt, magnesium, manganese, and calcium were monitored to ascertain the presence of these elements and discard possible interferences. Column recoveries for metals were checked by comparing metal contents between original sample, eluting solutions after sample injection and after blank injection.

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

For µHILIC HPLC-ESI MS and SEC-ESI MS, the HPLC systems were connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For analysis with SEC, a splitter allowed to divide by ten chromatographic flow before injection in ESI MS instrument. In both case, the coupling was achieved via a heated electrospray ionization source (H-ESI II) (Thermo Fisher Scientific). For both chromatographic setups, chromatographic separation conditions were identical as given above. For the post-column acidification experiments operated with SEC column, the solution added was formic acid:water (30/70%, *v*/*v*) delivered at 4 $\mu L.min^{-1}$ after splitting with a setting described elsewhere. The ion source was operated in the positive ion mode at 3.0 kV and in 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 Fischer Scientific) to screen spectra for heavy-element containing molecules. Once potential metalated species were observed, their fragmentations were performed during a second chromatographic run with high energy collision fragmentation (HCD) at 35% energy using an isolation width adapted to the number of isotopes present in the complex.

Copper reduction assays

Pea embryos were dissected with a surgical blade as fast as possible, and kept in 5 mM MES buffer pH = 5.5 for 30 minutes, representing the time to dissect and weight around 15 embryos. Embryos were then incubated in the assay solution. The copper reduction activity was estimated as the quantity of Cu-BCDS complexes formed in the assay solution calculated from the optical density measured at 463 nm (epsilon=12.25 mM.cm-1) with a Hitachi U-2800 spectrophotometer. OD463 was measured after incubation in the dark at 22-25°C with 250 RPM shaking, in an assay solution that contained 5 mM MES buffer pH = 5.5, 200 µM bathocuproine disulfonate (BCDS) and 100 µM $CuSO_4$.

Copper reduction activity measurement of exudates

Exudates consisted in 5 ml milliQ water in which 5 pea embryos were incubated, representing a total fresh weight of 0.7 to 1 g. 100 µM $CuSO_4$ and 200 µM BCDS were then added to exudates and allowed to react for 1 hour in the dark prior to OD463 determination. For the comparison of exudates activity with embryo activity, exudates were collected in milliQ water after 30 minutes incubation, and allowed to react with 100 µM CuSO4 and 200 µM BCDS for 1 hour in the dark. In the case of AOX treatment, 1.5 unit of AOX per ml of exudate were added 2 minutes before $CuSO_4$ and BCDS addition. All final values are mean results ± SD of three independent experiments.

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Table captions

Table 1. Metal complexes detected by HILIC ESI MS in xylem and ESL of pea.

Table 2. Metal complexes detected by SEC ESI MS in xylem and ESL of pea.

Figure captions

Figure 1. HILIC ICP MS (upper part) and HILIC ESI MS (bottom part) chromatograms of each main metallic elements studied in pea xylem (similar chromatograms were observed for pea ESL).

Figure 2. SEC ICP MS (upper part) and SEC ESI MS (bottom part) chromatograms of Zn, Cu and Fe in pea xylem (similar chromatograms were observed for pea ESL).

Figure 3. SEC ICP MS (upper part), SEC ESI MS (middle part) and SEC ESI MS after post column acidification (bottom part) chromatograms that are zoomed around elution time of $Fe(III)_3Cit_{(4-x)}Mal_x$ (x = 1 to 3) complexes. Citric and malic acid are detectable after sample acidification at complexes retention times whereas the free forms of these organic acids are observed to elute few minutes later by SEC chromatography.

Figure 4. Ferric reduction activity measured by BPDS method in presence of embryos (+embryo), after removing embryos (-embryo) or after removing embryo and adding ascorbate oxidase (-embryo + AOX). Results are means of 3 independent replicates ± standard deviation.

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Table 1

No	Complex	Formula (neutral form)	Theoretical mass	Experimental mass	Delta ppm	Retention time [min]	Ionistaion mode	Sample (xylem/endosperm)
1	$(Asn)_2Mg$	$C_8H_{14}O_6N_4Mg$	287,08365	287,08363	-0,080	30,50	pos	X
2	$(Asp)_2Mg$	$C_8H_{12}O_8N_2Mg$	289,05168	289,05183	0,511	31,95	pos/neg	X
3	$(Gln)_2Mg$	$C_{10}H_{18}O_6N_4Mg$	315,11495	315,11495	-0,011	29,86	pos/neg	X/E
4	$(citrate)_2Mg$	$C_{12}H_{14}O_{14}Mg$	407,03067	407,03063	-0,104	35,36	pos/neg	X/E
5	$(Asn)_2Na$	$C_8H_{15}O_6N_4Na$	287,09621	287,09593	-0,986	29,55	pos/neg	X/E
6	$(Asp)_2Na$	$C_8H_{13}O_8N_2Na$	289,06424	289,06397	-0,942	30,24	pos/neg	X/E
7	$(Gln)_2Na$	$C_{10}H_{19}O_6N_4Na$	315,12751	315,12761	0,306	29,06	pos/neg	X/E
8	(citrate)Ca	$C_6H_6O_7Ca$	230,98122	230,98132	0,444	34,30	pos	X/E
9	$(pipecolinic\ acid)_2Ca$	$C_{12}H_{20}O_4N_2Ca$	297,11217	297,11227	0,344	24,54	pos	E
10	$(Asn)_2Ca$	$C_8H_{14}O_6N_4Ca$	303,06120	303,06095	-0,817	29,43	pos/neg	X
11	$(Asp)_2Ca$	$C_8H_{12}O_8N_2Ca$	305,02923	305,02906	-0,547	30,24	pos/neg	X
12	$(Gln)_2Ca$	$C_{10}H_{18}O_6N_4Ca$	331,09250	331,09231	-0,587	30,19	pos/neg	X/E
13	$(aminoadipic\ acid)_2Ca$	$C_{12}H_{20}O_8N_2Ca$	361,09183	361,09208	0,679	22,33	pos	E
14	$(citrate)_2Ca$	$C_{12}H_{14}O_{14}Ca$	423,00822	423,00822	0,011	34,72	pos/neg	X/E
15	(citrate)K	$C_6H_7O_7K$	230,99016	230,98981	-1,524	33,22	pos	X/E
16	$(citrate)K_2$	$C_6H_6O_7K_2$	268,94604	268,94598	-0,220	33,28	pos	X/E
17	$(Asn)_2K$	$C_8H_{15}O_6N_4K$	301,05559	301,05534	-0,839	29,26	neg	X/E
18	$(Asp)_2K$	$C_8H_{13}O_8N_2K$	303,02362	303,02333	-0,952	30,21	neg	X/E
19	$(citrate)K_3$	$C_6H_5O_7K_3$	306,90192	306,90189	-0,086	33,41	pos	X/E
20	$(Gln)_2K$	$C_{10}H_{19}O_6N_4K$	331,10144	331,10094	-1,504	28,87	pos/neg	X/E

Table 1

21	$(Asn)_2Fe^{III}$	$C_8H_{13}O_6N_4Fe^{III}$	318,02573	318,02584	0,338	29,86	pos	X
22	$(Asp)_2Fe^{III}$	$C_8H_{11}O_8N_2Fe^{III}$	319,99376	319,99376	-0,006	30,56	pos/neg	X/E
23	$(malate)_2$-Fe^{III}	$C_8H_9O_{10}Fe^{III}$	321,96179	321,96181	0,058	29,80	pos/neg	X/E
24	$(Gln)_2Fe^{III}$	$C_{10}H_{17}O_6N_4Fe^{III}$	346,05703	346,05707	0,107	29,80	pos	X/E
25	$(citrate)_2Fe^{III}$	$C_{12}H_{13}O_{14}Fe^{III}$	437,97275	437,97284	0,201	35,22	pos/neg	X/E
26	$(malate)_2(citrate)_2$-Fe_3	$C_{20}H_{19}O_{24}Fe_3^{III}$	811,83873	811,83938	0,806	37,02	pos/neg	X/E
27	$(malate)(citrate)_3$-Fe_3	$C_{22}H_{21}O_{26}Fe_3^{III}$	869,84421	869,84526	1,212	39,22	pos/neg	X/E
28	$(Asn)_2Mn$	$C_8H_{14}O_6N_4Mn$	318,03666	318,03627	-1,230	31,41	pos	X/E
29	$(malate)_2Mn$	$C_8H_{10}O_{10}Mn$	319,95817	319,95747	-2,186	29,20	neg	X
30	$(Asp)_2Mn$	$C_8H_{12}O_8N_2Mn$	320,00469	320,00513	1,378	30,24	pos/neg	X
31	$(Gln)_2Mn$	$C_{10}H_{18}O_6N_4Mn$	346,06796	346,06799	0,098	30,24	pos	X/E
32	NA Mn	$C_{12}H_{19}O_6N_3Mn$	355,05816	355,05813	-0,076	31,17	neg	E
33	$(citrate)_2Mn$	$C_{12}H_{14}O_{14}Mn$	437,98368	437,98310	-1,314	35,25	pos	E
34	$(Asp)_2Fe^{II}$	$C_8H_{12}O_8N_2Fe^{II}$	318,98703	318,98667	-1,115	30,27	neg	X/E
35	$(malate)_2Fe^{II}$	$C_8H_{10}O_{10}Fe^{II}$	320,95506	320,95482	-0,753	29,05	neg	X
36	NA Fe^{II}	$C_{12}H_{19}O_6N_3Fe^{II}$	356,05505	356,05518	0,351	31,17	neg	E
37	$(citrate)_2$-Fe^{II}	$C_{12}H_{14}O_{14}Fe^{II}$	436,96602	436,96602	-0,011	32,63	neg	E
38	NA-Co^{III}	$C_{12}H_{18}O_6N_3Co^{III}$	360,06004	360,05972	-0,884	31,62	pos	X
39	$(Asn)_2Ni$	$C_8H_{14}O_6N_4Ni$	321,03396	321,03380	-0,503	29,67	pos/neg	X
40	$(Asp)_2Ni$	$C_8H_{12}O_8N_2Ni$	323,00199	323,00194	-0,157	30,62	pos/neg	X
41	$(malate)_2$-Ni	$C_8H_{10}O_{10}Ni$	324,97002	324,96987	-0,462	28,75	pos/neg	X
42	$(Gln)_2Ni$	$C_{10}H_{18}O_6N_4Ni$	349,06526	349,06528	0,051	28,35	pos	X

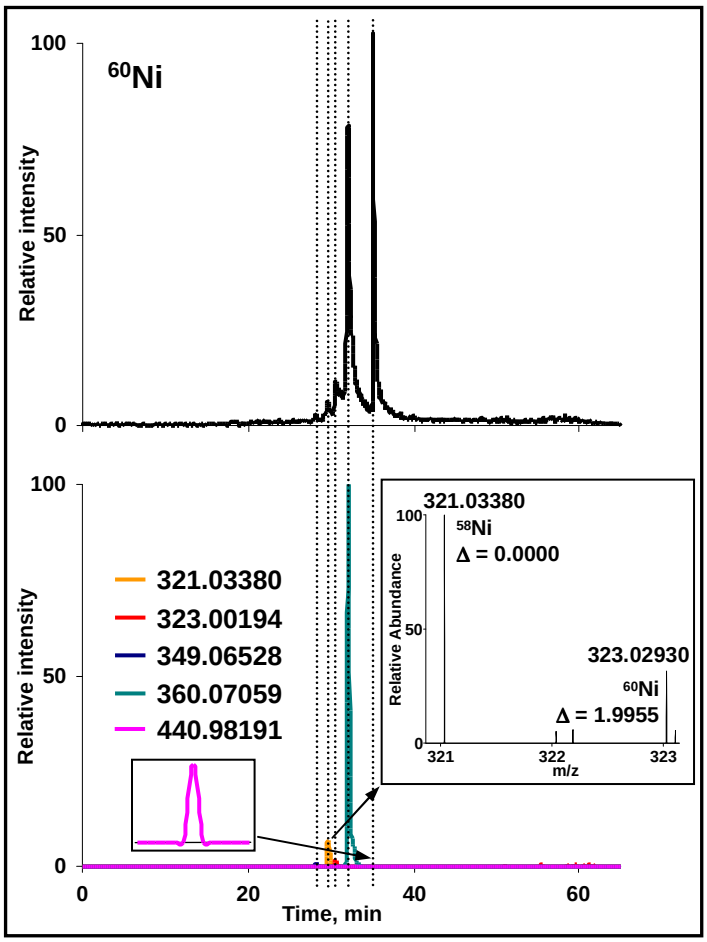
Table 1

43	NA Ni	$C_{12}H_{19}O_6N_3Ni$	360,07001	360,07059	1,615	32,08	pos/neg	X/E
44	$(His)_2Ni$	$C_{12}H_{16}O_4N_6Ni$	365,05137	365,05153	0,446	32,15	neg	X
45	$(Phe)_2Ni$	$C_{18}H_{20}O_4N_2Ni$	387,08493	387,08507	0,357	18,32	pos/neg	E
46	$(citrate)_2Ni$	$C_{12}H_{14}O_{14}Ni$	440,98098	440,98191	2,107	35,09	pos	X
47	$(Phe)_3$-Ni	$C_{27}H_{31}O_6N_3Ni$	552,16391	552,16422	0,567	18,32	pos/neg	E
48	$(Phe)_2(Trp)$-Ni	$C_{29}H_{32}O_6N_4Ni$	591,17481	591,17565	1,426	18,38	pos	E
49	NA Co^{II}	$C_{12}H_{19}O_6N_3Co^{II}$	361,06786	361,06824	1,058	31,22	pos/neg	X/E
50	$(citrate)_2Co$	$C_{12}H_{14}O_{14}Ni$	441,97883	441,97903	0,451	35,55	pos	X
51	$(Asn)_2Cu$	$C_8H_{14}O_6N_4Cu$	326,02821	326,02834	0,399	29,24	pos	X
52	NA Cu	$C_{12}H_{19}O_6N_3Cu$	365,06426	365,06415	-0,292	31,80	pos/neg	X/E
53	$(Phe)_2Cu$	$C_{18}H_{20}O_4N_2Cu$	392,07918	392,07921	0,079	18,21	pos	E
54	$(Phe)_3$-Cu	$C_{27}H_{31}O_6N_3Cu$	557,15816	557,15849	0,584	18,27	pos/neg	E
55	$(Phe)_2(Trp)$-Cu	$C_{29}H_{32}O_6N_4Cu$	596,16906	596,16998	1,536	18,21	pos	E
56	$(Gln)_2$-Zn	$C_{10}H_{18}O_6N_4Zn$	355,05906	355,05893	-0,361	30,41	pos	E
57	NA Zn	$C_{12}H_{19}O_6N_3Zn$	366,06381	366,06387	0,174	31,41	pos/neg	X/E
58	$(His)_2$-Zn	$C_{12}H_{16}O_4N_6Zn$	373,05973	373,05924	-1,310	31,93	pos/neg	E
59	$(citrate)_2Zn$	$C_{12}H_{14}O_{14}Zn$	446,97478	446,97502	0,535	35,07	pos	E

Table 2

No	Complex	Formula (neutral form)	Theoretical mass	Experimental mass	Delta ppm	Retention time [min]	Ionistaion mode	Sample (xylem/endosperm)
1	$(citrate)_2Fe^{III}$	$C_{12}H_{13}O_{14}Fe^{III}$	437,97275	437,97285	0,222	18,83	pos	X/E
2	$(malate)_3(citrate)\text{-}Fe_3^{III}$	$C_{18}H_{17}O_{22}Fe_3^{III}$	753,83324	753,83406	1,091	16,51	pos	X/E
3	$(malate)_2(citrate)_2\text{-}Fe_3^{III}$	$C_{20}H_{19}O_{24}Fe_3^{III}$	811,83873	811,84004	1,618	15,65	pos	X/E
4	$(malate)(citrate)_3\text{-}Fe_3^{III}$	$C_{22}H_{21}O_{26}Fe_3^{III}$	869,84421	869,84504	0,959	15,11	pos	X/E
5	$NA\text{-}Fe^{II}$	$C_{12}H_{19}O_6N_3Fe^{II}$	358,06960	358,06898	-1,743	21,24	pos	E
6	$(citrate)_2\text{-}Fe^{II}$	$C_{12}H_{14}O_{14}Fe^{III}$	438,98057	438,98033	-0,553	18,83	pos	E
7	NA-Cu	$C_{12}H_{19}O_6N_3Cu$	365,06426	365,06455	0,784	21,13	pos	E
8	Na-Zn	$C_{12}H_{19}O_6N_3Zn$	366,06381	366,06315	-1,814	21,13	pos	X/E
9	$(His)_2Zn$	$C_{12}H_{16}O_4N_6Zn$	373,05973	373,05967	-0,171	27,66	pos	E
10	$(citrate)_2\text{-}Zn$	$C_{12}H_{14}O_{14}Zn$	446,97478	446,97486	0,173	18,83	pos	E

Figure 1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60Ni
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Relative Abundance
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323
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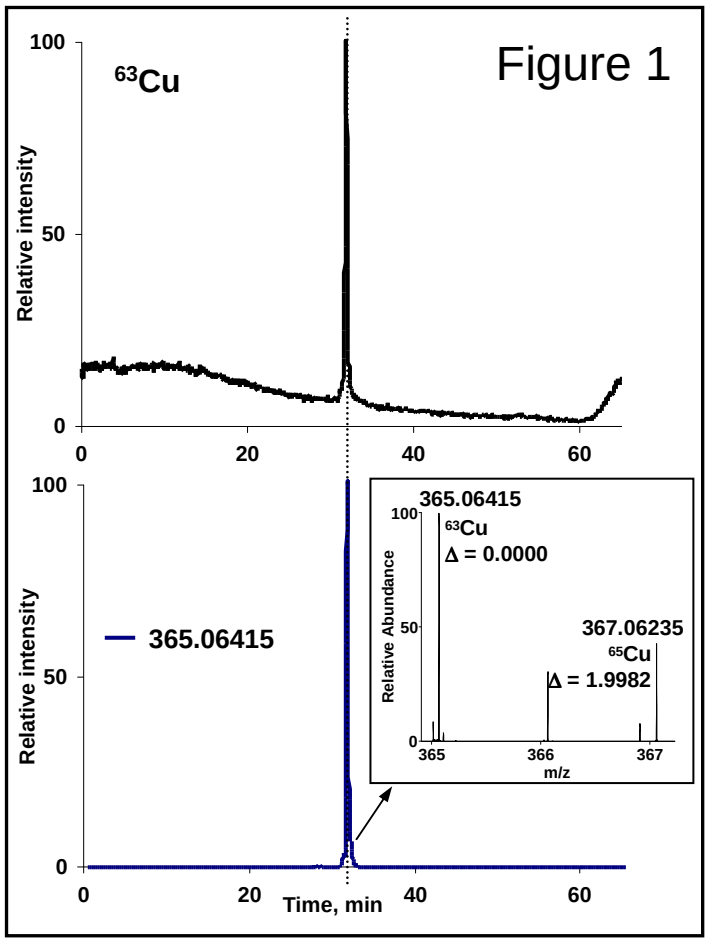
Time, min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365.06415
63Cu
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65Cu
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m/z
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366
367
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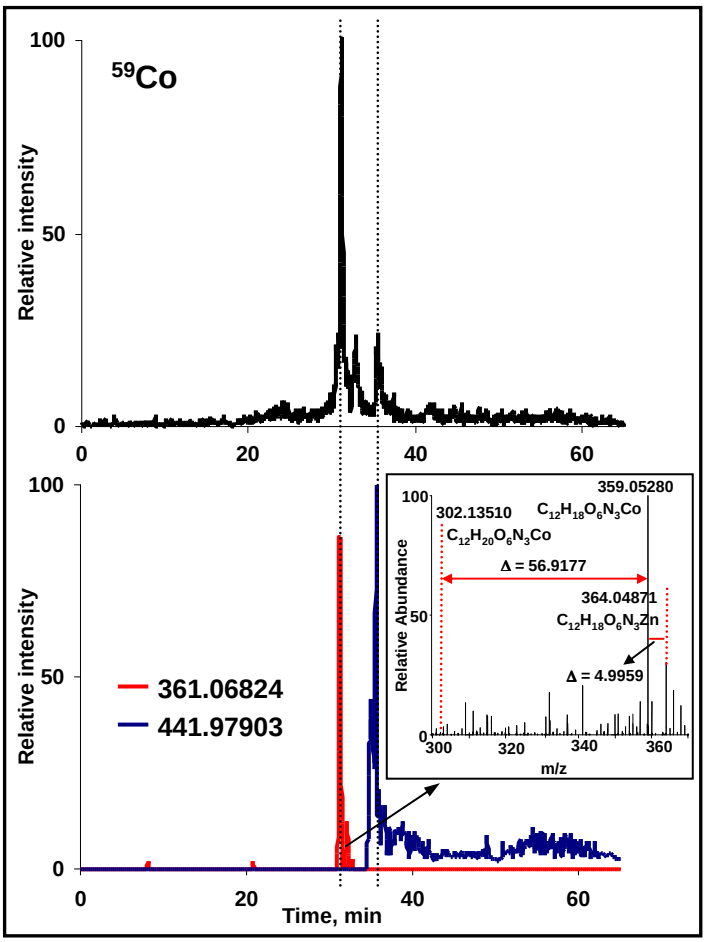
Time, min


59Co
Relative intensity
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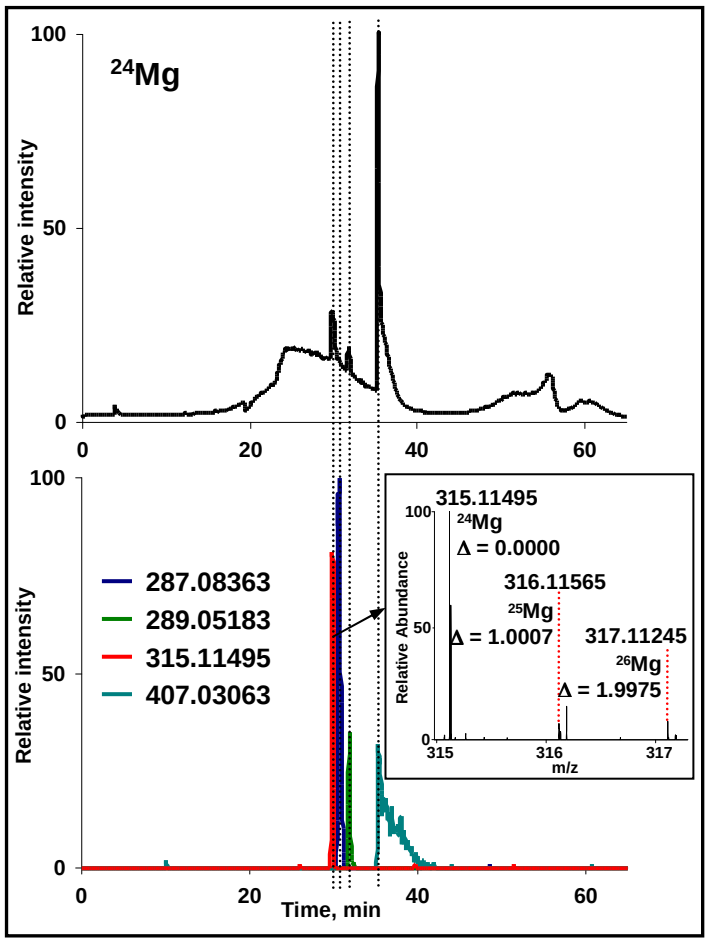
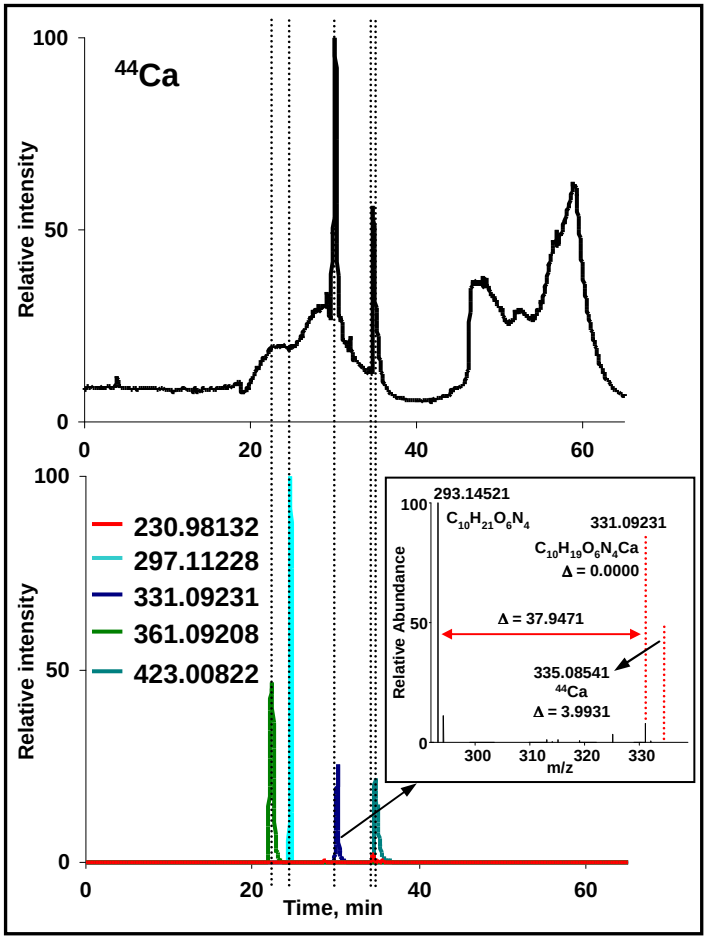
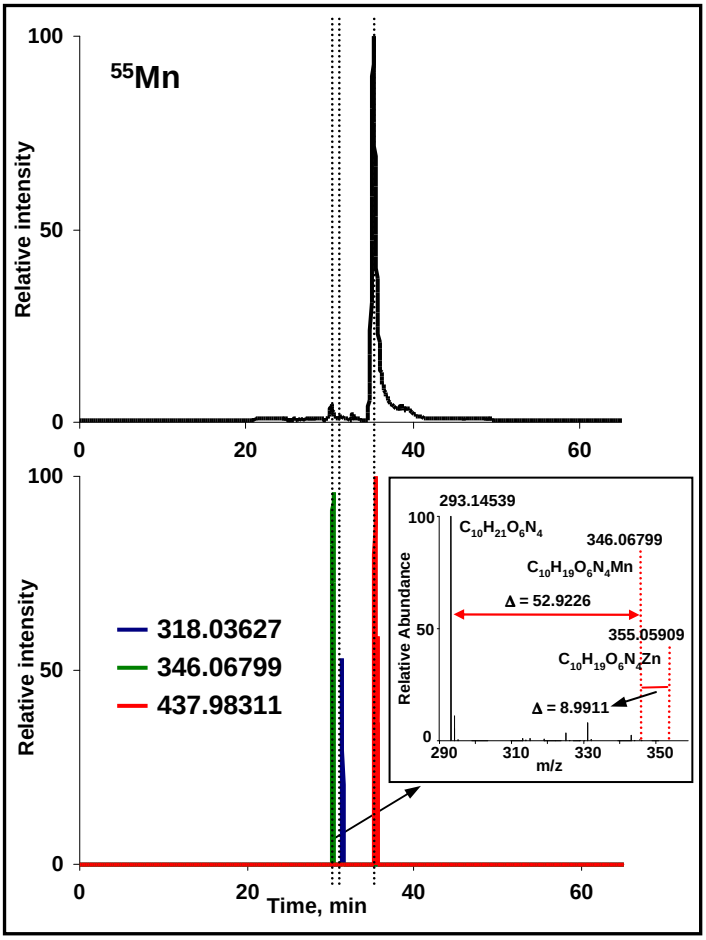
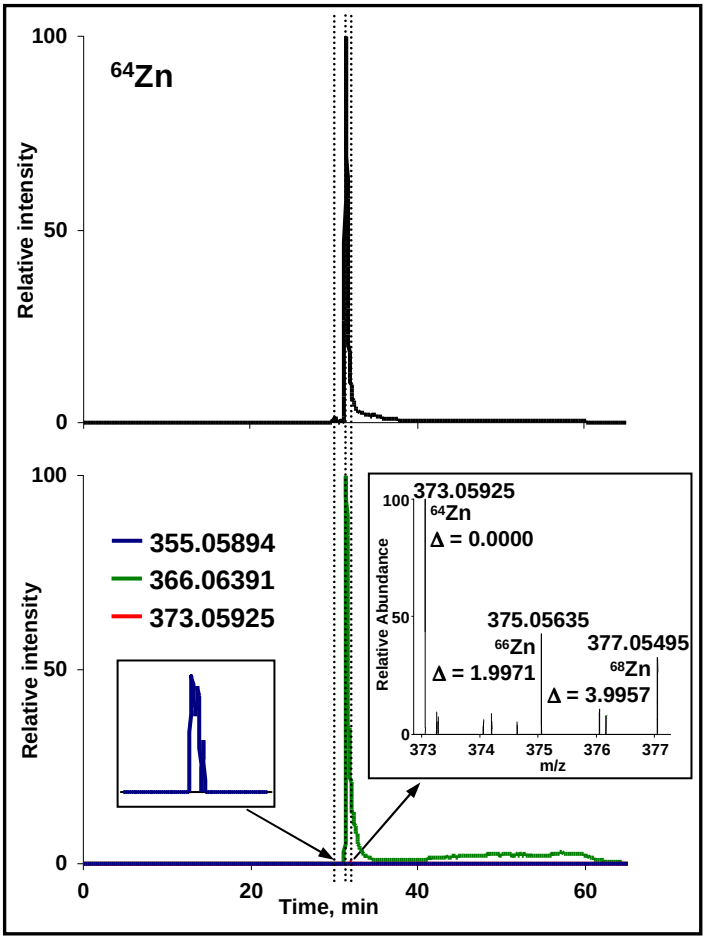
441.97903
359.05280
302.13510
C12H18O6N3Co
C12H20O6N3Co
Δ = 56.9177
364.04871
C12H18O6N3Zn
Δ = 4.9959
Relative Abundance
m/z
300
320
340
360
0
20
40
60
Time, min


24Mg
Relative intensity
100
50
0
287.08363
289.05183
315.11495
407.03063
315.11495
24Mg
Δ = 0.0000
316.11565
25Mg
Δ = 1.0007
317.11245
26Mg
Δ = 1.9975
Relative Abundance
m/z
315
316
317
0
20
40
60
Time, min


44Ca
Relative intensity
230.98132
297.11228
331.09231
361.09208
423.00822
293.14521
C10H21O6N4
331.09231
C10H19O6N4Ca
Δ = 0.0000
Δ = 37.9471
335.08541
44Ca
Δ = 3.9931
Relative Abundance
m/z
Time, min


55Mn
Relative intensity
318.03627
346.06799
437.98311
293.14539
C10H21O6N4
346.06799
C10H19O6N4Mn
Δ = 52.9226
355.05909
C10H19O6N4Zn
Δ = 8.9911
Relative Abundance
m/z
Time, min


64Zn
Relative intensity
355.05894
366.06391
373.05925
373.05925
64Zn
Δ = 0.0000
375.05635
66Zn
Δ = 1.9971
377.05495
68Zn
Δ = 3.9957
Relative Abundance
m/z
Time, min

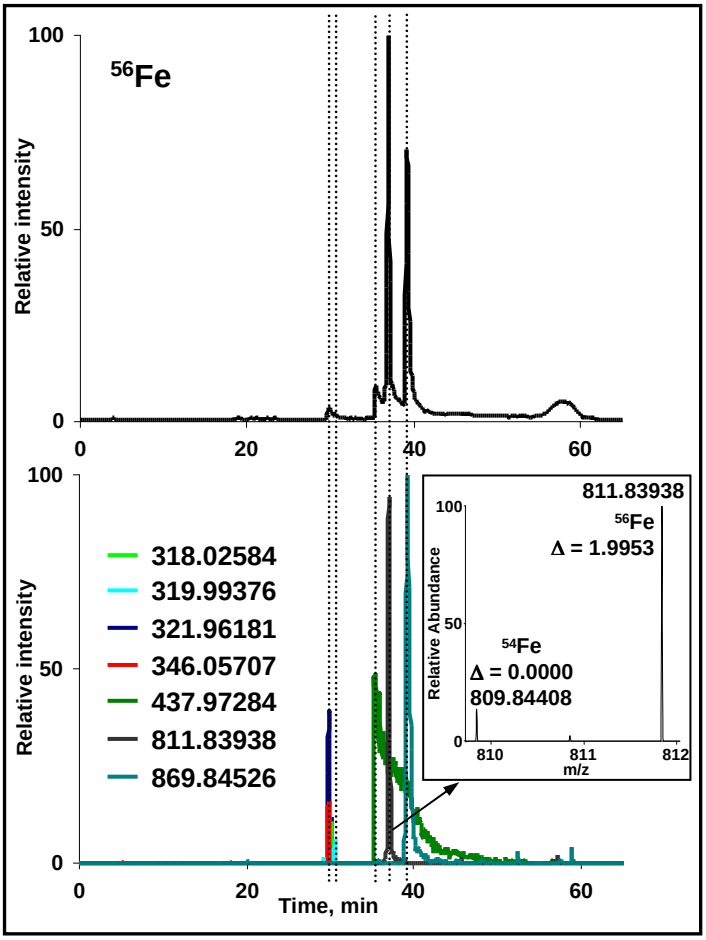
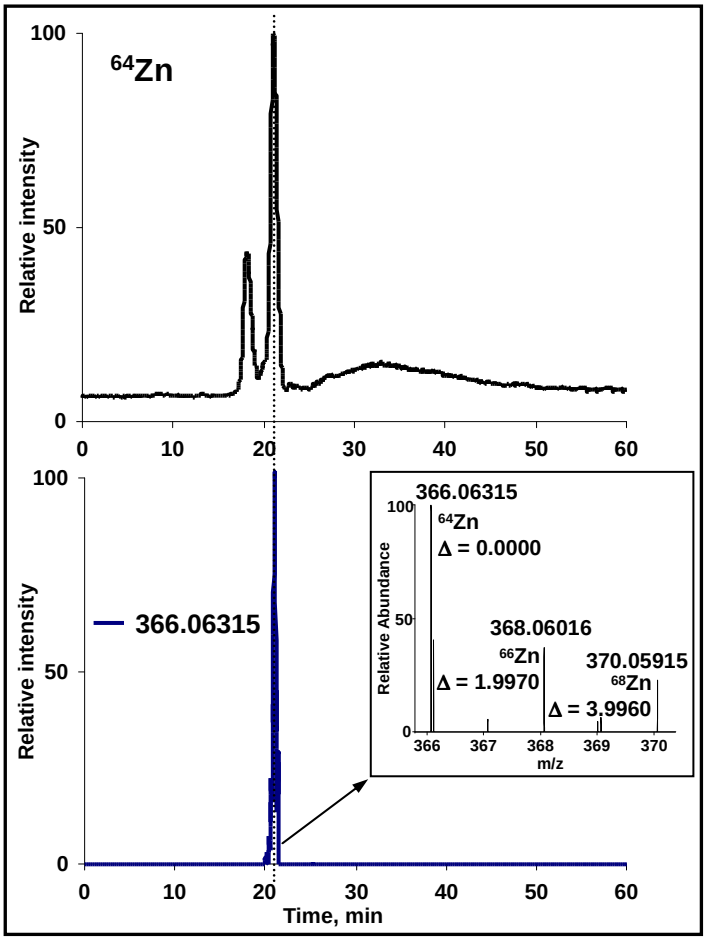

56Fe
Relative intensity
318.02584
319.99376
321.96181
346.05707
437.97284
811.83938
869.84526
811.83938
56Fe
Δ = 1.9953
54Fe
Δ = 0.0000
809.84408
Relative Abundance
m/z
Time, min

Figure 2


64Zn
Relative intensity
366.06315
366.06315
64Zn
Δ = 0.0000
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66Zn
Δ = 1.9970
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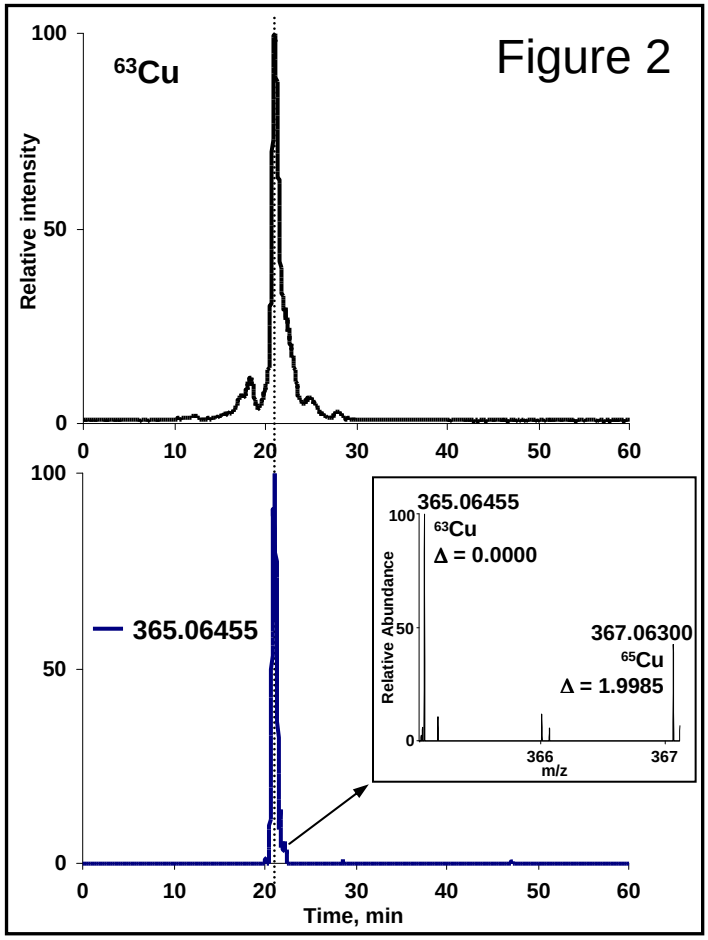
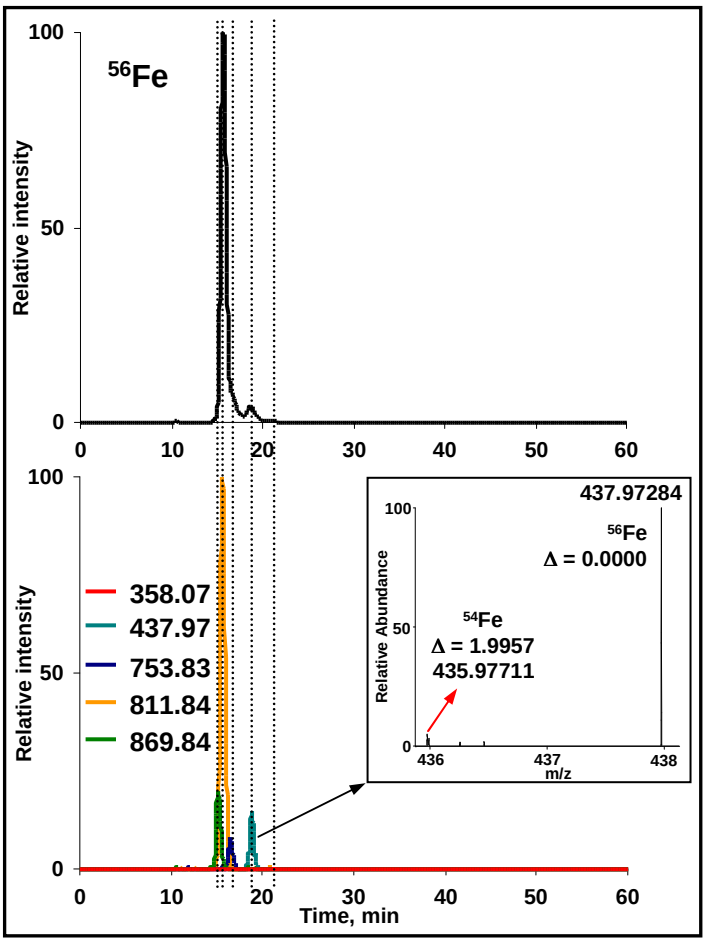
68Zn
Δ = 3.9960
Relative Abundance
m/z
Time, min


63Cu
Relative intensity
365.06455
365.06455
63Cu
Δ = 0.0000
367.06300
65Cu
Δ = 1.9985
Relative Abundance
m/z
Time, min


56Fe
Relative intensity
358.07
437.97
753.83
811.84
869.84
437.97284
56Fe
Δ = 0.0000
54Fe
Δ = 1.9957
435.97711
Relative Abundance
m/z
Time, min

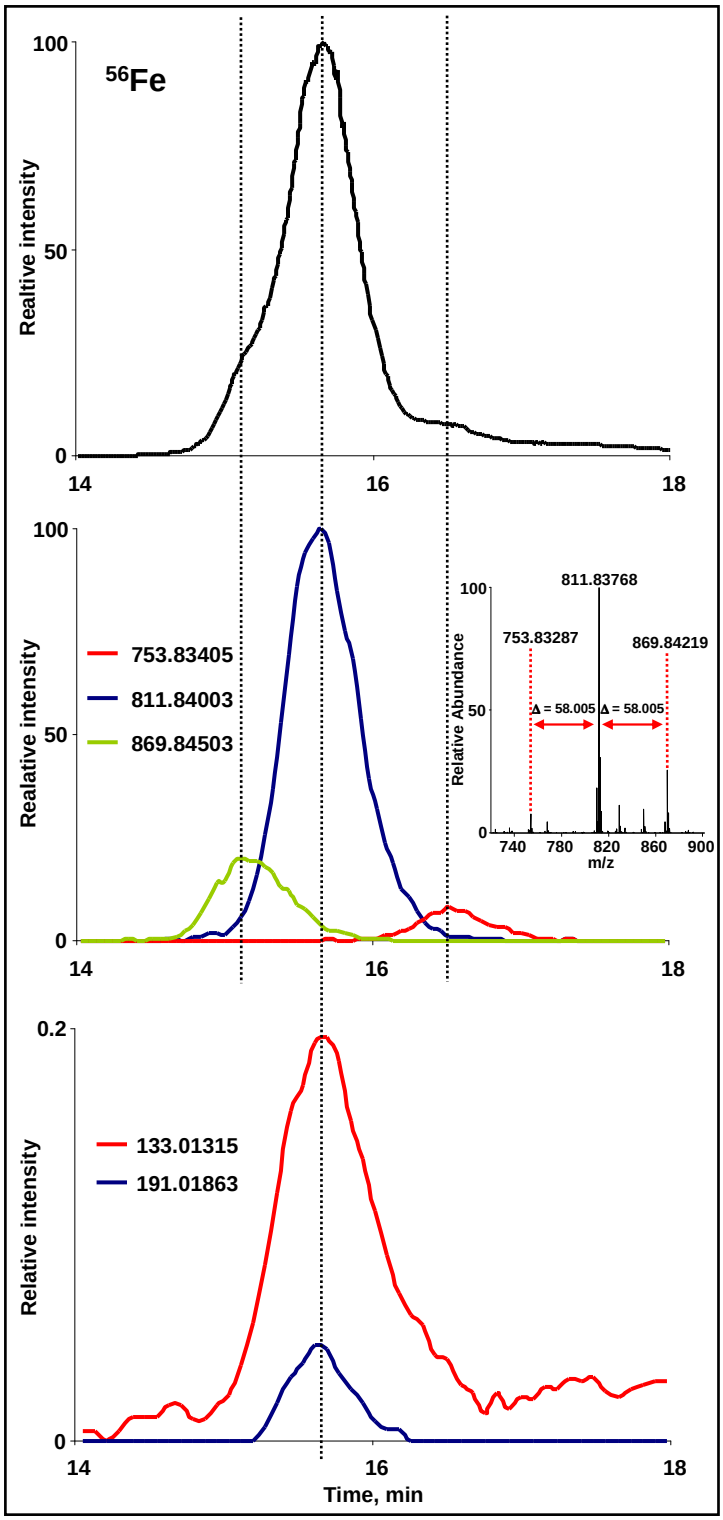

^{56}Fe
Realtive intensity
100
50
0
14
16
18
753.83405
811.84003
869.84503
Realative intensity
811.83768
753.83287
869.84219
Δ = 58.005
Δ = 58.005
Relative Abundance
740
780
820
860
900
m/z
133.01315
191.01863
Relative intensity
0.2
Time, min

Figure 3

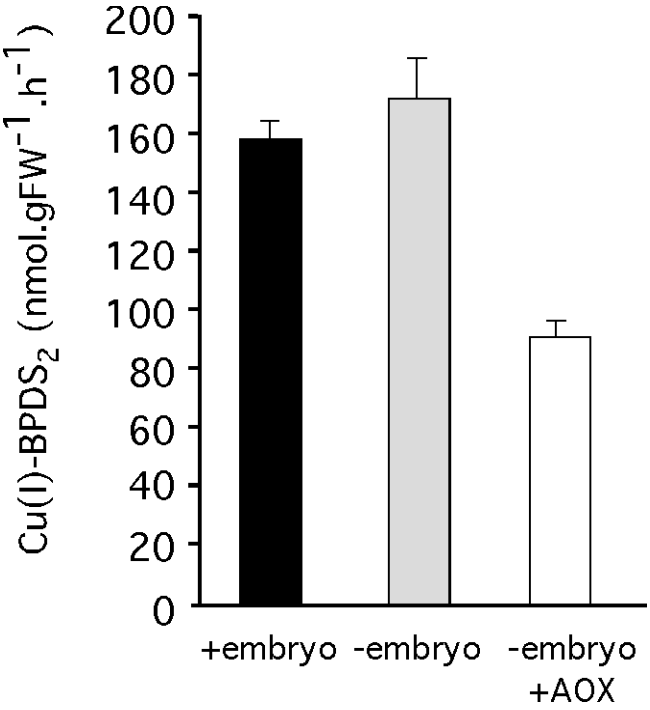

200
180
160
140
120
100
80
60
40
20
0
Cu(I)-BPDS2 (nmol.gFW-1.h-1)
+embryo
-embryo
-embryo
+AOX

Figure 4

Article II

Ascorbate efflux as a new strategy for iron reduction and transport within plants
(submitted)

Ascorbate efflux as a new strategy for iron reduction and transport in plants

Louis Grillet[a]*, Laurent Ouerdane[b]*, Paulina Flis[b], Marie-Pierre Isaure[b], Ryszard Lobinski[b], Catherine Curie[a], Stéphane Mari[a]

[a] Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, Institut de Biologie Intégrative des Plantes, Centre National de la Recherche Scientifique (UMR5004), Institut National de la Recherche Agronomique, Université Montpellier II, Ecole Nationale Supérieure d'Agronomie, F-34060 Montpellier Cedex 2, France. [b] Laboratoire de Chimie Analytique Bio-Inorganique et Environnement, Institut Pluridisciplinaire de Recherche sur l'Environnement et les Matériaux, Centre National de la Recherche Scientifique (UMR5254), Université de Pau et des Pays de l'Adour, F-64063 Pau Cedex 9, France

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Iron (Fe) is essential for virtually all living organisms. The identification of the chemical forms of Fe (the speciation) circulating in and between cells is crucial information to further analyze the mechanisms of Fe transport and delivery to its final targets. Here we have analyzed how iron is transported to the seeds, by the chemical identification of Fe complexes that are delivered to embryos, followed by the biochemical characterization of the transport of these complexes by the embryo, using pea (Pisum sativum) as a model species. We have found that Fe circulates as ferric complexes with citrate and malate (Fe(III)3Cit2Mal2, Fe(III)3Cit3Mal1, Fe(III)Cit2). Since dicotyledonous plants only transport ferrous iron, we have checked if embryos were capable of reducing these complexes. Indeed, to dissociate these complexes and take up Fe, embryos did express a constitutively high ferric reduction activity. Surprisingly, Fe(III) reduction is not catalyzed by the expected membrane-bound ferric chelate reductase, instead, embryos efflux high amounts of ascorbate that chemically reduce Fe(III) from citrate-malate complexes. In vitro transport experiments on isolated embryos using radiolabeled 55Fe demonstrated that this ascorbate-mediated reduction is an obligatory step for the uptake of Fe(II), at the embryo surface. Finally, the ascorbate efflux activity was also found in Arabidopsis embryos, suggesting that this new Fe transport system may be generic to dicotyledonous plants. Taken together, our results have identified a new Fe transport mechanism in plants that could play a major role in control of Fe loading in seeds.

iron | citrate | malate | ascorbate | seed

Introduction

Iron (Fe) is an essential micronutrient for plants, used as an enzymatic cofactor in a wide range of metabolic processes such as photosynthetic and respiratory electron transfer reactions, reduction of nitrate and sulfate, synthesis of fatty acids and branched amino acids. Iron was selected based on the redox capacity of the Fe^{2+}/Fe^{3+} couple that can exchange one electron. This chemical property is also responsible for the potential toxicity of iron in excess conditions since ferrous ions can promote, in the presence of oxygen, the generation of reactive oxygen species and oxidative stress. Plants, as sessile organisms, must maintain a strict iron balance to cope with deficiency or excess conditions. At the whole plant level this is achieved by the fine regulation of root absorption, allocation to the aerial parts, storage and remobilization.

Although very abundant in soils, Fe is often poorly available to plants since the main Fe form, Fe(III), has a very limited solubility and is prone to precipitate in alkaline and neutral conditions. Therefore, plants have developed strategies to efficiently acquire Fe. In roots of dicotyledonous species such as the model plant *Arabidopsis thaliana*, the uptake of Fe requires (*i*) the acidification of the rhizosphere by the proton pumping activity of the ATPase AHA2 (1) to release Fe^{3+} bound to humic substances and mineral phases, (*ii*) the reduction of ferric ions by the ferric chelate reductase encoded by FRO2 (2) and the subsequent uptake of Fe^{2+} by the metal transporter IRT1 (3-6).

Once in root cells, Fe atoms must reach the vascular system and enter the xylem vessels to be transported to the aerial parts. Xylem loading is controlled, at least in part, by the FRD3 protein that belongs to the Multidrug And Toxic compound Extrusion (MATE) family of transporters (7). FRD3 is a citrate effluxer expressed in root pericycle whose activity is required to solubilize iron in the xylem sap (8), where citrate was identified as the main Fe ligand (9), hence promoting Fe movement into the xylem. In leaves, unloading of Fe from xylem vessels and distribution in the surrounding cells also requires FRD3 (10). Likewise the OligoPeptide Transporter OPT3 (11) contributes to Fe movement in leaves but its substrate, whether it is a Fe ligand or a Fe complex, is still unknown (11). To date, the number of organic molecules shown to form complexes with Fe *in vivo* is extremely limited. Beside citrate, most of the information concerns nicotianamine (NA). This aminopropyl polymer, enzymatically synthesized from S-adenosylmethionine, has a high affinity for iron, copper and zinc. Several studies have shown that the chemical properties and the binding capacity of NA make it an ideal ligand for Fe in neutral, cytosolic, conditions whereas in acidic, apoplastic, conditions citrate will prevail (12, 13). *In planta*, NA is involved in the root-to-shoot transport of copper in tomato (14), and nickel and Zn in the hyperaccumulator species *Thlaspi caerulescens* (15, 16). For Ni, the formation of a complex between nicotianamine and nickel in xylem exudates was unambiguously

Significance

Seeds are a very important source of transition metals (Fe, Zn, Mn) for human nutrition. The understanding of how metals are transported into seeds is thus crucial to contend with human nutritional disorders. The added difficulty of metals in biology is that they are systematically bound to high-affinity ligands. The identification of these complexes is thus pivotal to understand how metals circulate in plants. Using pea seeds as a biochemical model, we have showed that Fe is delivered as ferric complexes with citrate and malate and we have discovered that ascorbic acid plays a key role for the reduction and transport of ferrous Fe in the embryo.

Reserved for Publication Footnotes

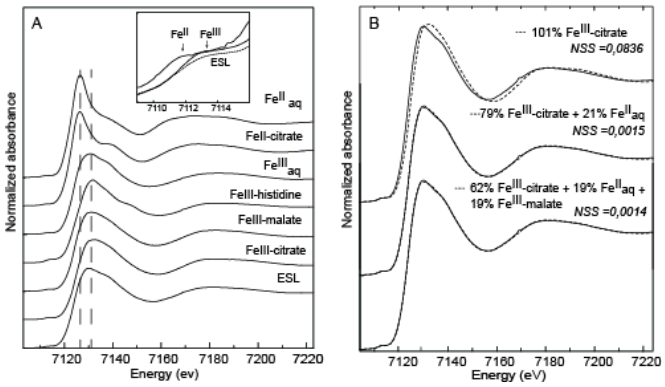


Fig. 1. : Fe K-edge XANES spectra of ESL and selected Fe model compounds (A) with the pre-edge structures of Fe^{II}_{aq}, Fe^{III}_{aq} and ESL (inset). One-, two-, and three-component fits (dotted lines) of ESL XANES spectrum (plain lines) (B). The quality of the fit was evaluated by the normalized sum-squares residuals, *NSS*=$\Sigma i(Xanes_{experimental}-Xanes_{fit})^2$ /$\Sigma i(Xanes_{experimental})$x100.

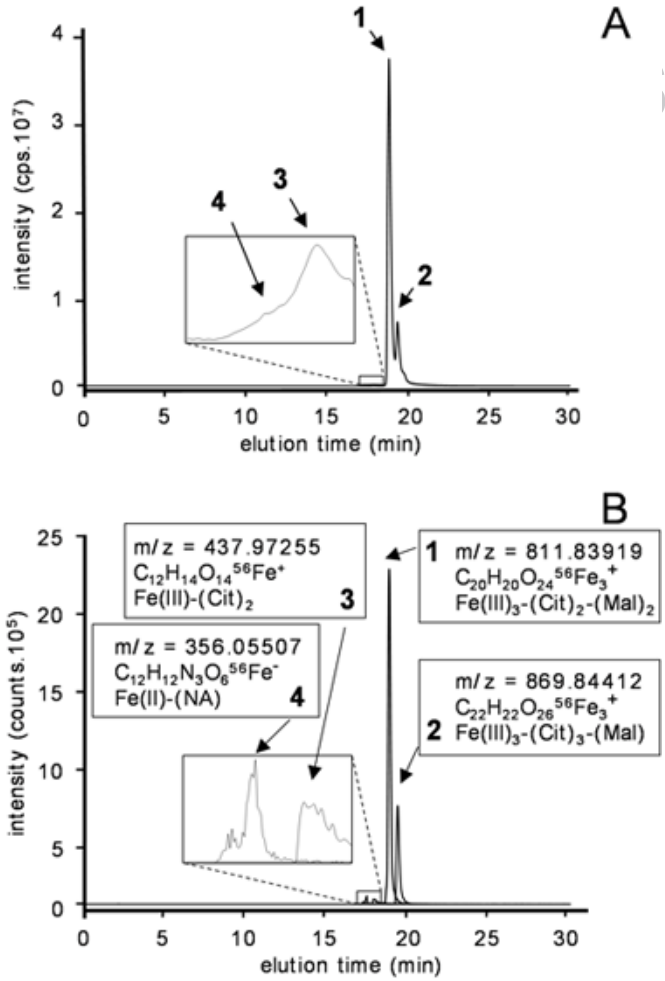


Fig. 2. : Analysis of Fe speciation in liquid endosperm by (A) HILIC ICP MS following ^{56}Fe and (B) HILIC ESI MS (in positive mode for compounds 1-3) following compound **1** at m/z 811.84, compound **2** at m/z 869.85, compound **3** at m/z 437.97 and compound **4** at m/z 356.05507 (in negative mode).

demonstrated by the coupling of chromatography and mass spectrometry (17, 18).

In *Arabidopsis thaliana*, several genetic approaches have allowed to pinpoint the role of NA in the transport of Fe. The

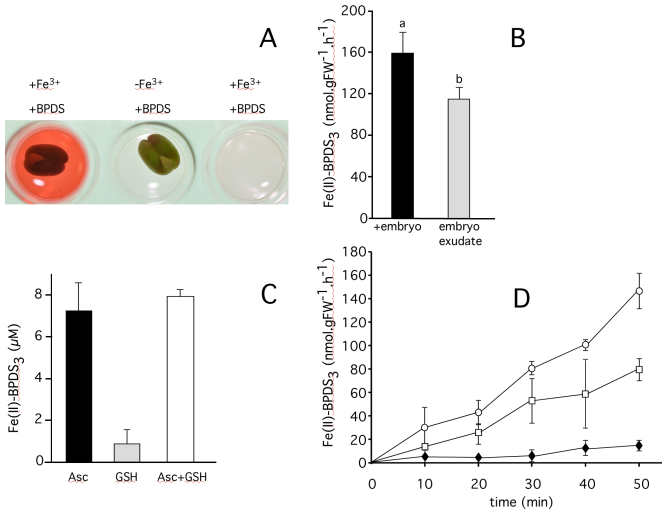


Fig. 3. : The ferric reduction activity of pea embryos is mediated by ascorbate efflux. (A) Isolated embryos were incubated for 2 h in a 5 mM MES buffer pH 5.5 with 100 µM Fe(III)EDTA and 300 µM BPDS (left well) and in a control medium without Fe(III)-EDTA (center well). In the right well, a complete assay medium without embryos was incubated in the same conditions. (B) Ferric reduction activity measured by BPDS method in presence of embryos (+embryo) or after removing embryos (embryo exudate). Results are means of 3 independent replicates ± standard deviation. Different letters indicate significant differences among means ($p < 0,05$ by Tukey's test). (C) *In vitro* reduction of ferric iron by ascorbic acid and glutathione. 100 µM Fe(III)-EDTA and 300µM BPDS were incubated with 5 µM ascorbic acid or 5 µM glutathione or both. OD_{535nm} was measured 30 minutes after addition of reductants. Results are means of 2 replicates ± SD. (D) Ferric reduction activity of pea embryos incubated in 5 mM MES pH 5.5 with 100 µM Fe(III)-EDTA and 300 µM BPDS without AOX (open circles), or in presence of 0.15 and 1.5 AOX Units .ml^{-1} (open squares and closed diamonds, respectively). Absorbance at 535 nm was measured every 10 minutes. Each curve has been built from means ± standard deviation of three independent experiments that consisted in measuring activities of 5 individual embryos for each treatment.

impairment of NA production, through the inactivation of all 4 NA synthase-encoding genes, provokes the expected phenotype of interveinal chlorosis described for the NA-free tomato mutant *chloronerva,* and, more interestingly, a reduced accumulation of Fe in seeds (19). Such an impact on seeds was also reported in mutants of the Yellow-Stripe1 Like (YSL) family of NA-metal transporters. The mutation in *AtYSL1* results in decreased Fe and NA accumulation in seeds (20), most likely caused by a reduced remobilization from old leaves, as shown for the Arabidopsis *ysl1ysl3* double mutant (21) (22). Taken together, these results indicate that disturbing long distance transport of iron has a major impact on the process of seed loading (reviewed in (23). Seeds represent the most important sink for plants and the end point of long distance circulation of nutrients (24, 25). Virtually nothing is known on the transport pathway that leads to iron loading in the seeds and on the chemical forms (*i.e.* the speciation) of Fe during this process. The speciation of a metal ion in a specific biological compartment is crucial information to further understand how the metal is kept soluble, transported across membranes and delivered to its final target.

In the work presented here, pea (*Pisum sativum*) was chosen as a biochemical model to study the mechanisms of Fe acquisition by the embryo. Grain legumes are particularly suited to study the processes of transport in the seeds. One specific feature of these seeds is that the bulk flow of nutrients delivered by the seed coat accumulates in large amounts, forming a liquid endosperm also termed Embryo Sac Liquid (ESL). This particular liquid is of high biological importance since it directly serves to feed the embryo (26). We have first analyzed the speciation of iron in

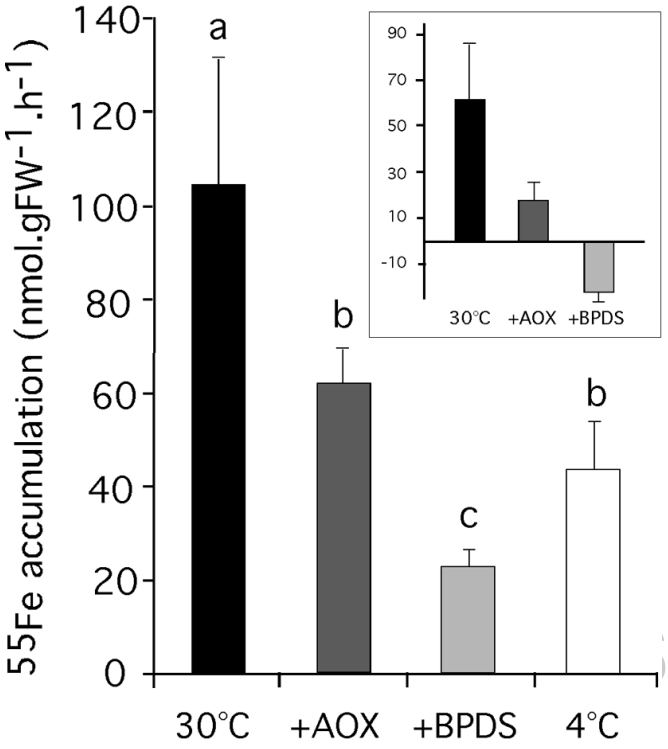


Fig. 4. Ascorbate-mediated ferric reduction is an obligatory step for Fe influx. ^{55}Fe influx was measured in isolated pea embryos. Embryos were incubated in uptake buffer containing 100 µM Fe(III)-EDTA and when stated 4 mM bathophenanthroline disulfonate (BPS) or 1.5 AOX Units. ml^{-1} (+AOX) were added. Incubations at 4 °C with the complete uptake medium were also realized to measure passive adsorption of Fe at the embryo surface. Insert, net influx of ^{55}Fe deduced by calculating the difference between Fe accumulation at 30 °C (complete, +AOX, +BPDS) and the 4 °C control incubation. Each measurement was performed on 4-5 embryos of 20-50mg, and experiments were repeated 3 times independently. Results are means ± SE (n = 3). Significant differences were determined with a non-parametric Kruskal-Wallis test ($p < 0,05$).

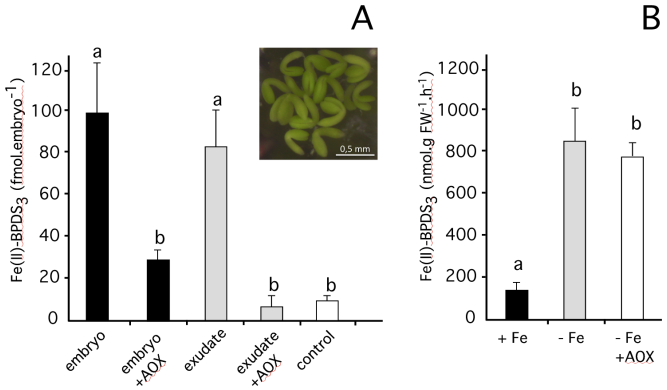


Fig. 5. The ascorbate efflux mechanism is conserved in *Arabidopsis thaliana* embryos. (A) Embryos at the bent torpedo stage were dissected and incubated to measure the ferric reduction activity, either with embryos in the medium or after removing the embryos to measure the activity on the exudates. When stated, AOX was added in the incubation medium. Results are means of 3 independent replicates ± standard deviation, each condition in an individual experiment was realized with 30 to 50 embryos. Different letters indicate significant differences among means ($p < 0,05$ by Tukey's test). (B) The root ferric chelate reductase activity is not inhibited by AOX. Ten day-old seedlings grown in 50 µM FeEDTA (+Fe) were transferred in a medium without Fe and supplemented with 30 µM ferroZine (-Fe) for 3 days and then assayed for ferric reductase activity. When stated AOX was added in the incubation medium. Values are means of 5 plants, different letters indicate significant differences among means ($p < 0,05$ by Tukey's test).

isolated ESL, using chromatography coupled to mass spectrometry and synchrotron radiation X-ray absorption spectroscopy. This information was crucial to further characterize the transport machinery that is expressed at the embryo surface to acquire Fe from maternal tissues.

Results

Fe speciation in the embryo sac liquid by XANES

The aim of these XANES spectroscopy measurements was to examine the redox status of Fe and the potential Fe complexes in the ESL with conditions minimizing the possible changes of Fe species during sample preparation and analyses. For that, the ESL sample was immediately frozen after collection, analyzed without any chemical additive and in frozen state to limit speciation change and beam radiation damage.

XANES spectra of Fe references show that the edge of Fe^{II} compounds is shifted to lower values compared to Fe^{III} compounds, as expected (Fig. 1*A*). Fe complexes involving malate and citrate, where the metal is coordinated by oxygen atoms from carboxyl groups, show similar spectral features but can be distinguished from Fe-histidine.

ESL spectrum edge mainly exhibits edge features of Fe^{III} compounds (Fig. 1*A*). However, the spectrum did not match with one Fe species only, and results of linear combination fitting showed that a mixture of 79% Fe^{III}-citrate and 21% Fe^{II}_{aq} gave a satisfactory fit (*NSS* = 0.0015, Fig. 1*B*). A correct match is also obtained for a combination of Fe^{III}-citrate and Fe^{II}-citrate (not shown, *NSS*= 0.0020) suggesting the presence of a minor Fe^{II} species, which was not clearly identified. Fits with Fe^{III}-malate instead of Fe^{III}-citrate gave poorer agreement (*NSS*=0,0022), and the improvement of the simulation when considered as a third component was not significant (*NSS* = 0,0014). We can thus infer that the major part of Fe is present as Fe^{III} in the ESL, probably as organic acids, while a minor Fe^{II} species can occur, but remains unidentified.

Fe speciation in the embryo sac liquid

The analysis of Fe speciation in the embryo sac liquid was performed by alternatively coupling hydrophilic liquid chromatography to two different mass spectrometry instruments, an ICP MS to determine the number of significant iron-containing species within this sample and quantify Fe and an ESI MS to identify the corresponding species (retention time matching), using the same chromatographic conditions. HILIC chromatography was recently established as an adequate technique to preserve and to separate metal complexes originating from raw samples allowing their identification and quantification by mass spectrometry (18). It was applied here to the liquid endosperm samples. Prior to analyses, iron recovery was tested during sample preparation (mixing with acetonitrile followed by centrifugation) and during chromatographic run. An overall 85 ± 8% of iron species were conserved after the two consecutive procedures, which meant that significant amounts of the original iron species were conserved by this method. After chromatographic coupling with ICP MS, two major Fe species as well as a minor Fe species were detected (Fig. 2*A*). By HILIC ESI MS in positive ion mode, these Fe-containing compounds were respectively identified as compound **1** at *m/z* 811.84, compound **2** at *m/z* 869.85 and compound **3** at *m/z* 437.97 (Fig. 2*B*). MS^2 fragmentation of compound **1** and **2** allowed to determine that organic molecules containing exclusively C, H and O with carboxylic acid groups were surrounding a nucleus constituted of 3 ions of Fe, which was consistent with the observed isotopic profile (Fig. S1). It was deduced that these two metal complexes were constituted of a mixture of citrate (Cit) and malate (Mal) binding three Fe(III)

ions. Each of these two complexes is then containing 4 organic acid molecules, 2 citrate molecules and 2 malate molecules in compound **1** ($Fe(III)_3Cit_2Mal_2$) and 3 citrate molecules and 1 malate molecule for compound **2** ($Fe(III)_3Cit_3Mal_1$). Compound **3** was found to be a complex between 2 citrate molecules and 1 Fe(III) ion ($Fe(III)Cit_2$). According to this, the chemical formula of each complex could be determined and experimental and theoretical masses could be compared: compound **1**, $C_{20}H_{20}O_{24}Fe_3^+$, m/z_{exp} 811.83919, m/z_{theo} 811.83873, δm = 0.6 ppm; compound **2**, $C_{22}H_{22}O_{26}Fe_3^+$, m/z_{exp} 869.84412, m/z_{theo} 869.84421, δm = -0.1 ppm; compound **3**, $C_{12}H_{14}O_{14}Fe^+$, m/z_{exp} 437.97255, m/z_{theo} 437.97275, δm = -0.5 ppm. The analysis of a standard mixture of Fe(III)-citrate-malate (0.2 : 1 : 5 ratios, buffered at pH 5.5 with ammonium acetate) definitively confirmed the formation of these Fe-citrate-malate complexes through the appearance of the above mentioned m/z (compound **1** and **2**) in MS spectra. Mixed complexes structures is unclear but is probably similar to $Fe(III)_3Cit_3$ complex structure as postulated by Fukushima *et al.* (2012), *i.e.*, a Fe_3 core is expected to occur with Fe ions bound to each other through α-hydroxy groups of organic acids and carboxylic groups are surrounding Fe ions. The absence of Fe_3Cit_3 complex in analyzed sample seems to indicate that the mixed Fe(III)-citrate-malate would be some even more stable species.

Traces of Fe(II)-nicotianamine (NA) complex (compound **4**, $C_{12}H_{18}N_3O_6Fe^-$, m/z_{exp} 356.05507, m/z_{theo} 356.05506, δm = 0.0 ppm) were also detected in negative ion mode (Fig. 2*B*) even if oxic (due to sampling and chromatography) and acidic (pH 5.5) conditions were not favorable to the presence of this form compared to Fe(III)-organic acids complexes (ref VonWiren 1999). Even if Fe(III) was preponderant Fe form in sample, Fe(III)NA could not be detected, which seemed to indicate that NA would have a relative higher affinity for Fe(II) than for Fe(III) in presence of citrate and malate, suggesting a differentiated speciation depending to Fe redox status.

Pea embryos are capable of ferric reduction through ascorbate efflux

Strategy I plants are not able to perform direct transport of ferric iron, but instead rely on a ferric reduction step prior to ferrous ion uptake. We therefore asked whether ferric chelate reduction was also part of the iron uptake machinery of the embryo. Embryonic ferric chelate reductase activity was assayed by incubating isolated immature pea embryos with the Fe^{2+}-chelator bathophenanthroline disulfonate (BPDS) in the presence or the absence of Fe^{3+}. With Fe^{3+} in the assay, the embryos were capable of generating Fe^{2+}, visualized by the formation of a pink Fe(II)-$BPDS_3$ complex (Fig. 3*A*). In contrast, no coloration was detected when Fe^{3+} was omitted in the incubation medium (Fig. 3*A* –Fe3+ +BPDS), thereby ruling out the possibility that production of Fe(II)-$BPDS_3$ complex is caused by the secretion of Fe^{2+} by the embryo. We therefore concluded that pea embryos are capable of reducing exogenous Fe^{3+}.

In order to check whether the ferric reduction activity was catalyzed by a FRO-like transmembrane reductase or mediated by the efflux of reductive compounds, we compared the ferric reduction activity measured with embryos in the medium to the activity of embryo exudates (*i.e.* by removing the embryos before measuring Fe reduction). Surprisingly, the reduction activity measured after removing the embryos was almost as high as the one obtained with embryos in the medium (Fig. 3*B*). This result indicated that, contrary to what had been described in roots, most of the reduction activity of pea embryos was due to the efflux of potent reductants in the medium.

Since the most obvious candidate reducing molecules in plants are ascorbic acid and glutathione, we then looked if these compounds were present in the embryo exudates, by electrospray ionization mass spectrometry (ESI-MS). As expected, ascorbate and glutathione could be detected by ESI-MS analysis of embryo exudates (Fig S2). To discriminate between these two molecules, we then tested their capacity to reduce ferric iron *in vitro*, in the conditions used with embryos (100 µM Fe^{3+}, 300µM BPDS). In these conditions, we could show that ascorbate, but not glutathione, was necessary and sufficient to spontaneously reduce iron *in vitro* (Fig. 3*C*). In order to confirm that the iron reducing molecule effluxed by embryos was ascorbate, we tested the effect of ascorbate oxidase (AOX) on the reducing activity of isolated embryos, over 50 minutes of incubation. The time course analysis of Fe reduction, linear for 50 minutes, was strongly inhibited by AOX, in a dose-dependent manner (Fig. 3*D*), confirming that ascorbic acid secreted by the embryos was responsible for the ferric reduction activity measured with isolated embryos.

Pea embryos take up ferrous iron

To assess the importance of ascorbate-mediated ferric reduction for embryo Fe uptake, we quantified the accumulation of the radioactive isotope ^{55}Fe, provided as Fe(III)-EDTA, into isolated embryos. To evaluate Fe^{2+} and Fe^{3+} uptake rates by the embryo, an uptake assay was set up by adding either AOX, in order to prevent the formation of Fe^{2+} due to ascorbate-mediated reduction, or BPDS, which is expected to scavenge newly formed Fe^{2+}. The rationale for this experiment was that if an ascorbate-mediated reduction step is required, then both BPDS and AOX should inhibit the uptake.

Untreated embryos accumulated 105 (± 27) nmol. g^{-1} FW.h^{-1} when incubated at 30°C, and 43 (± 11) nmol. g^{-1} FW.h^{-1} at 4°C. In the presence of AOX and BPDS, the Fe accumulation rates, not significantly different from the 4°C control, were respectively 62 (± 8) and 22 (± 4) nmol. g^{-1} FW. h^{-1} (Fig. 4). When expressed as net influx, by subtracting the Fe accumulation at 4°C, it appeared clearly that both BPDS and AOX significantly inhibited the uptake of Fe by isolated embryos (Fig. 4 insert). On the basis of these results it was concluded that ascorbate-mediated ferric reduction is an absolute requirement for iron uptake by pea embryos, thus uncovering a new role for ascorbate in plants.

Ascorbate efflux is not limited to pea

These new findings on pea embryos prompted us to check whether this ascorbate efflux activity was a general mechanism, found in other species such as the model plant *Arabidopsis thaliana*. Embryos were isolated (bent cotyledon stage) from developing siliques of Arabidopsis (Fig. 5*A*, insert) and Fe reduction activity was measured, as described previously for pea (see Fig. 3). Arabidopsis embryos were indeed capable of reducing Fe and this activity was significantly reduced by the addition of AOX in the incubation medium (Fig. 5*A*). This ferric reduction activity appeared to be fully attributable to ascorbate efflux since the rates obtained with embryos or with exudates (*i.e.,* after removal of embryos from the medium) were not significantly different. The addition of AOX to embryo exudates completely abolished Fe reduction, confirming the identity of ascorbic acid as the main reductant effluxed by Arabidopsis embryos (Fig. 5*A*). As a control for AOX specificity on the ascorbate-mediated reduction, we checked the effect of AOX on the well-established root ferric reductase activity mediated by the membrane protein FRO2. We thus assayed reductase activity of Arabidopsis roots isolated from Fe-sufficient and Fe-deficient plants (Fig. 5*B*). As previously shown, Fe-deficient roots exhibited higher ferric chelate reductase activity than Fe-replete roots (Fig. 5*B*). Interestingly, this activity was not significantly inhibited by AOX, indicating that ferric reduction in roots is biochemically different from that in embryos. Collectively, these data demonstrate that embryos acquire iron through a new strategy of Fe reduction and uptake, mediated by the efflux of ascorbate.

Discussion

In the present report, we have studied the mechanism of Fe entry into the seed through two approaches: a Fe speciation analysis at the interphase between seed coat and embryo in pea, associated with a biochemical dissection of Fe transport on isolated embryos of pea and Arabidopsis. We have established that (*i*) Fe is delivered by the maternal tissues as ferric complexes with citrate and malate, (*ii*) the embryos are capable of reducing the ferric ions of these complexes through the efflux of ascorbate and (*iii*) this reducing activity is an obligatory step for Fe transport as Fe(II). The analysis of iron speciation is by far the least documented aspect of Fe homeostasis, both in animals and plants. This is principally due to the difficulties of obtaining biologically reliable samples (*i.e.,* without mixing cellular compartments and creating artifact complexes) and to the low concentration of Fe in living tissues. To our knowledge, the Fe speciation analysis reported in the present study is one of the few unambiguous reports on the identification of iron complexes *in vivo*. Ferric-citrate complexes have long been proposed to occur in apoplastic compartments but only recently a speciation approach has led to the molecular identification of a tri iron(III) tri-citrate complex as the major Fe circulating form in the xylem of tomato plants (9). The embryo sac liquid (ESL) of pea seeds, although highly concentrated in nutrients (sugars, amino acids…) from symplastic origin (27), is characterized by an acidic pH (5.4), which is similar to the pH of an apoplastic compartment. Thus, our findings reinforce previous *in silico* (28) and *in vitro* studies (13), which demonstrated that in mildly acidic conditions ferric-citrate would be the most stable and abundant type of complex, despite the presence of other ligands like nicotianamine. Moreover, our data have identified malate as a new player in Fe speciation in plants. Although one of the metabolic responses to Fe deficiency is the accumulation of organic acids, including malate (29), a direct role of malic acid had never been clearly established. Instead, malate has been involved in the hyperaccumulation of nickel in *Thlaspi caerulescens* leaf extract (18) and *Alyssum serpyllifolium* with the formation of complexes with Ni in the xylem sap (30). Malate was also found to be also involved in the storage of Zn in the leaves of the hyperaccumulator *Arabidopsis halleri* (31). Malate is also a key molecule for the tolerance to aluminum, since it is excreted from roots by Al-activated efflux transporters (ALMT) to chelate aluminum in the rhizosphere (32-34). ALMT family members represent therefore putative candidate transporters, whose function could be to load malate in the embryo sac liquid. Belonging to the same MATE family of transporters as ALMT, FRD3 is a citrate efflux transporter that was recently proposed to play a critical role in the movement of iron between tissues that are not symplastically connected. A detailed analysis of the expression pattern of FRD3 and a phenotypical analysis of *frd3* knock-out mutants have demonstrated the importance of FRD3-mediated citrate efflux in Fe unloading in leaf (from xylem to adjacent cells) and in anther (from tapetum to pollen grains) (10). Interestingly, the seed coat and the embryo protodermis are also completely disconnected and FRD3 is highly expressed in both tissues in *Arabidopsis thaliana* (10). Thus citrate efflux by FRD3 could be important as well to load the ESL with citrate and subsequently induce the formation of iron-citrate complexes. More generally, our results strengthen the importance of citrate in the transport of Fe between cells and organs. Citrate may in fact be a ubiquitous and important intercellular iron ligand. Indeed, in mammals citrate is the main ligand of the non transferrin-bound iron (NTBI) species (35, 36), which appear to be the most abundant chemical form of Fe circulating in the extracellular medium of the brain,(37, 38) used as a source of iron for brain cells (39). Most of iron present in the liquid endosperm was found to be Fe^{3+} bound to a mixture of citrate and malate. It is the first observation *in vivo* of such mixed complexes. Compound **1** and **2** correspond to two iron complexes that most likely participate in binding Fe(III) to form a soluble, stable (contrary to the $Fe(III)Cit_2$ complex, for example) but low reactive species. Indeed, Fe(III) alone would precipitate leading to Fe deficiency whereas its presence in a weakly complexed form would probably lead to an increased stress for the plant because of expected frequent random exchanges of the Fe^{3+} ion with other non-specific binding sites in surrounding molecules. Therefore, these mixed complexes with organic acids probably participate in making Fe(III) available in the liquid endosperm for transport/reaction (such as reduction by ascorbate in the vicinity of the embryo) without inducing significant metal or oxidative stress.

The identification of Fe(II)-nicotianamine in the ESL represents the definitive proof of the *in vivo* role of nicotianamine in the movement of iron, although in our case the proportion of this complex is several orders of magnitude lower than the Fe bound to organic acids. Nevertheless, the presence of Fe-NA in the ESL could be interpreted as the signature of the activity of YSL transporters. The fact that Fe translocation to the seed is controlled in part by YSL transporters activity has been shown in Arabidopsis. The closely related members AtYSL1 and AtYSL3 have been involved in the loading of Fe, Zn and Cu to the seeds (20, 21). In particular, the mutation in *AtYSL1*, which is expressed in the chalazal endosperm of the seed, provokes a reduced accumulation of Fe and NA in mature seeds (20). Thus, the presence of Fe-NA complexes, the *bona fide* substrate of YSL transporters, in the ESL leads to the assumption that Fe is delivered from the seed coat to the ESL by YSL proteins.

Up to now, there was no direct functional link between the metabolism of ascorbate and Fe homeostasis. Instead, several reports had described the induction of ascorbate accumulation in response to Fe deficiency and proposed that it could represent a mechanism to cope with potential ROS production (40, 41). The most unexpected finding of this study was the discovery of ascorbate efflux as the mechanism to reduce and acquire Fe from the ferric complexes. Although other organic molecules such as phenolics can be excreted by roots of strategy I plants in response to Fe deficiency, these molecules are not capable of reducing Fe(III) in the medium (42). Furthermore, the Fe reduction activity had so far only been attributed to membrane proteins of the FRO family, such as the plasma membrane proteins FRO2, FRO4 and FRO5 (2, 43) and the chloroplastic protein FRO7 (44). In contrast, this report describes a new strategy of Fe acquisition, based on a reduction activity that is independent of the FRO proteins but relies instead on the property of ascorbate as a powerful reductant. This study thus establishes a direct role for ascorbate in Fe homeostasis, that is likely conserved in plants since it has also been observed in a non-legume species such as Arabidopsis (Fig. 5). Interestingly, a comparable Fe uptake mechanism has been proposed in mammals. Erythroleukemia K562 cells and astrocytes are capable of reducing Fe(III) using ascorbate efflux, generating Fe(II) and dehydroascorbate (DHA) that is reabsorbed to regenerate an ascorbate pool in the cytosol (45, 46). Transmembrane cycling of ascorbate coupled to ferrous transport by the divalent transporter DMT1 appears as one possible route for Fe accumulation in these cell types. Yet, in contrast to the constitutively high ascorbate efflux activity measured in embryos, ascorbate efflux was only triggered when cells were supplied with elevated DHA levels. The molecular identity of the efflux transport system remains unknown in both plants and mammals but the biochemical features of ascorbate efflux in mammalian K562 cells were compatible with the activity of an anion channel (45). Ascorbate efflux by pea embryos was insensitive to anion channel inhibitors (suppl fig) but required a pH gradient, suggesting that it could be mediated by an H^+/ascorbate antiporter. The importance of the proton motive force has already been suggested for the transport of nutrients in pea embryos

based on the specific expression in the outer cell layer of the cotyledons of genes encoding H^+/sucrose and H^+/amino acid co-transporters (47, 48).

Materials and methods

Plant material

Pea (*Pisum sativum* L., Dippes Gelbe Viktoria, DGV, cultivar) and *Arabidopsis thaliana* (cultivar Col-0) were used in this study. Plants were grown in a greenhouse 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, Geeste, Germany) irrigated with tap water. For root ferric chelate reductase experiments, *Arabidopsis thaliana* plants were cultivated *in vitro* on half strength Murashige/Skoog medium supplemented with 50 µM Fe-EDTA. After 10 days, plantlets were transferred in a MS medium without Fe and containing 30 µM FerroZin for 3 days.

Liquid endosperm sampling

Developing pods were dissected with a surgical blade. Two holes were pierced into each seed using a glass capillary tube. The liquid endosperm was then quickly extracted with another glass capillary using a peristaltic pump and gently blown at the bottom of an ependorff tube kept on ice. Pumping was carried out carefully in order to avoid bubbling. Samples were then maintained frozen in liquid nitrogen and kept at -80 °C until further analysis.

Fe speciation by X ray-absorption spectroscopy

See supporting text S1

Fe complexes by chemical analysis

See supporting text S1

Ferric reduction assays

Ferric-chelate reductase activity was estimated as the quantity of Fe-$BPDS_3$ complexes formed in the assay solution calculated from the optical density measured at 535 nm with a Hitachi U-2800 spectrophotometer. OD_{535} was measured after incubation in the dark at 22-25°C with 250 RPM shaking, in an assay solution that contained 5 mM MES buffer pH = 5.5, 300 µM bathophenanthroline disulfonate (BPDS) and 100 µM Fe(III)-EDTA unless otherwise stated. Pea embryos were dissected with a surgical blade as fast as possible, and kept in 5 mM MES buffer pH = 5.5 for 30 minutes, representing the time to dissect and weight around 15 embryos. Embryos were then incubated in the assay solution.

To measure root ferric-chelate reductase activity, whole root systems from individual Arabidopsis plants were excised, rinsed three times with demineralized water, and incubated in 50 ml assay solution following the same protocol. Comparison of embryo FCR activities has been repeated 5 times independently, and each experiment consisted in the incubation of 5 embryos in 5 ml assay solution with the usual protocol. Final results are means ± SD of the 5 values obtained.

Ferric reduction activity measurement of exudates

See supporting text S1

Time course of embryo Fe reduction

See supporting text S1

^{55}Fe accumulation in pea embryos

See supporting text S1

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Article III

Identification of the tri-Al tricitrate complex in *Plantago almogravensis* by hydrophilic interaction LC with parallel ICP-MS and electrospray Orbitrap MS/MS detection

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Identification of the tri-Al tricitrate complex in *Plantago almogravensis* by hydrophilic interaction LC with parallel ICP-MS and electrospray Orbitrap MS/MS detection†

Tomás Grevenstuk,[a] Paulina Flis,[b] Laurent Ouerdane,[b] Ryszard Lobinski[bc] and Anabela Romano*[a]

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The identification of the ligands binding Al is essential to understand the mechanisms by which plants detoxify Al internally. However, studies concerning the speciation of Al have been frustrated by its complex chemistry. This work describes the identification of the tri-Al tricitrate (Al_3cit_3) complex in *Plantago almogravensis*, encompassing an integrated mass spectrometry approach based on hydrophilic interaction liquid chromatography (HILIC) and parallel detection by ICP-MS and ESI-MS/MS. This work also reports that both Al and Fe are bound by tricitrate, sometimes simultaneously, and the consequences of this finding are discussed. Of the complexes separated by size exclusion chromatography, Al_3cit_3 is the most stable occurring in *P. almogravensis* as it was the only one recovered after HILIC. This approach provided new information on the mechanism of Al detoxification in *P. almogravensis*, namely that Al is bound by the organic acid citrate and that the relative concentration of the detected complexes is affected by the organ type and internal Al concentration, and has potential for studying the speciation of Al in less tolerant plants.

Introduction

Aluminium (Al) toxicity is one of the most serious limitations to plant growth in acidic soils. Most Al exists as oxides and aluminosilicates, which are harmless to plants; however, in acidic soils, Al is solubilized as the trivalent cation Al^{3+}. The precise targets at molecular and cellular levels remain elusive but it is known that free Al rapidly inhibits root growth and limits the subsequent uptake of water and nutrients.[1] The relevance of Al toxicity is aggravated by the fact that Al is the third most abundant element in the Earth's crust and that approximately 30% of the world's total ice-free land has a pH $<$ 5.5.[2] However, some plants are capable of performing despite taking up Al from soils. This occurrence is intriguing because even knowing that only a small percentage of Al is in its free state at physiological pH, such low concentrations can still be phytotoxic because of the strong affinity of Al for oxygen donor ligands.[3] *Plantago almogravensis* Franco thrives in soils with high free Al concentrations and its geographical distribution is restricted to a small area (10 ha) located at the SW of the Iberian Peninsula[4] and has thus been included in the UICN-Red List of critically endangered species.[5] *P. almogravensis* is known to accumulate Al,[4,6] however, the complexes that bind Al internally and thus protect vital biochemical processes are unknown.

Even if, to date, ^{27}Al nuclear magnetic resonance (NMR) spectroscopy has been used almost exclusively to study the speciation of Al in plants, as it can provide structural information about Al-complexes directly from intact samples, the inherent low sensitivity and low discriminating capacity of NMR spectroscopy for Al is a drawback that has prevented accurate speciation.[7] Mass spectrometry using electrospray ionization (ESI-MS) is a technique that offers increased sensitivity and great potential for identification of metal complexes but has found little application in the field of Al-speciation due to the lack of a suitable isotope. Because of the gentle transition from solution to the gas phase and the low collision energies, the dissociation of metal complexes is less likely and singly-charged

[a] IBB-CGB, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. E-mail: aromano@ualg.pt
[b] CNRS/UPPA, Laboratoire de Chimie Analytique Bio-inorganique et Environnement, UMR 5254, Helioparc, 2, Av. Pr. Angot, 64053 Pau, France
[c] Chair of Analytical Chemistry, Warsaw University of Technology, 00-664 Warszawa, Poland

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species with high mass resolution are generally produced, yielding simple mass spectra that facilitate assignment.[8] The lack of an isotope to aid with localization of Al-bearing complexes in mass spectra can be circumvented by parallel detection with an elemental detection technique, such as inductively coupled plasma (ICP)-MS, which allows determining the retention times of Al-complexes and pinpointing the region of the MS chromatogram that should be examined. Biological samples are too complex for direct infusion or flow injection, so chromatographic separation prior to ESI-MS measurements is required. Chromatography can pose a hurdle to the identification of Al-complexes because they can be dissociated as a result of the interaction with the stationary phase and the buffer content of the mobile phase can suppress ionization efficiency. These interactions are minimized when using size-exclusion chromatography (SEC) columns and hydrophilic interaction chromatography (HILIC) columns (that allow proper retention of highly polar complexes) and their corresponding buffers are compatible with ESI-MS.

The goal of this work is to study the speciation of Al in *P. almogravensis* plants to determine the ligands responsible for detoxifying Al using an MS-based approach. A previously established experimental design using *in vitro* produced plants[6a] was employed to eliminate extraneous factors and determine the intrinsic detoxification capacity of *P. almogravensis* without compromising natural populations.

Experimental

Reagents and standards

Analytical reagent grade chemicals such as acetonitrile, formic acid, acetic acid, nitric acid, ammonium acetate and ammoniac were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), metal standards from SCP Science (Canada) and citric acid from Acros Organics (Belgium). Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q system (Millipore, Bedford, MA).

Plant material and Al-treatment

Shoots of a selected *P. almogravensis* genotype were separated from *in vitro* axenic cultures proliferating for 6 weeks in Murashige and Skoog's medium[9] supplemented with 6-benzyladenine at 0.2 mg L^{-1}.[10] The obtained shoots were transferred to Murashige and Skoog's medium with macronutrients reduced to half containing 0.5 mg L^{-1} indole-3-acetic acid to induce root development. The produced plantlets were transferred aseptically to autoclaved Murashige and Skoog's liquid medium with macronutrients reduced to one quarter at pH 4.0 supplemented with 100, 200 or 400 μM Al for a 7-day treatment according to Martins *et al.*[6a] The chemical speciation program Geochem-EZ (http://www.plantmineralnutrition.net/Geochem/geochem%20home.htm)[11] was used to predict the Al^{3+} and Fe^{3+} activity values and Al and Fe speciation for each nutrient solution based on chemical equilibrium constants. In order to study the role of roots in the formation of Al-complexes, shoots without developed root system were also subjected to Al-treatment. The cultures were maintained at 25 ± 2 °C with a 16 h photoperiod (cool white fluorescent lamps, 69 μmol m^{-2} s^{-1}). Samples of fresh leaves, roots and shoots (~20 mg) were placed in eppendorfs, frozen in liquid nitrogen and ground with a glass rod. Extraction was carried out by adding 100 μL of water and mechanically stirring for 2 min at room temperature followed by centrifugation (3 min, 14 500 rpm). The supernatants were collected and immediately analysed. Apart from biological samples, standard solutions of Al–citric and –oxalic acid (3 : 1 ratio of organic acid to Al, buffered at pH 6) at an Al concentration approximate to that of roots were prepared for speciation analysis.

SEC-ICP-MS experiments

The SEC separations were conducted with a Dionex UltiMate 3000 LC-system (Thermo Fisher Scientific, Bremen, Germany) coupled to a 7500cs ICP-MS instrument (Agilent) equipped with a collision cell with hydrogen as collision gas. The interface between the LC system and the ICP-MS detector consisted of a quartz double pass Scott style spray chamber, a MicroMist EzyFit nebulizer (Glass Expansion, Australia), a 2.5 mm i.d. quartz torch and a set of nickel cones (Agilent Technologies, USA). The undiluted plant extracts and standard solutions (10 μL) were injected onto a Superdex Peptide HR 10/30 (300 × 10 mm i.d.; GE Healthcare Bio-Science AB, Sweden) SEC column using 5 mM ammonium acetate buffer at pH 6.2 as a mobile phase at a flow rate of 0.7 mL min^{-1}. The ICP-MS conditions were optimized daily for highest intensities and lowest interferences using a standard built-in software procedure. The H_2 collision cell mode was used to exclude polyatomic interferences that may occur for Fe and Al isotopes.

HILIC-ICP-MS/ESI-MS/MS experiments

The HILIC separations were performed using an Agilent 1100 capillary pump (Agilent, Tokyo, Japan) as a delivery system equipped with a 100 μL min^{-1} splitter module. The HILIC column was a TSK gel amide 80 (250 × 1 mm i.d.) (Tosoh Bioscience, Germany). The binary mobile phase consisted of (A) 10 mmol L^{-1} ammonium formate buffer at pH 5.5 and (B) acetonitrile. The optimized HILIC gradient started with 10% of A and was increased to 50% of A in 45 min. Samples were diluted with acetonitrile to obtain a 1 to 2 ratio (sample : acetonitrile), centrifuged and 7 μL of the supernatant was analyzed at the flow rate of 50 μL min^{-1}. The HPLC unit was coupled either to an ICP-MS or an ESI-MS/MS instrument. The interface between the LC module and the ICP-MS detector consisted of a glass Cinnabar cyclonic spray chamber (Glass Expansion, Australia), a 50 μL min^{-1} Micromist U-series nebulizer (Glass Expansion, Australia), a 1 mm i.d. injector torch (Agilent Technologies, Japan), a T-connector allowing the introduction of 11% O_2 and a set of platinum cones (Agilent Technologies, USA). The ICP MS conditions were optimized in the same way as for SEC-ICP-MS measurements. The ESI-MS/MS instrument was an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (H-ESI II) (Thermo Fisher Scientific).

The instrument was operated in the positive ion mode with the electrospray voltage set at 3 kV. The source and capillary temperature were 120 °C and 280 °C, respectively. The ESI-MS spectra were obtained in the 150–1200 m/z range with the resolution set at 100 000. The ions were fragmented by collision-induced dissociation (CID) at the energy level of 45%.

Total metal concentration determination

Triplicates of dried leaf and shoot samples were ground with a pestle and mortar, digested in 70% nitric acid for 6 h at 65 °C and then diluted before analysis 10 times with 2% HNO_3. The acidified sample and standard solution of metallic elements were analysed by ICP-MS (Agilent 7500ce, Tokyo, Japan).

Results

Analysis of *P. almogravensis* samples exposed to Al by SEC-ICP-MS allowed for the separation of five Al species numbered according to elution time (Fig. 1). The same complexes were observed in root and leaf samples of plants grown under controlled conditions exposed to 400 μM Al, albeit at lower concentrations in leaves (Fig. 1A). However, when plantlets with excised root systems (shoots) were subjected to the same concentration of Al, the complexes 1 to 4 could not be observed. Instead, a poorly resolved broad signal was detected at longer elution times. Complexes 1 to 4 were detected in plantlets exposed to the lowest concentration of Al (100 μM) as well, however variations in their relative concentrations were observed between plants exposed to 100 μM and 400 μM of Al (Fig. 1A and B). Monitoring of other elements during SEC-ICP-MS measurements allowed determining that Fe co-elutes with the detected Al complexes 1 to 4 and that phosphor co-elutes with the complex detected in shoots (Fig. 1C and D; respectively). Oxalate is a well-described ligand for Al in accumulating plants,[12] therefore an Al–oxalic acid standard solution was analyzed by SEC-ICP-MS. However, the elution time of the detected complex did not match any of the previously detected complexes, indicating that oxalate is not a major ligand for Al in *P. almogravensis* (Fig. S1A in the ESI†).

High-resolution MS experiments were employed in an attempt to identify the Al-complexes occurring in *P. almogravensis*. Because the chromatographic separation provided by SEC is not sufficient to obtain precise MS measurements, the separation was conducted by HILIC. The Al species identified so far in accumulating plants are complexes with organic acids. HILIC is an adequate method for the separation of these complexes considering that the resins guarantee a strong retention of highly polar complexes and the corresponding buffers are compatible with ESI-MS.[13] However, our results show that although a better resolved signal was obtained, as opposed to SEC, only one sharp Al peak could be detected after HILIC separation (Fig. 2). This result can be explained by the fact that the other minor complexes are eluting in less defined peaks because of a probably lower stability of these forms and higher interaction with chromatographic material.[8]

The obtained HILIC-ICP-MS chromatogram indicates the approximate retention time of the detected Al-complex, however,

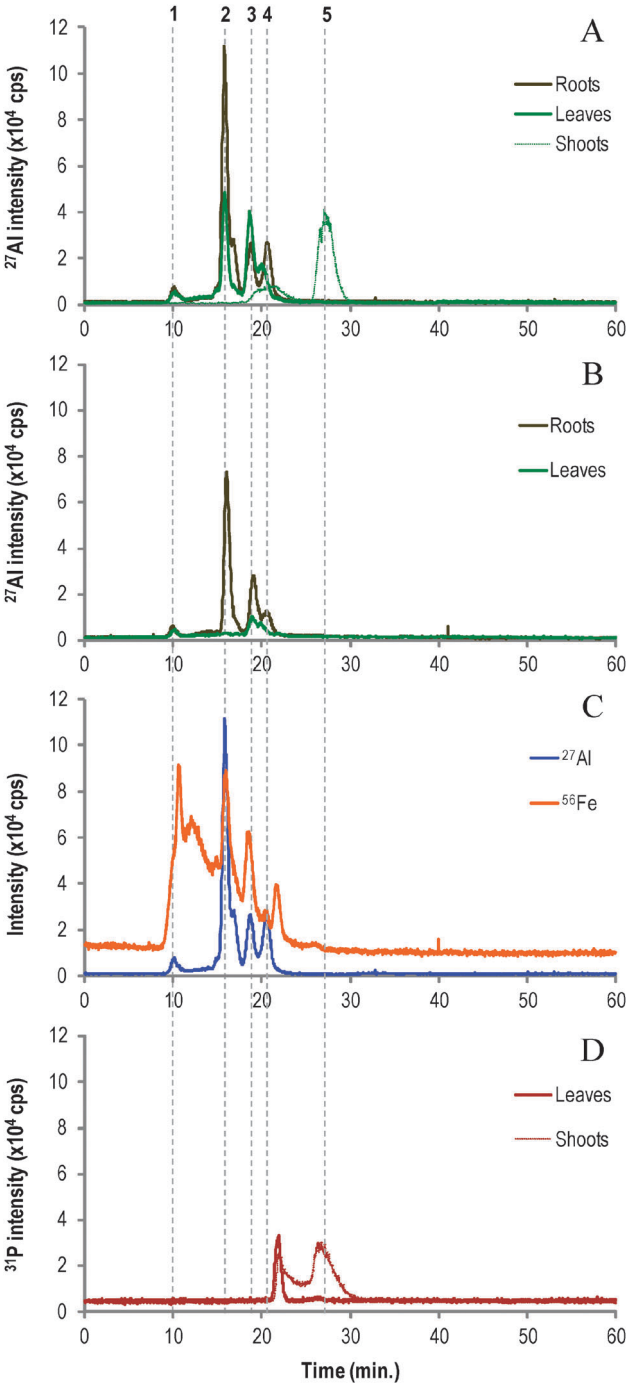

Fig. 1 SEC-ICP-MS chromatograms of *P. almogravensis* plants cultured in liquid Murashige and Shoog medium supplemented with Al: ^{27}Al signals detected in root, leaf and shoot samples exposed to 400 μM Al (A); root and leaf samples exposed to 100 μM Al (B); ^{56}Fe and ^{27}Al signals detected in the root sample exposed to 400 μM Al (C); and the ^{31}P signal detected in leaves and shoot samples exposed to 400 μM Al (D).

because Al is a mono-isotopic element it is not possible to examine the mass spectra obtained by HILIC-ESI-MS for molecular ions containing metals with specific isotopic signatures, as is the case for nickel[13] and cadmium complexes[14] for instance. A multitude of molecular ions is typically observed for

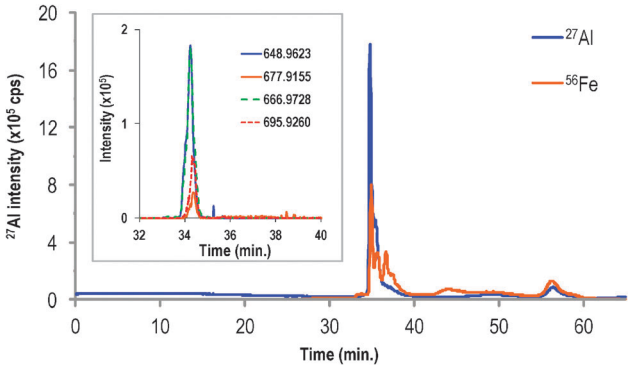

Fig. 2 ^{27}Al HILIC-ICP-MS chromatogram of the *P. almogravensis* root sample exposed to 400 μM Al. Inset depicts the extracted ion chromatogram of *m*/*z* 648.96, 666.97, 677.92 and 695.93 ions obtained by HILIC-ESI-MS.

each specific retention time and it is therefore unfeasible to identify *de novo* an Al-bearing complex without additional information. This is one of the limitations when pursuing the identification of metal complexes of mono-isotopic elements, especially in samples for which no prior information is available. In our case, because the Al-complex detected after HILIC also co-elutes with Fe (Fig. 2), the MS spectra were examined for molecular ions bearing the characteristic isotopic pattern of Fe at the retention time of the detected Al-complexes (Fig. 3). Two molecular ions with exact masses of *m*/*z* 677.92 and 695.93 were identified following this approach. Two other ions were observed at the same retention time with an exact mass difference of Δ 28.95 *m*/*z*, which corresponds to the difference in atomic weight between ^{56}Fe and ^{27}Al. This result indicates that the *m*/*z* 648.96 and 666.97 molecular ions bear at least one Al atom and that it is interchangeable with Fe. Exact mass determination indicated that the *m*/*z* 648.96 and 666.97 molecular ions are consistent with a tri-Al tricitrate (Al_3cit_3) and an Al_3cit_3 + H_2O complex, respectively. The assignment of these complexes was confirmed by analyzing a standard solution of Al and citric acid under the same conditions (Fig. S2 in the ESI†). This allowed for the assignment of the molecular ions at *m*/*z* 677.92 and 695.93 as Al_2Fecit_3 and Al_2Fecit_3 + H_2O (Fig. 3). Interestingly, the molecular ions corresponding to the $AlFe_2cit_3$ and Fe_3cit_3 complexes could also be detected at lower intensities (Fig. S3 in the ESI†). An extracted ion chromatogram indicated that the identified tricitrate complexes have the same retention time as the Al-complex detected by HILIC-ICP-MS (inset Fig. 2). To confirm the role of citric acid in Al complexation, a citric acid–Al standard solution was analyzed by SEC-ICP-MS. The resulting chromatogram shows a very good match between the complexes 2, 3 and 4 from root extract and the Al–citric acid standard solution (Fig. 1A and Fig. S1B in the ESI†) indicating that they are different forms of Al–citrate complexes, with Al_3cit_3 (peak 2) corresponding to the major peak as this complex was identified as the main Al complex by HILIC ESI MS. Regarding the remaining Al signals

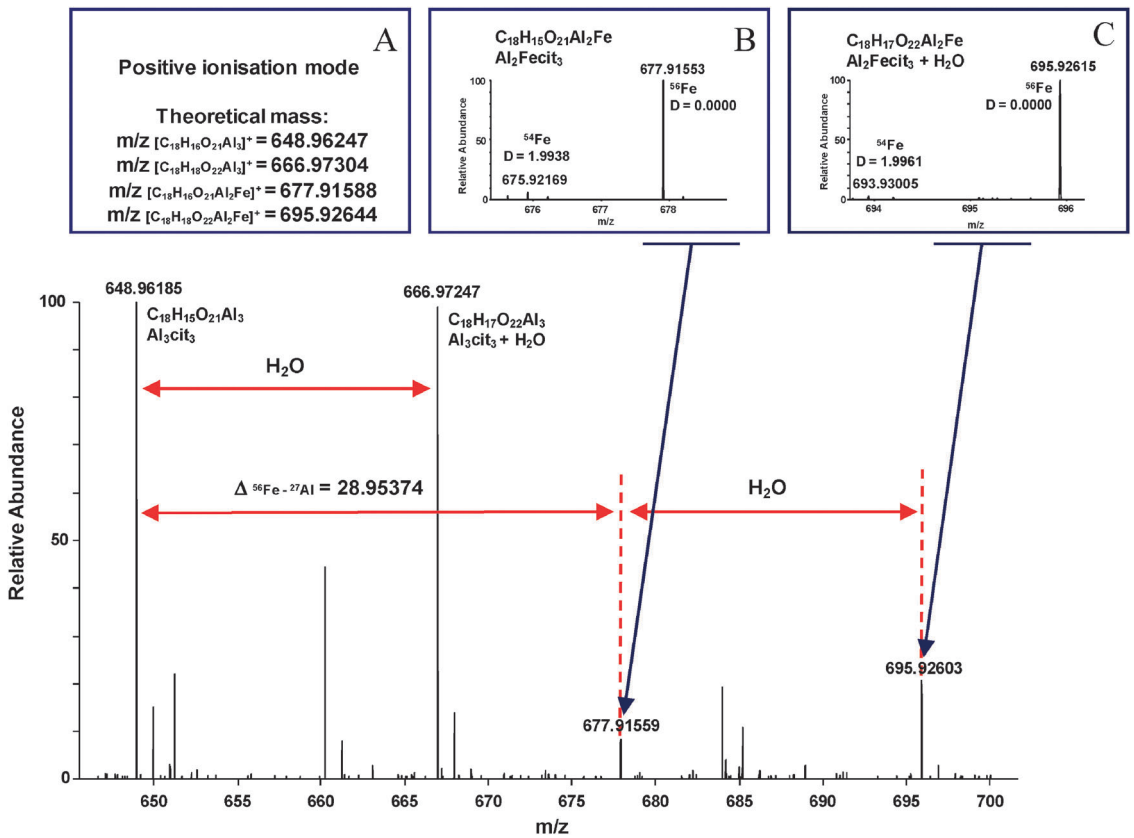

Fig. 3 ESI mass spectrum of the *P. almogravensis* root extract at a retention time of 34.7 min. The insets depict the theoretical masses of identified Al and Fe complexes in positive ionization mode (A) and ions with a characteristic isotopic signature of iron (B and C).

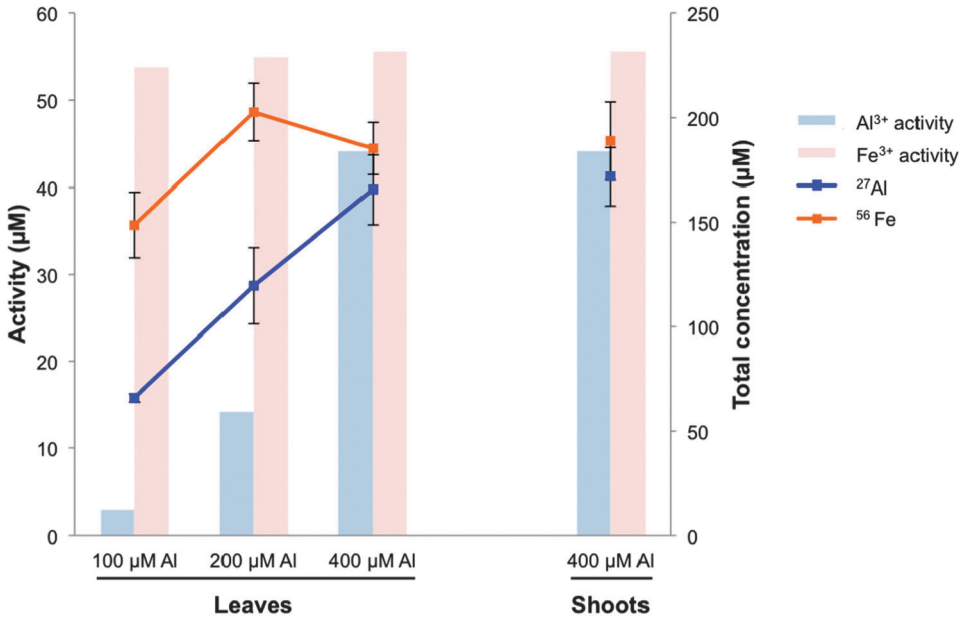

Fig. 4 Total Al and Fe concentration determination in leaves of *P. almogravensis* exposed to Al and calculated Al^{3+} and Fe^{3+} activity in the respective culture solution. Activity values were predicted using the Geochem-EZ speciation program based on chemical equilibrium constants.

detected in Fig. 1, peak 1 corresponds to the void due the Al entrapped with high molecular weight biomolecules and nanoparticles while peak 5 (detected exclusively in shoots) is likely to correspond to Al phosphate (matching peak shapes and migration times between Fig. 1A and D).

The total concentration of Al and Fe was determined in leaf samples and plotted together with the calculated Al and Fe activity in the culture media (Fig. 4). According to Parker *et al.*,[15] the activity of a free uncomplexed metal is in most cases the most relevant parameter to determine plant responses to changes in nutrient solution composition. The low pH and reduced macronutrients concentration in culture media allowed obtaining a significant amount of free Al in solution (34.6%, Table 1) because less Al is bound to SO_4 and EDTA. Our results also demonstrate that the concentration of Al found in plantlet leaves is two orders of magnitude higher than the Al^{3+} activity in culture solution (Fig. 4), indicating that *P. almogravensis* is able to concentrate Al in its upper tissues. Also, the determination of total Al indicates that its uptake is concentration-dependent and that the uptake capacity has not reached saturation at 400 μM Al (Fig. 4).

Table 1 Speciation of Al and Fe in Murashige and Skoog medium with macronutrients reduced to one quarter supplemented with 400 μM Al at pH 4.0. Values were calculated using Geochem-EZ and are presented as a percentage of the total Al and Fe present in solution

Species	Al^{3+} (%)	Fe^{3+} (%)
Free	34.60	92.38
Complexed with:		
PO_4	27.67	3.96
EDTA	23.16	0.15
SO_4	12.18	2.64
OH^-	1.94	0

Discussion

Identification of a tri-Al tricitrate complex following a mass spectrometry based approach

This work reports the first direct and unequivocal identification of an Al-complex in an accumulating plant using ESI-MS without prior information. Al speciation studies in plants have been frustrated by the technical constraints limiting the separation and identification of Al complexes.[16] The complex aqueous coordination chemistry of Al, the lack of a suitable multi-isotopic profile and the typical instability of metal complexes that renders concentration procedures unfeasible have slowed the understanding of the physiology of Al detoxification in tolerant plants. Most studies have been based on ^{27}Al NMR spectroscopy with a consequence that only plants accumulating extremely high concentrations could be examined. A 1 : 1 complex of Al and citrate has been detected in the xylem of several Al accumulating plants by ^{27}Al NMR spectroscopy.[17] ESI-MS experiments confirmed that in *Hydrangea macrophylla* cell sap an Al–citrate complex (*m*/*z* 215) occurs.[18] In this work we report that Al can be complexed by a tricitrate ligand. High-resolution MS experiments rendered a molecular ion at 648.96 *m*/*z* after separation by hydrophilic interaction LC. Exact mass determination and comparison with an Al-citrate standard solution allowed assigning the complex as tri-Al tricitrate. This complex is the most stable one occurring in *P. almogravensis* as it remained intact after both HILIC and SEC separation. The structure of a tri-Al tricitrate complex obtained by chemical synthesis has been characterized by NMR spectroscopy and X-ray crystallography[19] and a stability constant of 16.3 was determined by potentiometric studies,[20] which indicates that it is a quite stable complex. The results obtained for fragmentation of the *m*/*z* 648.96 molecular ion after CID-MS experiments

support to some extent that Al_3cit_3 has the same structure, *i.e.*, the loss of one citrate moiety could be observed indicating that the three Al atoms maintained in the central core were bound by the remaining two citrate molecules (Fig. S4 in the ESI†). The absence of observed loss of aluminum ions during fragmentation indicates that the Al_3 core is well buried into the complex as expected according to its structure. The knowledge that tricitrate is able to bind Al will allow determining parameters that are relevant to predict the fate of this complex *in planta*. Determination of stability constants and steric effects is important to predict to what extent the ligand prevents Al from binding to essential biomolecules, potential for membrane permeation and transport across the tonoplast that will determine the site of sequestration. Further investigation will have to be conducted to indicate the function of the tricitrate ligand regarding Al detoxification in *P. almogravensis*. It will be interesting to determine if the complex is used for transport only or if it can also be detected in intracellular spaces. *P. almogravensis* is a small woody hemicryptophyte that grows without a distinct stem,[21] so it will be unfeasible to isolate xylem and conversely, xylem will always be analysed when taking the leaves as a whole. In order to determine if the complex is found in xylem alone, separate analysis of cellular structures (protoplasts and vacuoles) will be required. The best studied Al-accumulating plants so far (*Fagopyrum esculentum*, *Camellia Sinensis* and *Melastoma malabathricum*) and *P. almogravensis* are considerably distinct from a taxonomical perspective, yet the involvement of organic acids in Al-detoxification seems to be universal. Because *P. almogravensis* has an unusual ecological behaviour compared to most Al-accumulating plants,[21] it was interesting to determine that Al is complexed by the organic acid citrate. The importance of organic acids in Al-detoxification might be explained by the high affinity of Al for electronegative donor groups like carboxyl but also due to the ubiquitous nature of these metabolites and the low metabolic investment that is required to synthesize them.[22]

Al and Fe are complexed by tricitrate

The relation between Fe and Al metabolism is interesting considering that Fe is essential for development and Al toxic for most plants, yet their similar chemical properties pose a significant challenge for selective uptake and incorporation into metabolism.[23] More interestingly, a tri-Fe(III) tricitrate complex involved in Fe transport has been recently identified in xylem sap of tomato plants[24] and our results show that Fe(III) and Al are interchangeable in this complex indicating that one of the ligands binding Al is the same ligand responsible for Fe transport. The Fe complex was modelled as having an oxo-bridged tri-Fe(III) (Fe_3Ocit_3) core and owing to its compact molecular geometry it is expected to permeate the plasmodesmata directly.[24] The authors also reported that while both Fe_3cit_3 and Fe_3Ocit_3 complexes could be identified in Fe-citrate standard solutions, only Fe_3Ocit_3 was found in xylem samples. However, the Fe_3cit_3 complex was detected in *P. almogravensis* root samples and represents the first report of this complex in biological samples (Fig. S3 in the ESI†). The fact that, of the biologically relevant elements, Fe has the closest ionic radius to the one of Al might explain their binding by the same ligand considering that the ionic radius is one of the most important predictive parameters relating to the biological behavior of metal ions.[25] The relative concentration ratios between the Al- and Fe-complex determined using the relative ^{27}Al and ^{56}Fe signal intensities (depicted in Fig. 1C) combined with the sensitivity of the instrument for these isotopes and the fact that the estimated free activity of Fe in the culture medium supplemented with 400 μM is higher than that of Al (Fig. 4) suggest that Al can outcompete Fe(III) for the tricitrate ligand. This observation has consequences concerning the effect of free Al concentration in soils on the Fe homeostasis in *P. almogravensis*. In fact, the results determined for the total Fe and Al concentrations indicate that the concentration of Al in the culture media affects the concentration of ^{56}Fe found in leaves (Fig. 4). While the concentration of ^{56}Fe increases when the concentration of Al in the culture solution is increased from 100 μM to 200 μM, an increment of the Al concentration from 200 μM to 400 μM leads to a reduction in ^{56}Fe concentration. In fact, a reduction of Fe uptake as a result of Al supplementation was also observed in *M. malabathricum*.[26] The authors observed that Al supplementation ameliorated Fe toxicity and speculated that, in *M. malabathricum*, the accumulation of Al could be an adaptive advantage to Fe-rich acid sulphate soils. The fact that *P. almogravensis* colonizes soils enriched in Fe and free Al,[4] makes it an interesting model plant to study the interaction of the uptake of both metals. However, further experiments will have to point out to what extent Al can compete with Fe for binding with citrate and how it affects Fe uptake. A recent study on Fe uptake in *Bacillus cereus* demonstrated for the first time that a protein, FctC, could act as a specific binder for the Fe_3cit_3 complex.[27] Even if the structure of Al_3cit_3 seems to be slightly different from the one of Fe_3cit_3,[27] it could be hypothesized that similar proteins could occur in higher organisms such as plants and for other metal complexes such as Al_3cit_3.

Speciation of Al is concentration-dependent

The results presented in Fig. 1 indicate that Al speciation in *P. almogravensis* is dependent on internal concentration. The Al_3cit_3 complex (complex 2) was not detected in leaves of plants exposed to the lowest concentration of Al while it was detected in roots (Fig. 1B) and in leaves of plants exposed to 400 μM Al (Fig. 1A). The Al^{3+} cation has a great affinity for negatively charged pectin[28] making it difficult to distinguish intracellular Al from Al adhered to the root cell wall. Therefore, instead of determining total concentration of Al in roots, a more precise measure of uptake is to consider the accumulation of Al in the upper organs. The quantification of total Al confirms that plants exposed to 400 μM Al accumulate more Al in leaves than plants exposed to 100 μM (165.4 ± 16.6 μM and 65.8 ± 1.8 μM, respectively). Shen *et al.*[29] reported that in buckwheat (*F. esculentum*) leaves different Al species were detected depending on the concentration of internal Al. At lower Al concentrations only Al–oxalate was detected while at higher concentrations Al–citrate complexes could also be observed. In buckwheat, Al that is bound to citrate in xylem undergoes a

ligand exchange in leaves, where it is converted to Al–oxalate. The authors proposed that when oxalate is limiting Al–citrate cannot be converted to Al–oxalate completely and both complexes are therefore detected simultaneously. Considering that an Al–citrate complex also occurs in *P. almogravensis*, the observed results could be an indication that a similar ligand exchange process is present and that it is halted at higher internal Al concentrations. However, it is more likely that the speciation of Al is dependent on the internal Al : citrate ratio considering that complexes 3 and 4 are also citrate complexes and that no Al–oxalate complexes could be detected (Fig. S1 in the ESI†). Also the Al–citrate speciation diagrams presented by Fukushima *et al.*[19,27,30] show that at typical physiological pH values the speciation of Al is dependent on the Al : citrate ratio and the formation of Al_3cit_3 is favoured as the Al : citrate ratio increases. Our results are consistent with the predicted speciation diagrams as the intensity of peak 2 (corresponding to the Al_3cit_3 complex) increases drastically between the extract of leaves exposed to 100μM Al (Fig. 1B) and 400μM Al (Fig. 1A) as a result of increasing internal Al concentration (Fig. 4). Accordingly, it could also be hypothesized that peaks 3 and 4 could respectively be related to $Alcit_2$ and Alcit complexes,[27] which would explain the presence of three main peaks in the Al–citric acid standard solution chromatogram (Fig. S1B, ESI†).

Finally, as stated above, competition with Fe certainly occurs during Al homeostasis. Actually, even if the structures of Al_3cit_3 and Fe_3cit_3 complexes have some dissimilarities,[27] they have comparable stability constants,[31] which would clarify why Al and Fe seem to enter into competition when present at similar concentration levels (Fig. 4). The actual equilibrium between Al, Fe and citrate (and other minor ligands) is therefore related to the Al(III) : Fe(III) : citrate ratio, to their total amount, to the pH and to the presence of competing metals/organic acids/salts in the different specific plant cell compartments, which means that further investigation would be needed to get a detailed and simultaneous view of Al and Fe speciation in *P. almogravensis*.

P. almogravensis roots and the uptake of Al

When cultured *in vitro*, *P. almogravensis* plants grow normally even in the absence of developed root systems. Consequently, the importance of the root system in the speciation of Al in the upper tissues could be examined. In terms of Al uptake, no differences were observed between plantlets and shoots, considering that similar amounts were quantified in leaves (165.4 ± 16.6 μM in plantlets and 171.8 ±14.0 μM in shoots; Fig. 4). Because the root systems were excised in shoots, the conducting vessels are directly exposed to the culture solution and Al uptake is a consequence of co-transport of culture solution driven by transpiration. The fact that shoots and plantlets accumulate the same amount of Al in leaves suggests that the root cell membrane does not pose a barrier to Al uptake. However, the speciation analysis provided interesting results. The obtained SEC-ICP-MS chromatograms showed that the complexes 1 to 4 were absent from shoot samples, yet a broad Al signal eluting at longer retention times was observed, suggesting that it is bound to a ligand with lower molecular weight. By comparing the elution time of this Al-complex with the signal of other elements a correlation was observed with ^{31}P (Fig. 1D). In fact, according to the calculated speciation values, over 25% of Al is complexed by PO_4 in the culture solution (Table 1), suggesting that Al does not undergo chemical transformation once taken up by shoots. The fact that the ^{31}P signal detected in shoots at 26.2 min was not observed in plantlet leaves (Fig. 1D) supports the hypothesis that Al is taken up directly as an $AlPO_4$ complex in shoots. The reason why Al found in shoots is not converted to Al_3cit_3 is unclear, considering that large amounts of citrate can be found in shoots[6a] and the Al_3cit_3 complex is formed spontaneously when Al and citrate are in solution. One hypothesis is that the Al_3cit_3 complex is formed at the roots because Al is absorbed as Al^{3+} and is available for binding with citrate. Information on the molecular form of Al that is able to permeate the plasma membrane is still fragmentary, but evidence has been presented indicating that Al^{3+} is the most membrane mobile species.[32] Because it was not expected for plants to have specific transporters for a non-essential element, it was hypothesized that Al^{3+} could permeate divalent cation channels.[25] However, a specific Al transporter named Nrat1 (Nramp aluminum transporter 1) was recently discovered in rice.[33] The protein is located on the plasma membrane of root cells and is specific for the Al^{3+} cation. Therefore it is possible that in *P. almogravensis* Al is taken up as Al^{3+} as well and immediately complexed by citrate. The Fe_3cit_3 complex has been identified only recently,[24] so it is yet unknown if it is a universal ligand for Fe transport in plants. If this is the case, the fact Al-accumulation has only been described for a limited amount of plants indicates that the Al-permeability at roots seems to be the main difference between accumulating and non-accumulating plants.

Conclusions

An integrated mass spectrometry approach encompassing parallel LC ICP-MS and ESI-MS measurements using the Fe signal as an internal tracer in MS spectra allowed for the direct identification of the Al_3cit_3 complex based on exact mass determination and retention time comparison. Our results also demonstrate that the same tricitrate ligand complexes Al and Fe and that the relation between Al accumulation and Fe homeostasis should be further investigated. In *P. almogravensis*, Al is mainly bound to complexes related to citrate, of which Al_3cit_3 is the major form. In a later stage the subcellular localization of these complexes will be crucial to better understand the mechanisms behind Al tolerance in *P. almogravensis* and in plants in general. The high sensitivity of the employed methodology allowed studying the speciation of Al using minute samples. Therefore it is envisioned that the described methodology may provide new insights not only into the mechanisms regarding Al tolerance because it provides a means to study the speciation of Al with greater organ specificity but it may also provide information about Al species occurring in susceptible plants and its relation to toxicity.

Acknowledgements

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Article IV

Comprehensive speciation of low-molecular weight selenium metabolites in mustard seeds by HPLC - electrospray linear trap/Orbitrap tandem mass spectrometry

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PAPER

Comprehensive speciation of low-molecular weight selenium metabolites in mustard seeds using HPLC: electrospray linear trap/orbitrap tandem mass spectrometry†

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Laurent Ouerdane,*[a] Federica Aureli,[b] Paulina Flis,[a] Katarzyna Bierla,[a] Hugues Preud'homme,[a] Francesco Cubadda[b] and Joanna Szpunar[a]

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An analytical methodology based on high-resolution high mass accuracy electrospray ionization (ESI) tandem MS assisted by Se-specific detection using inductively coupled plasma mass spectrometry (ICP MS) was developed for speciation of selenium (Se) in seeds of black mustard (*Brassica nigra*) grown on Se-rich soil. Size-exclusion LC-ICP MS allowed the determination of the Se distribution according to the molecular mass and the control of the species stability during extraction. The optimization of hydrophilic interaction of LC and cation-exchange HPLC resulted in analytical conditions making it possible to detect and characterize over 30 Se species using ESI MS, including a number of minor (<0.5%) metabolites. Selenoglucosinolates were found to be the most important class of species accounting for at least 15% of the total Se present and over 50% of all the metabolites. They were found particularly unstable during aqueous extraction leading to the loss of Se by volatilization as methylselenonitriles and methylselenoisothiocyanates identified using gas chromatography (GC) with the parallel ICP MS and atmospheric pressure chemical ionization (APCI) MS/MS detection. However, selenoglucosinolates could be efficiently recovered by extraction with 70% methanol. Other classes of identified species included selenoamino acids, selenosugars, selenosinapine and selenourea derivatives. The three types of reactions leading to the formation of selenometabolites were: the Se–S substitution in the metabolic pathway, oxidative reactions of –SeH groups with endogenous biomolecules, and chemical reactions, *e.g.*, esterification, of Se-containing molecules and other biomolecules through functional groups not involving Se.

Introduction

Selenium (Se) has been recognized as an environmental contaminant or as an essential micronutrient in animals and humans because of its speciation and concentration. The plant family *Cruciferae* (also called *Brassicaceae*) includes broccoli, Brussels sprouts and cabbage, and is referred to as primary or secondary Se-accumulators.[1] Se concentration in *Cruciferae* may vary considerably and, generally, plants grown on high Se areas or under experimental Se enrichment conditions showed concentrations between 100 and 1000 μg g^{-1} Se (dry weight).[2–8] The potential of *Brassica* plants for the purpose of phytoremediation of seleniferous areas on one hand,[9,10] and nutritional supplementation[11,12] of Se on the other hand, has stimulated research interest in the Se metabolism of this plant family resulting in a number of studies focusing on identification of the Se species present (Table 1).

The studies revealed that selenoaminoacids characteristic of the Se incorporation into proteins through the sulphur metabolic pathway, selenocysteine (SeCys) and selenomethionine (SeMet), were usually involved.[13–15] The other group of reported species were non-protein selenoaminoacids and oligopeptides, such as Se-methylselenocysteine (MeSeCys) and γ-glutamyl-Se-methylselenocysteine (γ-Glu-MeSeCys), whose biosynthesis is assumed to prevent the damaging effects on plant functions assumed by the incorporation of SeCys and SeMet into proteins.[14,16,17] Also, MeSeCys and γ-Glu-MeSeCys are referred to as precursors of methylselenol, a putative chemopreventive agent.[3,18,19]

[a] *Laboratoire de Chimie Analytique Bio-Inorganique et Environnement, CNRS UMR 5254, Université de Pau et des Pays de l'Adour, 64053 Pau, France. E-mail: laurent.ouerdane@univ-pau.fr; Tel: +33 540 175 024*

[b] *Istituto Superiore di Sanità, Department of Food Safety and Veterinary Public Health, Rome, Italy*

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Table 1 Se species identified in *Brassica* plants

Plant species	Tissue	Sample preparation	Species identified	Identification technique used	Ref.
Kale (*Brassica oleracea* A.)	Leaves, stems	Solvent extraction	Se-methylselenocysteine Selenomethionine Selenocysteine, HMW species	NanoESI-ITMS	43
Indian mustard (*Brassica juncea*)	Stem, shoots	Solvent extraction and enzymatic hydrolysis	Se-methylselenocysteine Selenomethionine, unknowns	RT matching in HPLC-ICP MS	44
Indian mustard (*Brassica juncea*)	Shoots, roots	Enzymatic hydrolysis	Se-methylselenocysteine Selenomethionine	RT matching in HPLC-ICP MS	2
Broccoli (*Brassica oleracea*)	Whole plant	Solvent extraction	Selenite, selenate Oxidised Se-methylselenocysteine and oxidised selenomethionine, Se-methylselenocysteine Methylseleninic acid, Se-methylselenocysteine, selenomethionine	RT matching in HPLC-ICP MS	45
Brassica napus, *Brassica rapa*	Seeds, meal	Enzymatic hydrolysis	Selenomethionine Selenate	RT matching in HPLC-ICP MS	31
Indian mustard (*Brassica juncea*)	Root exudates		Selenocysteine, selenosulfate	Off-line ESI MS	46
Indian mustard (*Brassica juncea*)	Roots, shoots	Enzymatic hydrolysis	Selenite, selenate, selenomethionine	RT matching in HPLC-ICP MS	7
Brassica oleracea capitata	Green leaves	Solvent extraction	Se-methylselenomethionine, selenocystathionine, Se-methylselenocysteine selenoxide, selenohomocystine, Se-methylselenocysteine, and selenomethionine	TLC (ninhydrin positive spots)	47
Indian mustard (*Brassica juncea*)	Roots, leaves	Solvent extraction	Se-methylselenomethionine, selenomethionine and dimethylselenide; Se-methylselenocysteine	RT matching in HPLC-ICP MS HPLC-ESI MS	48
Indian mustard (*Brassica juncea*)	Leaves	Enzymatic hydrolysis	Selenate, selenomethionine Se-methylselenocysteine	RT matching in HPLC-ICP MS	4
Indian mustard (*Brassica juncea*)	Leaves	Enzymatic hydrolysis	Se–S bridged seleno amino acid (*m*/*z* 347) Selenomethionine	RT matching in HPLC-ICP MSHPLC-ESI MS	6
Indian mustard (*Brassica juncea*) (wild type and transgenic)	Leaves	Solvent extraction	Se-methylselenocysteine and Se-methionine Se-cystathionine Se-methylselenomethionine Se-homocystine Unknown	RT matching in HPLC-ICP MSHPLC-ESI MS	5
Indian mustard (*Brassica juncea*)	Leaves	Solvent extraction	MeSeCys and MeSeMet SeMet, unknown	RT matching in HPLC-ICP MS	8
Broccoli (*Brassica oleracea*)	Roots, stem, fruit	enzymatic hydrolysis	Se-methylselenocysteine Se-methionine	RT matching in HPLC-ICP MS	49
Raphanus sativus	Whole plant	Solvent extraction	Selenohomolanthionine Selenate Se-methylselenocysteine	HPLC-ICP MSHPLC-ESI MS	50
Brassica rapa var. Hakabura *Brassica rapa* var. Peruviridis Indian mustard (*Brassica juncea*)	Roots, leaves	Solvent extraction	Selenite, Se-methylselenocysteine and Se-methionine	HPLC-ICP MS	51
Canola (*Brassica napus* var. Hyola 420) Indian mustard (*Brassica juncea* var. Pacific Gold) White mustard (*Sinapis alba* var. Ida Gold)	Seeds	Solvent extraction and enzymatic hydrolysis	Selenocystine Methylselenocysteine Selenomethionine Selenocysteine Selenate	HPLC-ICP MSμXANES	32
Broccoli (*B. oleracea* L. var. italica cv. 'Triathlon') Cauliflower (*B. oleracea* L. var. botrytis cv. 'Liberty'), Forage rape (*B. napus* cv. 'Maxima')	Florets, roots	Solvent extraction	4-(Methylseleno)butanenitrile, 5-(methylseleno)-pentanenitrile, 3-(methylseleno)propylisothiocyanate, 4-(methylseleno)butylisothiocyanate, and 5-(methylseleno)pentylisothiocyanate 3-(Methylseleno)propylglucosinolate (glucoselenoiberverin), 4-(methylseleno)butylglucosinolate (glucoselenoerucin), and 5-(methylseleno)-pentylglucosinolate (glucoselenoberteroin)	GC-MSHPLC-ESI MS	37

Considering that crucifers contain numerous other bioactive (and putatively chemopreventive) compounds, such as glucosinolates and isothiocyanates,[3] the overall number of the identified species in the *Brassica* selenometabolome is relatively small which may result from inadequate analytical methodology. Indeed, HPLC-ICP MS, with its inherent inconsistencies and pitfalls, was definitely the most widely used method for speciation of low molecular weight Se metabolites.[18,20–22] The approach suffers from the lack of identification of many peaks, the often ignored peak purity, and poor chromatographic

separations resulted in the impossibility of reliable identification by retention time matching. In terms of sample preparation, the studies often concerned proteolytic extracts only.

A momentum in Se metabolomic studies was created by the advent of high resolution high accuracy MS, readily combined with HPLC: electrospray linear trap/Orbitrap (tandem) mass spectrometry and ESI Orbitrap MS(/MS). It has become a powerful tool for identifying Se metabolites on-line down to the ppb levels.[23] HPLC–ESI Orbitrap MS(/MS) allowed comprehensive studies of Se metabolites in Se-rich yeast,[24–27] and cereal grains (rice, maize, and wheat).[28] A critical point is the choice and optimization of the chromatographic separation mechanisms allowing the separation of the analyte species from concomitants of easily ionizable matrix molecules. Indeed, under the ideal conditions, ESI Orbitrap MS can be more sensitive than ICP MS commonly used as a chromatographic detector in Se speciation studies.[24] Another problem, to which remarkably little attention has to date been paid, is the preservation of Se metabolites during extraction, avoiding enzymatic reactions leading to its volatilization.

The seeds of *Brassica nigra* (black mustard) are commonly employed as condiments for medical purposes. When grown on Se-rich soils, they become an attractive source of Se and can accumulate this element at higher proportions than pods and straw, with levels up to 670 μg g^{-1}.[29,30] The residual fraction obtained after oil processing of seeds, the meal, is an important protein source in animal diets and used in feed concentrates.[31] Information on Se speciation in the seeds is scarce and a recent work on that topic did not allow the direct identification of Se species but just a comparison with the standards of expected molecules.[32] The aim of this work was to characterize the low-molecular weight (<1 kDa) Se metabolites present in seeds of *Brassica nigra*. For this purpose, cation exchange (CE)-HPLC and hydrophilic ion interaction (HILIC) coupled in parallel to ICP MS and molecular MS instruments were investigated. Particular attention was paid to sample preparation and the control of the volatilization losses of Se using gas chromatography (GC) with the parallel ICP MS and atmospheric pressure chemical ionization (APCI) MS detection.

Experimental

Reagents, solutions and materials

Analytical reagent grade chemicals purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and water (18 MΩ cm), obtained using a Milli-Q system (Millipore, Bedford, MA) were used throughout unless stated otherwise. Selenious acid [Se(IV)], selenic acid (Se(VI)], seleno-L-methionine (SeMet) and dimethyldiselenide (DMDSe) were purchased from Sigma-Aldrich; methylseleno-L-cysteine was acquired from Fluka (Seelze, Germany). Instra-Analyzed HNO_3 (69–70%) and H_2O_2 (30%) were obtained from Baker (Deventer, Holland). The Se stock solution 1 mg mL^{-1} was obtained from SPEX CertiPrep (Metuchen, NJ); working solutions were prepared by its dilution with acidified (HNO_3) deionized water as necessary. Myoglobin (16.7 kDa), metallothionein-I (MT-1) (7 kDa), cobalamin (1.6 kDa) and SeMet (0.2 kDa) were used as molecular weight calibrants in size exclusion chromatography (SEC).

Instrumentation

Chromatographic separations were performed using an Agilent 1200 series HPLC pump (Agilent, Tokyo, Japan) as a delivery system for cation-exchange chromatography (CE) and size exclusion chromatography (SEC), whereas an Agilent 1100 capillary HPLC system was employed for HILIC. The exit of the column was directly connected by means of polyether-etherketone (PEEK) tubing to a MicroMist nebulizer (Glass Expansion, Romainmotier, Switzerland) for the HPLC-ICP MS coupling. The ICP MS instrument was an Agilent Model 7500cs equipped with a collision cell. A model Digi-Prep (SCP Science, Courtaboeuf, France) heated-block digester was used to perform the acidic digestion of samples and extracts for the total Se determination. An electrospray hybrid linear ion trap-orbital ion trap mass analyzer (Thermo H-ESI II LTQ Orbitrap Velos, Thermo Fisher Scientific, Waltham, MA, USA) was employed for HPLC ESI MS couplings. For GC analysis, a GC 6890 (Agilent) was coupled to either Agilent 7500cs-ICP MS or to a Xevo triple quadrupole (TQ) MS (Waters, Saint-Quentin-en-Yvelines, France) equipped with an APCI ion source (GC APCI TQ MS).

Procedures

Sample. Seeds of black mustard (*Brassica nigra*) grown in naturally Se-rich soils were employed in this study. The area where mustard was grown is mostly composed of some fine coarse loamy (Typic Haplustalfs) and coarse loamy (Typic Ustochrepts) soils and is present in the seleniferous belt in the Punjab region (India) where Se soil concentration is around 2 to 7 mg kg^{-1}.[33,34]

Total Se determination. 0.1 g of the seed sample (n = 5) was digested with 2.5 mL HNO_3, to which 1.25 mL H_2O_2 was added after an overnight pre-digestion step. The digestion program was: 30 min up to 80 °C and 240 min at 80 °C. After cooling down, the digests were diluted to 50 mL with water and analyzed by ICP MS. Quantitation and validation were performed as described elsewhere.[28] In brief, quantitation was performed by external calibration. Five Se isotopes were measured (76, 77, 78, 80 and 82) and Se determination was validated by the parallel digestion and analysis of the NIST 8436 Durum Wheat Flour reference material. A total Se concentration of 107 ± 7 μg g^{-1} in seeds was determined.

Extraction of Se compounds. Seed samples were ground in a mortar (around 50 mg) and quantitatively transferred into a 1.5 mL polypropylene (PP) tube where extraction was carried out by mixing crushed seeds for 5 min with either water or with 70% (v/v) methanol. The sample : extractant ratio was 1 : 4 (w/v). Samples were centrifuged for 5 min at 14 000 rpm. The supernatants were immediately analyzed by HPLC-ICP MS and ESI MS/MS.

For the Se determination in the extracts, aliquots (50 μL) were mixed with 1 mL of 2% HNO_3. After cooling down, the extract digests were made up to 25 mL and analyzed for total Se

content as described above. Three sample replicates were analyzed.

For GC experiments, a sample of *ca.* 20 mg of seeds was extracted each time with 500 μL of a mixture of pentane and diethyl ether (1 : 1 v/v). Two different protocols for solvent extraction were tested to analyze volatile Se species from mustard seeds: (i) seeds were placed in a glass vial with the extracting solvent, crushed, mixed, and the solvent was collected after a 5 min incubation; (ii) seeds were placed in a glass vial containing 500 μL of water and the extracting solvent on top, and only then the seeds were crushed (without mixing the two phases). The mixture was let to incubate for 5 min, then mixed and solvent was collected after decantation.

Liquid chromatography coupled to ICP MS and ESI MS/MS mass spectrometry. The optimized chromatographic conditions for cation-exchange (Hamilton PRP-X200, 150 mm × 2.1 mm, 10 μm) and size exclusion chromatography (Superdex Peptide 10/300 GL) were described elsewhere.[28] Concerning HILIC chromatography (TSK gel amide 80, 250 mm × 1 mm, 5 μm) similar settings were conserved but the gradient was modified to ensure better compound separation, especially for the most hydrophobic selenocompounds. The elution solvents used for HILIC were (A) acetonitrile and (B) 10 mM ammonium formate at pH 5.5; the gradient was: 0–5 min: 2% B, 5–50 min: 2–40% B, 50–55 min: 40% B, 55–57 min: 40–50% B, 57–60 min: 50% B, 60–65 min: 50–2% B. Each extract was diluted with 2 volumes of acetonitrile and centrifuged at 14 000 rpm prior to injection. SEC-ICP MS was carried out directly on a sample extract. Prior to CE HPLC, the extract was 5 kDa cut-off filtered (Ultrafree-CL Microcentrifuge Filters, NMWL 5000 Da, Sigma Aldrich) by centrifugation for 45 min at 4000 rpm. The ICP MS and ESI MS settings were as reported elsewhere.[28]

Gas chromatography tandem MS. The analyses were performed with a DB5-MS column (30 m × 0.25 mm × 0.25 μm, Agilent). The oven temperature gradient was: 0–13 min: 35 to 100 °C, 13–27 min: 100–240 °C, 27–30 min: 240 °C. The carrier gas, helium, was set at 3 mL min^{-1} in a splitless injection mode (delay 1 min). The injection volume was 2 μL. Injector temperature was set at 225 °C. During GC ICP MS coupling, ICP MS was set in a dry plasma mode (1050 W; carrier gas, Ar, at 0.48 mL min^{-1}) to which was added O_2 (9%) and Xe (50 ppm in argon) for internal calibration. For GC APCI TQ MS, the source was heated at 150 °C, corona discharge was set at 1 μA and a make-up gas, N_2, was added at 400 mL min^{-1}. Collision energy was set to 12 eV.

Results

Optimization of Se metabolite extraction

Sample preparation was optimized to maximize the recovery of Se from the mustard seed. Two main approaches were attempted: one using water or aqueous buffers [Tris-HCl buffer (pH 7.5) or ammonium acetate buffer (pH 6.5)] and one using 70% (v/v) methanol. The quantitative yield was measured by the determination of the total Se content in the extract related to the total Se content in seeds. The extraction efficiency with regard to the nature and the relative amounts of the extracted Se species was also evaluated by the observation of the differences/similarities between the chromatograms (SEC-ICP MS or HILIC-ICP MS) obtained after the different types of extraction.

Buffered extracting solutions were tested to check if the presence of salt and fixed pH would improve Se extraction yield. All the three types of the aqueous extractions (with or without buffers) gave similar results quantitatively and qualitatively. Consequently, extraction with water (without buffer) was chosen as it presents the lowest risk of sample pollution and facilitates extract preconcentration if necessary. No differences were observed between 5 min and 30 min extraction times indicating that the Se species were extracted immediately. The water extraction yield was 11 ± 3% of Se. SEC-ICP MS showed that *ca.* 10–20% of the extracted Se was distributed between the high molecular fraction (possibly proteins) and low molecular weight (LMW, <1 kDa) metabolites (Fig. 1B). This molecular weight distribution corroborates a literature report in which protease digestion increased Se extraction efficiency only by 5 to 6%[32] confirming the limited presence of Se in water soluble proteins.

Extraction with 70% methanol offered a twice higher yield. 24 ± 5% of Se was extracted and was only present as LMW metabolites (Fig. 1A). Extractants with 30% and 100% methanol were also tested but gave lower extraction yields. Summing up this and the water-soluble protein fraction non-extracted with methanol (*ca.* 2%) gives *ca.* 25–30% of extractable Se. This number is similar to the extraction yields reported for seeds of other Se-enriched mustard species (between 18% and 34%).[32] The remaining Se is expected to be membrane proteins as recently demonstrated for wheat[13] and rice.[15]

Optimisation of the HILIC and cation-exchange HPLC separations

Fig. 1A and B, show, to the best of our knowledge, the first record of molecular weight distribution of Se species in an aqueous extract of mustard seeds. However, size exclusion LC does not provide the resolution required for the species identification, although it can be considered as a first screening separation methodology before a more selective chromatography.[5] Therefore, HILIC (Fig. 1C and D) and cation-exchange (Fig. 1E) separations were optimized using ICP MS detection and electrospray ionization friendly buffers in order to obtain the best possible separation of each Se species by at least one chromatographic setup.

Identification of Se-containing metabolites using ESI Orbitrap MS(/MS)

HPLC-ICP MS allowed the determination of the number of the most intense Se species, their relative concentration and their retention times. The identification was carried out using HPLC under exactly the same conditions using ESI Orbitrap MS(/MS) as reported elsewhere for the Se speciation in plant extracts.[28]

The acquired Orbitrap data sets were searched for the presence of the characteristic isotopic profiles (inter-isotopic mass differences and isotopic ratios) in order to identify

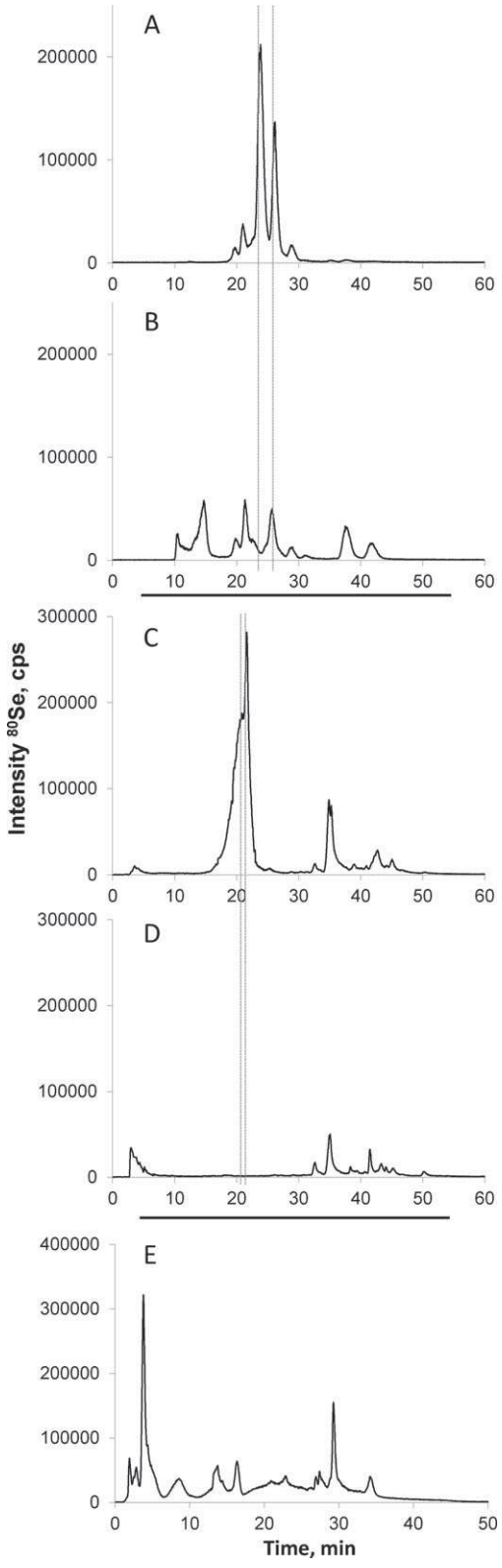

Fig. 1 Chromatograms of the ^{80}Se signal obtained using SEC-ICP MS for the analysis of 70% MeOH (A) and water (B) extracts, using HILIC-ICP MS for the analysis of 70% MeOH (C) and water (D) extracts and by CE-ICP MS for the analysis of water extracts (E). Note that, using HILIC, a slight variation in retention time between water and 70% MeOH extracts (checked by HILIC-ESI MS) could be noticed for a few compounds after 40 min of elution time because of the different extract matrices.

Se-containing molecules at retention times determined using HPLC-ICP MS as discussed elsewhere.[26,35] Note that ESI MS detection limits were comparable to ICP MS and even very low abundant species (<0.5% of total Se) could be detected. The identification was carried out by tandem MS/MS. Note that Orbitrap preserves the mass accuracy in the MS/MS mode facilitating the data interpretation. The latter was also facilitated by considering: (i) the structures of the known Se compounds with similar or identical mass in other matrices, such as yeast,[24–27] (ii) the sulfur compounds present in the plant matrix (especially *Brassica* seeds) because of the potential substitution of S by Se, and (iii) the other major expected species that could potentially react with selenol-containing species.

More than 30 different Se-containing molecules could be detected, identified as the molecular mass and largely characterized after chromatographic separation in positive and/or negative ionization mode using ESI Orbitrap MS/MS. The list of the identified species with their accurate masses is given in Table 2. MS/MS data are summarized in Table S1 (ESI†). The use of the different ionization modes also allowed the discrimination of salt adducts from original compounds. More than half of the detected molecules were never observed before. With regard to the most abundant species (13 compounds presented in bold in Table 2) HILIC and CE turned out to validate each other. For the low concentrated species, they were sometimes complementary due to the fact that each separation mechanism allowed the separation of different analytes from the concomitant matrix components.

Discussion

To date, among all studies performed in *Brassica* plants, not more than eight selenocompounds had ever been characterized in their extracts (Table 1) and mostly only through retention time matching with standards by HPLC-ICP MS without formal identification by molecular MS/MS. Moreover, the seeds studied in this work originated from plants grown on a naturally Se-rich soil in contrast with former works based on laboratory grown plants with artificial Se supplementation. From the chemical point of view, the detected species belong to different families and are discussed below.

Selenoglucosinolates

The most abundant Se compounds accounting for more than half of the amount of Se metabolites (*ca.* 15–20% of total Se in seed) belong to the family of glucosinolates. Glucosinolates are known to occur as secondary metabolites in almost all plants of the order *Brassicales.*[36] The main glucosinolates (**3** and **5**) account for the difference clearly seen in the SEC and HILIC (retention time 16 and 24 min) chromatograms between the water and methanol extracts. Fig. 2 shows the structures of the identified selenoglucosinolates. Every glucosinolate contains a central carbon atom which is bound *via* a sulfur atom to the thioglucose group and *via* a nitrogen atom to the sulfate group. In addition, the central atom is bound to a side group.

Table 2 Low molecular weight Se species identified in *Brassica nigra* seeds (main metabolites are presented in bold character). HILIC and CE retention times (when applicable), MS/MS fragments (for novel species not previously described in plants, Table 1, or in yeast[24,26,27]) and possible compound structures are detailed in Table S1 (ESI). Major Se species are presented in bold characters

No.	Exact mass (*m*/*z*)	Experimental mass (*m*/*z*)	Error value (ppm)	Molecular formula (monocharged)	Ionization mode	Putative compound name
1	**105.92014**	**105.92058**	**4.2**	**CNSe**	−	**(Iso)selenocyanate**
2	507.98555	507.98612	1.1	$C_{14}H_{22}NO_{10}S_2Se$	−	Methylselenoacetylgluconapin
3	**467.99061**	**467.98973**	**−1.9**	**$C_{12}H_{22}NO_9S_2Se$**	−	**Glucoselenoerucin**
4	404.13355	404.13367	0.3	$C_{18}H_{30}NO_4Se$	+	Methylselenosinapine
5	**453.97496**	**453.97436**	**−1.3**	**$C_{11}H_{20}NO_9S_2Se$**	−	**Glucoselenoiberverin**
6	268.08103	268.08101	−0.1	$C_{10}H_{20}NO_2Se$	+	Methylselenobutyrylcholine
7	**240.04973**	**240.04985**	**0.5**	**$C_8H_{18}NO_2Se$**	**+**	**Methylselenoacetylcholine**
8	**180.98756**	**180.98754**	**−0.1**	**$C_4H_9N_2OSe$**	**+**	**Se-allyl-*N*-hydroxy-selenourea**
9	**198.00278**	**198.00286**	**0.4**	**$C_5H_{12}NO_2Se$**	**+**	**Selenomethionine**
9′	195.98713	195.98733	1.0	$C_5H_{10}NO_2Se$	+	Oxidized selenomethionine
10	**408.07674**	**408.07698**	**0.6**	**$C_{12}H_{26}NO_9Se$**	**+**	**Methylseleno-Se-deoxypentose-hexose**
11	**183.98713**	**183.98732**	**1.0**	**$C_4H_{10}NO_2Se$**	**+**	**Methylselenocysteine**
12	**356.09714**	**356.09718**	**0.1**	**$C_{13}H_{26}NO_5Se$**	**+**	**Selenized choline ester**
13	**567.14587**	**567.14587**	**0.0**	**$C_{22}H_{35}N_2O_{10}Se$**	**+**	***N*-2,3-Dihydroxylpropionyl-selenocysteine-Se-sinapine**
14	313.02976	313.02953	−0.7	$C_9H_{17}N_2O_5Se$	+	γ-Glutamyl-Se-methylselenocysteine
15	**343.04029**	**343.04023**	**−0.2**	**$C_{10}H_{19}N_2O_6Se$**	**+**	**Se-glucosyl-Se-allyl-*N*-hydroxy-selenourea**
16	**286.01886**	**286.01889**	**0.1**	**$C_8H_{16}NO_5Se$**	**+**	**Deamino-hydroxy-selenohomolanthionine**
17	346.00362	346.00374	0.3	$C_9H_{16}NO_8Se$	+	2-Hydroxy-3-selenylpropanoic acid-*N*-2,3 -dihydro-propionyl-selenocysteine
18	**345.01969**	**345.01929**	**−1.2**	**$C_9H_{17}N_2O_7Se$**	**+**	***N*-2,3-dihydroxylpropionyl-selenolanthionine**
19	**304.02939**	**304.02917**	**−0.7**	**$C_8H_{18}NO_6Se$**	**+**	**5′-3-Selenylpropanoic acid-ribofuranose**
20	144.90455	144.90452	−0.2	$HSeO_4$	−	Selenate
21	198.98679	198.98686	0.4	$C_5H_{11}O_3Se$	+	Methylseleno carbohydrate
22	213.00244	213.00238	−0.3	$C_6H_{13}O_3Se$	+	Methylseleno carbohydrate
23	407.04546	407.04474	−1.8	$C_{12}H_{23}O_{10}Se$	+	Methylseleno-Se-pentose-hexose
24	674.04795	674.04864	−1.0	$C_{23}H_{32}NO_{13}S_2Se$	−	Methylselenosinapoylgluconapin
25	660.03303	660.03298	0.1	$C_{22}H_{30}NO_{13}S_2Se$	−	Methylselenosinapoylsinigrin
26	493.96974	493.96989	−0.3	$C_{13}H_{20}NO_{10}S_2Se$	−	Methylselenoacetylsinigrin
27	558.99576	558.99647	−1.3	$C_{17}H_{23}N_2O_{10}S_2Se$	−	Methylselenohydroxyglucobrassicin
28	573.01304	573.01213	1.6	$C_{18}H_{25}N_2O_{10}S_2Se$	−	Methylselenoneoglucobrassicin or methylseleno-4-methoxyglucobrassicin
29[a,b]	RT matching	—	—	$C_2H_6Se_2$	—	Dimethyldiselenide
30[b,c]	—	**163**	—	**C_5H_9NSe**	**+ (M^+)**	**4-(Methylseleno)butanenitrile**
31[b,c]	—	**177**	—	**$C_6H_{11}NSe$**	**+ (M^+)**	**5-(Methylseleno)pentanenitrile**
32[b,c]	—	**195**	—	**C_5H_9NSSe**	**+ (M^+)**	**3-(Methylseleno)propylisothiocyanate**
33[b,c]	—	**209**	—	**$C_6H_{11}NSSe$**	**+ (M^+)**	**4-(Methylseleno)butylisothiocyanate**

[a] Dimethyldiselenide was identified by retention time matching with the corresponding standard. [b] Compounds **29** to **33** were observed using GC ICP MS and GC APCI TQ MS coupling (low resolution). [c] Compounds **30** to **33** were identified through their specific mass transitions.

Fig. 2 indicates three incorporation patterns of Se:

(i) the presence of Se in the side chain as recently observed elsewhere for *Brassica* plants indicating that the presence of isothiocyanate and sulfate groups (and not isoselenocyanate and selenate) is required for the synthesis or stabilization of glucosinolates,[37] leading to glucoselenoerucin (**3**) or glucoselenoiberverin (**5**);

(ii) the acylation of the glucose hydroxyl group by, *e.g.*, methyl selenoacetic acid (**2**) or the Se-sinapinic acid metabolite of sinapine (**24** and **25**). Acylation of glucosinolates is not a common feature but several cases were confirmed;[38]

(iii) replacement of the S atom by Se in thiocyanate (**8** and **15**).

Surprisingly, the glucosinolates (that are soluble in water) were not found in the extract when only water (buffered or not) was used for extraction. It was therefore assumed that they might be degraded when seeds were crushed in the presence of water releasing myrosinase (there is a known activity of this enzyme in seeds after tissue disruption[36,38]) and volatilized because of the position of the methylselenol groups in these molecules.[37] In order to confirm such a hypothesis two extraction protocols were tested as described above: (i) direct extraction of seeds that were crushed in the presence of the pentane–diethyl ether mixture only, and (ii) seeds that were crushed in water while topped with the solvent mixture. The volatile compounds were then monitored in the solvent mixture by GC-ICP MS. The use of the latter protocol led to the observation of four major volatile species (Fig. 3A) whereas no Se-compounds were observed by the first protocol, thus indicating that degradation occurred during the exposure of crushed seeds to water.

The compounds detected in Fig. 3A were identified by GC APCI TQ MS/MS. According to the enzymatic degradation route of glucosinolates by myrosinase, the cleavage of the two main Se-glucosinolates detected should result in the presence of four volatile species, two Se-isothiocyanates and two Se-nitriles.[37] These species were characterized recently by MS in florets and roots of three *Brassica* species.[37] On the basis of the expected ions produced during their fragmentation in a quadrupole analyser, mass transitions (precursor ion → fragment ion)

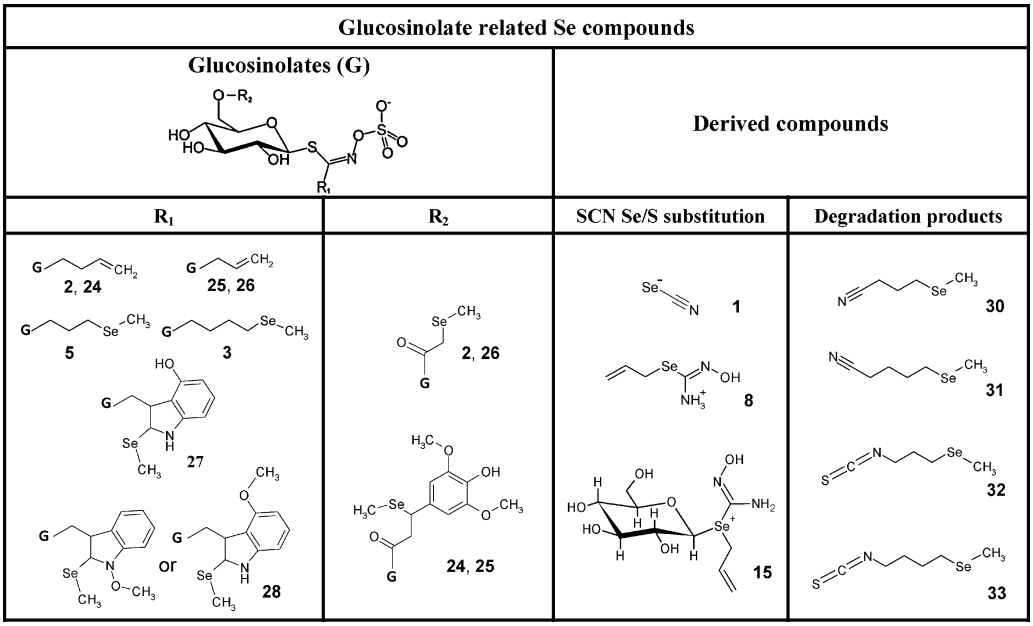

Fig. 2 Glucosinolates related Se compounds detected in *Brassica nigra* seed extracts and their putative structures.

specific to each of these expected compounds were screened for the main isotopologues containing ^{78}Se and ^{80}Se (Fig. 3B). The observed conservation of the isotopic ratio in the mass spectra and retention time matching with the GC-ICP MS chromatogram allowed the definitive confirmation of the presence of the four expected volatile Se compounds (**29–33**). Moreover, the non-degradation of Se-glucosinolates in the 70% methanol extract indicates an enzymatic (myrosinase is usually inactive in 70% methanol) and not simply chemical hydrolysis in water.

Minor forms of Se-glucosinolates could also be detected. Their identification was performed on the basis of the determined structure (*cf.* ref. 36 and 38) of the closely eluting abundant analogue forms lacking the methylselenol group (compounds **2** and **24** to **28**).

No Se-glucosinolates were detected in Se-enriched *Brassica* seeds in a recent work[32] using μXANES and anion-exchange HPLC-ICP MS. This shows the difficulties in the analysis of these compounds and limitations of even state-of-the-art analytical techniques when molecular MS is not employed to perform non-targeted speciation analysis.

Sinapine and choline metabolites

Sinapine (*cf.* Fig. 4) is a choline ester of sinapinic acid which is one of the main compounds found in *Brassica* seeds. The unsaturated bond on the alkyl chain present in the centre of its structure is a potential target of an oxidative attack of selenol-containing species. The reaction may lead to a selenized derivative of sinapine (compounds **4** and **13**). On the other side, the esterification reaction of choline could also occur with minor Se-carbohydrates and lead to the formation of other choline esters of Se-organic acids (compounds **6**, **7** and **12**). A selenol attack seems to be able to lead to its binding to either of the two carbon atoms of the central unsaturated bond of sinapine and, therefore, both types of binding should be expected. Therefore, compounds **2** and **7** could be the degradation products of compounds **24** and **4** through oxidative double bond cleavage, respectively. It is the first observation of this type of Se-metabolites in living organisms.

Se-containing amino acids

Se-amino acids (*cf.* Fig. 5) are typical of the non-specific incorporation of Se into the metabolic pathway of sulfur in organisms accumulating Se (yeast, plants, *etc.*). The presence of Se amino acids was frequently observed in plants accumulating Se, not only *Brassica* (Table 1) but also *Allium* species and Brazil nuts.[16,17] It is therefore not surprising to detect several Se-amino acids in *Brassica* seeds. Some of them, *e.g.* selenomethionine (**9**) and oxidized selenomethionine (**9′**), methylselenocysteine (**11**), and γ-glutamyl-Se-methylselenocysteine (**14**), which were frequently detected in plants and in *Brassica* species (Table 1), were also found in the analyzed seeds. Several other compounds, previously unreported in *Brassica* genus, such as selenolanthionine- and selenohomolanthionine derivatives (compounds **16**, **17**, **18** and **19**), were also identified.

Selenocarbohydrates

Until their recent discovery in plants,[28] Se-carbohydrates, and more specifically Se-sugars, were not expected to be synthesized in plants. These metabolites were identified in multiple forms (*cf.* Fig. 5), alone (compounds **10**, **23** and **25** for sugars and compounds **21** and **22** for general carbohydrates) or covalently bound to another compound (compounds **15** and **19** for sugars and compounds **2**, **6**, **7**, **12** and **26** for general carbohydrates). Their possible origin in plants, either through an enzymatic or an oxidative process, was discussed elsewhere for sugars.[28] Note that the determination of the exact structures of these

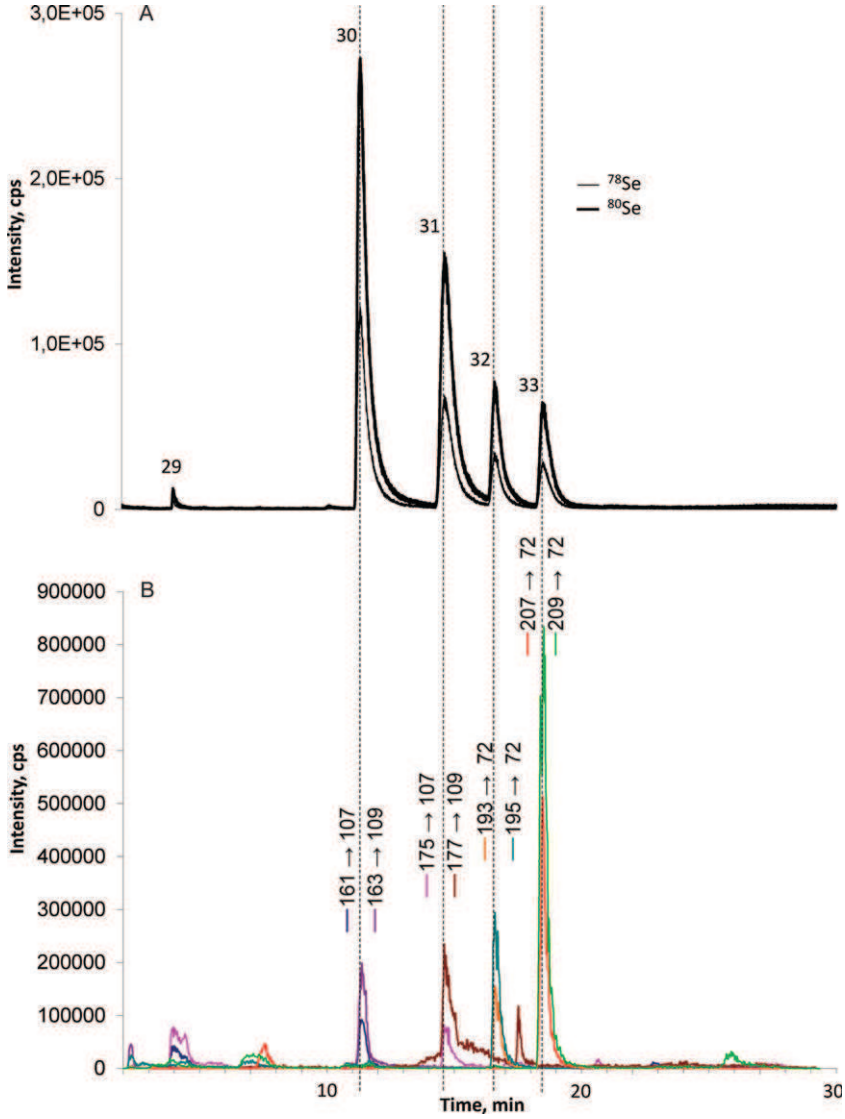

Fig. 3 Chromatograms obtained after analysis of pentane–diethyl ether extracts (in the presence of water) using GC ICP MS (A) and GC APCI TQ MS (B). Compound-specific mass transitions were screened for compound **30** (163 → 109 and 161 → 107), compound **31** (177 → 109 and 175 → 107), compound **32** (195 → 72 and 193 → 72) and compound **33** (209 → 72 and 207 → 72). Compounds numbers are given on top of peaks.

Se-carbohydrates related species is quasi-impossible by MS. The reasons are the large number of naturally occurring carbohydrates in the plant matrix which are potential targets for Se and, consequently, the very large number of possible combinations (even without considering enantiomers). For Se-carbohydrate side chain containing compounds, some possible structures matching the MS/MS data acquired were presented (compounds **6**, **12**, **19**, **21** and **22**) but the side chain structures remain highly hypothetical.

Other selenocompounds

Selenourea-containing compounds (**8** and **15**) and selenocyanate/isoselenocyanate (**1**) were putatively identified. Even if the presented structures of Se-urea compounds may remain questionable in some aspects (positions of sugar and –OH groups in molecules), they were consistent with MS/MS spectra in both positive (Table S1, ESI†) and negative (presence of selenourea fragment ion CHN_2Se^- at *m*/*z* 120.93) ionization mode. They allow further a hypothesis of the insertion of Se into the thiocyanate group instead of S (*cf.* Fig. 2). These two types of molecules could come from the degradation of glucosinolates either during their production in the seeds or during extraction.[36,38] The reaction of ammonium ions with the selenohydroxamic acid precursor could also lead to a cyclic structure for this selenourea as observed during chemical synthesis,[39] which could also explain the rearrangement of Se with regard to the side chain. These Se species are unusual compounds for plants and could be of great interest for cancer chemoprevention studies.[40]

Another minor Se compound could also be detected by GC-ICP MS (at 4 min) and it was identified as dimethyldiselenide (DMDSe, **29**, Fig. 5) by retention time matching with a standard compound. Its presence is not surprising as Se-mercaptans were

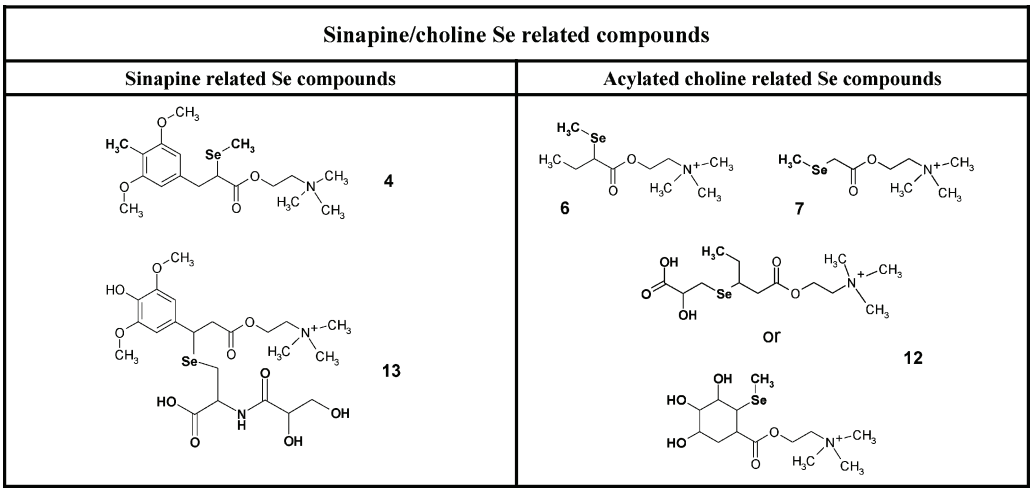

Fig. 4 Sinapine and choline related Se compounds detected in *Brassica nigra* seed extracts and their putative structures.

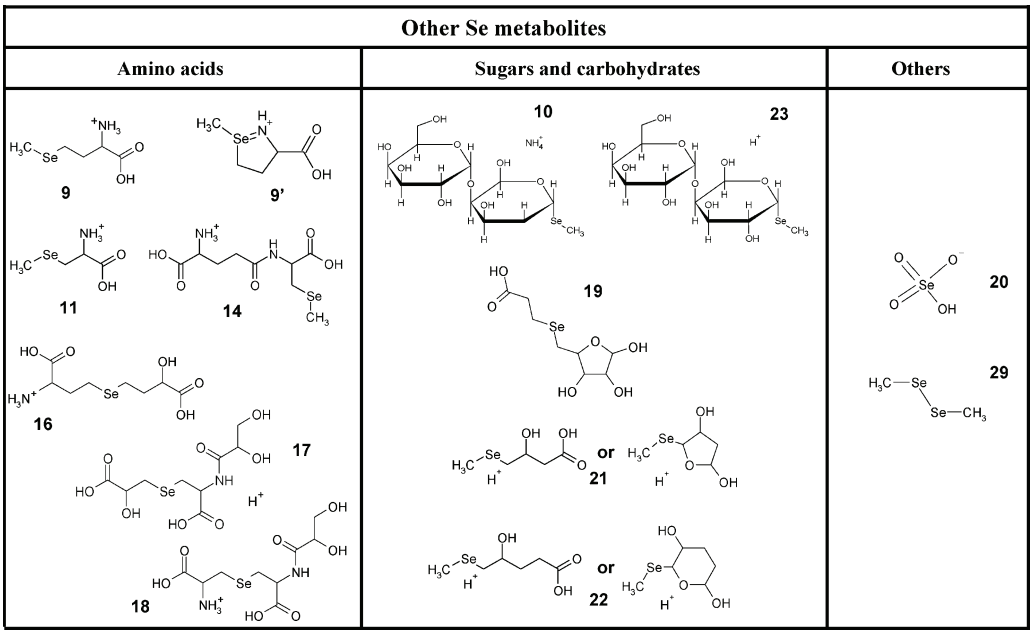

Fig. 5 Other Se compounds detected in *Brassica nigra* seed extracts and their putative structures.

commonly observed in plants.[41] It confirms that methylselenol can be released in the plant and react with endogenous plant compounds. Finally, selenate could also be detected at trace level (**20**, Fig. 5).

As observed in this study and by Matich *et al.*,[37] it is clear according to glucosinolate MS/MS fragments that Se is not incorporated into the isothiocyanate group of glucosinolates. However, the presence of isoselenocyanate- and selenourea-like molecules would suggest that precursors of glucosinolates incorporating Se instead of S into the isothiocyanate group could exist.

Quantitative aspects

The intensity of peaks in cation-exchange and HILIC-ICP MS chromatograms in Fig. 1C and E allows an assessment of the relative concentration of the different selenometabolites in the extracts. Most compounds contribute 0.1 to 5% of the total Se each with the exception of the two major glucosinolates (compounds **3** and **5**) that accounted together for 15 to 20% of the total Se in seeds.

Conclusion

In this study, several novel Se metabolites were observed for the first time in plants and more generally in living organisms such as compounds belonging to selenocyanate, selenosinapine or selenourea family. The optimization of HILIC and cation-exchange chromatography combined with ESI Orbitrap MS(/MS) allowed a comprehensive speciation of the Se metabolite

fraction in black mustard seeds grown on seleniferous area. The presence of proteinaceous and non-proteinaceous amino-acids and oligopeptides characteristic of *Brassica* plant genus was confirmed and a number of other compounds belonging to different families such as selenoglucosinolates, selenosinapine, selenoacylated choline, selenocarbohydrates and their metabolites selenourea, selenocyanates were identified. Sample preparation was found to be critical and enzymatic reactions during water extraction were responsible for the original species degradation leading to the formation of volatile Se species.

The main pathways of the incorporation of Se were: non-specific incorporation of Se instead of sulfur into amino acids and an oxidative attack of the selenol moiety from methylselenol or selenoamino acids on unsaturated bonds of alkyl chains or on reducing sugars. The targets of these attacks were the main compounds present in *Brassica nigra* seeds, such as sinapine, sinigrin and gluconapin, resulting in the formation of numerous Se metabolites. The potential beneficial effects of the Se-rich mustard seeds when consumed should be different depending on the chemical forms and concentrations of the remaining Se metabolites after food preparation. These Se species could have anti-inflammatory activity or even help cancer prevention[40] when assimilated because of specific redox chemistry or due to the release of reactive forms during enzymatic processes, *e.g.*, cysteine β lyase related enzymes can liberate *in vivo* reactive selenol-containing molecules.[42] This is why a complete Se speciation, as performed in this work, is essential for the further evaluation of the biological effects of the selenocompounds present in Se-enriched mustards seeds.

Acknowledgements

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Article V

Speciation of zinc in microliter volumes of plant sap by capillary HPLC-ICP-MS

Speciation of zinc in microliter volumes of plant sap by capillary HPLC-ICP-MS

Application note

Environmental

Author

Paulina Flis, Laurent Ouerdane, Ryszard Lobinski

CNRS-UPPA, Laboratoire de Chimie Analytique Bio-inorganique et Environnement, UMR5254, Hélioparc, 2, Av. Angot, 64053 Pau, France

Introduction

Many metals are very important for normal plant growth and development. One of these metals is zinc – typically the second most abundant transition metal in organisms and a substantial micronutrient that plays different roles in plant physiology. Zinc is an essential component of over 300 enzymes. It is responsible for gene regulation and stabilization of protein structure including Zn fingers, Zn clusters and RING finger domains. It is also involved in essential processes such as photosynthesis and CO_2 fixation. Excess or deficiency of zinc in plants leads to high plant mortality, reduced and stunted growth, chlorosis, necrosis, small leaves and delay in flowering. All of these symptoms may cause serious implications for food security because of the significant reduction of crop yields that is correlated to zinc availability [1-3].

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Better understanding of plant physiology through the identification of low molecular weight metal-containing compounds present in plant saps, and the determination of their exact function will further inform nutritional, agricultural and environmental studies. However, the study of complex biological matrices such as plant samples may cause several issues during sample preparation or chromatographic separation. The main challenges relate to the low concentrations of metal complexes and their huge diversity. In addition, metal complexes are often unstable and can be degraded during extraction, off-line preconcentration steps or even during chromatographic separation. Of the chromatographic techniques that have been investigated thus far, size exclusion chromatography (SEC), hydrophilic interaction chromatography (HILIC) and reverse phase (RP) chromatography have proved to be the most suitable techniques to avoid degradation of the metal complexes during analysis [4]. A chromatographic system consisting of a preconcentration column and HILIC or RP separation column seems to be ideal for this kind of application.

This work proposes an ICP-MS-assisted metallomic approach to the separation of zinc species present in post-phloem of *Pisum sativum*. The embryo sac liquid was analyzed by capillary HPLC-ICP-MS with on-line preconcentration. The use of capillary chromatography was essential due to the low amount of plant sap available.

Experimental

Sample

Embryonic sac liquid (post-phloem) obtained from the developing pods of *Pisum sativum* (green pea) was studied.

Sample preparation

The pods were perforated with a glass capillary and the liquid endosperm was extracted using a peristaltic pump and put into an Eppendorf tube kept on ice. After collection, the samples were immediately frozen in liquid nitrogen and stored at -20 °C until further analysis. Just before analysis, the samples were thawed, diluted with acetonitrile to obtain a 1:2 ratio (sample:acetonitrile) and then centrifuged for 2 minutes at 10000 rpm. The supernatant was collected and analyzed immediately.

HPLC-ICP-MS system

An Agilent 1100 LC fitted with a capillary pump and manual valve (loop size: 100 µL) was used. 30 µL of supernatant was loaded on the SeQuant zwitterionic (ZIC-)HILIC guard column (Merck KGaA, Darmstadt, Germany, 5 mm x 1 mm i.d., 5 µm) using an isocratic flow of 20 µL/min of 90% acetonitrile and 10 mM ammonium formate buffer (pH 5.5). The sample was washed with the mobile phase for 4 min and then back-flushed onto the SeQuant ZIC-HILIC capillary column (Merck KGaA, Darmstadt, Germany, 150 mm x 0.3 mm i.d., 3.5 µm) that was used for compound-separation. Gradient elution, at a flow rate of 4 µL/min, was carried out using eluent A, 10 mM ammonium formate buffer (pH 5.5), and eluent B, acetonitrile. The gradient program is given in Table 1.

Table 1. HPLC elution program

Step	Eluent [%B]	Time [min]
1	90	0-5
2	90-65	5-17
3	65-52	17-47
4	52-35	47-53
5	35	53-65
6	35-90	65-70
7	90	70-75

HPLC-ICP-MS and ESI-MS/MS

The outlet of the separating column was connected to the Agilent 7700x ICP-MS (via the Agilent capLC interface, G3680A, Figure 1) or ESI-MS/MS.

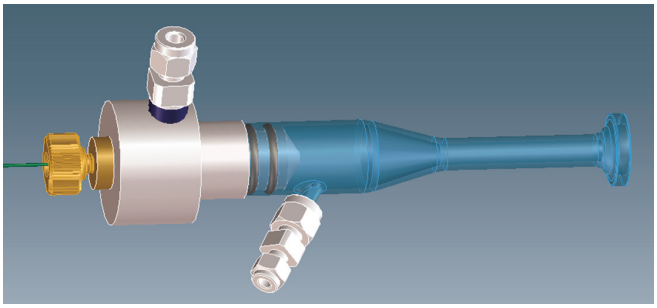

Figure 1. Agilent capillary LC interface kit (G3680A) which consists of a total consumption nebulizer inside a small quartz spray chamber

ICP-MS conditions were auto-optimized at the start of each day, using a tune solution containing 20 ppb of Y, Li, Tl, Ce in 2% nitric acid. The ORS^3 collision/reaction cell of the 7700x was operated in high energy helium mode to exclude polyatomic interferences that may occur on Zn isotopes. Signals for ^{64}Zn and ^{66}Zn were acquired using a dwell time of 60 ms. The ICP-MS operating conditions are given in Table 2.

Table 2. Agilent 7700x ICP-MS operating conditions

Parameter	Value
Nebulizer/spray chamber	Capillary LC Interface G3680A
Torch i.d.	1 mm
Cones	Platinum
RF power	1560 W
Sampling depth	7.5 mm
Carrier gas flow rate	0.78 L/ min
Optional gas (O_2) flow rate	0.04 L/ min
Lenses	
Extract 1	2.7 V
Extract 2	-180 V
Cell	
Octopole bias	-100 V
He flow	10 mL/ min
Kinetic energy discrimination	7 V

The ESI LTQ Orbitrap Velos mass spectrometer was operated in the positive ion mode at 3.0 kV. The vaporizer temperature of the source was set to 120 °C and the capillary temperature to 280 °C. The resolution in full MS mode was set at 100,000 (FWHM at *m/z* 400).

Results and discussion

The chromatogram obtained for ^{64}Zn for green pea post-phloem sample is shown in Figure 2. The use of capillary ZIC-HILIC ICP-MS with on-line preconcentration allowed us to obtain sharp and intense peaks and enabled the separation and detection of two zinc species (Figure 2a and 2b). Both metal complexes were identified using ESI Orbitrap MS/MS. ICP-MS detection was essential to determine the retention times of the different zinc species and to estimate the mass balance. This simplified the search for the zinc complexes in the ESI-MS mass spectra. The expanded parts of the mass spectra (Figure 2d and 2e) clearly show two ions containing the isotopic pattern for zinc. The retention times of these extracted ion chromatograms (EIC) are in agreement with the two zinc peaks observed via capillary ZIC-HILIC ICP-MS (Figure 2c). The data obtained allowed us to identify two zinc complexes: zinc-nicotianamine (NA) and zinc-(histidine)$_2$ (Table 3).

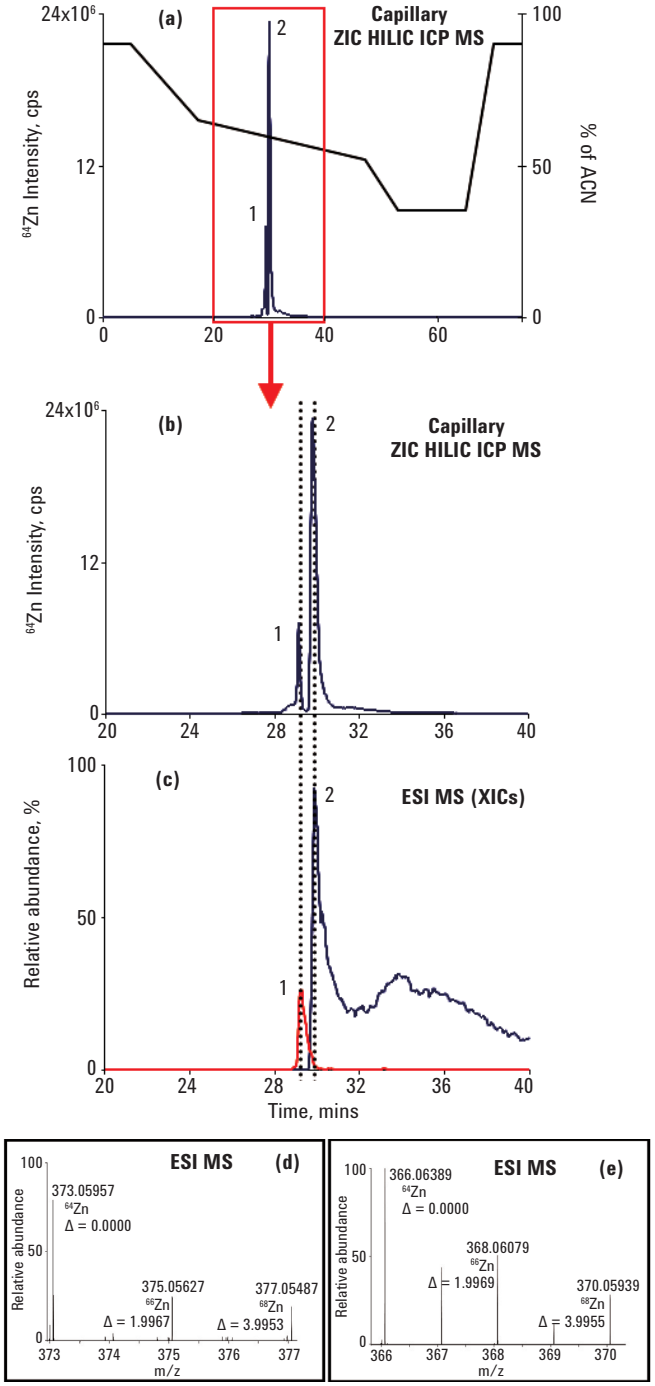

Figure 2. Chromatograms obtained by a) capillary ZIC-HILIC-ICP-MS; b) capillary ZIC-HILIC-ICP-MS – zoomed in section of chromatogram; c) capillary ZIC-HILIC-ESI-MS (selected ion chromatograms). d) and e) Zoomed in section of the ESI-MS spectrum containing zinc isotopic pattern.

Table 3. List of zinc containing complexes indentified in post phloem of *Pisum sativum* (green pea)

	Peak 1	Peak 2
Ligand	Histidine	Nicotianamine
Complex	$(His)_2$-Zn	NA-Zn
Formula (neutral form)	$C_{12}H_{16}O_4N_6Zn$	$C_{12}H_{19}O_6N_3Zn$
Theoretical mass	373.05973	366.06381
Experimental mass	373.05957	366.06389
Delta ppm	-0.437	0.216

The chromatographic system consisting of capillary HPLC and ICP-MS allowed us to achieve a detection limit of 75 ng/L for ^{64}Zn (~ 6 fmol of Zn-NA complex), calculated as 3x the standard deviation of 20 points of the base line. This value was compared with the signal of Zn-NA complex in respect to column recovery obtained for ^{64}Zn which was 70-80%.

Conclusions

The study shows an effective ICP-MS assisted metallomic approach for the separation and identification of zinc complexes present in post-phloem of *Pisum sativum*. The zinc species were preconcentrated using a ZIC-HILIC pre-column and then separated via a ZIC-HILIC capillary column. The combination of data obtained by coupling capillary HPLC to ICP-MS and ESI MS/MS instruments allowed the identification of different zinc complexes. The use of capillary chromatography was essential due to the low amount of plant sap available. Additionally the chromatographic system with on-line preconcentration is ideal to work with biological samples containing low concentrations of metal species that may also sometimes be unstable. This approach has been successfully used to identify two zinc species: zinc-nicotianamine (NA) and zinc-$(histidine)_2$ complexes proving that NA and histidine are two major ligands that complex zinc in post-phloem of green pea.

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Conclusion and perspectives

In order to investigate the metal/metalloid speciation in different plant samples a novel and efficient analytical approach, based on the combined techniques, was developed. It was based on couplings of size exclusion chromatography (SEC) and hydrophilic interaction chromatography (HILIC) to either mass spectrometer with ionization in inductively coupled plasma (ICP MS) or LTQ Velos Orbitrap with electrospray ionization (ESI) for elemental and molecular analysis, respectively. The combination and comparison of data obtained with high resolution ESI-MS (exact monoisotopic masses, characteristic isotopic signatures recognition and interisotopic mass defect) and with ICP-MS (retention times, shapes and intensities of chromatographic peaks of searched elements) allowed the development of a strategy to detect and to quantify systematically and unambiguously metal/metalloid species.

The developed approach was successfully applied in three projects that were carried out during this Ph.D. and aimed at metal/metalloid compound identification and characterization in plant material.

The first project aimed at the identification of metal complexes present in *Pisum Sativum* (green pea) saps and thus determination of ligands that are responsible for metal transport in this plant species. The developed analytical approach allowed the identification of over 60 different metal (e.g. Fe, Zn, Cu, Ni, Co, Mn, Ca and Mg) species in xylem and in embryo sac liquid of green pea. The main ligands responsible for metal transport were also determined as non-proteinogenic amino acids (e.g. nicotianamine), proteinogenic amino acids (e.g. aspartic acid, glutamine or phenylalanine) and organic acids (citric and malic acid). Additionally, the collaboration with biologists UMR Biochimie et Physiologie Moléculaire des Plantes AGRO-M/INRA in Montpellier (France) allowed the characterization of the mechanism of iron transport within this plant. It was determined that in xylem iron is present as a ferric cation and it is transported in form of $Fe(citrate)_2$, $Fe_3(citrate)_3(malate)$ and $Fe_3(citrate)_2(malate)_2$ complexes to the seeds.

The second project consisted in the identification of aluminum complexes present in different organs of *Plantago almogravensis* Franco. The use of hyphenated techniques permitted to investigate the speciation of aluminum – monoisotopic element with complex aqueous coordination chemistry. The novel methodology allowed the observation of the correlation between aluminum and iron. Therefore, Fe signal were used as internal tracer in high resolution mass spectra for identification of the tri aluminum tri citrate complex in an Al-accumulating plant without prior information. The developed approach permitted to identify

the $Al_3(citrate)_3$ complex and thus, determine that the citrate is the main ligand responsible for aluminum detoxification process in this plant species. It was confirmed by the analysis of Al – citrate standard. This approach allowed also identification of other complexes present in *Plantago almogravensis* including tri iron tri citrate complex – $Fe_3(citrate)_3$ and mixed aluminum-iron-citrate complexes – $Al_2Fe(citrate)_3$ and $AlFe_2(citrate)_3$.

In the third project, the above methodology aided by gas chromatography combined with element and molecule specific detection systems permitted to determine the comprehensive selenium speciation in the *Brassica nigra* (black mustard) seeds grown on a seleniferous area. More than 30 different low-molecular weight Se-containing compounds that belongs to different families, such as selenoglucosinates, selenosinapine, selenoamino acids, selenosugars, selenoacylated choline, selenocarbohydrates and their metabolites selenourea and selenocyanates were detected and characterized. Different pathways of selenium incorporation into these compounds were observed. In amino acids, selenium was incorporated nonspecifically instead of sulfur. In other compounds, selenium incorporation was based on oxidative attack of selenol moiety, a constituent of methylselenol and selenoamino acids, on either unsaturated bonds in alkyl chains or reducing sugars. It was determined that the most abundant Se compounds present in Brassica nigra seeds belong to the family of glucosinolates.

The obtained results show that the novel analytical approaches based on high resolution separation techniques combined with sensitive element specific and high resolution and mass accuracy molecule specific detection systems are promising tool to investigate metal speciation in complex biological matrixes.

The perspectives concerning presented topic include:

- investigation of speciation and distribution of metals in different parts of plant cell by analyzing fluids coming from extracted organelles;
- development of a chromatographic metal-free system;
- investigation of the exchange and the dynamic transport of metals in interface soil – root and root – plant using metal species enriched with stable isotopes;
- identifying model plants presenting an abnormal speciation (total and relative concentration of metals, different LC – ICP MS profile and/or different metal uptake mechanism compared to not mutated plants) and the characterization of the nature of these differences.

List of publications

1. **Exhaustive characterization of metal complexes in xylem and in liquid endosperm of pea;**
   (to be submitted)
   Paulina Flis, Laurent Ouerdane, Louis Grillet, Catherine Curie, Stéphane Mari, Ryszard Lobinski

2. **Ascorbate efflux as a new strategy for iron reduction and transport in plants;**
   (submitted)
   Louis Grillet, Laurent Ouerdane, Paulina Flis, Marie-Pierre Isaure, Ryszard Lobinski, Catherine Curie, Stéphane Mari

3. **Identification of the tri-Al tricitrate complex in *Plantago almogravensis* by hydrophilic interaction LC with parallel ICP-MS and electrospray Orbitrap MS/MS detection;**
   (Metallomics, 2013, 5, 1285-1293)
   Tomás Grevenstuk, Paulina Flis, Laurent Ouerdane, Ryszard Lobinski, Anabela Romano

4. **Comprehensive speciation of low-molecular weight selenium metabolites in mustard seeds by HPLC - electrospray linear trap/Orbitrap tandem mass spectrometry;**
   (Metallomics, 2013, 5, 1294-1304)
   Laurent Ouerdane, Federica Aureli, Paulina Flis, Katarzyna Bierla, Hugues Preud-homme, Francesco Cubadda, Joanna Szpunar

5. **Speciation of zinc in microliter volumes of plant sap by capillary HPLC-ICP-MS;**
   Agilent Application Note 5991-2415EN ; published May 3, 2013 ;
   Paulina Flis, Laurent Ouerdane, Ryszard Lobinski

6. **Intra- and inter-molecular isotopic mass defect as a powerful tool for the speciation of heavy elements by high resolution mass spectrometry;**
   (in preparation)
   Laurent Ouerdane, Paulina Flis, Ryszard Lobinski

Contribution to the articles

My contribution to the papers concerns the analytical part of each article presented in the thesis. This contribution includes samples preparation procedures, quantification of total metal concentrations, recovery experiments, HPLC-ICP MS analysis, HPLC-ESI MS(MS) analysis and data treatment – identification of metal complexes, tables and figures (chromatograms, mass spectra and compound structures).

The plant cultivation, collection of plant material and molecular biology experiments were performed in collaborating laboratories.

Presentations at international conferences

1. 13th Workshop on Progress in Trace Metal Speciation for Environmental Analytical Chemistry, TraceSpec, 18-20 May 2011, Pau, France;
Metal speciation in *Pisum sativum* (green pea) through the association of LC ICP-MS and LC ESI-LTQ Orbitrap MS couplings;
Paulina Flis, Laurent Ouerdane, Ryszard Lobinski
(poster)

2. 3rd International Symposium on Metallomics, 15-18 June 2011, Münster, Germany;
Metal speciation in model plants through the association of LC ICP-MS and LC ESI-LTQ Orbitrap MS couplings;
Laurent Ouerdane, **Paulina Flis**, Ryszard Lobinski
(oral presentation)

3. 7th Internationale Fraco–Spanish Workshop on Bio-inorganic Analytical Chemistry, 1-3 July 2012, Gijón, Spain;
Analytical approaches to metal speciation in model plants;
Paulina Flis, Laurent Ouerdane, Ryszard Lobinski
(oral presentation)

4. Plant Biology Congress, 29 July – 3 August 2012, Freiburg, Germany;
Aluminium speciation in the hyperaccumulating species *Plantago almogravensis*;
Tomás Grevenstuk, **Paulina Flis**, Laurent Ouerdane, Neusa Martins, Ryszard Lobinski, Anabela Romano
(poster)

5. European Winter Conference on Plasma Spectrochemistry; EWCPS; 10-15 February 2013, Kraków, Poland;
Combined techniques: LC-ICP MS and LC-ESI-LTQ Orbitrap MS as a powerful tool to investigate elements speciation in plants;
Paulina Flis, Laurent Ouerdane, Ryszard Lobinski
(poster)

6. 4th International Symposium on Metallomics, 8-11 July 2013, Oviedo, Spain;
Hyphenated techniques LC – ICP MS and LC – ESI-LTQ Orbitrap MS in investigation of metal speciation in *Pisum Sativum* and *Plantago almogravensis*;
Paulina Flis, Laurent Ouerdane, Louis Grillet[2], Stéphane Mari, Tomás Grevenstuk, Ryszard Lobinski
(oral presentation)

7. 4th International Symposium on Metallomics, 8-11 July 2013, Oviedo, Spain;
Iron (Fe) transport in plants: when speciation is crucial to reveal new biological functions;
Louis Grillet, Catherine Curie, Stéphane Mari, Laurent Ouerdane, **Paulina Flis**, Marie-Pierre Isaure, Ryszard Lobinski
(oral presentation)

8. 4th International Symposium on Metallomics, 8-11 July 2013, Oviedo, Spain;
Comprehensive study on speciation of low-molecular weight selenium metabolites in black mustard seed by chromatographic and MS-based techniques;
Laurent Ouerdane, Federica Aureli, **Paulina Flis**, Katarzyna Bierla, Hugues Preud'homme, Francesco Cubadda, Joanna Szpunar
(poster)

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