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LC-ICP-MS methods for speciation of phosphorus compounds in soil and plant related samples

The PhD thesis is presented for the fulfillment of the

requirements for the degree of *Dr.nat.tech*. at the

Vienna University of Natural Resources

and Life Sciences, BOKU-Vienna

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Vienna, 2016

Acknowledgements

I am grateful to my supervisor Stephan Hann, for all his support and guidance throughout my PhD studies. It was my great privilege to be a part of his outstanding working group with great diversity on research and social activities.

My co-supervisor Gunda Koellensperger, for not only being supervisor but a lot of time a stand by colleague with great support.

Gerhard Stingeder. the head of Division of Analytical Chemistry for all support during all these years.

My co-authors, Markus Puschenreiter, Stefan Neubauer, Lisa Fischer, Christina Troyer, Hedda Drexler, Chu Đinh Binh and Jakob Santner for their great cooperation, support and friendship during all these years.

To all of my former colleagues from Department of Chemistry, Faculty of Mathematics and Natural Sciences at University of Prishtina, Kosovo.

To all of my BOKU colleagues: Madeleine, Thomas, Gerrit, Marianna, Christof, Eveline, Kristaps, Raphaele, Daniela, Halimat, Yvonne, Karin, Theresa, Michaela, Simone, Anna, Sonja and Luzia for making it a pleasure coming at office every day.

And last but no means least, to my family for all their love and patience during all these years.

Without financial support from the Austrian Ministry of Science and Research, ÖAD - Bertha von Suttner–Kosovo foundation this PhD project would have never been realized.

Vienna, December 2016 **Ariana Rugova**

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SUMMARY

The objective of the presented work was the development of sensitive and selective methods for the analysis of organic phosphorus compounds in soil and plant-related samples, which can be of importance for studies aiming at the investigation of phosphorus mobilization and plant nutrition.

Chemical characterization as well as accurate quantification of the compounds present in the rhizosphere is a major pre-requisite for a better understanding of rhizosphere processes. In this context, there is a high number of organic phosphorus compounds present in the rhizosphere consisting in highly combined forms that remain still unidentified. This is due to the fact that representative sampling of phosphorus containing organic compounds from soil, their identification and accurate quantification as well as the interpretation of the obtained information in the context of bioavailability is very challenging. Thus, development and application of advanced sampling and extraction procedures in combination with highly selective and sensitive analytical techniques for quantification of organic phosphorus compounds in rhizosphere is foreseen as a necessity in order to ensure accurate quantification of organic phosphorus compounds of the rhizosphere processes.

An introduction to the role of phosphorus in the life cycle is presented and followed by a brief overview on organic phosphorus compounds in the environment. Special attention is paid to myo-inositol hexakisphosphate as the major representative of organic phosphorus compounds as one of the most important target analytes in this thesis.

Analytical techniques employed within all stages of method development are described in Chapter 2 followed by a review of selected publications. The major outcome of the presented work is a novel method based on high performance ion chromatography coupled to inductively coupled quadrupole plasma mass spectrometry based method employing strong anion exchange chromatography (HPIC-ICP-DRCMS) with HNO_3 gradient elution for simultaneous analysis of orthophosphate and myo-inositol hexakisphosphate (IP_6) in soil solution and plant extracts. This method is presented in publication II in Chapter 3. The developed HPIC-ICP-DRCMS method offers excellent repeatability and limits of detection based on 3σ criterion in the sub-micromole per liter range without the need for special sample pre-treatment.

A comprehensive literature research on application of mass spectrometry techniques in rhizosphere investigation documented in the form of a review paper within this thesis is presented as the first publication in Chapter 3. During the last years, targeted and non-targeted mass spectrometry based methods have emerged and have been successfully applied in

numerous studies related to the root exudates in the rhizosphere. Hence, elemental or molecular mass spectrometry emphasizing different separation techniques as gas chromatography, liquid chromatography, size exclusion chromatography or capillary electrophoresis applied in this research field are critically discussed within this review.

The third work, into which I was involved as a co-author (approximately 30% contribution) aimed at the development of a fast method for quantification of nucleotides in nucleotide enriched yeast extracts. The presented reversed-phase separation method combined with complementary ICP‑MS detection for quantification of free nucleotides in yeast products due to the absolute lack of reference materials could be implemented as a reference method for nucleotide quantification. To our best knowledge this is the first LC-ICP-MS method applied for the quantification of free nucleotides in biological samples as a method for measuring nucleotides orthogonal to LC-ESI-MS.

KURZFASSUNG

In der vorliegenden Dissertation wurde eine empfindliche und selektive Methode zur Analyse organischer Phosphorverbindungen in Boden- und Pflanzenproben entwickelt, welche für Studien zur Erforschung der Phosphormobilisierung und Ernährung der Pflanze von hoher Wichtigkeit sind.

Sowohl die chemische Charakterisierung als auch die akkurate Quantifizierung dieser Verbindungen in Rhizosphären (Wurzelraum der Pflanze) ist eine wichtige Grundvoraussetzung für ein besseres Verständnis der dort ablaufenden Prozesse. Bislang sind noch sehr viele der organischen Phosphorverbindungen nicht identifiziert, da sowohl eine repräsentative Probenahme phosphorhaltiger Verbindungen aus Böden als auch die Identifizierung und akkurate Quantifizierung sowie die Dateninterpretation hinsichtlich Bioverfügbarkeit eine große Herausforderung darstellen. Die Entwicklung und Etablierung fortgeschrittener Probenahme- und Extraktionsverfahren in Kombination mit nachweisstarken und äußerst selektiven analytischen Techniken ist eine Notwendigkeit, um die richtige Identifizierung und Quantifizierung dieser Verbindungen zu ermöglichen.

In der vorliegenden Arbeit wird einleitend in Kapitel 1 die Rolle von Phosphor für die Lebensdauer der Pflanze beschrieben sowie ein kurzer Überblick über organische Phosphorverbindungen in der Umwelt unter besonderer Berücksichtigung der Verbindung myo-Inositolhexakisphosphat, welche als bedeutendster Vertreter dieser Substanzklasse gilt und ein wichtiger Zielanalyt in dieser Arbeit war, erläutert. Sämtliche Phasen der Methodenentwicklung und deren Anwendung, unterstützt durch Bezugnahme auf ausgewählte wissenschaftliche Publikationen, werden in Kapitel II erläutert. Der im Rahmen dieser Dissertation entwickelte neue methodische Ansatz, basierend auf Hochleistungsionenchromatographie gekoppelt mit induktiv gekoppelter Quadrupol-Massenspektrometrie, erlaubt durch den Einsatz eines starken Anionenaustauschers als stationäre Phase und Gradientenelution mit Salpetersäure die gleichzeitige Bestimmung von o-Phosphat und myo-Inositolhexakisphosphat (IP_6) in Bodenlösungen und pflanzlichen Extrakten. Des Weiteren konnte eine exzellente Wiederholpräzision und Nachweisgrenzen – basierend auf dem 3s Kriterium – im niedrigen Mikromol pro Liter Bereich ohne aufwändiger Probenvorbereitung erreicht werden. Details zu dieser Methodik sind in Publikation I in Kapitel III dargestellt.

Publikation II aus Kapitel III enthält einen Review-Artikel aus der Zeitschrift Analytika Chimica Acta. Dieser enthält eine umfangreiche Literaturrecherche zur Anwendung

massenspektrometrischer Techniken in der Rhizosphärenforschung. In den vergangenen Jahren haben sich vor allem auf Massenspektrometrie basierende Methoden zur zielgerichteten und ungezielten Analyse von Wurzelexsudaten in der Rhizosphäre durchgesetzt und wurden erfolgreich in zahlreichen Studien angewendet. In diesem Übersichtsbeitrag wurden verschiedenste Trenntechniken wie Gaschromatographie, Flüssigchromatographie, Größenausschlusschromatographie oder Kapillarelektrophorese in Verbindung mit Element- bzw. Molekülmassenspektrometrischen Systemen kritisch betrachtet.

Die dritte Arbeit, an der ich als Co-Autor beteiligt war (ca. 30% Beitrag), beschäftigt sich mit der Entwicklung einer schnellen Methode zur Quantifizierung von Nukleotiden in Nukleotid angereicherten Hefeextrakten. Die vorliegende auf Umkehrphasenchromatographie basierende Trennmethode kombiniert mit der selektiven und empfindlichen ICP-MS-Detektion ermöglicht die genaue Quantifizierung von freien Nukleotiden in Hefeprodukten. Aufgrund des Fehlens von Referenzmaterialien könnte diese Methode als Referenzverfahren für die Nukleotid-Quantifizierung implementiert werden. Dies is, nach unserem Wissensstand, die erste Arbeit in welcher ein LC-ICP-MS-Verfahren als orthogonale Methode zur LC-ESI-MS für die Quantifizierung von freien Nukleotiden in biologischen Proben angewandt wurde.

1. Introduction

Phosphorus is a key nutritive soil element essential for all living organisms. Besides the fact that the amount of phosphorus in the soil is generally quite high (often between 400 and 5000 mg per kg soil) most of it exists in an insoluble form and is therefore not available to support plant growth [1].This phosphorus deficiency in agricultural fields most of the time is overcomed by application of phosphate fertilizers. However, this approach is not ideal since almost 75–90% of added amounts of these fertilizers is precipitated by metal cation complexes present in the soil and plants can only absorb less amounts of applied fertilizers and the rest is rapidly converted into insoluble organic phosphorus complexes in soil [2] with only 10-20 % of supplied phosphorus being absorbed by plants in the year of application [3].

Insoluble phosphorus is present as either an inorganic mineral such as apatite or as one of several organic forms including inositol phosphate as a phosphor-monoester, and phosphor di- and triesters [4,5]. The prevalent form of available phosphorus in the environment is the anionic phosphate [6]. Its chemistry is determined by the strength of ionic bonds to the surrounding atoms. Based on the relative strength of these ionic bonds an explanation can be withdrawn why phosphate behaves so differently to the common mineral forms of other nutrients, *e.g.* nitrate and sulphate [7]. The only form of phosphorus that most organisms take up is inorganic orthophosphate [8] besides the fact that organic compounds of phosphorus often dominate in soils and aquatic systems [9]. Although the total amount of phosphorus in the soil may be high as mentioned above, the preferred form for assimilation orthophosphate is not easily accessible to most plants and microbes, because of its adsorption to soil particles and clay minerals and surfaces of calcium (Ca) and magnesium (Mg) carbonates and its conversion to organically bound forms or the formation of insoluble precipitates with common cations like Fe, Al, and Ca [10,11].

Even though the importance of organic phosphorus in soil is so crucial due to the nutritional role in plant growth, our understanding of its dynamics and ecological function still remains unsatisfactory. Progress is limited in part by extraction methodology, because information on organic phosphorus species provided by modern analytical techniques can often not be linked to biological availability with the necessary degree of confidence [12].

Development and usage of improved detection methods has been necessary to develop in order to ensure accurate quantification of organic phosphorus compounds in soil for investigation of their behaviour [13]. Traditionally, soil organic phosphorus has been crudely

determined by the difference between total and inorganic phosphorus [14]. This method is commonly used because of the difficulties associated with direct measurement of organic phosphorus compounds. The main organic phosphorus compounds that have been investigated mostly are inositol phosphates with the major representative *myo*-inositol-hexakisphosphate (IP_6) a family of phosphoric esters of hexahydroxy cyclohexane [15] amongst phosphoric diesters are phospholipids and also nucleic acids [16]. Although separation of IP_6 in food samples is well documented in a major number of publications so far, were levels of IP_6 are in the mM range [Table 4], separation of IP_6 in soil solution and plant related samples were concentration of IP_6 is in the low µM range requires more sensitive techniques such as liquid chromatography (LC) coupled to Inductively coupled plasma mass spectrometry (ICP-MS) that offers a supreme detection in the field of elemental mass spectrometry. The aim of the presented thesis was to develop a more sensitive and selective method for quantitative analysis of *myo*-inositol-hexakisphosphate as an organic phosphorus containing compound in soil and plant related samples.

In addition to further investigations of organic phosphorus compounds and application of diverse separation techniques in phosphorus speciation a fast method for quantification of nucleotides in nucleotide enriched yeast extracts was developed. For this purpose a liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS) based method for separation of nucleotides, as less polar monoester phosphate compounds, was developed within this PhD project in addition to the pervious described method. To our best knowledge this is the first LC-ICP-MS method applied for the quantification of free nucleotides in biological samples as a method for measuring nucleotides orthogonal to LC-ESI-MS.

Moreover, comprehensive literature research about application of mass spectrometry techniques in rhizosphere research since the beginning of this century has been performed and documented in the form of a review paper within this thesis.

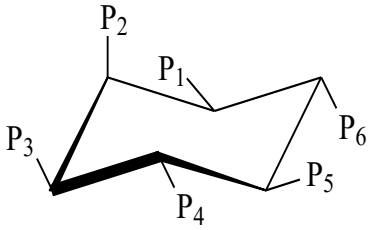
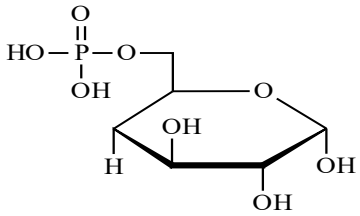
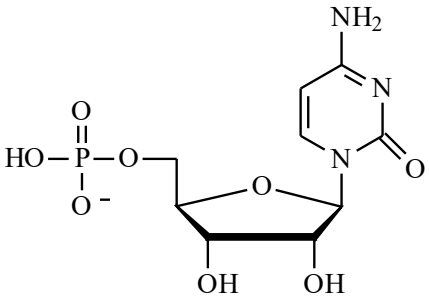
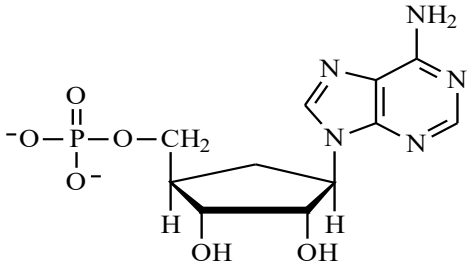
This critical review covers both elemental and molecular mass spectrometry as well as their combination with different separation techniques with discussion of the most important works by, at the same time, covering all methods which are essential for investigating soil/ plant related processes in the

.

1.1 Organic phosphorus compounds in the environment

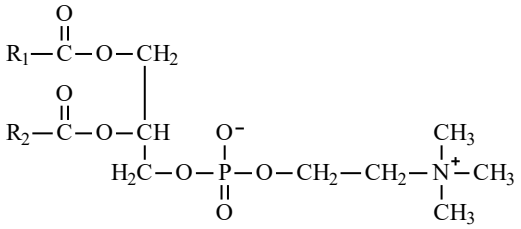
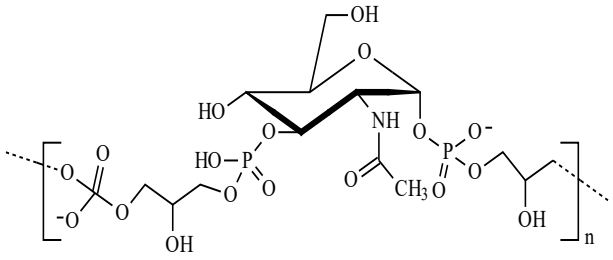
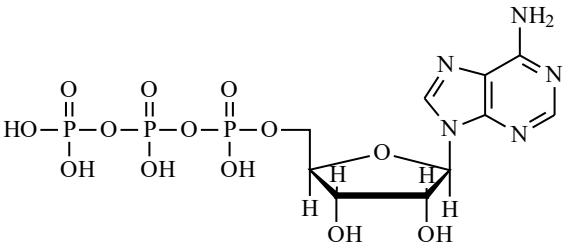
Organic phosphorus compounds play a significant role in plant nutrition and therefore understanding of their dynamics and ecological functions in the environment is highly important. Based on the nature of the phosphorus bond, soil organic phosphorus is classified into phosphate esters, phosphonates and phosphoric acid anhydrides. Phosphate esters are sub-classified according to the number of ester groups linked to each phosphate. Table 1 presents the main representatives of the phosphate monoesters as the dominant group of organic phosphorus compounds in most soils with inositol phosphates being as the major representative of this group. Nucleotides besides sugar phosphates and phosphoproteins presented in small amounts in soil, are composed of a phosphate moiety containing one to three phosphate groups bound to a pentose sugar on the one side and of a nucleobase moiety bound to the pentose sugar on the other side. They are referred as monomeric units of the polynucleotides such as RNA and DNA. Nucleotides have an acidic character and are negatively charged above pH=2 in a wide pH range. Nucleic acids present in less than 3% of the organic phosphorus in soil are associated with other compounds derived from the degradation of living organisms that allow rapid mineralization, also re-synthesizing and combination with other soil constituents. They can also be incorporated into microbial biomass.

Table 1. Major representatives of soil organic phosphate monoesters

Compound	Structure
Myo-inositolhexakisphospahte	
Sugar phosphate	
Cytidine monophosphate	
Monophosphate nucleotide	

Phosphate diesters include nucleic acids (DNA and RNA), phospholipids and teichoic acids and they are present in less than 10% of the soil organic phosphorus (Table 2).

Table. 2 Major representatives of soil organic phosphate

Compound (Functional class)	**Structure**
Phosphatidyl choline (Phospholipid)	
Teichoic acid from (Micrococcus species)	
Adenosine 5'-triphosphate (ATP) (polyphosphate, nucleotide)	

1.1.1 *Myo*-inositol hexakisphosphate

Myo-inositol hexakisphosphate (IP_6) is the six phosphate monoester of inositol and is also known as phytic acid representing the free acid form of IP_6. Salts of phytic acid, phytates are quite stable and accumulate in soils due to their affinity to bind with other minerals present in soil such as calcium, iron or even aluminum.

To the parent inositol can be linked up to six phosphate groups. *Myo*-inositol-hexakisphosphate is the most abundant form of phosphate monoesters in soil. It may represent 80% or more of the total organic phosphorus and exist in soil in several stereoisomeric forms: *myo-, scyllo-, D-chiro* and *neo*-inositol phosphates (Table 3) [17].

In terrestrial environment, IP_6 is synthesized in plants seeds and represents the major storage form of phosphorus in plants and organic soil components [18]. When present in soil, it is not directly available for uptake by plants until it's hydrolyzed to release phosphate and then becomes plant-available [19].IP_6 can be dephosphorylated by the enzyme phytase, thus producing inorganic phosphate that can be taken up by plants [11]. But still there is not much known about the mechanisms how plants make use of organic phosphorus and it is suspected that microorganisms play an important role for the degradation of organic phosphorus compounds. The uptake of phosphorus and its subsequent release by microorganisms is suspected to have a strong impact on the availability of phosphorus to plants [20].

Although it has been shown that IP_6 can be taken up by plant roots, the contribution of IP_6 to total phosphorus uptake is still unclear. One major reason for the poor understanding of IP_6 dynamics in the rhizosphere is the typically very low IP_6 concentration in soil solution and consequently the difficulty to obtain reliable information on concentration dynamics. Thus, methodological improvements in analyzing IP_6 in soil solution or soil extracts are highly emerging.

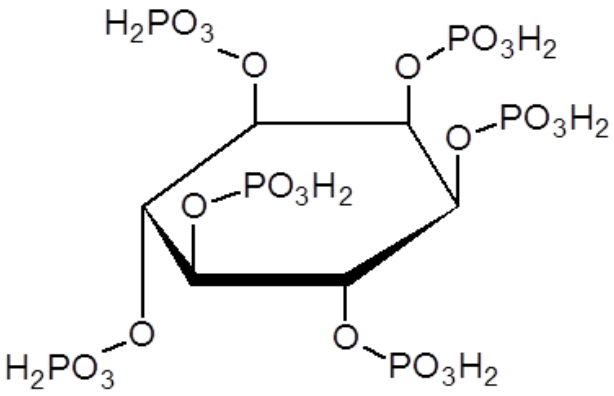
Table 3 Stereo-isomeric forms of phytic acid (IP_6)

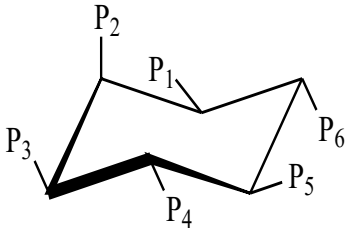
Myo-inositol-hexakisphosphate

Molecular formula $C_6H_{18}O_{24}P_6$

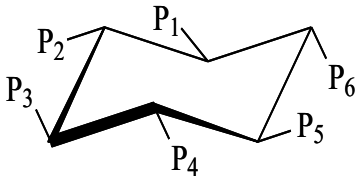
Molar mass 660. 035 Da

IUPAC name (1*R*,2*R*,3*S*,4*S*,5*R*,6*S*)-cyclohexane-1,2,3,4,5,6-hexaylhexakisdihydrogenphosphate

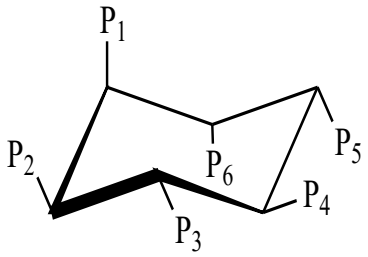




Myo



Scyllo



D-chiro

Neo

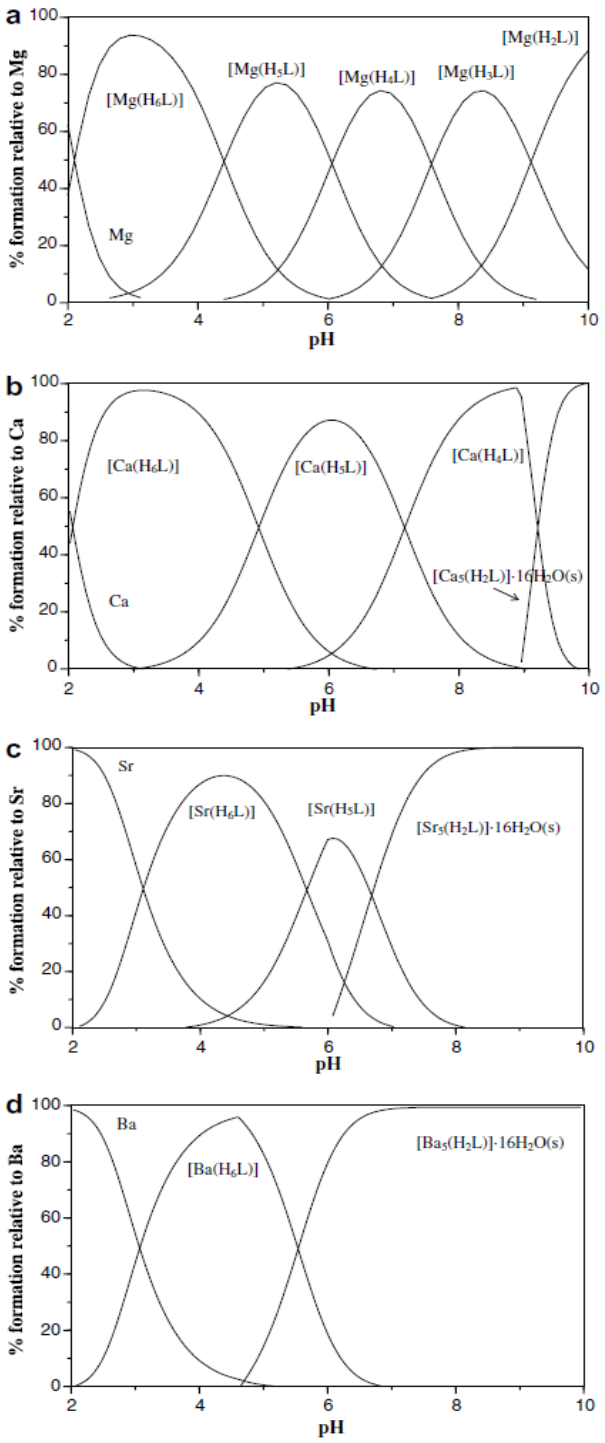


Figure 1 Diagram of comparative species distribution for the M^{2+}-$InsP_6$ systems: (a) Mg^{2+}, (b) Ca^{2+}, (c) Sr^{2+}, (d) Ba^{2+}. Permission requested [23].

The most outstanding feature of IP_6 is its powerful chelating function and this is due to the ability to strongly chelate with cations such as Ca, Mg, Zn, Cu, Fe and to form insoluble complexes and reduce the catalytic activities of these ions which makes IP_6 a potential natural antioxidant [21, 22].

Since IP_6 works in a broad pH region as a highly negatively charged ion (Figure 1) [23,24], when present in diet it has a negative impact on the bioavailability of divalent and trivalent mineral ions and therefore has a very high affinity to react with positive charged mineral food components of [25,26]. Due to this property, IP_6 has been considered as an anti-nutritional component in cereals, seeds and beans.

However, consumption of phytate seems not to have only negative aspects on human health, since results of epidemiological and animal studies in the past few years suggest that there are also beneficial effects, such as decreasing the risk of heart disease and colon cancer, but data from human studies are still lacking [27]. On the other hand, in animal studies phytic acid has shown a protective action in carcinogenesis [27].

Phytic acid may have health benefits for diabetes patients since it lowers blood glucose response [28]. Just recently IP_6 is presented as a novel type of selective inhibitor of osteoclasts and proved as very useful for the treatment of osteoporosis [29].

2. Analysis of organic phosphorus compounds in soil and plant related samples

Representative sampling of phosphorus containing organic compounds in soil, their identification and accurate quantification as well as the interpretation of the obtained information in the context of bioavailability is highly challenging.

Traditionally, soil organic phosphorus has been determined by the difference between total phosphorus and inorganic phosphorus and dates from the early 70ies [14] as mentioned in the introduction. This approach was a method of choice for a long time because of the difficulties associated with the selective measurement of different organic phosphorus compounds.

Accordingly, identification of organic phosphorus compounds in soils and soil extracts was accomplished using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Although NMR techniques have been developed and used successfully, their use is limited by the need for sample pre-concentration, which can eventually influence the pattern of phosphorus species, and long run times, which can be expensive and may cause sample degradation [30]. Quantifying NMR signal for individual species in the crowded and overlapping phosphate monoester region is a problem [31] and also the peak identification which is complicated by variations in chemical shift with pH and ionic strength is also contributing to the limitations of this method.

Analysis of soil solutions or plant extracts by molybdate colorimetry as a conventional method for phosphorus determination. However it is considered as a potential source of error in all procedures, because organic phosphorus is overestimated also in this method as in the above mentioned method in the presence of inorganic polyphosphates or complexes between inorganic phosphate and humic substances [12]. High performance liquid chromatography (HPLC) is a commonly used technique for qualitative and quantitative analysis of organic phosphorus compounds in soil, plants, foods and aqueous samples [32,33].

Table 4. Selected publications on organic phosphorus compounds analysis in different matrixes

Authors	Pub. Year	Journal	HPLC	Mobile phase	Detection	Matrix	$c_{measured}$	LOD/ [µmol L^{-1}]	Ref.
Phillippy et al.	2003	J. Agric. Food Chem.	IC post column deriva.	0.25 M HNO_3	UV vrs ELSD	roots and tubers	0.169 (missing unit)	**-**	**[34]**
Blaabjerg et al.	2010	J. of Chrom B	IC	A:Methanesulfonik acid B: H2O	ICP-MS	feedstuffs, diets, gastric and ileal digesta	40-100mg P L-1	**-**	**[35]**
Talmond et al.	2000	J. of Chrom A	IC	A:NaOH+H2O B:Isopropanol+H2O	chemicaly suppressed conductivity detector	cereals, oilseeds, legume seeds	1.25 g IP6 in 100 g dry matter	**0.962**	**[36]**
Henshall et al.	1992	J. of Chrom A	CE		photometric detection	physiological samples		**0.1**	**[37]**
Liu et al.	2009	Rapid Commun. Mass Spectrom.	IC	A: 200 mM ammonium carbonate B: 5% MeOH in H2O	MS/MS	almond, hazelnut and whole grain oat cultured cells	human cells: 0.03 µmol/mg	-	**[38]**
Tur et al.	2013	J. of Chrom B	UPLC-RP	TEAA/ ACN	MS/MS	rat, dog plasma	63.9-150.1 ng ml-1	**0.00006**	**[39]**
Munoz and Valiente	2003	Anal Chem	IC	50 mmol-1 HCL	ICP-MS	urine	1.5 mg L-1	**0.05**	**[40]**
Grases et al.	2004	Anal Chem Acta	IC	50 mM HCL	ICP-AES	urine	0.75-1.84 mg L-1	**0.01**	**[41]**
Neubauer et al.	2012	ABC	RP	A: H2O+0.1 fomric acid, B: ethanol 40%	ICP-MS	Feed supplements	>1.6 umol/g	**3-11 fmol**	**[42]**
Helfrich and Bettmer	2004	JAAS	IP-RP	A: H2O B: 10% MeOH +20 mmolL^{-1} citrate + 0.04 % TBA in H2O	ICP-SF-MS	nuts, cereals, legumes	0.53- 14 mg g-1	**0.0025**	**[43]**
Chen	2004	J. Agric. Food Chem.	IC	2 M HCL	spectrophotometry	food	3.98-14.28 mmol kg-1	**0.148**	**[44]**
Skoglund et al.	1997	J. Agric. Food Chem.	IC post column derivatization	5-98 % 0.5 M HCL	UV & ELSD	food	-	**1.5-3.4**	**[46]**

Speciation of organic phosphorus in soil involves analysing complex matrices, polar and non-volatile target analytes, which makes the speciation of these compounds not an easy task and all the associated difficulties are addressed to the molecular properties of organic phosphorus.

Table 4 summarizes selected publication on the IP_6 in different matrixes. The combination of selective liquid chromatographic (LC) separation techniques with elemental detection systems is therefore a promising method for accurate analysis of phosphorus containing compounds. Mass spectrometry is a fit for purpose quantitative tool, because of its sensitivity and selectivity. Additionally, this technique can exhibit a large dynamic range providing element specific detection by monitoring a single isotope mass of the element of interest, *i.e* phosphorus.
Therefore, development of analytical techniques for separation and detection of phosphorus in soil and plant related samples within this project is a contribution to further research related to the rhizosphere investigation in the context with plant-soil interactions.

2.1 Ion exchange chromatography of organic phosphorus compounds

Chromatography represents a separation technique, were the separation is performed based on the different distribution of components of analyte according to their affinity towards the stationary phase, and the mobile phase [45]. The chromatographic separation of ionic species from aqueous solutions is based on the ion exchange mechanism named also later as ion chromatography (IC). When flowing through the ion-exchange resin, the stationary phase, the analyte ions bind to the side groups of the resin, replacing consecutively H^+ or OH^- ions.
High performance ion chromatography (HPIC) is frequently used for the separation of analytes with a wide range of polarity and a very good example of such compounds are the objective of this PhD project - phosphorus containing compounds. A great part of these compounds are by nature organic and they are ionic in aqueous solution due to the presence of PO_4^{3-} in their molecule which makes them suitable for separation by ion exchange chromatography [46].
Most of the organic phosphorus compounds are highly charged and strongly retained in the IC stationary phase resin due to the attraction forces with the positively charged stationary phase providing the ion exchange mechanism. In such situation, a constant mobile phase composition during the analysis, *i.e.* isocratic conditions, may not provide an acceptable and sufficient separation. Early eluting analytes may be poorly resolved whilst other analytes may have unacceptably long retention times with poor peak shape and sensitivity. To resolve these disadvantages during separation, the composition of introduced mobile phase can be altered during elution (gradient elution), thus enabling better separation of the analysed species within a single and potentially shorter chromatographic run.

Organic phosphorus species are separated with ion chromatography columns usually made from polymer-based stationary phases. Among these columns, the latex-based anion-exchange columns with a basic eluent have been frequently used. This type of column resin is characterised by its gradient elution ability, enabling the simultaneous determination of low and highly retained anions in a single run.

Eelectrostatic interaction techniques are mainly applied for the separation of IP_6. In IP_6, six of twelve replaceable protons are strongly acidic (pKa <3.5) and the other six are weakly (pKa =4.6–10) acidic [47]. Electrostatic interaction and the changing protonation degree are the basis of the anion exchange separation with HNO_3 gradient elution. The reported HPLC methods so far (Table 4) are lacking for a better analytical approach for analysis of IP_6 in biological samples, and this is mainly related to poor spectrophotometric properties of the detection system applied. Therefore, improved analytical figures of merit in both terms, separation and detection for quantification of orthophosphate and IP_6 in soil and plants presented within this project could be mainly attributed to the detection part of the analytical technique applied, since ICP-MS has a high tolerance versus buffers and acids compared to other ionization sources and provides ruggedness regarding of long term measurements. .

2.2 ICP-MS for analysis of phosphorous

Inductively coupled plasma mass spectrometry (ICP-MS) is a hard ionization technique that provides stable isotope specific detection. One of the most important advantages of ICP-MS is its large dynamic range and linearity over up to nine orders of magnitude making it a quite flexible analytical method. Moreover, the technique offers a wide linear dynamic range and high robustness against matrices present in samples and chromatographic buffers [48,49]. Such hard ionization techniques can be employed to reduce organic phosphorus molecules to elemental phosphorus for quantitative analysis [50].

Elemental mass spectrometry usually requires low resolution analyzers and at plasma temperatures of 5 000 - 10 000 ^{O}C all elements are ionized at a certain degree (approx. 1.5 %) [50]. Regarding phosphorus however, the low resolution of elemental MS due to the relatively high first ionization potential (10.5 eV) limits ionization efficiency leading to a lower sensitivity and higher matrix suppression effects as compared to other elements. In addition, as mentioned before, phosphorus has only one stable isotope and does not allow application of isotope dilution mass spectrometry for absolute quantification and the only stable isotope ^{31}P exhibits

several spectral interferences of polyatomic ions, such as $^{15}N^{16}O^{+}$, $^{14}N^{16}O^{1}H^{+}$, and $^{12}C^{1}H^{3}\ ^{16}O^{+}$. This results in a higher background and detection limits than those corresponding to other ions. To resolve this issue the application of high resolution sector field mass spectrometry (ICP-SF-MS) is also one of the options. Another approach to remove the isobaric interferences is by using reaction cell device or dynamic reaction cell technology (DRC), [50] that is deemed as a state-of-the-art and, same as ICP-SFMS, mandatory for obtaining accurate and reliable results especially in analysis of elements suffering from isobaric interferences.

ELAN DRC II by Perkin Elmer, used during this work, includes a dynamic reaction cell (DRC) technique. The cell consists of a low constant rf amplitude quadrupole [51], located between the ion optics and the actual mass analyzer. The DRC is operated as a mass-filtering device (bandpass to remove unwanted secondary interferences) and can be used with gases such as oxygen, ammonia or methane as a reaction gas for elimination of interfering species via interference dissociation or discharge, or by transferring the interfered analyte to a higher mass via reaction with oxygen [52].The reaction gas is selected based on its ability to perform a gas phase chemical reaction with the interfering species and remove the interference itself. Interference removal can occur through various processes, including: collisional dissociation, electron transfer, proton transfer and oxidation. These processes are happening because the charged ions can interact with the dipole moment of a polar molecule or induce a dipole in a non-polar molecule. Ion-dipole interactions are effective at long inter-atomic distances so the collision rate between ions and neutral molecules is high. The ion dipole interaction is usually sufficiently strong to exceed the activation energy potential barrier to exothermic reactions thus thermodynamically allowed ion molecule reactions are usually fast. The ability to use more reactive gases including the above mentioned as well as CH_3F and CO_2 provides superior interference reduction and improved detection limits in a wide variety of sample types [50].

Several groups have applied ICP-AES and ICP-MS as a powerful elemental detection methods in combination with selective enrichment of IP_6 by ion chromatography either in off-line ion exchange or solid phase extraction mode or after fraction collection after high performance ion chromatography [35]. In this context Munoz and Valiente developed an ICP-MS method for determination of phytic acid in human urine based on the total phosphorous measurement of purified extracts of phytic acid by solid phase extraction (SPE) with a limit of detection of 5 $\mu g L^{-1}$ (8 nmol L^{-1}) [40]. Grases *et al.* have reported a method for quantification of Na-IP_6 in urine samples after enrichment via ion exchange offering an LOD of 64 $\mu g\ L^{-1}$ (68 nmol L^{-1}) [41].

In the presented work, oxygen was used as a reaction gas oxidizing phosphorus into the molecular ion PO^{+} and therefore P was detected at m/z=47 circumventing all isobaric

interferences at m/z = 31. The LOD calculated based on the threefold SD of the baseline signal was 0.3 µmol L^{-1}, while the LOQ (tenfold SD of the baseline signal) was 1 µmol L^{-1} for both compounds that corresponds to an on-column LOD of 3 pmol (10 µL injection volume). This is an improvement by several orders of magnitude in comparison to alternative detection methods, which have been used in connection with anion exchange chromatography [34].

2.3 IC-ICP-MS for speciation of phosphorous

Ion chromatography with conductivity detection coupled to inductively plasma mass spectrometry (IC-ICP-MS) represents still the most frequent approach for the phosphorus speciation in the environmental field of research [53]. Numerous ion chromatography (IC) applications for analysis of phosphorus compounds involve determination of orthophosphate, either as the specific analyte or as the final product of the sample treatment procedure in speciation studies. An ion paring method coupled to ICP-SFMS [43] was developed by Helfrich and Bettmer and they could obtain an LOD of 0.35 µmol L^{-1}. Even though phosphorus is not amenable to soft ionization techniques Liu *et al*. by using LC-MS/MS to analyze IP_6 in biological matrixes [38] were able to obtain an absolute limit of detection of 0.25 pmol.

However up to date there is no IC method combined with ICP-MS for analyzing IP6 in soil samples. One of the main advantages of this strategy (IC coupled to ICP-MS) is a high selectivity for phosphorus containing compounds and a low vulnerability to ion suppression and above all the easily handling and straightforward interface between IC and ICP-MS. Another great advantage of this mode of coupling is also the acidic gradient applied in the separation of high polar organic compounds which goes in favour for the ionisation in the argon plasma. In this context ICP-MS is quite tolerant and highly compatible with IC acidic mobile phases.

Despite the advantages of coupling IC to ICP-MS for separation and detection of polar organic compounds, such as low detection limits, wide linear range, and specificity, this approach has not been very popular for phosphate determination because of the presence of some inherent difficulties. As mentioned before (Chapter 2.2.), determination of phosphorus by ICP-MS is difficult, due to its high ionisation potential and its consequent low ionisation efficiency in argon plasma. In addition, if high concentrations of dissolved salts in the mobile phase are present then this is the major drawback for the ionisation source of the mass spectrometry, as they lead to ionization suppression of analytes.

However, a certain degree of ionisation suppression in gradient acidic elution is unavoidable in ion chromatography during a long chromatographic run especially for the late eluting analytes.

Within this work, this event was monitored during method development and quantification and intensity loss of approx. 10% was observed for PO^+ (*m/z*= 47) (Chapter 3, Figure S1). This is not the case if DRC is not used. This is explained by the fact that with increasing HNO_3 concentration during the gradient run, the N-based interferences are also increased leading to an increased signal intensity at *m/z* = 31.

Thus, ion chromatography with gradient elution with a strong acid in combination with inductively coupled plasma mass spectrometry with dynamic reaction cell is the method of choice for analysis of high polar phosphate compounds since this method offers stronger retention providing both chromatographic selectivity and efficiency and is compatible with plasma ionisation source.

2.4 LC-ICP-MS for speciation of phosphorous

In addition to further investigations of organic phosphorus compounds and application of diverse separation techniques in phosphorus speciation, another liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS) based method for separation of nucleotides, was developed within this PhD project within a collaboration.

This method was developed for quantification of nucleotides in nucleotide enriched yeast extracts. Yeast autolysates enriched in nucleotides, particularly 5'-GMP, and 5'-IMP are commercially used as flavour enhancers in foods and beverages [54,55]. The benefits of nucleotide enriched yeast products in feeding industry are currently under investigation for its beneficial physiological effects. The supplements are mostly produced by autolysis and the characterization of the final composition of the yeast extracts is important. The metabolites investigated in this work were ribonucleotides CMP, UMP, AMP and GMP – the breakdown products of RNA – and IMP as well as nucleosides and nucleobases, the breakdown products of the nucleotides [54, 56].

Nucleotides are negatively charged in a wide pH range, thus anion exchange mechanism is the method of choice of HPLC traditionally used for their separation. However, since these separations rely on the use of non-volatile Na- and K-phosphate buffers, they are not useful for LC-MS analysis. Moreover, volatile eluents are generally ruled out in LC-ESI-MS because of vast background and ion source pollution. In reversed phase (RP) liquid chromatography analysis, nucleotide separation with a C_{18}-based column is limited because of the inherently poor interaction of the highly polar nucleotides. Good separation of nucleotides on conventional C_{18} phases can be achieved by addition of ion pair (IP) reagents into the mobile phase. An

improved retention and selectivity can be achieved, however combination of IP-RP-LC with ICP-MS, implicates always the drawbacks of ion suppression and contamination since the counter ion effect of the ion pair reagent can compromise sensitivity and lead to contamination as already mentioned before.

Almost all analytical methods for measuring nucleotides include a liquid chromatography technique for their separation and due to the fact that nucleotides are not volatile and instable during derivatization, gas chromatography is not the preferred method of choice. Techniques for the analysis of nucleotides other than liquid chromatography include enzymatic assays, capillary electrophoresis, and capillary electrochromatography [57,58,59].

Mass spectrometry plays a major role in the study of nucleobases, nucleosides, and nucleotides that represent monomers of nucleic acid structures [60] and has gained a wide range of application for small biomolecules, due to development of soft ionization techniques such as electrospray ionization (ESI). The ionic and non-volatile characteristics of nucleotides make them potential candidates for liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) or inductively coupled plasma mass spectrometry. Application of negative ionization is more suitable for measuring nucleotides due to their negative charged phosphate groups in ESI-MS. A special detection system is represented by tandem mass spectrometry (MS-MS) in which the precursor ion of each analyte undergoes a fragmentation and one of the fragment ions represents the product ion.

Publication III in Chapter 3 presents a novel method for quantitative profiling in nucleotide enriched yeast extracts used as additives in animal feedstuff is presented. The analytical study concerned the development of a rapid chromatographic separation, which could be combined with both molecular and elemental mass spectrometric detection. Since there are no certified reference materials available for this type of bioanalytical application, the orthogonal ICP-MS method may be implemented as a reference method for nucleotide quantification and prove the validity of LC-MS-MS based quantification developed in our working group. Nucleotide measurement via ICP-MS until recently, concerned only extracted DNA samples, aiming at quantification of deoxy nucleotides dCMP, dTMP, dAMP, and dGMP and in all this work ICP-MS was combined to μHPLC and capillary electrophoresis [61,62,63]. For the first time, LC-ICP-MS is applied to the quantification of free nucleotides in biological samples as a method for measuring nucleotides orthogonal to LC-ESI-MS.

References:

[1] Sanyal, S. K. and De Datta, S. K., Adv. Soil Sci. 1991, 16, 1–120.

[2] Stevenson F.J., Cycles of soil carbon, nitrogen, phosphorus, sulphur micronutrients, Wiley, 1986, New York.

[3] Mclaughlin, MJ., Alston, AM., Martin, JK., Aust J Soil Res, 1998, 26:333–342.

[4] M. S. Khan, A. Zaidi, and P. A. Wani (2007), "Role of phosphate-solubilizing microorganisms in sustainable agriculture—A review," Agronomy for Sustainable Development, 27, 1, 29–43.

[5] Glick, B.R. The enhancement of plant growth by free-living bacteria, Canadian Journal of Microbiology, 41, 2, 1995, 109–117.

[6] Rausch, C., Bucher, M. Molecular mechanisms of phosphate transport in plants. Planta 216: 2002, 23–37.

[7] Holford, ICR Soil phosphorus: its measurement and its uptake by plants. Aust J Soil Res 35, 1997, 227–239.

[8] Sentenac, H. and Grignon, C. Effect of pH on orthophosphate uptake by corn roots. Plant Physiol 77, 1985, 136–141.

[9] Bünemann, E., Bossio, D.A, Smithson, P.C, Frossard, E., Roberson, A. Microbial community composition and substrate use in a highly weathered soil as affected by crop rotation and P fertilization. Soil Biol Biochem. 2004, 36:889-901.

[10] Rodríguez, H., Fraga, R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances. 1999, 17: 319–339.

[11] Richardson, A. E., Hadobas, P. A. Soil isolates of Pseudomonas spp. That utilise inositol phosphates. Canadian Journal of Microbiology, 1997, 43: 509–516.

[12]. Turner, B-L., Papha´zy, M.J., Haygarth, P.M., McKelvie, I.D., Trans. R. Soc. Lond. B. 2002, 357, 449–469

[13] Leytem, A. B., Mikkelsen, R. L., Comm. Soil Scien. and Plant Anal. 2003, 34, 9, 1393 — 1406.

[14]. Castro, C.L.; Rolston, D.E. Am. J. Soil Sci. Soc. 1977, 41, 1085–1092.

[15] Turner, B.L, Cade-Menun, B.J, Condron, L.M, Newman ,S., Talanta.2005, 66, 294–306.

[16] Anderson, G., Williams, E., G., Moir, J., J. of Soil Science. 1974, 25, 1.

[17] Cosgrove, D.J.; Inositol Phosphates: Their Chemistry, Biochemistry and Physiology, Elsevier Scientific,.Amsterdam, Netherlands, 1980.

[18] Turner B.L., E. Frossard, D.S. Baldwin, Organic phosphorus in the Environment, CABI Publishing, Wallingford, UK, 2004.

[19] Lung , and Lim, B., Plant and Soil. 2006, 279, 187-199.
[20] Richardson, A.E, Hadobas P.A., Hayes, J.E., O'Hara, C.P., Simpson, R.J. Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil microorganisms. Plant Soil, 2001, 229: 47–56.
[21] Raboy, V., Myo-inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry, 2003, 64.
[22] Thompson, L., U. and Zhang, L., Carcinogenesis. 1991, 12 (11):2041-5.
[23] Torres, J., Veiga, N., Gancheff, J.S., Domínguez, S., Mederos, A., Sundberg, M., Sánchez, A., Kremer, C., Journal of Molecular Structure, 2008, 874 (1-3), 77-88.
[24] Brigando, C., Mossoyan, J. C., Favier, F., Benlian, D., J. Chem. Soc., Dalton Trans. 1995, 575–578.
[25] Fredlund, K., Isaksson, M., Rossander-Hulthén, L., Almgren, A., & Sandberg, A. S., J. of Trace elem. in Med. and Biology. 2006, 20, 1, 49–57.
[26] Lopez, H. W., Leenhardt, F., Coudray, C., & Rémésy, C. International Journal of Food Science and Technology, 2002, 37, 727–739.
[27] Kumar, V., Sinha, A.,K., Makkar, P.S., Becker, K., Food Chemistry. 2010, 120, 945–959.
[28] Larsson, O. Barker, C.J., Sjoholm, A., Carlqvist, H., Michell, R.H., Bertorello, A. Inhibition of phosphatases and increased Ca^{2+} chanel activity by inositol hexaphosphate. Science, 1997. 278, 471-474.
[29] Del Mar Arriero, M., Ramis, J.M, Perelló, J., Monjo, M., Cells and Human Primary Osteoclasts, 2012, 7, 8:43187.
[30] Heighton, L., Schmidt, W.F., Siefert, R.L., J. Agric. Food Chem. 2008, 56, 9543-9547.
[31] Doolette, A.L., Smernik, R.J., Dougherty, W.J. Overestimation of the importance of phytate in NaOH–EDTA soil extracts as assessed by ^{31}P NMR analyses Organic Geochemistry, 2011, 42,8: 955-964.
[32] Sandberg, A.S. and Ahderinne, R. J. Food Sci. 1986, 51, 547–550.
[33] Rounds, M.A.; Nielsen, S.S. J. Chromatogr A. 1993, 653, 148–152.
[34] Phillippy, B. Q., Bland J.M., Evens T.J., J.Agric. Food Chem. 2003, 51, 350-353.
[35] Blaabjerg, K., Hansen-Møller, J., Poulsen, H.D., J. of Chromatogr. B. 2010, 878, 3–4, 347-354.
[36] Talamond, P., Doulbeau, S., Rochette, I., Guyot, J.P., Treche, S., J. of Chromatogr. A. 2000, 871, 1-2, 7-12.
[37] Henshall, A., Harrold M.P., Tso, J.M.Y., J. of Chromatogr. A. 1992, 608, 1–2, 413-419.
[38] Liu, X., Villalta P.W., Sturla S., J. Rapid Commun Mass Spectrom. 2009, 23, 5, 705-12.

[39] Tur, F., Tur, E., Lentheric, I., Mendoza, P., Encabo, M., Isern, B., Grases, F., Maraschiello, C., Perelló, J., J. of Chromatogr. B. 2013, 928, 146–154.

[40] Muñoz, J.A, Mesas M-L., Valiente M., Anal. Chim. Acta. 2003, 75, 6374-6378.
[41] Grases, F., Perelló, J., Isern, B., Prieto, R.M., Anal. Chim. Acta. 2004, 510,1, 41-43.
[42] S. Neubauer, A. Rugova, D. B. Chu, H. Drexler, A. Ganner, M. Sauer, D. Mattanovich, S. Hann, G. Koellensperger. Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements. Analytical and Bioanalytical Chemistry August 2012, 404,3: 799-808.
[43] Helfrich, A. and Bettmer, J., J. Anal. At. Spectrom. 2004, 19, 10, 1330-1334.
[44] Chen QC. Determination of phytic acid and inositol pentakis phosphate in foods by HPLC. Agric Food Chem. 2004;52:4604–4613.
[45] Niessen W.M.A. Liquid Chromatography - Mass Spectrometry. Second Edition, Revised and Expanded. 1999, Marcel Dekker, Inc.
[46] Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S., J. Agric. Food Chem. 1998, 46, 1877-1882.
[47] Brigando, C., Mossoyan, J. C., Favier, F., Benlian, D., J.Chem. Soc., Dalton Trans. 1995, 575–578.
[48] Tromp, J. W., Pomares, M., Alvarez-Prieto, M., Cole, A.,Ying, H., Salin, E. D., Spectrochim. Acta Part B 2003, 58,1927–1944.
[49] Shah, M., Caruso, J., J. Sep. Sci. 2005, 28, 1969–1984.
[50] Bandura, DR, Baranov, VI, Tanner, SD. Anal Chem. 2002, 74, 7, 1497-502.
[51]
http://shop.perkinelmer.com/content/relatedmaterials/articles/atl_dynamicreactioncellicpms.pdf
[52] S. D. Tanner, V. I. Baranov, D. R. Bandura, Reaction cells and collision cells for ICP-MS: a tutorial review, Spectrochimica Acta Part B. 57 (2002) 1361–1452.
[53] Popp, M.; Hann, S.; Koellensperger, G.; Anal. Chim. Acta. 2010, 668, 2, 87-214.
[54] Zhao J, Fleet GH Separation of twenty isomers of ribonucleotides and deoxyribonucleotides by reversed-phase ion-pairing high-performance liquid chromatography. J Chromatogr A. 1996, 732:271-275
[55] Yamaoka N, Kudo Y, Inazawa K, Inagawa S, Yasuda M, Mawatari K, Nakagomi K, Kaneko K Simultaneous determination of nucleosides and nucleotides in dietary foods and beverages using ion-pairing liquid chromatography-electrospray ionisation-mass spectrometry. J Chromatogr B. 2010, 878:2054-2060.
[56] Zhao J, Fleet GH Degradation of RNA during the autolysis of Saccharomyces cerevisiae

produces predominantly ribonucleotides. J Ind Microbiol Biot. 2005, 32:415-423.
[57] Roy B, Beuneu C, Roux P, Buc H, Lemaire G, Lepoivre M.; Simultaneous Determination of Pyrimidine or Purine Deoxiribonucleoside Triphosphates Using a Polymerase Assay. Anal Biochem 1999, 296:403-409.
[58] Tuytten R, Lemière F, Van Dongen W, Esmans EL, Siegers H Short capillary ion-pair high-performance liquid chromatography coupled to electrospray (tandem) mass spectrometry for the simultaneous analysis of nucleoside mono-, di-, and triphosphates. Rapid Commun Mass Spec. 2002, 16:1205-1215.
[59] Chen X, Yang F, Wang Y, Li S CE and CEC of nucleosides and nucleotides in food materials. Electrophoresis, 2010, 31:2092-2105.
[60] Dudley, E. and Bond, L., Mass Spectrometry analysis of nucleosides and nucleotides, Mass Spectrom. Rev. 2014, 33, 302–33.
[61] Fujii S, Inagaki K, Takatsu A, Yarita T, Chiba K Determination of phosphorus using capillary electrophoresis and micro-high-performance liquid chromatography hyphenated with inductively coupled plasma mass spectrometry for the quantification of nucleotides. J Chromatogr A. 2009, 1216:7488-7492.
[62] Fujii S, Inagaki K, Chiba K, Takatsu A Quantification of phosphorus in DNA using capillary electrophoresis hyphenated with inductively coupled plasma mass spectrometry. J Chromatogr A. 2010, 1217:7921-7925.
[63] Wrobel K, Landero Figueroa JA, Zaina S, Lund G, Wrobel K Phosphorus and osmium as elemental tags for the determination of global DNA methylation – A novel application of high performance liquid chromatography inductively coupled mass spectrometry in epigenetic studies. J Chromatogr B, 2010, 878:609-614.

3. PUBLICATIONS

Publication I

A. Rugova, M. Puschenreiter, G. Koellensperger, S. Hann, *Elucidating rhizosphere processes and root exudate dynamics based on mass spectrometric techniques*, Analytica Chimica Acta, 2017, in press, accepted manuscript.

This article contains a critical and comprehensive review of mass spectrometry based methods for rhizosphere research, which have been developed and applied since the beginning of this century. Comprehensive literature research and discussion with both, scientists from the field of rhizosphere research and analytical chemistry is critically reviewed in this publication. This review covers both elemental and molecular mass spectrometry as well as their combination with different separation techniques with discussion of the most important works by, at the same time, covering all methods which are essential for investigating soil/plant related processes in the rhizosphere.

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ARTICLE IN PRESS

Analytica Chimica Acta xxx (2017) 1–13

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

Elucidating rhizosphere processes by mass spectrometry – A review

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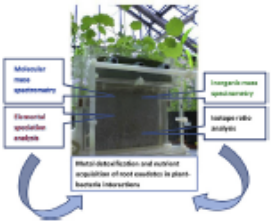
HIGHLIGHTS

- State-of-the-art mass spectrometry methods developed and applied in rhizosphere research are reviewed.
- Elemental and molecular mass spectrometry emphasizing different separation techniques (GC, LC or CE) are discussed.
- Case studies on metal detoxification and nutrient acquisition of root exudates in plant-bacteria interactions are discussed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form
27 December 2016

ABSTRACT

The presented review discusses state-of-the-art mass spectrometric methods, which have been developed and applied for investigation of chemical processes in the soil-root interface, the so-called rhizosphere. Rhizosphere soil's physical and chemical characteristics are to a great extent influenced by a complex mixture of compounds released from plant roots, i.e. root exudates, which have a high impact on nutrient and trace element dynamics in the soil-root interface as well as on microbial activities or soil

Abbreviation: APCI, atmospheric pressure chemical ionization; CE, capillary electrophoresis; CE-ESI-MS, capillary electrophoresis coupled to electron ionization mass spectrometry; CE-ICP-MS, capillary electrophoresis coupled with inductively coupled plasma mass spectrometry; CE-ICP-SFMS, capillary electrophoresis coupled to (double focusing) sector field mass spectrometry; CRM, certified reference material; DMA, 2'-deoxymugineic acid; ESI-MS, electrospray ionisation mass spectrometry; GC, gas chromatography; GC-APIC, gas chromatography coupled with atmospheric pressure chemical ionisation; GC-c-IRMS, gas chromatography combustion isotopic ratio mass spectrometry; GC-ICP-MS, gas chromatography coupled with inductively coupled plasma spectrometry; GC-ICP-TOF-MS, gas chromatography inductively coupled plasma time of flight mass spectrometry; HF-LPME, hollow fibre liquid-phase micro extraction; HILIC, hydrophilic interaction liquid chromatography; HPIC, high performance ion chromatography; HPLC, high performance liquid chromatography; HPLC-ICP-MS, high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry; IC-ICP-MS, ion chromatography inductively coupled plasma mass spectrometry; IC-ICP-QMS, ion chromatography coupled to inductively coupled plasma quadrupole mass spectrometry; IC-ICP-SFMS, ion chromatography coupled to inductively coupled plasma sector field mass spectrometry; ICP, inductively coupled plasma; ICP-AES, inductively coupled plasma atomic emission spectrometry; ICP-DRC-MS, inductively coupled plasma mass spectrometer equipped with dynamic reaction cell; ICP-MS, inductively coupled plasma mass spectrometry; ICP-QMS, inductively coupled plasma quadrupole mass spectrometry; ICP-SFMS, inductively coupled plasma sector-field mass spectrometry; ICP-TOF-MS, inductively coupled plasma time of flight mass spectrometry; IRMS, Isotope ratio mass spectrometry; LC, liquid chromatography; LC-ESI-MS or LC-ESI-MS/MS, liquid chromatography coupled with electrospray ionization mass spectrometry; LC-ESI-Q-TOF-MS, liquid chromatography-electrospray ionization-quadrupole-time of flight mass spectrometer; LC-ESI-TOF-MS, liquid chromatography coupled with ESI with time of flight MS; LC-ICPMS, liquid chromatography inductively coupled plasma mass spectrometry; LC/IRMS, liquid chromatography isotope ratio mass spectrometry; LOD, limit of detection; MS, mass spectrometry; MALDI-TOF/TOF-MS, matrix assisted laser desorption ionisation tandem time of flight mass spectrometry; MC-ICPMS, multicollector inductively coupled plasma mass spectrometry; Py-FIMS, pyrolysis-field ionization mass spectrometry; QMS, quadrupole mass spectrometry; RP-HPLC, reversed phase high performance liquid chromatography; RP-HPLC-ICP-DRC-MS, high performance liquid chromatography coupled to inductively coupled plasma dynamic reaction cell mass spectrometry; SEC, size exclusion chromatography; SFMS, (double-focusing) sector field mass spectrometry; TOF-MS, time of flight mass spectrometry; TOF-SIMS, time of flight secondary ion mass spectrometry; UHPLC-TOF-MS, ultra-high-performance liquid chromatography time-of-flight mass spectrometry.

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http://dx.doi.org/10.1016/j.aca.2016.12.044
0003-2670/

Please cite this article in press as: A. Rugova, et al., Elucidating rhizosphere processes by mass spectrometry – A review, Analytica Chimica Acta (2017), http://dx.doi.org/10.1016/j.aca.2016.12.044

Accepted 28 December 2016
Available online xxx

Keywords:
Rhizosphere
Root exudates
Mass spectrometry
Separation techniques

physico-chemical characteristics. Chemical characterization as well as accurate quantification of the compounds present in the rhizosphere is a major prerequisite for a better understanding of rhizosphere processes and requires the development and application of advanced sampling procedures in combination with highly selective and sensitive analytical techniques. During the last years, targeted and non-targeted mass spectrometry-based methods have emerged and their combination with specific separation methods for various elements and compounds of a wide polarity range have been successfully applied in several studies. With this review we critically discuss the work that has been conducted within the last decade in the context of rhizosphere research and elemental or molecular mass spectrometry emphasizing different separation techniques as GC, LC and CE. Moreover, selected applications such as metal detoxification or nutrient acquisition will be discussed regarding the mass spectrometric techniques applied in studies of root exudates in plant-bacteria interactions. Additionally, a more recent isotope probing technique as novel mass spectrometry based application is highlighted.

Contents

1. Introduction

Plant root exudates comprise an enormous range of low and high molecular weight compounds that are released into the close environment of soil, the so-called rhizosphere [1]. The rhizosphere, as described first by Lorenz Hiltner in 1904, is the "soil influenced by roots" [2] representing the highly dynamic interface between soils and roots as well as between roots and soil microbes, invertebrates, and root systems of competitors [3]. The rhizosphere itself is different from bulk soil due to a range of biological, biochemical, chemical and physical processes that occur as a consequence of root growth, water and nutrient uptake, respiration, rhizo-deposition and enhanced microbial activities [4].

Plant root exudates consist of a complex mixture of hundreds of different compounds [5] which can be categorized according to their molecular mass [6] along to their solubility in water. The water-soluble fraction comprises low molecular weight carbohydrates, amino acids, organic acid anions, inorganic ions (*e.g.* HCO_3^-, OH^-, H^+), gaseous molecules (CO_2, H_2) and various secondary metabolites (*i.e.* natural compounds). On the other hand the more hydrophobic fraction is largely composed of polymeric carbohydrates, enzymes, plant mucilages or mucigel [7]. Amongst low molecular weight root exudates organic acids represent the most abundant fraction playing a crucial role involved in different processes, thus they are of great interest for detailed investigation.

A large range of low molecular weight exudates are pivotal for nutrient acquisition [8] based on chelation and/or ligand exchange by organic acids (citrate, malate and oxalate) [9–11]. It is well known that organic acids [12–14], as well as amino acids and phenolic surfactants [8] are involved in iron and phosphorous acquisition. Vitamins [15] and sugars [16] are considered as promoters of plant and microbial growth, while, e.g. detoxification of aluminium is attributed only to organic acids [17] and phenolic compounds [16]. Furthermore, several complex interactions with soil microorganisms mediated by root exudates are taking place in the rhizosphere, as illustrated in Fig. 1 [18]. Roots produce chemical signals that attract or repel bacteria and fungi via the secretion of flavonoids, strigolactones or antimicrobials, phytotoxins, nematicidal and insecticidal compounds [18].

Even though plant root exudates and the biological processes triggered within the rhizosphere have a crucial role in plant development, there is still a lack of information about their characterization and function [19]. Comprehensive chemical characterization as well as quantification of exudates present in the soil root interface is to a great extent an analytical challenge due to their localized deposition as well as the low concentration in the soil solution and the associated difficulties of proper sampling of exudates and/or rhizosphere soil and soil solution. In the recent years the development of mass spectrometric techniques contributed significantly to an increased understanding of the rhizosphere. The method portfolio offered by mass spectrometry (MS) combined with diverse separation methods for volatile and polar compounds

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together with ^{13}C and ^{15}N - isotope labeling strategies and total element analysis by inorganic MS was unrivalled in terms of sensitivity and selectivity. Hence, it can be clearly stated that these methods were pivotal for the advancement of rhizosphere research since MS offered tools for addressing global analysis of root exudates in the soil solution.

The aim of this review is to critically highlight the advances in the rapidly developing mass spectrometric techniques applied in characterization of root exudates, with particular emphasis on novel research strategies developed in the last ten years, aiming at compound identification, quantification and investigation of their specific functions.

2. Analytical methodologies

2.1. Inorganic mass spectrometry for total element analysis

Elemental MS has been used extensively to determine the (trace) elemental composition of soil and rhizosphere-related samples. Inductively coupled plasma MS (ICP-MS) is undoubtedly the most powerful mass spectrometric technique for the collection of elemental information, offering a limit of detection (LOD) in the low- to sub-ng L^{-1} range for most of the elements [20]. The use of high-resolution sector field systems or collision/reaction cells for reduction of polyatomic interferences has become routine. Total metal quantitative analysis is usually performed using internal or external calibration procedures, which are validated through the use of certified reference materials, if appropriate materials exist for the matrices of interest. In most of the work which has been performed with environmental samples, acid digestion was applied facilitating the analysis of complex matrices such as soil and soil related samples. It is noteworthy that ICP-MS has become a routine method within the end of the last century and that current publications presenting technical or methodological novelties are rare. Relevant studies are discussed in the applications part of this review (see Chapter 3.2).

In general, although ICP-MS is regarded as a mature technique, in the case of complicated matrices as present in rhizosphere derived samples, spectral and non-spectral interferences hampering accurate quantification remain the major challenge, and demand for careful method development and validation.

2.2. Isotope ratio analysis

The development of plasma source mass spectrometers has enabled the measurement of the stable isotope ratios with high precision and accuracy (0.1% for quadrupole and 0.01% for single collector sector field mass spectrometers) to detect significant variations of isotopic systems in the environment and to apply them to biogeochemical studies [21]. Multi collector MC-ICP-MS) system on the other hand is the best ICP based technique to perform isotopic analysis of various elements with the high precision of approx. 0.001%, and the ability of the ICP source to ionize nearly all elements in the periodic table, have resulted in an increased use of MC-ICP-MS [22].

Thermal ionization MS (TIMS) is a non-plasma based ionisation technique that uses thermal energy as a ionisation source from a heated surface and is the method of choice for achieving the highest accuracy and precision (approx. 0.0001%) for isotope ratio measurements and is frequently applied in the soil-plant related samples and other environmental samples [23].

Similar as ICP-MS, gas source isotope ratio MS became a mature technique during the recent years. By performing a separation prior to isotope ratio analysis, hyphenated techniques such as GC-IRMS and LC-IRMS can provide isotopic analysis for selected compounds in complex mixtures, thereby enabling additional information and higher discriminatory power. IRMS offers the potential of unlimited applications for non-volatile and volatile compounds

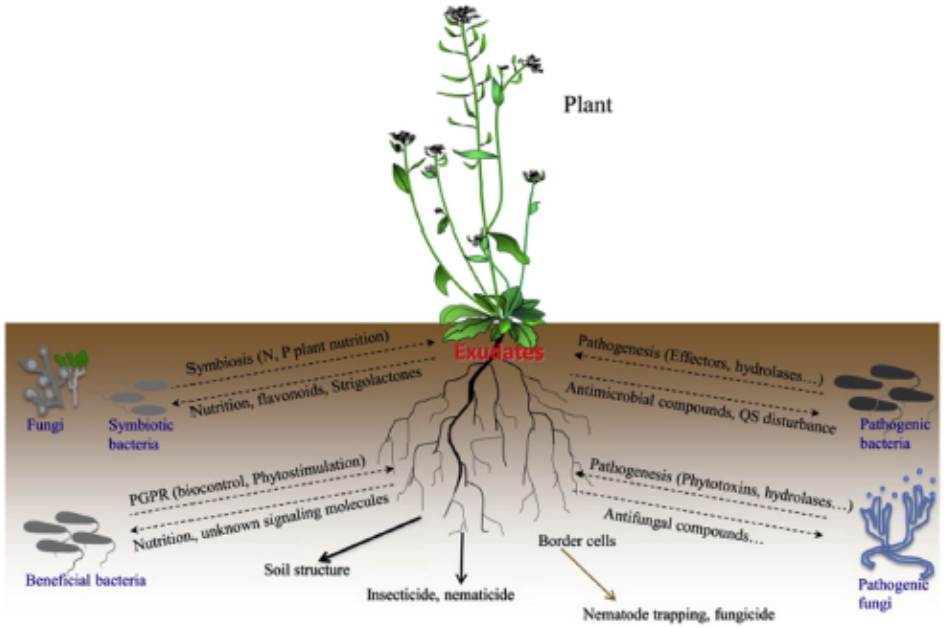

Fig. 1. Overview on root exudate driven processes in the rhizosphere between plant roots and microrganisms mediated by root exudates. With permission from Elsevier [18].

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while achieving higher accuracy and precision via increased automation [24]. Fig. 2 depicts schematics of an IRMS instrument [24]. Several different interfaces are used to introduce samples into the IRMS and the most common separation/sample introduction system is gas chromatograph interface (GC-IRMS) shown in the figure on the left. Because the isotope ratios for questioned samples are reported relative to the reference gas standard, best results are obtained when the signal intensities for the two samples are of similar magnitude and they are analysed as close as possible together in time. Actual applications employing gas source MS are discussed in Chapter 3.3.

Although both LC-IRMS and GC-IRMS are reliable techniques for compound-specific stable carbon isotope analysis in case when derivatization is complete and the calibration requirements are met, Moerdijk-Poortvliet et al. just recently suggested that LC-IRMS is the technique of choice [25]. The reasons mentioned are improved precision, simpler sample preparation, and straightforward isotopic calibration. Indeed, the data presented were associated with excellent precision of ±0.08‰ and ±3.1‰ at natural abundance and enrichment levels, respectively, for the glucose standards and proved to be superior to GC-IRMS (±0.62‰ and ±19.8‰ at natural abundance and enrichment levels). Although there is no application of LC-IRMS documented in rhizosphere research so far, this improved precision is considered as a future perspective for application of IRMS in isotopic analysis in this field.

As already mentioned, MC-ICP-MS matured during the last decades as a valuable alternative to IRMS and TIMS as a technique for accurate isotope ratio measurement. The isotopic fractionation of magnesium was investigated using MC-ICP-MS in the soil-plant continuum. According to Black and co-workers the process involves both abiotic and biological mechanisms [26]. By using a standard-sample bracketing technique the exudate experiments suggested that the mechanism of heavy isotope accumulation is in part due to processes taking place during the uptake of magnesium from roots to shoots. This work is one of the first reported cases of a higher plant using the heavy isotopes of an essential mineral nutrient.

Jouvin et al. investigated the isotopic fractionation of Cu and Zn during uptake by higher plants [27] using MC-ICP-MS with standard-sample bracketing technique developed by Mason et al. [28,29]. The results expressed with the combined uncertainty on $\delta^{65/63}$Cu and on $\delta^{66/64}$Zn were ±0.07‰ (±2σ) demonstrating the excellent suitability of MC-ICP-MS. According to the authors combination of Cu and Zn isotopes has two distinct advantages: first, a previously developed preliminary model for Zn isotope fractionation in higher plants enables constraining the potential controls such as speciation, complexation, adsorption and diffusion. The second advantage: Zn is not subjected to changes in the oxidation state during plant uptake, thus allowing a comparison with Cu, which may be involved in electron transfer reactions. From the presented work of the fractionation patterns of these two micronutrients authors concluded that both elements are decoupled during the transport from nutrient solution to root.

2.3. Elemental speciation analysis

IUPAC defines an elemental species as *the specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure* [30]. Accordingly, speciation analysis involves the use of analytical methods that can provide information on the abundance of elemental species in a sample. The major challenge hereby is sensitivity, selectivity and the preservation of the original, native distribution of the species in the samples during sample preparation and analysis.

Information about the physicochemical forms of the elements is a necessity for understanding their chemistry and role in biological and environmental processes. Lately, a great deal of interest has been focused on the speciation of trace metal elements and micronutrients in environmental samples and the development of sophisticated analytical strategies has enabled gathering more information about element bio distribution, toxicological and nutritional interactions of these elements [31,32] in biological systems.

The development of hyphenated methods combining diverse separation techniques with mass spectrometric detectors has generated an enormous number of applications of speciation analysis in environmental studies. The most common combination is LC coupled to quadrupole based ICP-MS, but also gas chromatography (GC) and capillary electrophoresis (CE) are frequently used as separation techniques, although the GC- and CE-interfaces are technically more complex and not as robust as the LC-ICP-MS connection. Several of the above mentioned techniques have been employed in the context with rhizosphere research.

Phytosiderophores as 2'-deoxymugineic acid (DMA) are known to enable the transport of iron (II) and other metals through the soil/plant interface. Schindlegger et al. [33] have developed a method that allowed the separation and quantification of metal-

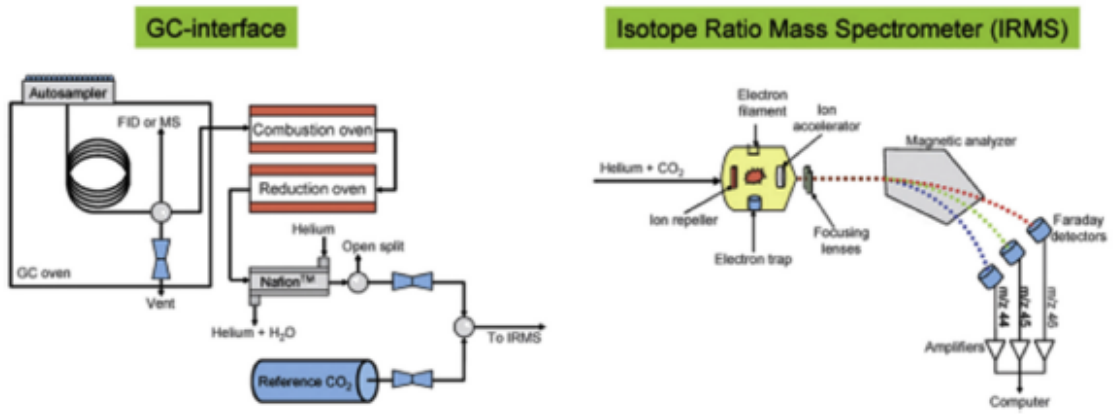

Fig. 2. Schematics of IRMS instrument, with gas chromatography used as the most common separation/sample introduction system (GC-IRMS). With permission from The Royal Society of Chemistry [24].

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DMA complexes with Fe, Co, Cu, Ni, Mn and Zn in soil related matrices in order to investigated strategy I iron acquisition (see Chapter 3.1) by plants utilizing LC-ICP-MS. Limits of detection for the different metal-DMA complexes were obtained in the range of 10–120 nM and in the soil extracts metal complexes in a concentration range of 0.4–30 μM were detected.

A phosphorus speciation method was developed for simultaneous detection and quantification of orthophosphate and organic fraction of *myo*-inositolhexakisphosphate (IP_6) in soil and plant related samples by employing an ion chromatography coupled to ICP-MS [34]. As ICP-MS analysis of phosphorus at $m/z = 31$ was hampered by N-based interferences, ^{31}P was measured as $^{31}P^{16}O^+$ at $m/z = 47$ employing dynamic reaction cell technique with O_2 as reaction gas. Orthophosphate and IP_6 were separated within a total chromatographic runtime of 12 min revealing an LOD of 0.3 μmol L^{-1}. The method has been successfully applied to extracts from three different soils as well as root and shoot extracts of *Brassica napus* L. This is the first method to quantify simultaneously orthophosphate and *myo*-inositol hexakisphosphate (IP_6), both inorganic and organic phosphorus compounds in a single chromatographic run.

The use of orthogonal separation methods as well as the complementary use of elemental and molecular MS is a modern strategy in metallomics, which "refers to the entirety of research activities aimed at the understanding of the molecular mechanisms of metal-dependent life processes" [35]. In this context, selenium-containing root exudates were investigated in Indian mustard (*Brassica juncea*) addressed by ion-pairing IP-HPLC coupled to ICP-MS [36,37]. Von der Heide et al. were able to identify several selenium-containing compounds by retention time matching with standards in the exudate-containing solution, *e.g.* selenocystine and the selenosulfate ($SSeO_3^{2-}$) ion. In a complementary approach, chromatographic peaks that could not be identified by retention matching were collected for analysis by electrospray ionization MS (ESI-MS). In a multi-platform approach, Mounicou et al. used a combination of diverse separation and MS techniques for the investigation of selenium-containing exudates [37]. Solid phase micro-extraction was employed for sampling the plant head-space and combined on-line with GC-ICP-MS and GC-TOFMS. This study is a good example how combination of diverse separation and detection techniques can give a more complete overview of the investigated species and the outcome of this study is the detection of primary selenium volatiles and elemental mercury.

In a further orthogonal approach, Dytrtova and her working group performed a study on the determination of heavy metals complexed by low molecular-weight-organic acids (LMWOA) in soil solutions. The authors combined the sensitivity of differential pulse anodic stripping voltammetry (DPASV) with the molecular insight gained by ESI- MS [38,39]. The combination of a sensitive electrochemical approach with molecular MS permits to achieve some insight about the composition of cadmium-oxalic acid complexes. Voltammetric analysis was superior in sensitivity compared to ESI-MS. However, the application of ESI-MS was crucial to attribute any specific stoichiometry to the complexes formed. The greatest disadvantage of the proposed methods lied on the pH of the model solution which was kept at about 4.5. The pH was not adjusted to the pH of the soil solution, because the addition of a buffer would change significantly the patterns of ion signals in ESI-MS. The authors justified this by the fact that in such case the spectra of this measurement would be dominated by alkali cations as well as their anionic clusters.

By using size exclusion chromatography combined with an inductively coupled plasma-mass spectrometer (SEC-ICP-MS) and an ultrafiltration technique, Takeda et al. studied the influence of plant-derived dissolved organic matter (DOM) on the mobility and bioavailability of metals in soil solution [40]. The authors presented a comparison between two detection techniques coupled to the SEC, *i.e.* UV and ICP-MS for assessing the size distribution of metal-organic complexes in rhizosphere and bulk soil extracts. Indeed, they found a different size distribution between the two sampled compartments. The results indicated that metals in bulk soil solution were mainly in smaller labile forms, including free ions or organic and inorganic complexes. In contrast, the percentages of metals in the low molecular weight fraction in rhizosphere soil solution were significantly lower than those in the bulk soil solution for Al, Mn, Zn, Pb, Y, La and U. The authors suggested that plant growth enhanced the dissolution of metals via complexation with organic ligands from the solid phase in rhizosphere soil.

2.4. Molecular mass spectrometry

2.4.1. GC-MS

GC is a high-resolving chromatographic separation technique that is commonly applied for the separation of volatile and semi-volatile compounds. Derivatisation reactions are also used additionally prior to analysis directly or indirectly making a large number of non-volatile and more polar compounds amenable to GC separation. GC instrumentation is compatible with most of the mass spectrometers such as quadrupole MS, tandem MS, orbitraps, TOF and QTOF-MS and can offer several advantages compared to LC-MS, such as higher chromatographic efficiency and selectivity and lower LODs, depending on the analytes of interest.

GC-MS has enabled numerous studies investigating for example root exudates as ligands with metal ions [41], detection and identification of fatty acids of maize, lupin and wheat in root mucilage [42], or fatty acid methyl esters and fatty acid amides and their function in stimulating nitrogen removal of denitrifying bacteria [43], sugars as primary metabolites [44] as well as volatile isoprenoids [45]. Derivatization reactions commonly used in root exudate analysis by GC coupled with mass spectrometers are esterification [54,46], silylation [46–49], acetylation [49,56,57] and alkylation [48,57]. Trimethyl silylation of sugars is presented as advantageous regarding rapid sample preparation and is fit for purpose. On the other hand regarding the separation of selected sugars after derivatization prior to analysis, Derrien et al. address difficulties on the potential co-elution of some analytes and the risk of misinterpretation especially in the presence of several anomers [49]. GC-MS was extensively used by many authors in studies addressing identification and quantification of organic acids [50]. However, all these methods of analysis are mainly qualitative studies of plant root exudates and do not offer concentration based data of the secreted compounds. Quantification is challenging due to the low repeatability of derivatization yields, instable derivatives and matrix suppression. ^{13}C labeled internal standardization is correcting for these errors and is considered as the state-of-the-art for quantification of primary metabolites in biological samples [51,67].

GC separation and quantification of amines is to a great extent hampered by their polarity causing tailing in the capillary column. As already mentioned several derivatisation methods are available for solving such analytical problems. Innovative approach comprising hollow fibre liquid-phase micro extraction (HF-LPME) was proposed by Sun et al. as an excellent invention to overcome the challenges in sample preparation for the determination of compounds with high polarity [52,53]. The derivatisation reagent acetic anhydride is protected within the hollow fibre, which allows simultaneous derivatization and extraction of the analytes prior to GC-MS analysis revealing excellent limits of detection from 0.057 to 0.096 μg mL^{-1}. Recently a study demonstrating a convenient and non-destructive sampling technique for root-exuded metabolites has been published [54]. A mesocosm was designed in which volatile

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rhizosphere chemicals were sampled passively (by diffusion) without air and water flow on polydimethylsiloxane tubes. After separation of volatiles and derivatized sugars fifteen volatiles were retrieved from the sorptive material by thermal desorption for analysis by GC-MS operating in electron impact mode. Furthermore, three sugars were collected from the rhizosphere substrate by aqueous extraction and derivatized prior to GC-MS analysis.

2.4.2. LC-MS

HPLC is the most often used separation technique for the determination of nonvolatile root exudates [55]. In earlier days, detection techniques as refractive index [56], evaporative light scattering detectors [57] and pulsed amperometric detection [58] have been used for analysis of carbohydrates in soil and plant related samples. Clearly the above mentioned detectors are limited by low sensitivity and selectivity. As an alternative, the analytical approach employing LC-ESI-MS allows the soft ionization of a wide range of polar and moderately polar compounds in both positive and negative ionization mode [59] and offers high mass selectivity and sensitivity thus making ESI-MS an attractive technique in rhizosphere research. Nevertheless, ESI has some drawbacks regarding the compatibility with HPLC mobile phases, as high buffer contents *e.g.* ion exchange chromatography of hydrophilic interaction LC (HILIC) are suppressing ionization and mitigate the robustness of these systems. However, recent developments combining electro-chemical suppressors led to more flexibility in IC-MS applications.

Single stage quadrupole mass filters are the cheapest instrumental set-up, but if more selectivity, higher dynamic range and lower LODs are desired, tandem mass spectrometers enabling collision induced dissociation and multiple reaction monitoring are preferably used. Indeed, tandem MS systems are most frequently employed, when it comes to quantification of selected compounds (*i.e.* targeted analysis or profiling). Combining ESI with accurate MS (*e.g.* TOFMS or orbitraps) has enabled the analysis of complex environmental samples due to the benefit of high mass resolution (up to 600 000 m/Δm) and accurate mass measurement (<1 ppm mass accuracy). With such systems, the elemental sum formula of the detected analyte can be obtained, but in many cases several formulas are possible representing a multitude of chemical structure or compounds. In this situation, fragmentation techniques such as collision induced fragmentation prior to accurate mass measurement can further support identity confirmation of the detected analytes which is in most cases achieved with dedicated databases as METLIN or DNP.

Carboxylic acids are highly abundant, important compounds within rhizosphere exudates and therefore they have been extensively studied in the last decade. Common LC methods for analysis of these exudates are ion exclusion or anion exchange based separations. However, the composition of eluents used is often incompatible with ESI-MS due to their poor volatility of *e.g.* H_2SO_4 or other additives. Chen et al. quantified 10 organic acids (pyruvic, lactic, malonic, maleic, fumaric, succinic, malic, tartaric, trans-aconitic, and citric acid) from plant root exudation by reversed phase chromatography on a C_{18} column with ESI-MS detection [60]. One year later, the same authors published an ion exclusion chromatographic application and were able to identify only nine organic acids that were separated within 8 min, revealing an LOD of 100 nmol L^{-1} [61]. Jaitz and coworkers, developed a more sensitive LC–ESI–TOFMS method for quantification of citric, fumaric, malic, malonic, oxalic, trans aconitic, and succinic acid in soil- and root-related samples [62]. The method compared to previous method from Chen *et al.* [60], comprises a fast and simple in situ esterification as a derivatization step with benzyl alcohol. The excellent detection limits are improved in particular due to the derivatization step which improves ESI efficiency. The method provided procedural detection limits of 1 nmol L^{-1} for citric, 47 nmol L^{-1} for fumaric, 10 nmol L^{-1} for malic, 10 nmol L^{-1} for malonic, 16 nmol L^{-1} for oxalic, 15 nmol L^{-1} for succinic, and 2 nmol L^{-1} for aconitic acid, utilizing 500 μL of liquid sample for derivatization. Quantification of the analytes in soil-related samples was performed via external calibration of the entire procedure utilizing ^{13}C-labeled oxalic and citric acid as internal standards. Erro et al. used LC coupled to highly selective tandem MS for determination of organic acids [63]. Even though, based on this method citrate and isocitrate were not well separated by the chromatographic method, selective MS-transitions enabled to distinguish between the two isomers. The main advantage of this method is that it does not require sample purification before HPLC analysis or sample derivatization to improve quantification results.

Identification of carbohydrates as a major group of root exudates by GC techniques requires tedious derivatization techniques and can result in multiple peaks from each carbohydrate, making data interpretation difficult according to McRae and Monreal [64]. The detection of carbohydrates using MS allows the distinction between carbohydrate homologues of different masses; however, isobars such as mannose, glucose, and galactose cannot be differentiated by mass alone. However, they were able to quantify 15 further carbohydrate compounds in soil solutions using 1-phenyl-3-methyl-5-pyrazolone derivatization prior to LC-ESI-MS/MS analysis including monosaccharides ranging from three carbon to six-carbon as well as two disaccharides, in a single method.

LC coupled to ESI-MS/MS is also used for analysis of phytosiderphores (PS) in root exudates. A powerful quantitative method for analysis of PS-complexes and their free ligands is reported by Dell'mour and co-workers [65]. Chromatographic separation was established on a non-commercially available mixed-mode RP/weak-anion exchange stationary phase (RP/WAX) under neutral conditions in combination with MS/MS detection. Tsednee et al. [66] performed another UPLC-ESI-TOFMS method for separation and identification of two new PS in roots of *Hordeum vulgare cv Himalaya* exudates under iron deficiency. Fig. 3 depicts fragmentation pattern of avenic acid (AVA), hydroxyavenic acid (HAVA) and 3″-oxo acid exuded by barley roots under iron deficient conditions. The two identified PSs could be responsible for Fe acquisition under Fe deficiency because of their relative abundance and ability to form iron complexes in the rhizosphere. In this example it is shown that LC-ESI-QTOFMS method greatly facilitates the identification of free PSs and PS-Fe complexes in one plant sample. However, due to the high polarity of PS the application of reversed phase chromatography is limited, as most of the investigated compounds identified in this study are eluting in or close to the void volume of the chromatographic system. Accordingly, Schindlegger et al. suggested a more selective LC separation technique and for the first time the PS-DMA could be determined via LC-MS/MS in plant and soil related samples employing graphitized carbon as stationary phase. Accurate quantification was performed in the low and sub-micromolar range using ^{13}C for labelling four C atoms in DMA for internal standardization [67].

Dell'mour and co-workers used HILIC, as a validated method and very good compatible to ESI method for retention and separation of underivatized highly polar amino acids. In this study they have combined this separation mechanism with triple quadrupole MS in multiple reaction monitoring mode. The method was validated and successfully applied in plant culture in nutrient solution and detection of total of 16 amino acids was achieved in the fmol detection range after off-line enrichment via ion exchange [68].

2.4.3. CE-MS

Capillary electrophoresis coupled with MS (CE-MS) is the

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method of choice for simultaneous separation of anionic, cationic and neutral plant metabolites, using only a small quantity of samples without chemical derivatization [69]. Prior to CE-MS measurement some authors prefer to conduct ion-exchange solid phase extraction (SPE) especially in environmental samples with a high concentration of inorganic ions. Influential factors may hamper CE leading to high detection limits and low robustness against high ionic strength matrices. With such an application Oikawa et al. detected 17 cationic metabolites in the soil solution and in addition, the authors claim that these ions were present in different concentrations in soil with and without plants present [70].

A fit-for-purpose method for investigation of phytosiderophore chemistry in rhizosphere related samples has been developed by Dell'mour et al. [71]. The developed capillary electrophoresis method coupled with ICP-MS (CE-ICP-MS) together with CE-ESI-MS was ideally suited for gaining a more fundamental understanding for mobilization properties of mugineic acids (MA) with different metals. While the CE-ESI-MS approach allows measuring the free versus the metal bound ligands, the CE-ICP-MS reveals the ratio of free metals versus the complexed metals. According to the authors of this work, although ICP-MS is known for a substantially higher sensitivity for metal containing molecules when compared with ESI-MS, only a slight improvement regarding the LODs was obtained. In this case, for the Fe(III)-DMA complex when analyzed with CE-ESI-MS authors of this work obtained an LOD of 0.87 μM, whereas CE-ICP-SFMS revealed an LOD of 0.35 μM.

Sato et al. used capillary electrophoresis coupled with UV/VIS diode array detection and mass spectrometric detection to analyze water-soluble intracellular metabolites from rice leaves. By using this method, the authors have successfully measured the levels of 88 main metabolites involved in glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, photorespiration, and amino acid biosynthesis [69].

To summarize, CE–MS methods have been used in very few attempts performing metabolome analysis in higher plants, despite the availability of such superior techniques with a sensitivity loss as a major drawback of this method.

2.4.4. Py-FIMS

Pyrolysis-field ionization MS (Py-FIMS) produces almost exclusively non-fragmented molecular ions of thermal degradation products enabling qualitative chemical characterization of eluted root exudates. Py-FIMS was successfully applied for the analysis of peptides and other N-containing compounds and carbohydrates from nonsterilized soils [72], root exudates from maize plants [73] and on a study about molecular-chemical diversity of potato plant rhizodeposits [74]. Py-FIMS analysis is performed on the leached and freeze-dried rhizodeposits that requires a large sample volume (500 μL), is time consuming and perhaps can alter the composition of the rhizodeposits. Therefore Leinweber et al. [75] have developed a new, rapid micro-method of direct Py-FIMS that is pretreatment-free and is based on sample volumes as small as 5 μL. This new profiling method is suitable for rhizosphere research since it is highly sensitive for detecting and quantifying a wide range of organic substances considered as indicators of modified root environment and rhizosphere processes.

3. Application of MS-based techniques in rhizosphere research

Within this chapter we highlight selected studies which made use of the methods discussed in Chapter 2 in elucidating the role of root exudates in the overall rhizosphere processes, such as nutrient and trace element acquisition, plant-microbial interactions mediated through root exudates and metal detoxification. Moreover, other relevant new strategies to rhizosphere research are discussed such as ^{13}C labeling and metabolite profiling techniques together with MS imaging as a new analytical approach in protein studies.

3.1. Nutrient and trace element acquisition

Plant growth is controlled mainly by internal signals that depend on the adequate supply of mineral nutrients by soil root uptake [8]. These minerals are present mainly as insoluble compounds in the soil solid phase and they are thus not available directly to the plant. Via the roots plants can take up only the soluble fraction of these nutrients and various processes are triggered by root exudates to increase the soluble fraction in the soil solution.

Amongst plant nutrients, phosphorus is probably the most widely studied macronutrient for plants [76] due to its very low solubility in many soils of the world. Mechanisms for accessing phosphate sources include the acidification of the rhizosphere and secretion of low molecular weight organic anions as well as phosphatase enzymes into the soil [9]. At concentrations commonly found in the rhizosphere (10–100 μM) [77], citrate and oxalate

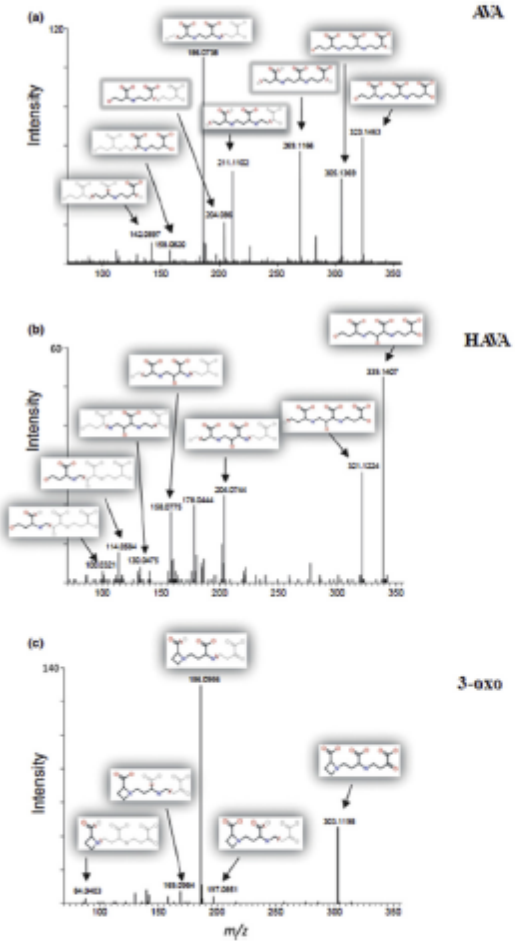

Fig. 3. Mass fragmentation patterns of the detected avenic acid (AVA) (a), hydroxyavenic acid (HAVA) (b) and 3''-oxo acid (c) in iron -deficient barley (*Hordeum vulgare cv Himalaya*) roots obtained by tandem MS/MS in positive electrospray ionization mode. With permission from Wiley [66].

were found to have a greater potential for P mobilisation compared to other organic anions. In such low level of concentration of exudates there is a need for improvement of analytical strategies to identify and quantify these low molecular organic molecules. Application of MS techniques coupled to separation chromatographic methods has enabled ways to get more ideas about classification of exudates in their crucial role in plant nutrient accessibility [62–64].

Capillary electrophoresis time-of-flight MS (CE-TOF-MS) was the method of choice for Tawaraya and co-authors to study common bean (*Phaseolus vulgaris* L.) root exudates under phosphorus deficiency using the non-targeted metabolomics approach [78]. The non-target CE-MS method was employed to separate and detect ionic metabolites, including amino acids, organic acids, nucleotides and sugar phosphates, without chemical derivatization and more than 150 metabolites were detected. These results suggest that more than 10% of primary and secondary metabolites are induced to be exuded from roots of common bean under P deficiency. With application of HPLC analysis coupled to electrospray MS, P starvation was also found to stimulate exudation of salicylic acid and citramalic acid by sugar beet roots and it was found that both metabolites solubilize soil P in Oxisol soil fertilized with calcium phosphate [79].

Like phosphorus, iron has a very low solubility and plant productivity can be strongly limited by iron deficiency. Plants have evolved two different strategies for Fe acquisition, the acidification-reduction strategy and the chelation strategy [80], also called 'Strategy I' and 'Strategy II' respectively. Until nowadays there is a major gap in our understanding of the first strategy if there is a potential role of phenolic compounds secreted by the roots in Fe acquisition. Schmidt et al. performed a study on roots of *Arabidopsis thaliana* plants grown under Fe-deficient and Fe-sufficient conditions and searched for compounds that increased their abundance under Fe deficiency [81]. By combining comprehensive metabolome analysis by GC-MS with ultra performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS) they were able to identify various coumarins. They were able to conclude that coumarin biosynthesis in roots is enhanced under conditions of Fe deficiency and the secretion of coumarins is an essential component of *A. thaliana* Fe acquisition in alkaline soil.

A study from Oburger et al. addressed the investigation of phytosiderophore exudation from wheat (*Triticum aestivum cv Tamaro*) grown on a calcareous soil [82]. It was shown for the first time that 2′-deoxymugineic acid (DMA) release rates of soil-grown plants were significantly lower than exudation rates observed for plants grown in zero-Fe nutrient solutions. In a parallel study, Schenkeveld et al. showed based on a series of interaction experiments with calcareous clay soil and the phytosiderophore DMA, in which metal and DMA speciation were examined as a function of time and DMA concentration [83,84]. Based on this a conceptual model has been developed that takes into account the chemical kinetics of phytosiderophores mediating Fe acquisition. The authors observed that over time the adsorption of the Fe decreased, but did not prevent Fe mobilization by DMA even though due to the microbial activity DMA content was decreasing in the soil solution over time.

Neumann and coworkers hypothesized that different soil types with different physicochemical properties, including varying nutrient availability, will influence root growth patterns and root exudation [85]. By applying online derivatization with methoxyhydroxy- dimethylamine with GC analysis coupled to MS they investigated the effects of soil type on root development and root exudation of lettuce (*Lactuca sativa* L. *cv. Tizian*). A total of 33 compounds were identified: 17 amino acids and amides, 8 sugars, and sugar alcohols, 5 organic acids as well as ornithine, urea, and phosphate. Authors of this work conclude that even under controlled conditions on well-fertilized soils mutual interactions between plant roots and soil specific microbiomes seems to be important factor influencing the root shape, root exudation and thereby the establishment of rhizosphere microbial communities.

3.2. Metal mobilization and detoxification

Exposure of plants to enhanced metal concentrations may be the main cause for production and secretion of low molecular weight organic acids (LMWOAs) into the rhizosphere. These root exudates may contribute to the metal tolerance of plants via complexation in rhizosphere soil solution, rendering the metals less toxic and less available.

Al toxicity in very acidic soils (pH < ~4.5) is an important agricultural problem limiting crop production and thus causing substantial yield losses for many different crops [17,86]. Recently there has been a lot of research was carried out in the progress of external Al detoxification in higher plants.

To investigate Al tolerance in seedlings of six *Eucalyptus* species Silva et al. used GC-MS and ion chromatography (IC) analyses and their study indicates that root exposure to Al led to a greater than 200% increase in malic acid concentration in the root tips of all eucalypt species [87]. Although malate and citrate exudation by roots may partially account for the overall high Al tolerance of these eucalypt species, it appears that tolerance is mainly derived from the internal detoxification of Al by complexation with malic acid. Studies about plant physiological response to Cd, Cu and Pb toxicity in the rhizosphere soil of tobacco and sunflower have been performed to test the ability for phytoremediation [88,89]. LMWOAs exudates were analyzed by GC-MS and ICP-MS was used for analysis of concentration of total heavy metals in case of Cd. The obtained results suggest that the different levels of LMWOAs detected in rhizosphere soil play an important role in metal solubilization processes via complexation prior to the uptake by plants.

ICP-MS was applied by several other authors to gain information regarding the elemental composition of rhizosphere-related samples. A validated ICP-MS method was used by Marwa et al. for a field survey with the objective of studying the contamination of potentially toxic elements As, Pb, Cr and Ni in Tanzanian agricultural soils and their uptake and translocation in maize [90]. Microwave digestion using nitric acid–hydrogen peroxide was implemented as sample preparation for the analysis of Cr, As, Pb and Ni from maize shoot and grain. The analysis of certified reference materials (CRMs), *i. e* long rice from Arkansas USA (NIST 1568a) and certified reference soil (ZC 73007) from the China National Analysis Centre for iron and steel was an integral part of the study showing the validity of the applied methodological approaches.

Wiseman et al. used ICP-MS to analyse soil, plant tissue and plant rhizosphere samples for a range of traffic-related elements, including Cr, Mn, Cu, Ni, Cd, As, Sb and Pb [91]. The authors were dealing with insufficient extraction recoveries especially in case of Mn, As and Pb and Sb in the used CRMs (trace metals in spinach leaves (NIST 1570a) and San Joaquin soil (NIST 2709a)) and did not overcome the problem of spectral interferences for some elements, which is a common problem when using single quadrupole low mass resolution mass spectrometer without appropriate cell technology. Indeed, the authors were reporting extreme recovery values especially for As (up to 500%), which could be explained by poor spectral resolution.

Residues from use of depleted uranium munitions pose a lasting environmental impact through persistent contamination of soils and therefore an understanding of the factors determining the fate

Please cite this article in press as: A. Rugova, et al., Elucidating rhizosphere processes by mass spectrometry – A review, Analytica Chimica Acta (2017), http://dx.doi.org/10.1016/j.aca.2016.12.044

of depleted uranium in soil is necessary. Using soil contaminated with uranium Duquène et al. found that one month's growth of either Indian mustard or rye grass produced soil solution concentrations of U twenty fold higher in sandy soil and two fold higher in clay soil than in non-planted soil [92]. Tandy et al. on the other hand investigated the effects of root exudates on depleted uranium dissolution by using the Single-Cell-Sampling-and-Analysis (SiCSA) technique for the first time in soil [93]. DU fragments were sampled and analyzed using SiCSA and CE-ICP-MS for organic acids and uranium and by means of high spatial and temporal resolution in the rhizosphere they were able to conclude that root exudates can solubilize DU giving high localized soil solution concentrations. An interesting study by Mihalik and coworkers was conducted about the acceptability of sunflowers in remobilization of U and Fe in soil via citric acid as root exudate [94]. As the authors conclude the sunflower uptake of U was greatly enhanced, whereas the uptake of Fe was not affected – in accordance with the high regulation of this nutrient in the plant. Dynamics of remobilization in soil were assessed using Diffusive Gradient in Thin-film (DGT) measurements and DGT resin was then eluted with HNO_3 and U and Fe were quantified via ICP–AES or ICP-MS depending on the concentration. All treatments induced a remobilization of U and Fe from the soil solid-phase to the soil solution.

For investigating metal mobilization processes induced by metal-accumulating plants metabolomics were applied to analyse the variation of root exudates from *S. alfredii* a Cd hyperaccumulator under different Cd exposure concentrations and times by GC-MS after derivatization with methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide [95]. It was found that the Cd hyperaccumulator *S. alfredii* could be able to adjust the secretion of root exudates to increase Cd tolerance and accumulation. By applying such metabolomics analysis 15 compounds were identified (oxalic acid, 2-hydroxyacetic acid, octanol, Benzoic acid, 2-Hydroxypentanoic acid, succinic acid, fumaric acid, L-serine, decanoic acid, putrescine, fructose, mannitol, 1-monooctadecanoylglycerol, octacosanol beta-sitosterol).

3.3. Plant-microorganism and plant–plant interactions via root exudates

Plants release numerous secondary metabolites through root exudates into the rhizosphere for the attraction of beneficial soil microorganisms and the defense against pathogens [96]. In return, plants benefit from the microbial turnover of root exudates and other soil organic and inorganic matter.

In the recent years, there has been an increasing interest to better understand the interaction between plants and rhizosphere bacteria and thus getting more information about the biological properties of the rhizosphere, *e.g.* by using proteomic techniques (characterization of a set of proteins under specific conditions) to elaborate the ways in which plants and bacteria influence protein expression in their partner organism, nutrient exchange and the alteration of plant development by microbes [97,98].

Plant–microbe proteomics poses additional challenges, such as the need to differentiate between plant and microbial proteins [97]. Jayaraman and co-workers have reviewed the techniques that have been applied to the study of plant microbe interactions [99]. MS has been widely applied to plant microbe proteomics and is the most common technique for unbiased protein identification [100].

The plant growth promoting rhizobacteria classified as acid-producing rhizosphere bacteria have been intensively studied, regarding their plant growth-promoting capacity of releasing phosphorus from insoluble phosphates. Jones and Oburger have reviewed the research that has been conducted in identification of P solubilizing organisms (PSM) that exist in soil, types of P that they can utilize and the mechanisms by which this occurs and the potential for managing them in an agricultural context [101]. This review highlights a major problem when interpreting the results of PSM field trials that still remains the lack of consideration of quantification of P dynamics in the soil. The plant microbe interaction to enhance trace element availability in the rhizosphere has been studied by a numerous authors and was intensively reviewed by Sessitsch and colleagues [102]. As these authors claim, by employing these types of plant-associated bacteria phytoextraction through enhancement of metal bioavailability to the plants it's being possible to be performed.

The first in-situ study where the $^{15}N/^{13}C$ leaf-labelling technique has been followed by compound specific ^{13}C phospholipid fatty acid (PLFA) analysis to investigate plant microbial interactions under undisturbed field conditions, was performed by Kusliene et al. [103]. Stable dual $^{15}N/^{13}C$ labelling as good tool for targeted tracing of C and N in mixed microbial communities in combination with analysis of biomarkers (PLFA) by GC-c-IRMS of soil microorganisms enables estimations of the contribution of various types of root exudates to C and N dynamics in soil. Plants (*Trifolium repens* L. and *Lolium perenne* L) showed very fast below-ground allocation of ^{13}C and ^{15}N, showing ability to fix atmospheric N and hence considered to be a potential for improving soil fertility via rhizodeposition.

When roots are under stress or they experience challenges in the rhizosphere, they react by releasing exudates such as amino acids, organic acids, and phenolics as well as proteins. These root exudates for some types of plants can mediate chemical interferences and act as phytotoxins in mediating chemical interference, i.e. allelopathy [104] which is considered to be as one of the mechanisms by which plants may gain an advantage over their neighboring competitors [1]. On the other hand they can also exhibit symbiotic responses as a positive form of interaction by increasing herbivore resistance in neighboring plants [105]. Allelopathic crops are of great interest because they may be used to effectively suppress weeds, and invasive weeds could rely, in part on allelopathic interactions to facilitate their establishment and wider distribution [106] and MS techniques can contribute to a great extent for a better characterization of these plants.

A GC-MS with silyliation method using N,O-bis (trimethylsiyl) acetamide (BSA) was used by Zhang et al. who designed a laboratory bioassay to determine the allelopathic potential of root exudates of three fruit tree species [107]. Their work provides data that root exudates of apple (*Malus pumila* L.) and peach (*Prunus persica* L.) inhibited germination and radicle growth of apple seeds.

Martínez-Cortés a et al. used nano LC matrix assisted laser desorption ionization tandem time of flight MS (nanoLC-MALDI-TOF/TOF-MS/MS) for identifying 47 proteins in root exudates of *Physcomitrella patens* [108]. The identified proteins were classified as a response to stress, response to stimulus, oxido-reduction, cell wall modification, photosynthesis and carbohydrate metabolism transport, DNA metabolic process and regulation/signaling. The identified defense related proteins are a good example of application of hyphenated MS techniques in elucidating the plant interactions with the surrounding environment through exudates.

Not only plants (as described in Chapter 3.2) but also associated rhizosphere microbes may contribute to the accumulation efficiency of metal-hyperaccumulating plants. The weathering capacities of Ni mobilization by *Arthrobacter* strains: LA44 as indole acetic acid (IAA) producer or SBA82 (siderophore producer, PO_4^{3-} solubilizer, and IAA producer) was studied by Becerra-Castro et al. [109]. The role of selected strains on metal availability and plant uptake was performed in a pot experiment by growing plants in ultramafic soil and adding bacterial inoculants. The organic acid exudation specifically oxalate, was associated with LA44 strain which is found to be more efficient in Ni mobilization compared to

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the other strain SBA82. Authors recommend also that the concurrent mobilization of Fe and Si indicates preferential weathering of Fe oxides and serpentine minerals, possibly related to the siderophore production capacity of these strains. Carboxylic acids were separated by reversed-phase LC and analyzed by LC-ESI-TOF-MS. Quantification of selected phenolic compounds by LC-MS/MS in negative ionization mode on triple-quadrupole mass spectrometer equipped with an ESI interface while Ni concentrations were measured by ICP-OES. This study revealed that Ni-mobilizing inoculants could be useful for improving Ni uptake by hyperaccumulator plants.

3.4. ^{13}C isotope labelling technique for studying the dynamics of root-derived carbon in the rhizosphere

Plants rapidly release photo-assimilated carbon to the soil via direct root exudation and associated mycorrhizal fungi, with both pathways promoting plant nutrient availability. The release of carbon into the soil by growing roots is an important part of the terrestrial carbon cycle and the benefit to plants from this carbon loss is the alteration of the rhizosphere environment [110]. However, the in situ nature and dynamics of root-derived carbon in the soil is still poorly understood. Application of recently developed $^{13}CO_2$ pulse labelling and stable isotope probing (SIP) methods offer the possibility to track ^{13}C-labelled plant photosynthates enabling studies on examination of the relationship between soil microbial diversity and carbon flow in situ. The pulse labeling is conducted through injections of few mL either as single or repeated pulses from few minutes to days at different growing stage of plants, while continuous flow isotopic labeling techniques involves the use of labeled gas to expose the plants during the complete growing season [111]. Griffiths and coworkers performed a pioneering work on this field that deals with identification of microorganisms utilizing plant exudates using stable isotope probing methodologies [112] with a protocol developed by Manefield et al. about separation and analysis of ^{13}C-enriched ribonucleic acid (RNA) [113].

An interesting application of GC-IRMS was performed by Derrien et al. in a study for investigation of nature and dynamics of root-derived carbon in the soil by coupling pulse-chase isotope labelling of photo assimilated $^{13}CO_2$ with the subsequent quantitative analysis of labelled carbohydrates in the soil after trimethylsilyl derivatization [48,49]. After a two-step separation of the soil from the roots of wheat, (*Triticum aestivum* L.), the amount of total organic ^{13}C as well as the quantities of ^{13}C in arabinose, fructose, fucose, glucose, galactose, mannose, rhamnose and xylose were determined with GC-IRMS. Authors concluded that 40% of the root-derived carbon was present in the form of neutral sugars, and exhibited a time-increasing signature of microbial sugars and that the composition of rhizospheric sugars rapidly tended towards bulk soil organic matter.

Using nanoscale secondary ion MS (NanoSIMS) imaging and ^{13}C-phospho and neutral lipid fatty acids, Kaiser et al. [114] traced in-situ flows of recently photo assimilated C of $^{13}CO_2$- exposed wheat (*Triticum aestivum*) through arbuscular mycorrhiza (AM) into root- and hyphae-associated soil microbial communities. They have traced the fate of recently plant-assimilated ^{13}C in both root- and hyphae-associated microbial communities via phospholipid fatty acid and neutral lipid fatty acid (NLFA) stable isotope probing and in dissolved organic carbon pools via isotope-ratio MS. Their findings indicate that a significant fraction of recently photo-assimilated C delivered to soil microbes leaves plant roots via mycorrhizal hyphae already upstream of the place of passive root exudations. Arbuscular mycorrhiza extra radical hyphae which enlarge root areas may thus not only improve nutrient uptake by plants, but also act as an effective distributor of recently assimilated plant C to the soil microbial community.

3.5. Metabolite profiling techniques

Metabolomics is one of the most advanced –omics strategies and has been applied to various scientific fields [115]. Rhizosphere metabolomics aims at the analysis of the entire metabolome of the root-soil interface addressing root exudates, *i.e.* primary metabolites and plant natural products involved in chemical signalling, exudates of the root associated microbiome and fungi. Different strategies such as metabolic fingerprinting that includes non-targeted analysis as well as metabolic profiling aiming in targeted quantitative analysis of selected metabolites are often utilized in rhizosphere metabolomics.

To explore the chemical composition of root exudates of the model plant *Arabidopsis thaliana* a workflow for non-targeted metabolite profiling of the semipolar fraction of root exudates was developed by Strehmel et al. [116]. Using this setup for the main cultivation period, the volume of the nutrient solution per plant was reduced to a manageable amount in order to simplify the subsequent enrichment of exuded components. Based on the presented workflow, more than 100 compounds, i.e. mainly secondary metabolites, were detected and structurally characterized by UPLC/ESI-QTOF-MS techniques of which more than 90 were structurally characterized or classified and among them 42 compounds were rigorously identified using an authenticated standard. The development of such methods should facilitate a significant reduction of the sampling volume of the nutrient or soil solution.

The same UHPLC-TOF-MS based metabolomics approach was used by Marti et al. to evaluate local and systemic herbivore-induced changes in maize leaves, sap, roots and root exudates without any prior assumptions about their function [117]. Authors of this work were able to identify 32 differentially regulated compounds from *Spodoptera littoralis*-infested maize seedlings and isolated for structure assignment by micro flow nuclear magnetic resonance (CapNMR). To measure absolute concentrations of elicited compounds in crude plant extracts, a method based on direct nanoinfusion MS/MS was developed and nine compounds were quantified by this method.

In a different approach, matrix assisted laser desorption ionization MS imaging (MALDI-MSI) has been used as a tool to identify and map antibiotics synthesized by root-associated bacteria and more generally, to investigate plant–microbe interactions at the molecular level [118] and for the characterization of the novel constitutively secreted and inducible phytochemicals that directly repel, inhibit, or kill pathogenic microorganisms in the rhizosphere [119]. Thus, MS contribution in identification and mapping of bacterial processes represents a very important milestone. In the most recent published work of Kierul and co-authors proteomic studies gave more evidence on the bacterial responses to maize root exudates [120]. Out of the 121 proteins secreted by *Bacillus amyloliquefaciens* FZB42, identified by MALDI-TOF/MS, 61 were predicted to contain secretion signals and this study reveals conclusion that approximately 34 proteins were differentially secreted in response to root exudates during either the late exponential or stationary phase and hypothesise that they were mainly involved in nutrient utilization and transport.

4. Conclusion

Chemical characterization as well as quantification of exudates present at the soil root interface is to a great extent an analytical challenge due to their localized deposition as well as the low concentration in the soil solution and the associated difficulties of

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proper sampling of exudates and/or rhizosphere soil (solution). Development and application of advanced sampling procedures in combination with highly selective and sensitive analytical techniques with targeted and non-targeted MS based methodologies is a key approach for elucidating plant-soil-microbe interactions, since only a more comprehensive knowledge on the quality and quantity of root exudates can provide the basis for better understanding of rhizosphere processes. Progress in MS has enabled accurate quantification of many different compounds released by roots. However, our understanding of rhizosphere dynamics is still incomplete due to the difficulty of assessing processes at the root-soil interface under controlled but also realistic conditions.

Clearly, the solvation of these challenges and difficulties will be driven by future improvements of the detection power and selectivity of the analytical technologies. Looking back to the last three decades, it can be estimated that these improvements will mainly address the developments in molecular MS. The detection power of elemental ultratrace analysis is mostly hampered by contamination and background, so that a further improvement of sensitivity will not lead to more significant results in the context with rhizosphere research.

Also, in-situ sampling and analysis will emerge in the field, *e.g.* LA-ICP-MS (most importantly with TOF mass spectrometers) or MALDI based imaging. Both methods are powerful, however, currently obtained data is often misinterpreted or lacks quality control. As a major drawback, absolute quantification is still not achieved by these techniques.

Thus, developing novel methodologies to study rhizosphere ecology under natural conditions is indispensable for better understanding of root-soil interactions, which might allow the optimization of plant performance in terms of plant health and yield as well as in the context of improving phytoremediation efficiency.

References

[1] H.P. Bais, T. Weir, L. Perry, S. Gilroy, J.M. Vivanco, The role of root exudates in rhizosphere interactions with plants and other organisms, Annu. Rev. Plant Biol. 57 (2006) 233–266.
[2] A. Hartmann, M. Rothballer, M. Schmid, Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research, Plant Soil 312 (2008) 7–14.
[3] A.M. Hirsch, W.D. Bauer, D.M. Bird, J. Cullimore, B. Tyler, J.I. Yoder, Molecular signals and receptors: controlling rhizosphere interactions between plants and other organisms, Ecology 84 (2003) 858–868.
[4] P. Hinsinger, G.R. Gobran, P.J. Gregory, W.W. Wenzel, Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes, New Phytol. 168 (2005) 293–303.
[5] E.A. Curl, B. Truelove, The Rhizosphere,Springer-Verlag, Springer-Verlag, Berlin-Heidelberg-New York-Tokyo, New York, 1986, p. p 288. ISBN 3-540-15803-0.
[6] X.F. Huang, J.M. Chaparro, K. Reardon, R. Zhang, Q. Shen, J.M. Vivanco, Rhizosphere interactions: root exudates, microbes, and microbial communities, Botany 92 (2014) 267–275.
[7] N.C. Brady, R.R. Weil, Nature and Properties of Soils, Prentice Hall, Upper Saddle River, New York, 2002.
[8] F.D. Dakora, D.A. Phillips, Root exudates as mediators for mineral acquisition, in low-nutrient environments, Plant Soil 245 (2002) 35–47.
[9] E. Oburger, G.D.J. Kirk, W.W. Wenzel, M. Puschenreiter, D.L. Jones, Interactive effects of organic acids in the rhizosphere, Soil. Biol. Biochem. 41 (2009) 449–457.
[10] D.L. Jones, P.G. Dennis, A.G. Owen, P.A.W. Van Hees, Organic acid behavior in soils – misconceptions and knowledge gaps, Plant Soil 248 (2003) 31–41.
[11] M. Fomina, S. Hillier, J.M. Charnock, K. Melville, I.J. Alexander, G.M. Gadd, Role of oxalic acid over excretion in transformations of toxic metal minerals by beauveria caledonica, Appl. Environ. Microbiol. 71 (1) (2005) 371–381.
[12] D.L. Jones, P.R. Darrah, Role of root derived organic-acids in the mobilization of nutrients from the rhizosphere, Plant Soil 166 (1994) 247–257.
[13] L. Ström, A.G. Owen, D.L. Godbold, D.L. Jones, Organic acid behavior in a calcareous soil: sorption reactions and biodegradation rates, Soil. Biol. Biochem. 33 (2001) 2125–2133.
[14] L. van Scholl, E. Hoffland, N. van Breemen, Organic anion exudation by ectomycorrhizal fungi and *Pinus sylvestris* in response to nutrient deficiencies, New Phytol. 170 (2006) 153–163.
[15] A.D. Rovira, J.R. Harris, Plant root excretions in relation to the rhizosphere effect. V. The exudation of B-group vitamins, Plant Soil 14 (1961) 199–214.
[16] A.L. D'Arcy, Study of soja and lentil exudates I. Kinetics of exudation of phenolic compounds, amino acids and sugars in the first days of plant growth, Plant Soil 68 (1982) 399–403.
[17] J.F. Ma, J. Furukawa, Recent progress in the research of external Al detoxification in higher plants: a minireview, J. Inorg. Biochem. 97 (2003) 46–51.
[18] F.Z. Haichar, C. Santaella, Th. Heulin, W. Achouak, Root exudates mediated interactions belowground-A review, Soil Biol. Biochem. 77 (2014) 69–80.
[19] Y. Kuzyakov, G. Domanski, Carbon input by plants into the soil, review, J. Plant. Nutr. Soil. Sci. 163 (2000) 421–431.
[20] Simon Nelms (Ed.), Inductively Coupled Plasma Mass Spectrometry Handbook, Wiley-Blackwell, 2006.
[21] F. von Blanckenburg, N. von Wirén, M. Guelke, D.J. Weiss, T.D. Bullen, Fractionation of metal stable isotopes by higher plants, Elements 5 (2009) 375–438.
[22] L. Yang, Accurate and precision determination of isotopic ratios by MC-ICP-MS: a review, Mass Spectrom. Rev. 28 (2009) 990–1011.
[23] O.T. Butler, W.R.L. Cairns, J.M. Cook, Ch. M. Davidson, Atomic spectrometry update. Environmental analysis, J. Anal. At. Spectrom. 28 (2013) 177–216.
[24] Z. Muccio, G.P. Jackson, Isotope ratio mass spectrometry, Analyst 134 (2009) 213–222.
[25] T.C.W. Moerdijk-Poortvliet, H. Schierbeek, M. Houtekamer, T. van Engeland, D. Derrien, L.J. Stal, H.T.S. Boschker, Comparison of gas chromatography/isotope ratio mass spectrometry and liquid chromatography/isotope ratio mass spectrometry for carbon stable-isotope analysis of carbohydrates, Rapid Commun, Mass Spectrom. 29 (2015) 1205–1214.
[26] J. Black, E. Epstein, W. Rains, Q. Zhuyin, W. Casey, Magnesium-isotope fractionation during plant growth, Environ. Sci. Technol. 42 (2008) 7831–7836.
[27] D. Jouvin, D.J. Weiss, T.F.M. Mason, M.N. Bravin, P. Louvat, F. Zhao, F. Ferec, P. Hinsinger, M.F. Benedetti, Stable isotopes of Cu and Zn in higher plants: evidence for Cu reduction at the root surface and two conceptual models for isotopic fractionation processes, Environ. Sci. Technol. 46 (2012) 2652–2660.
[28] T.F.D. Mason, D.J. Weiss, M. Horstwood, R.R. Parrish, S.S. Russell, E. Mullane, B.J. Coles, High precision Cu and Zn isotope analysis by plasma source mass spectrometry: Part 1: spectral interferences and their correction, J. Anal. At. Spectrom. 19 (2004) 209–217.
[29] T.F.D. Mason, D.J. Weiss, M. Horstwood, R.R. Parrish, S.S. Russell, E. Mullane, B.J. Coles, High precision Cu and Zn isotope analysis by plasma source mass spectrometry: Part 2: correcting for mass bias discrimination effects, J. Anal. At. Spectrom. 19 (2004) 218–226.
[30] IUPAC, Compendium of Chemical Terminology, in: A.D. McNaught, A. Wilkinson (Eds.), The "Gold Book", second ed., Blackwell Scientific Publications, Oxford, UK, 1997.
[31] O.T. Butler, W.R.L. Cairns, J.M. Cook, C.M. Davidson, Atomic spectrometry update-a review of advances in environmental analysis, J. Anal. at Spectrom. 31 (2016) 35–89.
[32] M. Popp, S. Hann, G. Koellensperger, Environmental application of elemental speciation analysis based on liquid or gas chromatography hyphenated to inductively coupled plasma mass spectrometry—a review, Anal. Chim. Acta 668 (2010) 114–129.
[33] Y. Schindlegger, E. Oburger, M. Puschenreiter, G. Stingeder, G. Koellensperger, S. Hann, Speciation of 2′-deoxymugineic acid-metal complexes in top soil extracts by multi-modal stationary phase LC-ICP-MS, J. Anal. At. Spectrom. 30 (2015) 1345–2135.
[34] A. Rugova, M. Puschenreiter, J. Santner, L. Fischer, S. Neubauer, G. Koellensperger, S. Hann, Speciation analysis of orthophosphate and myo-inositol hexakisphosphate in soil- and plant-related samples by high-performance ion chromatography combined with inductively coupled plasma mass spectrometry, J. Sep. Sci. 37 (2014) 1711–1719.
[35] S. Mounicou, J. Szpunara, R. Lobinski, Metallomics: the concept and methodology, Chem. Soc. Rev. 38 (2009) 1119–1138.
[36] A.P. Von der Heide, S. Mounicou, J. Meija, H.F. Henry, J.A. Caruso, J.R. Shann, Investigation of selenium-containing root exudates of Brassica juncea using HPLC-ICP-MS and ESI-qTOF-MS, Analyst 131 (2006) 33–40.
[37] S. Mounicou, M. Shah, J. Meija, J.A. Caruso, A.P. Vonderheide, J. Shann, Localization and speciation of selenium and mercury in Brassica juncea—implications for Se–Hg antagonism, J. Anal. At. Spectrom. 21((2006) 404–412.
[38] J.J. Dytrtova, M. Jakl, I. Sestakova, E.L. Zinsa, D. Schrodera, T. Navratil, A new approach to study cadmium complexes with oxalic acid in soil solution, Anal. Chim. Acta 693 (2011) 100–105.
[39] J.J. Dytrtova, M. Jakl, D. Schroder, Complexation of malic acid with cadmium(II) probed by electrospray ionization mass spectrometry, Talanta 90 (2012) 63–68.
[40] A. Takeda, H. Tsukada, Y. Takaku, S. Hisamatsu, Fractionation of metal complexes with dissolved organic matter in a rhizosphere soil solution of a humus-rich Andosol using size exclusion chromatography with inductively coupled plasma-mass spectrometry, Soil Sci. Plant Nutr. 55 (2009) 349–357.
[41] T.W.M. Fan, A.N. Lane, M. Shenker, J.P. Bartley, D. Crowley, R.M. Higashi, Comprehensive chemical profiling of gramineous plant root exudates using high-resolution NMR and MS, Phytochem 57 (2001) 2209–2221.
[42] D.B. Read, A.G. Bengough, P.J. Gregory, J.W. Crawford, D. Robinson, C.M. Scrimgeour, I.M. Young, K. Zhang, X. Zhang, Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil, New Phytol. 157 (2003) 315–326.

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[43] Y. Lu, Y. Zhou, S. Nakai, M. Hosomi, H. Zhang, H.J. Kronzucker, W. Shi, Stimulation of nitrogen removal in the rhizosphere of aquatic duckweed by root exudate components, Planta 239 (2014) 591–603.
[44] K. Suzuki, K. Okazaki, K. Tawaraya, M. Osaki, T. Shinano, Gas chromatography-mass spectrometry associated global analysis of rice root exudates under aseptical conditions, Soil Sci. Plant Nutr. 55 (2009) 505–513.
[45] Ch. Lin, S.M. Owen, J. Peñuelas, Volatile organic compounds in the roots and rhizosphere of Pinus spp., Soil Biol. Biochem. 39 (2007) 951–960.
[46] G. Neumann, S. Bott, M.A. Ohler, H.P. Mock, R. Lippmann, R. Grosch, K. Smalla, Root exudation and root development of lettuce (lactuca sativa l. Cv. Tizian) as affected by different soils, Front. Microbiol. 5 (2014) 2.
[47] T. Toyama, T. Furukawa, N. Maeda, D. Inoue, K. Sei, K. Mori, S. Kikuchi, M. Ike, Accelerated biodegradation of pyrene and benzo[a]pyrene in the Phragmites australis rhizosphere by bacteria–root exudate interactions, Water Res. 45 (2011) 1629–1638.
[48] D. Derrien, C. Marol, J. Balesdent, The dynamics of neutral sugars in the rhizosphere of wheat. An approach by ^{13}C pulse-labelling and GC/C/IRMS, Plant Soil 267 (2005) 243–253.
[49] D. Derrien, J. Balesdent, C. Marol, C. Santaella, Measurement of the $^{13}C/^{12}C$ ratio of soil-plant individual sugars by gas chromatography/combustion/isotope-ratio mass spectrometry of silylated derivatives, Rapid Commun. Mass Spec. 17 (2003) 2626–2631.
[50] S. Nardi, E. Sessi, D. Pizzeghello, A. Sturaro, A. Rella, G. Parvoli, Biological activity of soil organic matter mobilized by root exudates., Chemosphere 46 (2002) 1075–1081.
[51] S. Neubauer, C. Troyer, K. Klavins, H. Russmayer, M.G. Steiger, B. Gasser, M. Sauer, D. Mattanovich, S. Hann, G. Koellensperger, U^{13}C cell extract of Pichia pastoris – a powerful tool for evaluation of sample preparation in metabolomics, J. Sep. Sci. 35 (2012) 3091–3105.
[52] H. Sun, Y. Wang, Hollow fiber liquid-phase microextraction combined with gas chromatography-mass spectrometry for determination of root exudative potential allelochemicals in capsicum annuum (chili), Anal. let. 45 (2012) 1566–1580.
[53] H. Sun, Y. Wang, Hollow fiber liquid-phase microextraction with in situ derivatization combined with gas chromatography-mass spectrometry for the determination of root exudate phenylamine compounds in hot pepper (Capsicum annuum L.), J. Agr. Food Chem. 61 (2013) 5494–5499.
[54] E.J. Eilers, G. Pauls, M.C. Rillig, B.S. Hansson, M. Hilker, A. Reinecke, Novel set-up for low-disturbance sampling of volatile and non-volatile compounds from plant roots, J. Chem. Ecol. 41 (2015) 253–266.
[55] K. Fischer, Environmental analysis of aliphatic carboxylic acids by ion-exclusion chromatography, Anal. Chim. Acta 465 (2002) 157–173.
[56] J.M. Langemeier, D.E. Rogers, Rapid method for sugar analysis of doughs and baked products, Cereal. Chem. 72 (1995) 349–351.
[57] B.M. Miguez, J.M. De la Montana, J.O. Garcia, HPLC determination of sugars in varieties of chestnut fruits from Galicia (Spain), J. Food. Compos. Anal. 17 (2004) 63–67.
[58] Y.C. Lee, High-performance anion exchange chromatography for carbohydrate analysis, Anal. Biochem. 189 (1999) 151–162.
[59] E. Rosenberg, The potential of organic (electrospray- and atmospheric pressure chemical ionisation) mass spectrometric techniques coupled to liquid-phase separation for speciation analysis, Review article, J. Chromatogr. A 1000 (2003) 841–889.
[60] Z. Chen, X. Jin, Q. Wang, Y. Lin, L. Gan, C. Tang, Confirmation and determination of carboxylic acids in root exudates using LC-ESI-MS, J. Sep. Sci. 30 (2007) 2440–2446.
[61] Z. Chen, K.R. Kim, G. Owens, R. Naidu, Determination of carboxylic acids from plant root exudates by ion exclusion chromatography with ESI–MS, Chromatographia 67 (2008) 113–117.
[62] L. Jaitz, E. Oburger, M. Puschenreiter, G. Koellensperger, S. Hann, Hydrophilic interaction LC combined with electrospray MS for highly sensitive analysis of underivatized amino acids in rhizosphere research, J. Sep. Sci. 33 (2010) 911–922.
[63] J. Erro, A.M. Zamarreno, J.C. Yvin, J.M. Garcia-Mina, Determination of organic acids in tissues and exudates of maize, lupin, and chickpea by high-performance liquid chromatography-tandem mass spectrometry, J. Ag. Food Chem. 57 (2009) 4004–4010.
[64] G. McRae, C.M. Monreal, LC-MS/MS quantitative analysis of reducing carbohydrates in soil solutions extracted from crop rhizospheres, Anal. Bioanal. Chem. 400 (2011) 2205–2215.
[65] M. Dell'mour, W. Schenkeveld, E. Oburger, L. Fischer, S. Kraemer, M. Puschenreiter, M. Lämmerhofer, G. Koellensperger, S. Hann, Analysis of iron-phytosiderophore complexes in soil related samples: LC-ESI-MS/MS versus CE-MS, Electrophoresis 33 (2012) 726–733.
[66] M. Tsednee, Y.-W. Mak, Y.-R. Chen, K.-C. Yeh, A sensitive LC-ESI-Q-TOF-MS method reveals novel phytosiderophores and phytosiderophore-iron complexes in barley, New Phytol. 195 (2012) 951–961.
[67] Y. Schindlegger, E. Oburger, B. Gruber, W.D.C. Schenkeveld, S.M. Kraemer, M. Puschenreiter, G. Koellensperger, S. Hann, Accurate LC-ESI-MS/MS quantification of 2′-deoxymugineic acid in soil and root related samples employing porous graphitic carbon as stationary phase and a $^{13}C_4$-labeled internal standard, Electrophoresis 35 (2014) 1375–1386.
[68] M. Dell'mour, L. Jaitz, E. Oburger, M. Puschenreiter, G. Koellensperger, S. Hann, Hydrophilic interaction LC combined with electrospray MS for highly sensitive analysis of underivatized amino acids in rhizosphere research, J. Sep. Sci. 33 (2010) 911–922.
[69] S. Sato, T. Soga, T. Nishioka, M. Tomita, Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection, Plant J. 40 (2004) 151–163.
[70] A. Oikawa, N. Fujita, R. Horie, K. Saito, K. Tawaraya, Solid-phase extraction for metabolomic analysis of high-salinity samples by capillary electrophoresis-mass spectrometry, J. Sep. Sci. 34 (2011) 1063–1068.
[71] M. Dell'mour, G. Koellensperger, J.P. Quirino, P.R. Haddad, Ch. Stanetty, E. Oburger, M. Puschenreiter, S. Hann, Complexation of metals by phytosiderophores revealed by CE-ESI-MS and CE-ICP-MS, Electrophoresis 31 (2010) 1201–1207.
[72] Y. Kuzyakov, S. Siniakina, A novel method for separating root derived organic compounds from root respiration in non-sterilized soils, J. Plant Nutri. Soil Sci. 164 (2001) 511–517.
[73] A. Melnitchouck, P. Leinweber, K.-U. Eckhardt, R. Beese, Qualitative differences between day- and night-time rhizodeposition in maize (Zea mays L.) as investigated by pyrolysis-field ionization mass spectrometry, Soil. Biol. Biochem. 37 (2005) 155–162.
[74] A. Schlihting, P. Leinweber, New evidence for the molecular-chemical diversity of potato plant rhizodeposits obtained by pyrolysis-field ionisation mass spectrometry, Phytochem. Anal. 20 (2009) 1–13.
[75] P. Leinweber, K.-U. Eckhardt, H. Fischer, Y. Kuzyakov, A new rapid micromethod for the molecular-chemical characterization of rhizodeposits by field-ionization mass spectrometry, Rapid Com. Mass Spec. 22 (2008) 1230–1234.
[76] K.G. Raghothama, Phosphate acquisition, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 665–693.
[77] D.L. Jones, Organic acids in the rhizosphere – a critical review, Plant Soil 205 (1998) 25–44.
[78] K. Tawaraya, R. Horie, S. Saito, T. Wagatsuma, K. Saito, A. Oikawa, Metabolite profiling of root exudates of common bean under phosphorus deficiency, Metabolites 4 (2014) 599–611.
[79] R. Khorassani, U. Hettwer, A. Ratzinger, B. Steingrobe, P. Karlovsky, N. Claassen, Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus, BMC Plant Biol. 11 (2011) 12.
[80] T. Kobayashi, N.K. Nishizawa, Iron uptake, translocation, and regulation in higher plants, Annu. Rev. Plant Biol. 63 (2012) 131–152.
[81] H. Schmidt, C. Günther, M. Weber, C. Spörlein, S. Loscher, C. Böttcher, R. Schobert, S. Clemens, Metabolome analysis of Arabidopsis thaliana roots identifies a key metabolic pathway for iron acquisition, PLoS One 9 (2014) 7.
[82] E. Oburger, B. Gruber, Y. Schindlegger, W.D.C. Schenkeveld, S. Hann, S.M. Kraemer, W.W.M. Puschenreiter, Root exudation of phytosiderophores from soil-grown wheat, New Phytol. 203 (2014) 1161–1174.
[83] W.D.C. Schenkeveld, E. Oburger, B. Gruber, Y. Schindlegger, S. Hann, M. Puschenreiter, S.M. Kraemer, Metal mobilization from soils by phytosiderophores - experiment and equilibrium modelling, Plant Soil 383 (2014) 59–71.
[84] W.D.C. Schenkeveld, Y. Schindlegger, E. Oburger, M. Puschenreiter, S. Hann, S.M. Kraemer, Geochemical processes constraining iron uptake in strategy II Fe acquisition, Environ. Sci. Technol. 48 (2014) 12662–12670.
[85] G. Neumann, S. Bott, M.A. Ohler, H.P. Mock, R. Lippmann, R. Grosch, K. Smalla, Root exudation and root development of lettuce (lactuca sativa l. Cv. Tizian) as affected by different soils, Front. Microbiol. 5 (2014) 2.
[86] L.V. Kochian, O.A. Hoekenga, M.A. Piñeros, How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorus efficiency, Annu. Rev. Plant Biol. 55 (2004) 459–493.
[87] I.R. Silva, R.F. Novais, G.N. Jham, N.F. Barros, F.O. Gebrim, F.N. Nunes, J.C.L. Neves, F.P. Leite, Responses of eucalypt species to aluminum: the possible involvement of low molecular weight organic acids in the Al tolerance mechanism, Tree Physiol. 24 (2004) 1267–1277.
[88] P.N. Chiang, M.K. Wang, C.Y. Chiu, S.Y. Chou, Effects of cadmium amendments on low-molecular-weight organic acid exudates in rhizosphere soils of tobacco and sunflower, Environ. Toxicol. 21 (2006) 479–488.
[89] F. Qin, X. Shan, B. Wei, Effects of low-molecular-weight organic acids and residence time on desorption of Cu, Cd, and Pb from soils, Chemosphere 57 (2004) 253–263.
[90] E.M.M. Marwa, A.A. Meharg, C.M. Rice, Risk assessment of potentially toxic elements in agricultural soils and maize tissues from selected districts in Tanzania, Sci. Total. Environ. 416 (2012) 180–186.
[91] C.L.S. Wiseman, F. Zereini, W. Püttmann, Traffic-related trace element fate and uptake by plants cultivated in roadside soils in Toronto, Canada, Sci. Total. Environ. 442 (2013) 86–95.
[92] L. Duquène, H. Vandenhove, F. Tack, E. Meers, J. Baeten, J. Wannijn, Enhanced phytoextraction of uranium and selected heavy metals by Indian mustard and ryegrass using biodegradable soil amendments, Sci. Total. Environ. 407 (2009) 1496–1505.
[93] S. Tandy, S.R. Brittain, B.M. Grail, C.W. Mcleod, E. Paterson, A.D. Tomos, Fine scale measurement and mapping of uranium in soil solution in soil and plant-soil microcosms, with special reference to depleted uranium, Plant Soil 368 (2013) 471–482.
[94] J. Mihalik, P. Henner, S. Prelon, V. Camilleri, L. Février, Citrate assisted phytoextraction of uranium by sunflowers: study of fluxes in soils and plants and resulting intra-planta distribution of Fe and U, Environ. Exp. Bot. 77 (2012) 249–258.

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[95] Q. Luo, L. Sun, H. Wang, X. Hu, Metabolic profiling analysis of root exudates from the Cd hyper accumulator Sedum alfredii under different Cd exposure concentrations and times, Anal. Methods 7 (2015) 3793–3800.
[96] A.L. Neal, S. Ahmad, R. Gordon-Weeks, J. Ton, Benzoxazinoids in root exudates of maize attract Pseudomonas putida to the rhizosphere, PloS ONE 7 (2012) 35498.
[97] U. Mathesius, Comparative proteomic studies of root-microbe interactions – a review, J. Proteom 72 (2009) 353–366.
[98] K. Mukerji, Rhizosphere biology, in: K.G. Mukerji, C. Manoharachary, J. Singh (Eds.), Microbial Activity in the Rhizosphere, Springer- Verlag, Berlin Heidelberg, Germany, 2006, pp. 1–39.
[99] D. Jayaraman, K. Forshey, P.A. Grimsrud, J.M. Ané, Leveraging proteomics to understand plant– microbe interactions, Plant Sci. 3 (2012) 44.
[100] U. Mathesius, N. Imin, S. Batera, B.G. Rolfe, Proteomics as a functional genomics tool, Methods Mol. Biol. 236 (2003) 395–414.
[101] D. Jones, E. Oburger, Solubilization of phosphorus by soil microorganism, in: E.K. Bünemann, et al. (Eds.), Phosphorus in Action, Soil Biology, vol. 26, Springer-Verlag Berlin Heidelberg, 2011.
[102] A. Sessitsch, M. Kuffner, P. Kidd, J. Vangronsveld, W.W. Wenzel, K. Fallmann, M. Puschenreiter, The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils, Soil Biol. Biochem. 60 (2013) 182–194.
[103] G. Kusliene, J. Rasmussen, Y. Kuzyakov, J. Eriksen, Medium-term response of microbial community to rhizodeposits of white clover and ryegrass and tracing of active processes induced by ^{13}C and ^{15}N labelled exudates, Soil Biol. Biochem. 76 (2014) 22–33.
[104] T.L. Weir, S.W. Park, J.M. Vivanco, Biochemical and physiological mechanisms mediated by allelochemicals, Curr. Opin. Plant Biol. 7 (2004) 472–479.
[105] R. Glinwood, J. Pettersson, E. Ahmed, V. Ninkovic, M. Birkett, J. Pickett, Change in acceptability of barley plants to aphids after exposure to allelochemicals from couch-grass (Elytrigia repens), J. Chem. Ecol. 29 (2003) 261–274.
[106] Inderjit L.A. Weston, S.O. Duke, Challenges, achievements and opportunities in allelopathy research, J. Plant Interac 1 (2007) 69–81.
[107] J.H. Zhang, Z.-Q. Mao, L.-Q. Wang, H.-R. Shu, Bioassay and identification of root exudates of three fruit tree species, J. Integr. Plant Biol. 49 (2007) 257–261.
[108] T. Martínez-Cortéisa, F. Pomara, F. Merinoa, E. Novo-Uzalb, A proteomic approach to Physcomitrella patens rhizoid exudates, J.Plant Physiol. 171 (2014) 1671–1678.
[109] C. Becerra-Castro, P. Kidd, M. Kuffner, A. Prieto-Fernández, S. Hann, C. Monterroso, A. Sessitsch, W. Wenzel, M. Puschenreiter, Bacterially induced weathering of ultramafic rock and its implications for phytoextraction, Appl. Environ. Microbiol. 79 (2013) 5094–5103.
[110] D.V. Badri, J.M. Vivanco, Regulation and function of root exudates, Plant Cell Environ. 32 (2009) 666–681.
[111] E. Liljeroth, J.A. van Veen, H.J. Miller, Assimilate translocation to the rhizosphere of two wheat lines and subsequent utilization by rhizosphere microorganisms at two soil nitrogen concentrations, Soil Biol. Biochem. 22 (1990) 1015–1102.
[112] R.I. Griffiths, M. Manefield, N. Ostle, N. McNamara, A.G. O'Donnell, M.J. Bailey, A.S. Whiteley, $^{13}CO_2$ pulse labelling of plants in tandem with stable isotope probing: methodological considerations for examining microbial function in the rhizosphere, J. Microbiol. Method 58 (2004) 119–129.
[113] M. Manefield, A.S. Whiteley, R.I. Griffiths, M.J. Bailey, RNA stable isotope probing, a novel means of linking microbial community function to phylogeny, Appl. Environ. Microbiol. 68 (2002) 5367–5373.
[114] Ch. Kaiser, M.R. Kilburn, P.L. Clode, L. Fuchslueger, M. Koranda, J.H. Cliff, Z.M. Solaiman, D.V. Murphy, Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation, New Phytol. 205 (2015) 1537–2155.
[115] K. Saito, M.Y. Hirai, K. Yonekura-Sakakibara, Decoding genes with coexpression networks and metabolomics – "majority report by precogs", Trends Plant Sci. 13 (2007) 36–43.
[116] N. Strehmel, C. Böttcher, S. Schmidt, D. Scheel, Profiling of secondary metabolites in root exudates of Arabidopsis Thaliana, Phytochemistry 108 (2014) 35–46.
[117] G. Marti, M. Erb, J. Boccard, G. Glauser, G.R. Doyen, N. Villard, C.A.M. Robert, T.C.J. Turlings, S. Rudaz, J.-L. Wolfender, Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots, Plant Cell Environ. 36 (2013) 621–639.
[118] D. Debois, E. Jourdan, N. Smargiasso, Ph. Thonart, E. De Pauw, M. Ongena, Spatiotemporal monitoring of the antibiome secreted by bacillus biofilms on plant roots using MALDI mass spectrometry imaging, Anal. Chem. 86 (2014) 4431–4438.
[119] U. Baetz, E. Martinoia, Root exudates: the hidden part of plant defense, Trends Plant Sci. 19 (2014) 90–98.
[120] K. Kierul, K.B. Voigt, D. Albrecht, X.H. Chen, L.C. Carvalhais, R. Borriss, Influence of root exudates on the extracellular proteome of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42, Microbiology 161 (2015) 131–147.

Ariana Rugova graduated in Chemistry at University of Prishtina, Kosovo. She joined Stephan Hann's group in Division of Analytical Chemistry at University of Natural Resources and Life Sciences in Vienna for her PhD studies. Her research is focused in LC–ICP-MS method development for phosphorous speciation in soil and plant related samples.From 2014, for more than two years she worked within Environmental Sample Laboratory-Mass Spectrometry Team, Safeguards Analytical Service at International Atomic Energy Agency in Vienna.

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Publication II

A. Rugova, M. Puschenreiter, J. Santner, L. Fischer, S. Neubauer, G. Koellensperger, S. Hann*, Speciation analysis of orthophosphate and myo-inositol hexakisphosphate in soil- and plant-related samples by high-performance ion chromatography combined with inductively plasma mass spectrometry.* Journal of Separation Science, 2014, 37 (14):1711-9.

A novel method to quantify orthophosphate and *myo*-inositol hexakisphosphate (IP_6), both inorganic and organic phosphorus compounds in a single chromatographic run is presented in publication II. Detection of free orthophosphate is strongly limited in LC-ESI-MS based methods both due to the low retention in the reversed phase separations and low sensitivity in electrospray ionization. It is the first time that HIC-ICP-MS was employed for simultaneous analysis of orthophosphate and *myo*-inositolhexakisphosphate (IP_6) in soil solution and plant extracts. Orthophosphate and IP_6 are separated within a total chromatographic runtime of 12 minutes revealing a limit of detection of 0.3 µmol L^{-1}. This method was successfully applied to extracts from three different soils as well as root and shoot extracts of *Brassica napus L*.

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Received January 22, 2014
Revised March 20, 2014
Accepted April 13, 2014

Research Article

Speciation analysis of orthophosphate and *myo*-inositol hexakisphosphate in soil- and plant-related samples by high-performance ion chromatography combined with inductively coupled plasma mass spectrometry

A novel method based on high-performance ion chromatography inductively coupled plasma mass spectrometry employing strong anion exchange chromatography with HNO_3 gradient elution for simultaneous analysis of orthophosphate and *myo*-inositol hexakisphosphate (IP_6) in soil solution and plant extracts is presented. As inductively coupled plasma mass spectrometry analysis of phosphorus at m/z 31 is hampered by N-based interferences, ^{31}P was measured as $^{31}P^{16}O^+$ at m/z 47 employing dynamic reaction cell technique with O_2 as reaction gas. Orthophosphate and IP_6 were separated within a total chromatographic runtime of 12 min revealing a limit of detection of 0.3 μmol/L. The coefficients of determination obtained in a working range of 1–100 and 1–30 μmol/L were 0.9991 for orthophosphate and 0.9968 for IP_6, respectively. The method was successfully applied to extracts from three different soils as well as root and shoot extracts of *Brassica napus L.* The precision of three independently prepared soil extracts was in the range of 4–10% relative standard deviation for PO_4^{3-} and 3–8% relative standard deviation for IP_6. Soil adsorption/desorption kinetics for IP_6/orthophosphate were performed for investigating the sorption behavior of the two P species in the experimental soils.

Keywords: Orthophosphate / Phytic acid / Plant extracts / Soil extracts / Soil adsorption
DOI 10.1002/jssc.201400026

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1 Introduction

Phosphorus is essential for life and plays an important role in plant nutrition. The element exists in soil in the form of inorganic and organic compounds, where the percentage of organic phosphorus compounds is ranging between 20 and 80% [1]. Among soil organic phosphorous compounds, such as phosphate mono- and diesters, phosphonates, and phosphoric acid anhydrides, *myo*-inositol hexakisphosphate (IP_6, see Fig. 1) is the most abundant form of phosphate monoesters. Through degradation of plant litter, IP_6 is introduced into soils, where it is highly stable. Even though the diversity of phosphorous-containing compounds is quite broad, most of the soil P is strongly bound to Fe- and Al-oxyhydroxides or precipitated as sparingly soluble minerals, resulting in very low (0.1–1 μmol/L) soluble P concentrations [2]. Higher plants take up P only as dihydrogen phosphate [3] where only the fraction present in soil solution is directly available for root uptake. The contribution of IP_6 to the total P uptake is still unclear. One major reason for the poor understanding of IP_6 dynamics in the rhizosphere is the very low concentration in soil solution and consequently the difficulty to obtain reliable information on concentration dynamics. Thus, methodological improvements in simultaneous analysis of IP_6 and orthophosphate in soil solution or soil extracts are highly necessary.

Traditionally, most of the analytical chemistry strategies addressing organic P compounds in soils and soil extracts have employed ^{31}P NMR spectroscopy as a detection technique [4]. Although these methods have been developed and

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Abbreviations: **CAL**, calcium acetate lactate; **HPIC**, high-performance ion chromatography; **ICP-MS**, inductively coupled plasma mass spectrometry; **DRCMS**, dynamic reaction cell MS; **SFMS**, sector field mass spectrometry; **IP_6**, *myo*-inositol hexakisphosphate

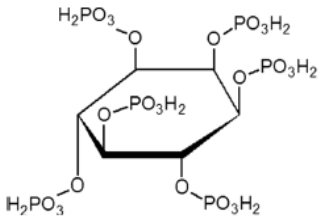


Figure 1. Two-dimensional structure of *myo*-inositol hexakisphosphate.

applied successfully, the use of NMR spectroscopy is limited by the requirement for sample preconcentration due to low sensitivity and by long run-times, which is tedious on the one hand and may be hampered by analyte degradation, on the other hand [5]. However, the development of alternative separation/detection methods is challenging, as the determination of organic P in soil involves complex matrices as well as polar, nonvolatile target analytes, which do not contain any UV-light-absorbing chromophores. Consequently, most analytical approaches employ chromatographic separations with postcolumn derivatization and spectrophotometric detection. LC, mainly based on electrostatic interaction techniques, was most frequently applied for the separation of IP_6. In this context, Leytem and Mikkelsen developed a RP ion-pairing method for separation of soil organic phosphorous compounds such as nucleotides (AMP, ADP, and ATP) as well as IP_6 [5]. IP_6 was detected in an isocratic system with a refractive index detector. The authors encountered several problems with this separation method since the mobile phase was sensitive to changes in sample pH causing difficulties with peak separation efficiency under the selected conditions. Moreover, the method has not been validated to provide quantitative data regarding the level of IP_6 in NaOH- and EDTA-containing soil extracts.

Phillippy et al. have used an ion chromatography under isocratic conditions for separation of IP_6 in roots and tubers. A comparison of IP_6 detection by evaporative light scattering versus absorbance detection following postcolumn derivatization revealed absolute LODs of 1 μg and 0.5 μg (1.52 and 0.76 nmol), respectively [6].

Blaabjerg et al. developed an ion chromatography method for separation and quantification of IP_6 in feedstuffs, employing methanesulfonic acid as an eluent on a Dionex CarboPac column (Dionex, Sunnyvale, CA, USA) with UV absorbance detection after postcolumn derivatization [7]. An anion exchange chromatographic method with chemically suppressed conductivity detection in food analysis has been described by Talamond et al. [8]. The authors compared two methods, i.e. high-pressure ion chromatography (HPIC) and classic absorption spectroscopy. The study showed that the HPIC method was more precise and could be applied to samples without prepurification offering a LOQ of 0.1 μmol/L. CE was applied by Henshall et al. for determination of IP_6 in physiological samples employing indirect photometric detection [9]. The developed CE method was performed within 10 min providing baseline separation of monophosphate *myo*-inositol with an LOD of 200 ng/mL (corresponding to submicromol per liter range LODs, depending on the type of IP_1 and IP_2 salt determined) using an electrolyte containing phthalate.

MS represents a valuable alternative as detection method for organic phosphorous compounds in complex sample matrixes. The key advantage of MS compared to the abovementioned methods is a substantial increase of selectivity, which is offered by both, elemental and molecular MS. The latter has been employed in terms of GC–MS for IP_6 determination in variety of biological samples based on purification by anion exchange chromatography, enzymatic hydrolysis of IP_6 to *myo*-inositol, and derivatization to a trimethylsilyl derivative, revealing an excellent LOD of 9 μg/L (14 nmol/L) [10]. LC–MS has been applied more frequently in the context with IP_6 detection. Liu et al. have presented an anion exchange MS/MS method for the separation and simultaneous quantification of different naturally occurring phosphorylated compounds offering an absolute LOD of 0.25 pmol [11]. Vats et al. also presented a method involving separation of various enzymatically dephosphorylated derivatives of IP_6 on a C_{18} column and their identification using ESI-MS in a positive-ion mode [12]. Just recently, a validated bioanalytical LC–MS method has been published enabling the quantification of phytate levels in plasma matrixes directly, with a detection limit in the range 30–80 ng/mL (50 and 120 nmol/L) [13].

Several groups have applied inductively coupled plasma (ICP) atomic emission spectrometry and ICP-MS as powerful elemental detection methods in combination with selective enrichment of IP_6 by ion chromatography either in off-line ion exchange or SPE mode or after fraction collection after HPIC [7]. In this context, Munoz and Valiente developed an ICP-MS method for determination of phytic acid in human urine based on the total phosphorous measurement of purified extracts of phytic acid by SPE with LOD of 5 μg/L (8 nmol/L) [14]. Grases et al. have reported a method for quantification of Na-IP_6 in urine samples after enrichment via ion exchange offering an LOD of 64 μg/L (68 nmol/L) [15]. The LODs obtained from RP-LC–ESI-MS/MS were approximately 2.5 nmol/L for the investigated compounds, while those of LC–ICP-MS were 1–2 orders of magnitude higher, which is mainly due to suppression effects of the organic solvent employed for the RP separation [16].

The capabilities of LC–ICP-MS regarding the speciation of phosphorous were reviewed recently by Popp et al. [17]. It is advantageous to use ICP-MS detection as it represents a generic phosphorus detector with high specificity and compound independent sensitivity. Moreover, the technique offers a wide linear dynamic range and high robustness of the ICP against matrices present in samples and chromatographic buffers [18, 19]. The high robustness allows the use of high buffer or acid concentrations, which are typically necessary in ion and size-exclusion chromatography. However, ICP-MS analysis of P and P-containing compounds is also challenging due to the fact that the only naturally occurring, stable phosphorus isotope (^{31}P) exhibits several spectral

interferences (e.g. $^{14}N^{16}O^{1}H^{+}$ and $^{15}N^{16}O^{+}$). Moreover, the first ionization potential of phosphorus is relatively high (10.5 eV) and limits ionization efficiency leading to lower sensitivities for P as compared to other elements. Interferences from polyatomic ions that occur at m/z 31, result in higher background and detection limits than those corresponding to other elements. However, these interferences can be removed by using reaction/collision cell technique or high-spectral resolution [17]. The dynamic reaction cell (DRC) is an enclosed quadruple containing a certain concentration of reaction gas and is situated between the ion lens and quadrupole mass analyzer. The DRC is operated as a mass-filtering device (*bandpass* to remove unwanted secondary interferences) and can be used with ammonia or methane for elimination of interfering species via interference dissociation or discharge, or by transferring the interfered analyte to a higher mass via reaction with oxygen. In our case phosphorous is oxidized into PO^{+} and therefore appears at m/z of 47 circumventing isobaric interferences at $m/z = 31$.

Several groups have made use of ion chromatography or ion pair chromatography in combination with ICP-MS detection for analysis of IP_6, as the method offers higher retention and is highly compatible with the ionization source. Helfrich and Bettmer have developed a method for determination of IP_6 in food samples by employing ion pair chromatography coupled to double focusing ICP sector field mass spectrometry (SFMS) [20]. Phosphorous species were detected in medium mass resolution via the $^{31}P^{+}$ signal revealing an LOD of 230 ng/g (0.35 μmol/L) for IP_6. Degradation products were identified, but not quantified by recording a comparative chromatogram of an IP_6 hydrolysate. However, phosphate could not be retained under the selected conditions and the authors have reported an additional severe drawback of the use of ion pair reagents, i.e. the contamination of the LC system led to impaired suitability of the instrumentation for other applications. The method described above has been adapted by another group for phosphorous speciation analysis in barley grain tissues employing ICP-MS with DRC technique. In the same work, size-exclusion chromatography was implemented for assessing the stoichiometry of Fe bound to IP_6 [21].

The aim of the presented work was to develop a robust, sensitive, and selective method for speciation and quantification of phosphorus-containing compounds. As a major drawback, the detection of free orthophosphate is strongly limited in LC–ESI-MS-based methods both due to low retention in RP separations and low sensitivity in ESI. Accordingly, we have developed and validated a method based on strong anion exchange chromatography and ICP-MS detection, which was applied for the first time for speciation of orthophosphate and IP_6 in plant- and soil-related samples.

2 Materials and methods

2.1 Chemicals

A reagent I grade water (>10 MΩ/cm resistance according to ISO 3696 water specifications) purification system (Ultra Clear basic Reinstwassersystem, SG Wasseraufbereitung und Regenerierstation, Barsbüttel, Germany) was used to obtain purified water, which was further purified in a quartz sub-boiling system (Milestone-MLS, Leutkirch, Germany). Ultrapure water was obtained by sub-boiling distillation of reverse osmosis purified water by using ultraclear system SG Water. Analytical grade nitric acid (65%, Normapur, p.a., VWR International, Vienna, Austria) and hydrochloric acid (37%, Merck, Darmstadt, Germany) were additionally cleaned by sub-boiling distillation (twice for HNO_3, once for HCl) in an ultrapure quartz apparatus (Milestone-MLS,). $Ca(NO_3)_2{\cdot}4H_2O$ (Riedel-de-Haën) used for incubation of soil was purchased from Sigma–Aldrich Handels, Vienna, Austria. The syringe filters (0.45 μm) were purchased from VWR International and consisted either of cellulose acetate membrane or nylon. The analytical workflow was carried out on metal-free class 100 work benches inside a class 100 000 clean room laboratory.

2.1.1 Standards

Myo-inositol hexakisphosphate calcium salt was obtained from Sigma–Aldrich Handels. Standard solutions of IP_6 were always prepared freshly in ultrapure water prior to analysis. For quantification of total phosphorus via ICP-MS and SFMS, the phosphorus atomic absorption standard solution was purchased from Alfa Aesar, Karlsruhe, Germany.

For total P analysis, all necessary standard and soil extract dilutions were performed in 2% sub-boiled HNO_3. Calibration standards were prepared by serial dilution of a 100 μM stock of each standard. Sodium triphosphate pentabasic ($Na_5P_3O_{10}$; Sigma–Aldrich Handels) was used as internal standard for phosphorous speciation analysis. Indium was used as internal standard for quantification of P via ICP-SFMS.

Element standards used for ICP-SFMS multielement analysis were certified 1000 mg/L single element ICP standards for trace analysis and were purchased from Merck. Certified reference materials were integrated in all measurements for quality assurance. The NWTM 27.3 low-level fortified standard prepared from Lake Ontario water (National Water Research Institute NWRI, Burlington, Ontario Canada) was purchased at LGC Promochem, Germany. The soil solution A reference material was from high-purity standards (Charleston, SC, USA). The results of the two reference materials were in agreement with the certified ranges given for the element concentrations.

2.2 Sample preparation

In the present study, three soils were investigated, i.e. Lassee 1 (L1) and Lassee 3 (L3; position 48° 13′ 47″N, 16° 49′ 52″O), and Wiesen–Lindengasse (W; position 47°43′ 53″N, 16° 20′ 28″O). Soil samples were collected from the topsoil layer (0–20 cm). After sampling, the soils were air-dried and sieved to <2 mm. The content of sand, silt, clay, and organic

carbon (C_{org}) was determined according to the method by Blum et al. [22] Calcium acetate lactate (CAL)-extractable P was determined according to the method from Austrian standards for soil investigation [23]. Organic phosphorus (P_{org}) was determined according to Kuo [24].

2.2.1 Soil extraction

Two different soil extraction schemes were used within this work. The first scheme was the extraction of 2 g soil in 0.25 mol/L NaOH and 0.005 mol/L EDTA for 2 h at a soil/solution ratio of 1:10. This extraction scheme was adapted from Bowman and Moir [25] with modification regarding temperature of extraction. The second scheme was the extraction of 10 g of soil with a 1 mmol/L citrate solution for seven days. The extractant solution contained 2 mmol/L $Ca(NO_3)_2$ as a background electrolyte. Two hundred milligrams per liter of NaN_3 was partly added to the citrate extractions as biocidal treatment. All extractions were done in triplicates. After extraction, the samples were syringe filtered (0.45 μm), and transferred to polyethylene HNO_3 washed vials and stored at 4°C. The extracts were further diluted when necessary to match within the calibration working range.

2.2.2 *Aqua regia* digestion of soil

Aqua regia extraction of smoothly crushed soil samples was performed in a closed polytetrafluorethylen vessel microwave unit (MLS 1200 mega). To approximately 100 mg of sample, 3 mL *aqua regia* (3:1 HCl/HNO_3) has been added. The digestion procedure was performed by a power-controlled temperature program (25 min, maximum 600 W, maximun temperature 160°C, maximum pressure 25×10^5 Pa). The obtained digestion solutions were quantitatively transferred into precleaned 10 mL polyethylene vials, filled up with ultrapure water to 10 mL and centrifuged (Sigma laboratory centrifuge 2–5, 3900 rpm, 30 min). The supernatant was further diluted 1:10 000 and 1:1000 (with 1% *aqua regia*) to match within the calibration range of the ICP-SFMS. Quantification of total element concentrations from acid extract solution was carried out by an eight-point external calibration and internal standardization with indium.

2.2.3 Adsorption experiment

2.2.3.1 Kinetic study

The adsorption kinetics of IP_6 with three soils were investigated by shaking 10 g soil in 100 mL solutions containing different IP_6 concentrations (100 and 500 mg/L) in 2 mmol/L $Ca(NO_3)_2$. At time intervals of 10, 30, 60, 120, 180, and 240 min, 10 mL aliquots were taken, syringe filtered, and stored at 4°C and measured in three replicates by HPIC–ICP-MS.

2.2.3.2 Adsorption isotherm

The kinetic study showed that equilibrium between solution and soil-bound IP_6 was reached within 10 min. W soil was the only soil that did not show complete sorption of IP_6 in the kinetic experiment. Therefore, only this soil was used to determine the adsorption equilibrium for IP_6. Ten grams of soil was shaken in 100 mL solutions containing 750, 1000, 1500, and 1750 mg/L IP_6 in 2 mmol/L $Ca(NO_3)_2$. The samples were syringe filtered (0.45 μm), transferred to polyethylene HNO_3-washed vials, stored at 4°C, and diluted in further steps to match within calibration working range.

2.2.4 Plant extraction

Analysis of IP_6 concentrations in plant samples is often done in acid extracts of fresh or dried plant material. We adapted the method of Philippy et al. [6] to extract IP_6 from root and shoot samples of plants grown in hydroponic culture from two rapeseed (*Brassica napus*) cultivars, Caracas and CR 1886. Harvested plant material was divided in to roots and shoots and consecutively washed with tap and deionized water. One to six grams of root and 7–16 g shoot plant fresh material was extracted in 100 mL of 0.75 mol/L HCl solution for 20 h. The extraction was performed with five replicates. Each extract was additionally analyzed in three measurement replicates. After extraction, the samples were transferred to polyethylene HNO_3 washed vials and stored at +4°C until analysis by HPIC–ICP-MS.

2.3 Separation by ion-exchange chromatography

For speciation analysis, a metal-free chromatographic system ICS-3000DP with dual pump system and the Chromeleon Chromatography Management System (version 6.40) all from Dionex was employed together with a PAL autosampler from Thermo Scientific. Chromatographic separation was performed by anion exchange chromatography employing IonPac® AS7 Anion-Exchange Column from Dionex (4 × 250 mm, 10 μm particle size) with a 4 × 50 mm guard column.

As eluent A, sub-boiled H_2O was used, while eluent B was 500 mmol/L HNO_3. The method was adapted from an isocratic method published by Philippy et al. [6] and further optimized for retention and separation of orthophosphate, sodium triphosphate pentabasic (internal standard), and IP_6 by implementing gradient elution. The flow rate was kept at 1 mL/min with an injection volume of 10 μL. The acidic gradient was as follows: 0–4 min: 100% eluent A, 4–14 min: linear increase of eluent B to 100%, 14–15 min: 100% B, 15–20 min: reequilibration at 100% eluent A. An internal standard, sodium triphosphate pentabasic, was added to the samples and calibration standards at a final concentration of 10 μmol/L. The signal suppression of P during gradient elution was determined by injecting a 50 μg/L P at different HNO_3 concentrations (10, 50, 100, 200, and 300 mmol/L).

2.4 Instrumentation

ICP-DRCMS (Elan 6100 DRC II, Perkin Elmer SCIEX, Ontario, Canada) measurements of the PO^+ signal were performed after a daily tuning procedure. The final operating parameters after method development are listed in Supporting Information Table S1. The system was connected to HPLC via polyether ether ketone tubing with id 0.13 mm to a PFA nebulizer (Elemental Scientific, Omaha, NE, USA), which was connected to a cyclonic spray chamber (PE-SCIEX). The interface was equipped with an aluminium sampler cone and a nickel skimmer cone.

Total metal concentrations in soil samples were measured using an ICP-SFMS (ELEMENT 2, Thermo Fisher, Bremen, Germany) after microwave-assisted sample digestion described above. Sample introduction was performed by a particle tolerant slurry type nebulization setup employing a V-groove nebulizer (Glass Expansion, Melbourne, Australia) with an id of 145 μm and a PC_3 cyclonic quartz chamber (ESI) operated at 4°C. Sample transport to the nebulizer was enabled by a peristaltic pump with a sample uptake rate of 1.0 mL/min. A detailed description of the ICP-SFMS method can be found in Ref. [26].

Generation and export of HPIC–ICP-DRCMS chromatograms was carried out using Chromlink (Version 2.1, PE SCIEX) in combination with Totalchrom (Version 6.2, PE-SCIEX). Chromeleon software (Version 6.7, Dionex) was used for integration of all chromatographic data.

3 Results and discussion

3.1 Ion chromatographic separation of IP_6

Speciation of orthophosphate and IP_6 in soil and plant extracts was performed by high-performance anion exchange chromatography with HNO_3 gradient elution in combination with ICP-DRCMS detection. In IP_6, six of 12 replaceable protons are strongly ($pK_a < 3.5$) and six are weakly ($pK_a = 4.6–10$) acidic [27]. Electrostatic interaction and the changing protonation degree are the basis of the anion exchange separation with HNO_3 gradient elution. Analysis of IP_6 in soil solution and plant extracts requires a robust and sensitive method, as the analyte is present in the samples in the low micromoles per liter range together with a complex matrix. The presented strong anion exchange separation method offers baseline separation of orthophosphate ($t_r = 4.7$ min), P impurities, and IP_6 ($t_r = 11.1$ min) within 12 min. Figure 2 depicts a HPIC–ICP-MS chromatogram obtained from an aqueous solution containing 30 μmol/L IP_6 and 10 μmol/L internal standard ($Na_5P_3O_{10}$). The peaks in the chromatogram in Fig. 2 listed from 6.9 to 8.63 min are not identified and are impurities present in the commercially available IP_6 standard. The relative area of IP_6 in the obtained chromatogram is 76 ± 4% (after correction for signal suppression by the HNO_3 gradient, see below), which is not in accordance with the 90% declared purity of the standard available from the supplier.

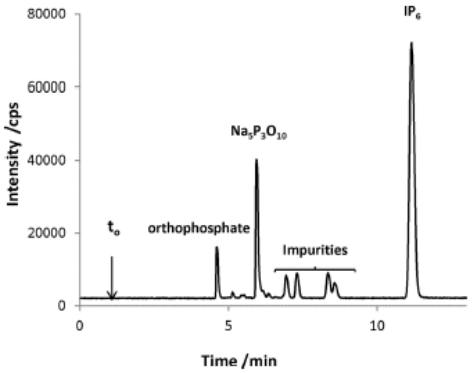


Figure 2. Chromatogram of a 30 μmol/L IP_6 standard solution spiked with 10 μmol/L internal standard ($Na_5P_3O_{10}$). The impurities eluting between the internal standard and IP_6 are degradation products present in commercially available IP_6. According to the literature the impurities are dephosphorylated hydrolization products of IP_6 [11, 21, 27]. Accordingly, the orthophosphate ions originate from dephosphorylation of the IP_6 molecules.

Table 1. Analytical figures of merit of HPIC–ICP-MS method development for separation and quantification of orthophosphate and IP_6

	PO_4^{3-}	IP_6
Retention time (RSD, %; $n = 30$)	4.7 min (0.2)	11.1 min (0.2)
Peak asymmetry	1.21	1.14
LOD (3 σ)	0.3 μmol/L	0.32 μmol/L
LOQ (10 σ)	0.99 μmol/L	1.1 μmol/L
LOD absolute (10 μL)	3 pmol	3 pmol
LOQ absolute (10 μL)	9.9 pmol	11 pmol
Working range	1–100 μM	1–30 μM
R^2	0.9991	0.9997
Long-term repeatability RSD (%) $n = 9$ in 30 h	2.7	1.7

Long-term repeatability of the retention times of the two analytes of interest, i.e. orthophosphate and IP_6 was 0.2% in a measurement sequence of 24 h ($n = 9$). A peak asymmetry of 1.14 ± 0.09 for IP_6 and 1.20 ± 0.10 for orthophosphate was calculated ($n = 5$).

3.2 Quantification

The analytical figures of merit of the presented method are listed in Table 1. Quantification was performed by external calibration. Internal standardization was performed in blanks, calibration standards, and samples after extraction in order to compensate the variation of the injection volume as well as the long time drift of the HPIC–ICP-DRCMS system. Linear calibration curves with coefficients of determination of 0.9991 and 0.9997, respectively, could be obtained over

the working range from 1 to 100 μmol/L for PO_4^{3-} and 1–30 μmol/L for IP_6. The LOD calculated based on the threefold SD of the baseline signal was 0.3 μmol/L, while the LOQ (tenfold SD of the baseline signal) was 1 μmol/L for both compounds. This corresponds to an on-column LOD of 3 pmol (10 μL injection volume) and is an improvement by several orders of magnitude in comparison to alternative detection methods, which have been used in connection with anion exchange chromatography [6]. Repeatability precision calculated from three consecutive measurements revealed an RSD <3% for PO_4^{3-} and 2% for IP_6, respectively. The method was successfully applied to soil extracts of two soil types with different soil characteristics as well as root and shoot extracts of *B. napus L.* The precision of three independently prepared soil extracts was in the range of 4–10% RSD for PO_4^{3-} and 3–8% RSD for IP_6 in a total measurement time of 30 h.

LODs for IP_6 in so far published studies in food samples are 0.96–4.7 μmol P L^{-1} by ICP-MS after fraction collection [7], 0.35 μmol/L by IP-ICP-SFMS [20], or in urine samples 0.06 μmol/L by ICP atomic emission spectrometry after off-line ion exchange [15] and 0.01 μmol/L by ICP-MS after SPE [14]. In biological samples, an absolute LOD of 0.25 pmol has been obtained by LC–MS/MS [11]. As already mentioned, phosphate is not amenable to LC–MS/MS, which is a clear drawback in the context of soil and rhizosphere research.

The influence of the sample matrix on signal suppression mitigating the accurate quantification of IP_6 was assessed by a standard addition experiment employing soil solutions. A comparison of the slopes obtained for the standard addition curve and external (aqueous) calibration curve revealed no significant difference, proving that the method is robust with regard to the investigated sample matrices. Moreover, the impact of the HNO_3 concentration gradient on sensitivity was investigated. Supporting Information Fig. S1 demonstrates an intensity loss of approximately 11% over the gradient for PO^+ ($m/z = 47$), which is due to ionization suppression. It is noteworthy that the ICP-MS signal is increasing at $m/z = 31$ with increasing HNO_3 concentration because of the increased formation of N-based interferences, if DRC is not employed. This clearly indicates the absolute necessity of interference removal in order to obtain reliable results with the proposed method.

3.2.1 Improvement of LOD by a large volume injection

Investigation of IP_6 in soil is challenging due to the very low concentrations in soil extracts. Enhancement of sensitivity by enrichment of IP_6 at the column head was possible due to the high-capacity factor provided by the selected stationary phase and chromatographic conditions. With the presented method, sensitivity could be improved linearly by increasing the injection volume from 12.5 to 150 μL (data not shown). As the background signal and noise were not influenced by the higher injection volume, this corresponds to a decrease of the LOD of approximately one order of magnitude. It is noteworthy that increasing the injection volume did not affect the chromatographic characteristics of the method.

3.3 Application

Representative sampling of phosphorus-containing organic compounds from soil, their identification and accurate quantification as well as the interpretation of the obtained information in the context of bioavailability is highly challenging. The developed separation method was applied to different types of soil that differ in pH and carbonate content, as these characteristics influence the behavior of selected organic phosphorous compounds in soil significantly [1]. The investigated soils were also characterized for their elemental composition by ICP-SFMS analysis after *aqua regia* digestion.

3.3.1 Soil characteristics

The investigated soils were characterized to get more information about the parameters controlling the solubility of phosphate and inositol phosphates. These characteristics are summarized in Supporting Information Table S2. W soil is a carbonate-free Cambisol with pH_{CaCl_2} 5.4 and pH_{H_2O} 6.3, respectively. L_1 and L_3 are calcareous czernosems with pH values of pH_{CaCl_2} 7.7 and pH_{H_2O} 8.4. The CAL-extractable P (P_{CAL}) is reflecting the potentially plant available P. According to the Austrian guideline for P fertilization [28], the P_{CAL} concentration is very low in soil W, but high in L_1 and very high in L_3. This suggests that P deficiency might occur for crops growing on soil W, but not on L_1 and L_3. The content of organic carbon (C_{org}) is very similar for all soils. For total metal concentrations, significant differences between the three experimental soils were found, especially regarding Ca, Mg, and Sr (L3>L1>>W).

3.3.2 Soil extraction

The three soils were extracted with different extraction solutions at different incubation times as described in Section 2.2.1. Detectable amounts of IP_6 were only obtained after extraction with NaOH/EDTA, whereas in the citrate extracts the IP_6 concentration was below the LOD (Table 2).

The separation of IP_6 from other P species in the NaOH/EDTA extracts is shown in the chromatogram in Fig. 3. The combination of NaOH and EDTA has been suggested by Condron et al. [29] for assessing organic phosphorus in soils. It is noteworthy that the injection peak is derived from C- or Na-based interferences from the extraction solution of NaOH/EDTA and does not relate to phosphorus-containing compounds. Extraction of soil solution by use of an in-house method based on a preliminary study utilizing citrate was performed over a relatively long extraction time of seven days. The citrate solution was selected as extractant to simulate the solubilizing activity (ligand exchange, ligand-promoted dissolution [5]) of root exudates. NaN_3 was partly added for excluding the influence of microbial activity on the

Table 2. Orthophosphate and IP_6 concentrations obtained for three investigated soils extracted with different extraction solutions

Soil	Total P		Extractable P					P extracted %	PO_4^{3-} (μmol/g)		IP_6 (μmol/g)	
	Aqua regia digestion (μmol/g) soil		Extraction solution	Extraction time	Total extractable P (μmol/g)							
	Conc.	SD			Conc.	SD			Conc.	SD		SD
Wiesen (W)	15.4	0.2	1 mM citrate	7 days[a)]	0.08	0.002	0.5		<LOD	–	<LOD	–
	15.4	0.2	1 mM citrate	7 days[b)]	0.31	0.04	2.1		<LOD	–	<LOD	–
	15.4	0.2	NaOH/EDTA	2 h	8.74	0.42	56.7		4.98	0.21	0.34	0.01
Lasse 1 (L_1)	31.9	0.5	NaOH/EDTA	2h	12.0	0.88	37.5		9.04	0.97	3.04	0.24
Lasse 3 (L_3)	36.1	1.0	NaOH/EDTA	2h	11.2	0.53	31.0		5.36	0.54	2.91	0.25

Note: Total phosphorous concentrations in *aqua regia* digests were determined by ICP-SFMS while extractable P concentrations were measured with HPIC–ICP-MS. The SD was derived from three consecutive measurements of three replicates of the same sample solution.
a) Extracts with no added NaN_3.
b) Extracts with added NaN_3.

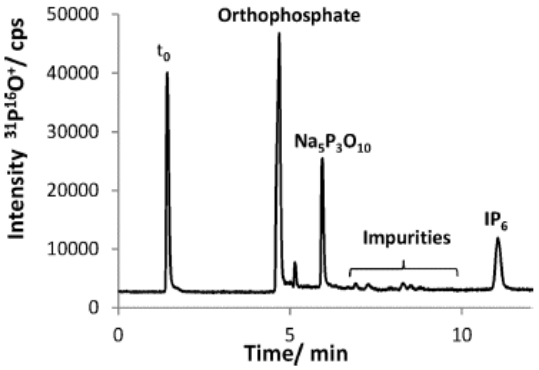


Figure 3. Chromatogram of L_3 soil extracted with NaOH/EDTA solution spiked with 10 μM internal standard ($Na_5P_3O_{10}$). The injection peak (t_0) presents a blank peak originating from the extraction solution and is therefore not considered for data interpretation. The impurities are products of IP_6 degradation.

extraction process. It can be seen in Table 2 that the presence of NaN_3 increased the extractability of P from the experimental soils, probably because P uptake by soil microbes was inhibited. IP_6 concentrations in citrate extracts below LOD, along with the sorption behavior of IP_6 on the experimental soils (see Section 3.3.3) indicate strong adsorption to the soil solid phase.

3.3.3 Adsorption study

Investigation of time-dependent IP_6 adsorption has been carried out to get more information about the solubility of IP_6 in calcareous soils compared to a carbonate-free acidic soil. With six phosphate groups, IP_6 has a high charge density and is thus strongly adsorbed to Fe- and Al-oxyhydroxides, clay minerals, and soil organic matter [30] and it also forms highly insoluble precipitates with metal ions. As can be seen in Supporting Information Fig. S2, the IP_6 concentration in the suspensions of the calcareous soils (L_1 and L_3) decreased rapidly and after 60 min reaction time no IP_6 was detectable in the filtered suspensions. In the W soil, the IP_6 concentration initially also decreased fast, but reached the equilibrium after approximately 60 min. The IP_6 concentration in solution remained stable at 200 μmol/L until the end of the experiment (240 min). In the case of soils L_1 and L_3, the decrease of IP_6 in the incubation solution is likely caused by precipitation of IP_6 with Ca^{2+} in these calcareous soils (Supporting Information Table S2). The complete loss of IP_6 is a strong indication for a precipitation rather than an adsorption reaction. In contrast, the slower concentration decrease and the equilibrium concentration of IP_6 in the W soil suspensions, indicate a sorption process on oxide surfaces, clay, or organic matter.

The IP_6 concentration dependent adsorption has been carried out for W soil with an equilibration time of 240 min, based on the results of the time-dependent adsorption experiment. The W soil had a high capacity for IP_6 adsorption

Table 3. Separation of IP_6 in plant root and shoot extracts of two different cultivars of *B. napus*

	Caracas				CR 1885			
	Root		Shoot		Root		Shoot	
	μmol/g	SD	μmol/g	SD	μmol/g	SD	μmol/g	SD
Total P (FI)	3.48	0.5	1.6	0.4	2.51	0.3	1.10	0.7
PO_4^{3-}	2.87	0.5	1.45	0.3	2.27	0.9	0.94	0.3
IP_6	0.02	0.01	0.01	0.001	0.02	0.01	0.09	0.001

Note: The SD was calculated from three consecutive measurements of three independently prepared plant extracts of each plant cultivars and compartment, respectively.

and even at 130 μmol IP_6 g^{-1} complete saturation was not achieved (Supporting Information Figs. S3a and b). The high adsorption capacity is likely due to the content of Al and Fe hydroxides, indicated by the high total content of Fe and Al (Supporting Information Table S2). Since IP_6 is competing for the same binding sites as PO_4^{3-}, orthophosphate is increasingly released with higher amounts of IP_6 adsorbed [31] (Supporting Information Fig. S3b).

3.3.4 Quantification of IP_6 in *B. napus L.* extracts

The suitability of the method for analysis of plant extracts was assessed for *B. napus L.* IP_6 was detected in all plant extracts, ranging from 10 to 90 μmol/kg fresh weight (Table 3). These values are low compared to those reported by Phillippy et al. [6], but these authors analyzed mainly tubers, which serve as nutrient storage organs and can therefore be expected to show higher IP_6 concentrations than shoots and roots. However, our data show that the HPIC–ICP-MS method is suitable for analyzing acidic extracts of fresh plant material, especially of plant parts that contain low amounts of IP_6. In addition, the new method simultaneously provides orthophosphate concentrations in the samples, if required.

4 Concluding remarks

For speciation of phosphorus compounds, ICP-MS is an ideal detector as it offers species-unspecific quantification, robustness against buffers and acids, and good selectivity and sensitivity. However, formation of matrix-based spectral interferences has to be carefully investigated and suitable strategies for interference separation have to be applied. The developed HPIC–ICP-DRCMS method offers excellent reproducibility and LODs in the submicromol per liter range without the need of special sample pretreatment. As an advantage, simultaneous quantification of orthophosphate and IP_6 is feasible. This can be of importance for studies aiming at the investigation of P mobilization and plant nutrition.

A. R. would like to thank the Austrian Federal Ministry of Science and Research, ÖAD- Bertha von Suttner/Kosova fund for financial support.

The authors have declared no conflict of interest.

5 References

[1] Turner, B. L., Frossard, E., Baldwin, D. S., *Organic Phosphorus in the Environment*, CABI Publishing, Wallingford, UK 2004.

[2] Hinsinger, P., *Plant and Soil* 2001, *237*, 173–195.

[3] Ullrich-Eberius, C. I., Novacky, A., Van Bel, A. J. E., *Planta* 1984, *161*, 46–52.

[4] Heighton, L., Schmidt, W. F., Siefert, R. L., *J. Agric. Food Chem.* 2008, *56*, 9543–9547.

[5] Leytem, A. B., Mikkelsen, R. L., *Commun. Soil Sci. Plant Anal.* 2003, *34*, 1393–1406.

[6] Phillippy, B. Q., Bland, J. M., Evens, T. J., *J. Agric. Food Chem.* 2003, *51*, 350–353.

[7] Blaabjerg, K., Hansen-Møller, J., Poulsen, H. D., *J. Chromatogr. B.* 2010, *878*, 347–354.

[8] Talamond, P., Doulbeau, S., Rochette, I., Guyot, J. P., Treche, S., *J. Chromatogr. A* 2000, *871*, 7–12.

[9] Henshall, A., Harrold M. P., Tso, J. M. Y., *J. Chromatogr. A.* 1992, *608*, 413–419.

[10] March, J. G, Simonet, B. M, Grases, F., *J. Chromatogr. B.* 2001, *757*, 247–255.

[11] Liu, X., Villalta, P. W., Sturla, S., *J. Rapid Commun. Mass Spectrom.* 2009, *23*, 705–712.

[12] Vats, P., Bhushan, B., Chakarborti, A. K., Banerjee, U. A., *J. Sep. Sci.* 2008, *31*, 3829–3833.

[13] Tur, F., Tur, E., Lentheric, I., Mendoza, P., Encabo, M., Isern, B., Grases, F., Maraschiello, C., Perelló, J., *J. Chromatogr. B* 2013, *928*, 146–154.

[14] Muñoz, J. A, Mesas, M-L., Valiente, M., *Anal. Chim. Acta.* 2003, *75*, 6374–6378.

[15] Grases, F., Perelló, J., Isern, B., Prieto, R. M., *Anal. Chim. Acta.* 2004, *510*, 41–43.

[16] Neubauer, S., Rugova, A., Binh Chu, D., Drexler, H., Ganner, A., Sauer, M., Mattanovich, D., Hann, S., Koellensperger, G., *Anal. Bioanal. Chem.* 2012, *404*, 799–808.

[17] Popp, M., Hann, S., Koellensperger, G., *Anal. Chim. Acta.* 2010, *668*, 87–214.

[18] Tromp, J. W., Pomares, M., Alvarez-Prieto, M., Cole, A., Ying, H., Salin, E. D., *Spectrochim. Acta Part B* 2003, *58*, 1927–1944.

[19] Shah, M., Caruso, J., *J. Sep. Sci.* 2005, *28*, 1969–1984.

[20] Helfrich, A., Bettmer, J., *J. Anal. At. Spectrom.* 2004, *19*, 1330–1334.

[21] Persson, D. P., Hansen, T. H., Laursen, K. H., Schjoerring, J. K., *Metallomics* 2009, *1*, 418–426.

[22] Blum, W. E. H., Spiegel, H., Wenzel, W. W., *Bodenzustandsinventur. Konzeption, Durchführung und Bewertung*, Universität für Bodenkultur, Vienna 1996.

[23] Schüller, H., *Pflanzenernaehr. Bodenkd.* 1969, *123*, 48–63.

[24] Kuo, S., in: Sparks, D. L. (Ed.), *Phosphorous, Methods of Soil Analysis*, Soil Science Society of America, Madison 1996, pp. 869–919.

[25] Bowman, R. A., Moir, J. O., *Soil Sci. Soc. Am. J.* 1993, *57*, 1516–1518.

[26] Popp, M., Koellensperger, G., Stingeder, G., Hann, S., *J. Anal. At. Spectrom.* 2008, *23*, 111–118.

[27] Brigando, C., Mossoyan, J. C., Favier, F., Benlian, D., *J. Chem. Soc., Dalton Trans.* 1995, *4*, 575–578.

[28] BMLFUW: Richtlinien für die sachgerechte Düngung. *Anleitung zur Interpretation von Bodenuntersuchungsergebnissen in der Landwirtschaft. 6. Auflage. Hrsg*, BMLFUW, Vienna 2006.

[29] Condron, L. M., Turner, B. L., Cade-Menun, B. J., in: Sims, J. T., Sharpley, A. N. (Eds.), *Phosphorus: Agriculture and the Environment*, ASA, CSSA and SSSA, Madison 2005, pp. 87–122.

[30] Celi, L., Barberis, E., in: Turner B. L., Frossard, E., Baldwin, D. S. (Eds.) *Organic Phosphorus in the Environment*, CABI Publishing, Wallingford 2004, pp. 113–132.

[31] Doolettea, A. L., Smernika, R. J., Dougherty W. J., *Eur. J. Soil Sci.* 2010, *61*, 563–575.

Journal of Separation Science

Electronic Supplementary Material

A. Rugova, M. Puschenreiter, J. Santner, L. Fischer, S.Neubauer, G.Koellensperger, S.Hann

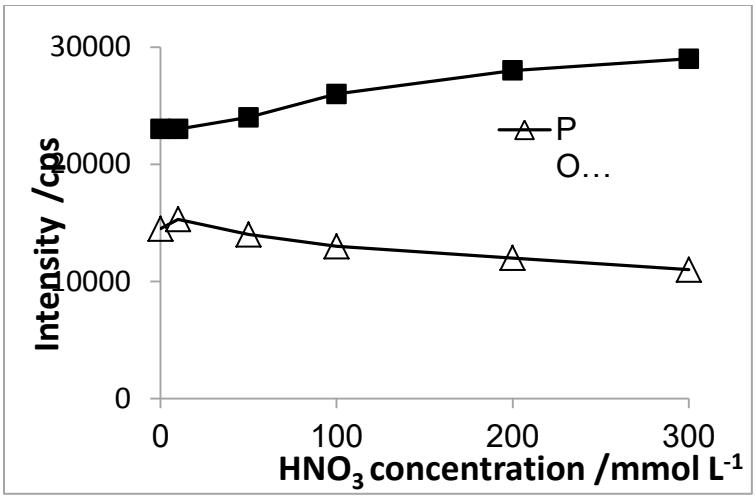


Figure S1: Impact of increasing HNO_3 concentration in the eluent on the sensitivity of ICP-MS detection: if P is measured as PO^+ at m/z = 47 by employing dynamic reaction cell technique with O_2 as cell gas, an intensity loss of 11% was observed. If P is measured at m/z = 31 (without employing the dynamic reaction cell) nitrogen based polyatomic interferences are not eliminated and are increasing with increasing HNO_3 concentration, which leads to an apparently increased sensitivity of the system.

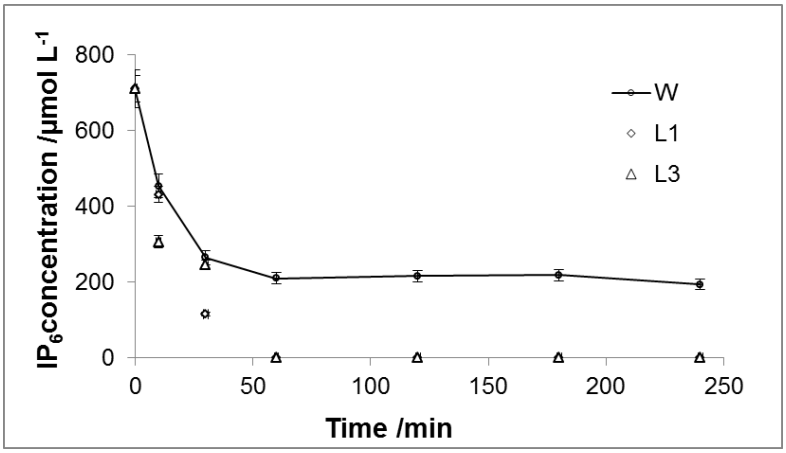
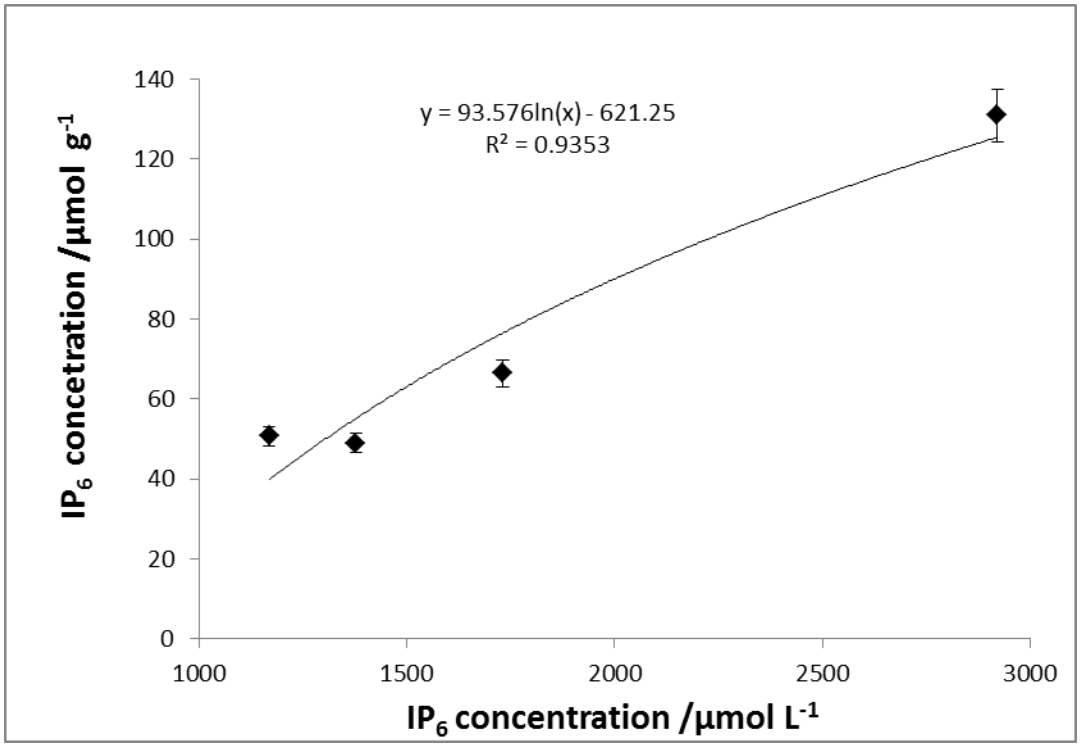


Figure S2: Time-dependent adsorption study of IP_6 in the three experimental soils. 100 g of soil was incubated with 716 µmol L^{-1} P_6 (500 mg L^{-1}) in 2 mmol L^{-1} Ca $(NO_3)_2$ solution. IP_6 is quickly and quantitatively adsorbed by L_1 and L_3 soils, while in W soil fast adsorption is reaching equilibrium with approximately 30% of the initial IP_6 amount being present in the liquid phase from 60 min to the end of the experiment (240 min). Error bars represent the relative standard deviation of three independently performed experiments.



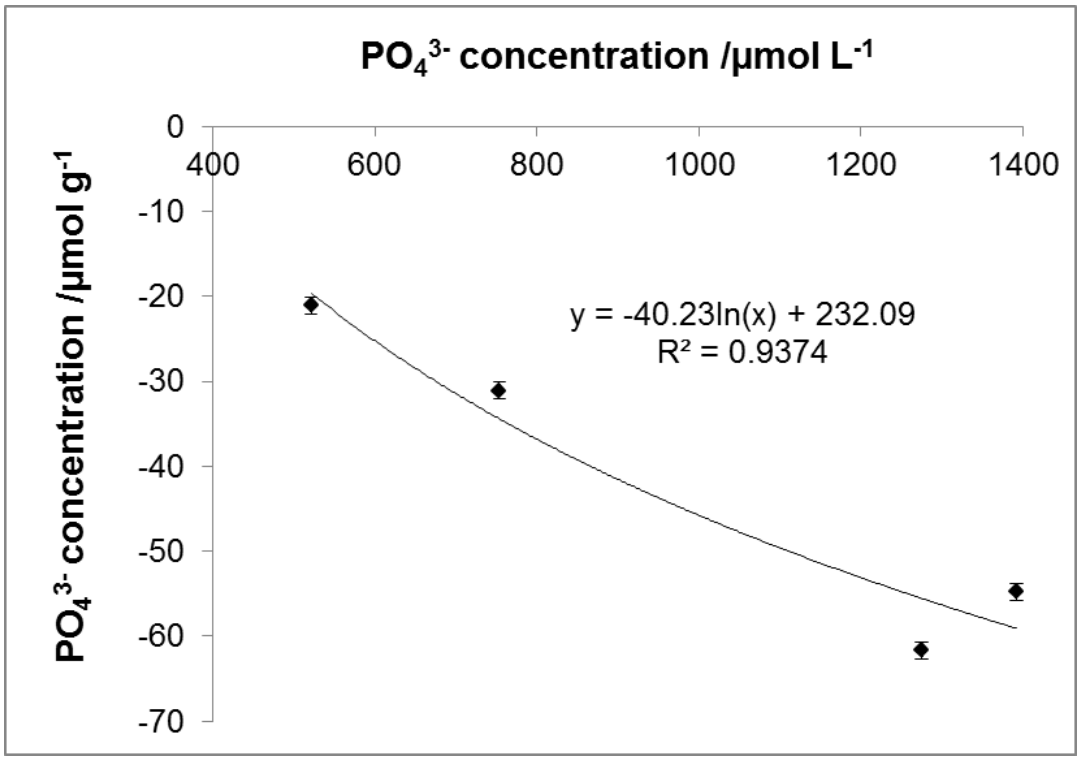


Figure S3. (a) Adsorption of IP_6 from solutions of 1.1, 1.4, 1.7, 2.1 and 2.5 mmol L^{-1} and (b) concomitant release of PO_4^{3-} (b) in soil W for a reaction time of 240 min. Error bars indicate the standard deviation of three independently performed experiments.

Table S1. Operating conditions of ion exchange chromatography combined with inductively coupled plasma-dynamic reaction cell-quadrupole mass spectrometer (ICP-DRC-QMS)

Ion Chromatography	
Flow rate	1 mL min^{-1}
Mobile phase A	H_2O
Mobile phase B	500 mmol L^{-1} HNO_3
Separation Column	Ion Pac AS7, 4 x 250 mm with guard column AS7, 4 x 50 mm 10 µm particle diameter
Column temperature	20 °C
Injection volume	10 µL
ICP-DRC-MS	
Nebulizer	Meinhard
Spray chamber	Cyclonic, 5 °C
Nebulizer gas flow	1.1 L min^{-1}
Auxiliary gas flow	1.2 L min^{-1}
Plasma gas	16 L min^{-1}
Cell gas flow (O_2)	0.8 L min^{-1}
ICP RF power	1300 W
Lens voltage	6.1 V
Dwell time	50 ms
Rpq	0.3
m/z measured	$^{31}P^{16}O^{+}$

Table S2. Soil characteristics with elemental composition of the investigated soils derived from *aqua regia* digestion. The uncertainty of the values is below 10 % relative standard deviation (n = 3)

Soil characteristics		**W**	**L1**	**L3**
pH_{H2O}		6.3	8.4	8.4
pH_{CaCl2}		5.4	7.7	7.7
Sand	g kg^{-1}	386	222	308
Slit	g kg^{-1}	501	655	480
Clay	g kg^{-1}	113	123	212
C_{org}	g kg^{-1}	49.4	47.6	52.2
Sr	mg kg^{-1}	12.1	167	295
Mg	mg kg^{-1}	3500	15500	21100
Al	mg kg^{-1}	18500	20500	20800
Ca	mg kg^{-1}	1500	70000	86100
Fe	mg kg^{-1}	20300	22300	20000
P_{total}	mg kg^{-1}	478	988	1100
P_{org}	mg kg^{-1}	158	492	173
P_{CAL}	mg kg^{-1}	8.53	71.6	150

Publication III

S. Neubauer, A. Rugova, D. B. Chu, H. Drexler, A. Ganner, M. Sauer, D. Mattanovich, S. Hann, G. Koellensperger. Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases - application to feed supplements. Analytical and Bioanalytical Chemistry. 2012, 404, 3:799-808.

Accurate method for quantitative profiling in nucleotide enriched yeast extracts used as additives in animal feedstuff is presented in publication III. The analytical study concerned the development of a rapid chromatographic separation, which could be combined with both molecular and elemental mass spectrometric detection. Since there are no certified reference materials available for this type of bioanalytical application, the orthogonal ICP-MS method may be implemented as a reference method for nucleotide quantification and prove the validity of LC-MS-MS based quantification developed in our working group.

Anal Bioanal Chem (2012) 404:799–808
DOI 10.1007/s00216-012-6170-9

ORIGINAL PAPER

Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements

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Received: 12 March 2012 / Revised: 9 May 2012 / Accepted: 30 May 2012 / Published online: 30 June 2012

Abstract In this work, accurate MS-based methods for quantitative profiling of nucleotides, nucleosides, and nucleobases in yeast extracts used as additives in animal feedstuff are presented. Reversed-phase chromatography utilizing a stationary phase compatible with 100 % aqueous mobile phases resulted in superior analytical figures of merit than HILIC or ion-pair reversed-phase separation. The novel separation method was combined with both molecular and elemental mass spectrometry. By use of RP–LC–MS–MS, excellent limits of detection <1 μmol L^{-1} could be obtained for all the compounds investigated. The elemental speciation analysis approach enabled determination of nucleotides by phosphorus detection. Sensitivity of LC–ICP–MS was 1–2 orders of magnitude lower than that of LC–MS–MS. Quantitative analysis of yeast products using complementary MS detection furnished values in good agreement.

Keywords Nucleotide · Nucleoside · Nucleobase · LC–MS–MS · LC–ICP–MS · Feed supplement

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Electronic supplementary material The online version of this article (doi:10.1007/s00216-012-6170-9) contains supplementary material, which is available to authorized users.

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Abbreviations

ACN	Acetonitrile
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
As	Adenosine
ATP	Adenosine triphosphate
Cd	Cytidine
CE	Capillary electrophoresis
CMP	Cytidine monophosphate
DNA	Deoxyribonucleic acid
DRC-QMS	Dynamic reaction cell–quadrupole mass spectrometry
ESI	Electrospray ionization
FV	Fragmentor voltage
GMP	Guanosine monophosphate
Gs	Guanosine
HILIC	Hydrophilic-interaction liquid chromatography
HPLC	High-performance liquid chromatography
IC	Ion chromatography
ICP	Inductively coupled plasma
IMP	Inosine monophosphate
IP	Ion pair

IS	Internal standard
Is	Inosine
k′	Capacity factor
LC	Liquid chromatography
LOD	Limit of detection
MeOH	Methanol
MRM	Multiple reaction monitoring
MS-MS	Tandem mass spectrometry
N	Number of theoretical plates
NDP	Nucleoside diphosphate
NMP	Nucleoside monophosphate
NTP	Nucleoside triphosphate
RP	Reversed phase
tR	Retention time
Ud	Uridine
UMP	Uridine monophosphate

Introduction

Nucleotide-enriched yeast products, used as flavour enhancers in foods and beverages (high concentrations of 5′GMP, and 5′ IMP) [1–3] are currently used for their beneficial effects as feed supplements. Feed supplementation was mainly proposed because of reported evidence of the effect of nucleotides on the metabolism of fatty acids and the contribution of nucleotides to iron absorption in the gut [1, 4].

The production of nucleotide-enriched yeast is based either on chemical treatment or, more commonly, on autolysis. Yeast cells contain a range of different nucleases, and autolytic degradation of polynucleotides—basically RNA—yields different isomers of nucleotides, and nucleosides and nucleobases. As a consequence, optimization of nucleotide enrichment and characterization of the yeast products requires quantitative methods including not only nucleotides, but also nucleosides and nucleobases. More specifically, the metabolites of interest are the ribonucleotides CMP, UMP, AMP and GMP—the breakdown products of RNA—and IMP, and the corresponding nucleobases and nucleosides. In the past, different nucleotide patterns have been found in yeast autolysates depending on yeast strain or autolysis conditions [1, 5]. Moreover, yeast autolysis resulted not only in the well-known 5′ NMPs but also in isomers phosphorylated at other positions (3′ NMPs and 2′ NMPs) [1, 5].

Table S1 (Electronic Supplementary Material) gives an overview of the state of the art of chromatography-based bioanalytical methods and applications for nucleotide, nucleobase, and nucleoside quantification [1–32]. Alternative techniques include enzymatic assay [6] or separation by capillary electrophoresis and capillary electrochromatography [7]. A comprehensive overview of recent liquid chromatographic methods for determination of endogenous nucleotides in cancer therapy was recently published [8]. Since the advent of mass spectrometry, its combination with chromatography has developed into an important technique for quantitative profiling of metabolites. Accordingly, the chromatographic separations initially developed for UV detection [1, 4, 9–11], for example ion chromatography or reversed-phase chromatography, utilizing non-volatile, saline buffers, have been replaced by separation methods compatible with electrospray ionization. As can be readily observed, the predominant separation principle used for nucleotide analysis in combination with MS detection was, and still is, ion-pair chromatography. Nucleotides—comprising phosphate esters—are negatively charged in a wide pH range. Accordingly, tetraalkylammonium or trialkylammonium salts could be used as cationic ion-pair reagents, especially in combination with UV detection. Different volatile ion-pair reagents, for example dibutylammonium formate [12], dihexylammonium acetate [2], triethylamine [3], *N*,*N*-dimethylhexylamine [13], and dibutylamine [14], have been used for MS detection. As a general drawback, the combination of IP-RP-LC with ESI–MS, resulted in adverse effects, for example limited robustness regarding the stability of retention times. Moreover, compromised sensitivity by ion suppression and contamination is often regarded as a limitation of this technique. The parallel use of negative and positive ionization for optimum detection of nucleotides, nucleosides, and nucleobases is not possible, because of suppression/interference by the introduced counter ion. However (also reflected in Table S1), few studies have focused on alternative modes of chromatographic separation. As a matter of fact, ion-pair chromatography is unrivalled in terms of separation efficiency, and reversed-phase chromatography, the most common separation method combined with ESI–MS was limited to the analysis of nucleobases and nucleosides [15]. Alternatively, HILIC [16] or adsorption chromatography utilizing porous graphitic carbon as stationary phase [17, 18] could be applied to nucleotides, nucleosides, and nucleobases. Preinerstorfer et al. showed the potential of HILIC in a comprehensive metabolomic study using HILIC at two different pHs [16]. In adsorption chromatography high buffer concentrations up to 100 mmol L^{-1} ammonium acetate were needed for elution of nucleotides. This, again, limited the compatibility of the separation method with electrospray ionization techniques.

Table S1 also lists recent elemental speciation methods based on ICP–MS. Evidently, this type of analysis was limited to phosphorus-containing nucleotides and was only rarely applied. The studies concerned extracted DNA samples, with the objective of quantification of deoxy nucleotides dCMP, dTMP, dAMP, and dGMP. ICP–MS was combined with μHPLC and capillary electrophoresis [19–21]. Previous LC–ICP–MS studies had focused on

analysis of DNA adducts [22, 23] or use of the octapole collision cell for interference-free measurement of phosphorus by CE–ICP–MS and LC–ICP–MS applied to calf thymus DNA [24]. Pioneering work in 1999 utilized complementary LC–ICP–MS and LC–ESI–MS for quantification of DNA adducts [25].

In this work, quantitative profiling of nucleotides, nucleobases and nucleosides in nucleotide-enriched yeast extracts used as additives in animal feedstuffs was addressed by use of LC–MS–MS. Recent developments in RP phases, for example materials with lower ligand density tolerating highly aqueous conditions, offered unique selectivity for analysis of these polar compounds compared with conventional C_{18} phases. Hence, as a novelty, reversed-phase separation of nucleotides, nucleosides, and nucleobases could be obtained. Analytical figures of merit of RP–LC–MS–MS are discussed and compared with those of ion-pair chromatography–MS–MS and HILIC–MS–MS. Moreover, because of the absolute lack of reference materials, a reference method for nucleotide quantification was implemented. The reversed-phase separation was combined with complementary ICP–MS detection. For the first time, LC–ICP–MS has been used for quantification of free nucleotides in yeast products.

Experimental

Chemicals and standards

Acetonitrile and methanol of LC–MS-grade were purchased from Fisher Scientific (Loughborough, UK). Water was filtered and deionized in two steps. After reverse osmosis, filtration, and UV treatment as the first step, the electrical conductivity of the water was <0.2 μS cm. The second step was use of a high-purification system from Millipore (Billerica, USA), which furnished water with electrical resistance >18 MΩ. Suprapur formic acid (98–100 %, *v*/*v*) and glacial acetic acid (100 %) from Merck (Darmstadt, Germany) and tributylamine (p.a. ≥99.5 %) from Fluka (Vienna, Austria) were used for chromatography. Sodium chloride, tris (hydroxymethyl)aminomethane (Tris), and hydrochloric acid (30 % Ultrapur), used for sample preparation, were purchased from Merck (Vienna, Austria).

Cytosine, uracil, adenine, guanine, cytidine, uridine, adenosine, guanosine, inosine, 5′CMP (cytidine 5′-monophosphate disodium salt), 5′UMP (uridine 5′-monophosphate disodium salt), 5′AMP (adenosine 5′-monophosphate sodium salt), 3′AMP (adenosine 3′-monophosphoric acid), 2′AMP (adenosine 2′-monophosphate), 5′GMP (guanosine 5′-monophosphate disodium salt hydrate), 2′GMP and 3′GMP (guanosine 2′ and 3′-monophosphate, mixed isomers 63:36, supplied as the sodium salts), and 5′IMP (inosine 5′-monophosphate disodium salt) were purchased from Sigma or Fluka (Vienna, Austria). Adenosine $^{15}N_5$5′AMP ($^{15}N_5$ 5′-monophosphate, supplied as the sodium salt) and $^{15}N^{13}C_2$ uracil from Euriso-top (Saint-Aubin Cedex, France) were used as internal standards. Each substance (0.5 to 2.0 mg) was weighed and 1 mL HPLC-grade water was added (for guanine, guanosine, 3′AMP and 2′AMP 1 mL 0.1 mol L^{-1} NaOH was added instead of water) resulting in 5 mmol L^{-1} stock solutions. Further dilution was performed with pipettes with disposable tips from Eppendorf (Vienna, Austria). The final dilution step was conducted by diluting with water. Stock solutions were kept at −20 °C.

Sample preparation

The samples investigated were provided by the Biomin research center (Tulln, Austria). These preparations were from a variety of companies; most are used as additives in animal feedstuff. Dry powdered feed supplement (50 mg) was dissolved in 50 mL saline buffer (150 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris adjusted to pH 7.4 with hydrochloric acid) and treated ultrasonically at room temperature for 15 min. Centrifugal filtration was conducted using Amicon Ultra filter units (0.5 mL, 10 kDa cut off, cat no: UFC501096) from Millipore and the MiniSpin plus micro centrifuge from Eppendorf (Hamburg, Germany). Filter units were loaded with 0.5 mL sample solution and centrifuged for 5 min at 14,100 rcf. For LC–ICP–MS analysis, the filtrate was diluted by a factor of 10, and five standard additions were prepared, resulting in additional concentrations of 1, 2, 5, 10, and 20 μmol L^{-1} of 5′NMPs. For LC–MS–MS analysis $^{15}N_5$ 5′AMP and $^{15}N^{13}C_2$ uracil were added to each sample as internal standards before filtration, resulting in final concentrations of 2 μmol L^{-1}, and samples were diluted by a factor of 100 after filtration. For standard addition experiments with LC–MS–MS four spikes were prepared resulting in additional concentrations of 0.5, 1, 2, and 5 μmol L^{-1} NMPs.

Chromatography

HILIC–MS–MS was performed with a ZicHILIC separation column (150 mm×4.6 mm, 3.5 μm particle size, 100 Å pore size) from SeQuant (Marl, Germany) and a ZicHILIC guard column (20 mm×2.1 mm, 5 μm particle size). The mobile phase was a gradient prepared from water–ACN–formic acid 98:1:1 (%*v*/v) (eluent A) and ACN–water–formic acid 98:1:1 (%*v*/v) (eluent B). Mobile phase composition was held constant at 90 % B for 2 min, then B was reduced to 10 % within 7 min and was held for 1 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 90 % B for 9.9 min resulted in a total analysis time of 20 min. The flow rate was 0.6 mL min^{-1}, the injection volume 3 μL, and the column temperature 40 °C.

IP-RP-LC–MS–MS was performed as published elsewhere [26]. In brief a Synergi Hydro-RP column (150 mm×2.0 mm, 3 μm particle size, 100 Å pore size) from Phenomenex (Aschaffenburg, Germany) was used. The mobile phase was a gradient prepared from an aqueous 10 mmol L^{-1} solution of tributylamine, pH adjusted to 4.95 with acetic acid (eluent A) and methanol (eluent B). Mobile phase composition was held constant at 0 % B for 5 min, then B was increased to 10 % within 15 min and was held for 2 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 0 % B for 12.9 min resulted in a total analysis time of 35 min. The flow rate was 0.2 mL min^{-1}, the injection volume 3 μL, and the column temperature 40 °C.

RP-LC–MS–MS and RP-LC–ICP–MS were performed with an Atlantis T3 C_{18} reversed-phase column (150 mm× 4.6 mm, 3 μm particle size) from Waters (Milford, USA) with an Atlantis T3 guard column (20 mm×4.6 mm, 3 μm particle size). The mobile phase was a gradient prepared from water containing 0.1 % (*v*/*v*) formic acid (eluent A) and methanol (eluent B). The gradient used for LC–MS–MS was: 0 % B held constant for 2 min, then increased to 40 % within 8 min and held for 2 min. Subsequent increase to 100 % within 0.1 min and held for 1.9 min to flush the column, subsequent reconstitution of the starting conditions within 0.1 min, and re-equilibration with 0 % B for 5.9 min resulted in a total analysis time of 20 min. The gradient used for LC–ICP–MS was: 0 % B held constant for 2 min, then increased to 40 % within 8 min and held for 2 min. Subsequent reconstitution of the starting conditions within 0.1 min and re-equilibration with 0 % B for 7.9 min resulted in a total analysis time of 20 min. For both detection methods the flow rate was 1.0 mL min^{-1}, the injection volume 12.5 μL, and the column temperature 40 °C.

LC–MS–MS system

Agilent Technologies G1312A series 1200 binary pump, G1367B high-performance autosampler, and G1316A column compartment were used for HPLC. MS detection was performed with an Agilent Technologies 6410 LC–MS–MS featuring an ESI interface. Source conditions in negative and positive-ionization modes for IP-RP-LC and HILIC were: drying gas temperature 300 °C, drying gas flow 10 Lmin^{-1}, nebulizer pressure 25 psi, and capillary potential 4000 V. Source conditions in negative and positive-ionization modes for RP-LC using the Atlantis T3 were: drying gas temperature 350 °C, drying gas flow 10 Lmin^{-1}, nebulizer pressure 50 psi, and capillary potential 4000 V. MRM transitions of four nucleobases, five ribonucleosides and nine ribonucleoside monophosphates were determined by flow injection of 20 μmol L^{-1} single standards (isocratic conditions: 0.05 % formic acid in MeOH–H_2O 50:50 *v*/*v*). For optimization, Mass Hunter Optimizer Software from Agilent was used in four steps, in the order:

1. optimization of isolation of the selected precursor ion by varying the fragmentor voltage;
2. determination of the four most abundant product ions;
3. optimization of the collision energies for each of these product ions; and
4. determination of the exact *m*/*z* value of the product ions.

The transition with the highest signal-to-noise ratio was chosen for quantification. For some cases the transition with the second highest signal-to-noise ratio was chosen for identification (qualifier). Precursor and product ions, and the specific values of the fragmentor voltage and collision energy are listed in Table S2 in the Electronic Supplementary Material.

Phosphorus speciation via LC–ICP–DRC-QMS

For speciation analysis a metal-free chromatographic system consisting of an AS 50 autosampler (including a custom-made temperature-control device) and an ICS-3000DP dual pump system, all from Dionex (Sunnyvale, CA, USA), was used. Chromatographic separation was performed on an Atlantis T3 C_{18} reversed-phase column by using the water–methanol gradient described above. HPLC was connected to the ICP-QMS (Elan 6100 DRC II; Perkin Elmer Sciex, Ontario, Canada) The Meinhard nebulizer (Elemental Scientific, Omaha, NE, USA) with the cyclonic spray chamber (PE-SCIEX) were used as the sample-introduction system. Phosphorus was measured as PO^+ at *m*/*z*=47 by use of the dynamic reaction cell (DRC) technique with oxygen at a flow rate of 0.8 mL min^{-1}. Finally, a downscaling experiment with 2.1 and 1 mm Atlantis T3 columns and the PFA nebulizer (Elemental Scientific) was used for accommodating HPLC flow rates of 50 μL min^{-1} and 200 μL min^{-1}.

Data evaluation

Generation and export of HPLC–ICP–QMS chromatograms was performed by use of Chromlink (Version 2.1; PE Sciex) in combination with Totalchrom (Version 6.2, PE-Sciex). Chromeleon software (Version 6.7; Dionex, Sunnyvale, CA, USA) was used for integration of all chromatographic data from ICP–MS detection. Agilent LC–ESI–MS–MS data were evaluated by use of Agilent Mass Hunter Qualitative and Quantitative Analysis software modules.

Results and discussion

Chromatographic separation

Chromatographic separation of nucleotides, nucleobases, and nucleosides was studied for analysis of yeast-derived samples which have been processed to enrich nucleotides. The major

objective was the development of a novel separation method suitable for complementary combination with molecular and elemental mass spectrometry, avoiding use of ion-pair reagents. In the past, ion-pair reversed-phase chromatography was the method of choice for analysis of polar (and charged) nucleotides by LC–MS. However, a severe drawback of the use of ion-pair reagents is the impaired sensitivity of LC–MS instrumentation for other applications, because of contamination. In a first step, two orthogonal chromatographic separation methods were compared with ion-pair chromatography using tributylammonium acetate as ion-pair reagent: HILIC using a zwitterionic phase and a novel reversed-phase separation using a 100 % wettable C_{18} phase were tested in combination with MS–MS-based detection. Retention times and capacity factors of the three implemented separations principles are listed in Table 1. As can be readily observed, the ion-pair RP-LC separation resulted in the expected good separation efficiency and capacity factors ranging from approximately 1 to 10. However, for some analytes (adenine, NMPs) peak tailing was observed. By use of the Atlantis T3 column for reversed-phase separation, not only could ion-pair reagents be avoided, but also separation time could be significantly reduced. By adopting a linear water–methanol gradient with initial conditions of 100 % aqueous mobile phase, an excellent separation could be achieved, increasing the number of compounds studied from 14 to 18 (compare Table 2 with Table 3, and Figs. 1 and 2). As an outstanding feature, excellent separation of the 5′, 3′, and 2′ isomers of nucleotides could be achieved. The isomers of AMP eluted after 6.2, 7.3, and 8.2 min and the isomers of GMP eluted after 6.5, 8.1, and 9.2 min. The isomers of CMP and UMP were not included in the analysis because standards were not available. Nevertheless, in analysis of samples of nucleotide-enriched yeast we found signals corresponding to isomers of CMP (t_R=3.7, 6.0, and 6.2 min) and isomers of UMP (t_R=5.9, 7.3, and 7.6 min). The separation efficiency of HILIC was, however, relatively poor, because all the compounds investigated eluted in the time window from 7.3 to 9.1 min.

In this specific application, separation of nucleosides from corresponding nucleobases and nucleotides is a crucial criterion. Because of in-source fragmentation, a phenomenon which often occurs in electrospray ionization, ESI–MS based unambiguous detection and, consequently, accurate quantification could be hampered. More specifically, in-source fragments of a nucleoside could simulate the respective nucleobase and in-source fragments of nucleotides could simulate the corresponding nucleosides, if not chromatographically separated. This criterion is met by ion-pair chromatography and reversed-phase chromatography. However, using HILIC, adenine and adenosine were not separated to baseline and the two compounds uracil and cytosine could not be detected.

As a matter of fact, compared with the other separations studied, RP chromatography was also superior in respect of the sensitivity obtained, which ultimately resulted in superior signal-to-noise ratios. Indeed, significantly lower LODs by 1–2 orders of magnitude were obtained compared with the other two methods investigated after MS optimization. Finally, the water–methanol gradient used in RP-LC enables straightforward combination with both ESI–MS and ICP–MS detection.

Optimization of ESI–MS-MS detection in combination with reversed-phase chromatography

In principle both, negative and positive ESI ionization modes with corresponding MS-MS transitions could be used for nucleobases, nucleosides, and nucleotides (cytosine and

Table 1 Comparison of three separation methods for nucleobases, nucleosides, and nucleotides

Compound	t_R (min)			k'		
	ZicHILIC	Ion pair	RP (Atlantis)	ZicHILIC	Ion pair	RP (Atlantis)
Cytosine	–	1.5	2.1	–	0.07	0.21
Uracil	–	2.5	5.0	–	0,79	1.83
Adenine	8.3	5.5	4.1	1.86	2.93	1.33
Guanine	8.6	3.1	4.5	1.97	1.21	1.56
Cytidine	8.9	2.6	4.3	2.07	0.86	1.47
Uridine	7.0	3.6	7.2	1.41	1.57	3.09
Adenosine	8.2	12.4	7.6	1.83	7.86	3.36
Inosine	7.3	6.7	8.2	1.52	3.79	3.66
Guanosine	8.0	8.5	8.2	1.76	5.07	3.66
5′CMP	9.1	14.2	3.7	2.14	9.14	1.10
5′UMP	8.2	15.2	5.9	1.83	9.86	2.40
5′AMP	8.7	16.1	6.2	2.00	10.50	2.57
5′IMP	8.3	15.5	7.0	1.86	10.07	3.01
5′GMP	8.8	15.5	6.8	2.03	10.07	2.87

Table 2 Analytical figures of merit for RPLC–MS–MS and RPLC–ICP–MS method development for separation and detection of nucleobases, nucleosides, and nucleotides in aqueous solution

Compound	t_R (min)		k'		N		S/N[a]		LOD (nmol L^{-1})		LOQ (nmol L^{-1})		Precision of LC–MS–MS, RSD (%; n=6)	
	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS	LC–MS–MS	LC–ICP–MS
Cytosine	2.1	–	0.21	–	2260	–	11900	–	0.30	–	1.0	–	13	–
Uracil	5.0	–	1.83	–	8750	–	133	–	38	–	130	–	15	–
Adenine	4.1	–	1.33	–	4970	–	3030	–	1.4	–	4.7	–	15	–
Guanine	4.5	–	1.56	–	6240	–	4990	–	0.87	–	2.9	–	8	–
Cytidine	4.3	–	1.47	–	5650	–	48690	–	0.082	–	0.27	–	5	–
Uridine	7.2	–	3.09	–	44180	–	7180	–	0.67	–	2.2	–	3	–
Adenosine	7.6	–	3.36	–	45230	–	19060	–	0.17	–	0.56	–	8	–
Inosine	8.2	–	3.66	–	49670	–	9360	–	0.33	–	1.1	–	6	–
Guanosine	8.2	–	3.66	–	52680	–	20020	–	0.20	–	0.67	–	7	–
5′CMP	3.7	3.2	1.10	0.92	4790	2890	1800	30	2.6	170	8.7	550	3	4
5′UMP	5.9	5.1	2.40	2.08	18110	5030	5750	21	0.94	240	3.1	790	3	5
5′AMP	6.2	6.0	2.57	2.57	31680	15760	5070	81	0.93	61	3.1	200	2	6
3′AMP	7.3	7.1	3.18	3.26	42840	43400	12450	73	0.39	68	1.3	230	2	5
2′AMP	8.2	8.0	3.69	3.78	54330	57480	16910	101	0.28	49	0.93	160	3	5
5′IMP	7.0	6.7	3.01	3.02	33610	24070	2880	44	1.8	110	5.9	370	2	5
5′GMP	6.8	6.5	2.87	2.89	37370	25490	3980	52	1.3	96	4.3	320	3	6
3′GMP	8.1	7.7	3.60	3.61	43400	41840	3410	25	0.98	130	3.3	430	3	6
2′GMP	9.2	8.7	4.24	4.22	53330	41100	2400	8	0.85	220	2.8	730	4	5

[a] Signal-to-noise ratio from 10 µmol L^{-1} standards except 3′GMP (6.3 µmol L^{-1}) and 2′GMP (3.6 µmol L^{-1})

uracil transitions were found in positive mode only and 5′ IMP transitions in negative mode only). However, optimum sensitivity was obtained by use of the positive-ionization mode for nucleobases and nucleosides, and the negative mode for nucleotides, resulting two methods. In both methods, the optimum transitions of quantifier and qualifier ions of analytes and internal standards were segmented into three time intervals, as illustrated in Figs. 1 and 2. Table S2 in the Electronic Supplementary Material summarizes the optimized measurement routine used in this study. With regard to MRM settings, for all nine nucleobases and nucleosides the selected precursor ions corresponded to $[M+H]^+$ ions. Product ions for nucleoside measurement corresponded to the entire or fragmented nucleobase. For the nucleotides, all selected precursor ions corresponded to $[M-H]^-$ ions. Selected product ions were derived from the nucleobase, from the phosphate (m/z=79 or 97), or from the ribose phosphate (m/z=211). Quantifier and qualifier fragments could be implemented for most of the compounds investigated. Moreover, for internal standardization, isotopically labelled $^{15}N^{13}C_2$ uracil and $^{15}N_5$ 5′AMP were included in the MS method as given in Table S2. Dwell times were optimized, depending on the number of concurrent transitions per time segment required to achieve 15 to 30 time points per peak. Figs. 1 and 2 show chromatograms resulting from optimized RP–LC–MS–MS determination of a standard mixture.

Table 3 Downscaling of normal bore RP–LC–ICP–DRC–MS to microbore RP–LC–ICP–DRC–MS

Compound	Column diameter	Flow (µL min^{-1})	S/N	LOD (nmol L^{-1})	LOQ (nmol L^{-1})
AMP	1	50	6	1683	5612
	2.1	200	35	310	1032
	4.6	1000	81	61	203
CMP	1	50	2	4832	16106
	2.1	200	8	1240	4134
	4.6	1000	30	170	567

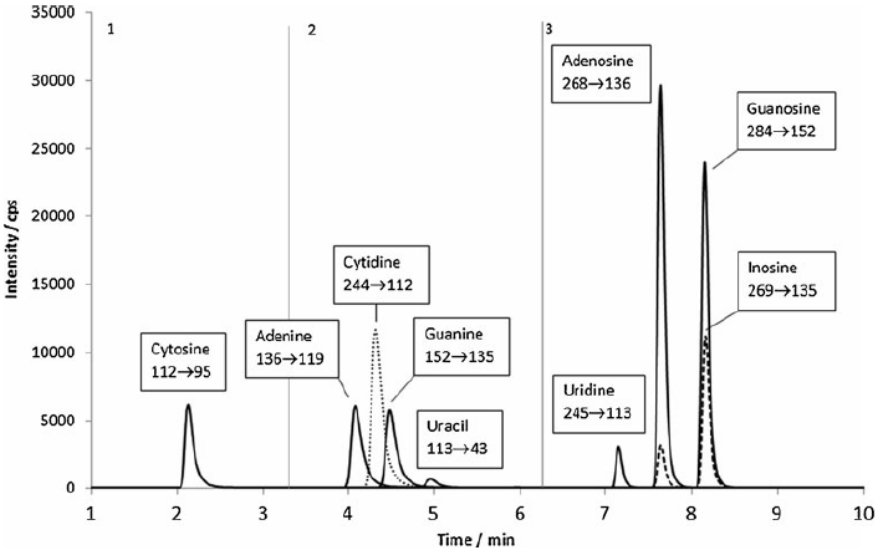

Fig. 1 Extracted chromatograms of nine MRM transitions obtained by RPLC–MS–MS in positive-ionization mode from aqueous standard mixtures of four nucleobases and five nucleosides. Concentrations are 10 µmol L^{-1} for uracil, 1 µmol L^{-1} for adenosine, and 2 µmol L^{-1} for the other substances

As already mentioned above, interferences arising from in-source fragments were avoided by chromatographic separation. In nearly all cases, interferences from compounds differing in one mass unit and with analogous quantifier ions could also be avoided by reversed-phase chromatographic separation. In these cases, the isotopologue M+1 of the lighter analyte could simulate the heavier analyte. A number of pairs of analytes differ by one mass unit—cytosine and uracil, cytidine and uridine, adenosine and inosine, CMP and UMP, and AMP and IMP. After reversed-phase separation, only one problematic case was left. At high concentrations of 3′AMP, the separation between the 3′AMP and 5′ IMP was not sufficient, as can be observed in Fig. 2. As a consequence, the isotopologue M+1 of 3′AMP (m/z=347, t_R=7.3) simulated 5′IMP (m/z=347, t_R=7.0) having the corresponding transition 347→79. Moreover, two nucleosides, i.e. inosine and guanosine, were not separated by the developed reversed-phase chromatographic method (Fig. 1). However, in this case, specific fragments enabled interference-free detection and accurate quantification.

Excellent signal-to-noise ratios could be obtained, resulting in LODs in the sub-nmol L^{-1} range for all the investigated nucleosides, nucleobases, and nucleotides summarized in Table 2. More specifically, for nucleobases (measured in positive-ionization mode) LODs were between 0.3 and 1.4 nmol L^{-1} with the exception of uracil (LOD 40 nmol L^{-1}). LODs between 0.08 and 0.7 nmol L^{-1} and between 0.3 and 2.6 nmol L^{-1} were obtained for nucleosides (measured in positive-ionization mode) and nucleotides (in negative-ionization mode), respectively.

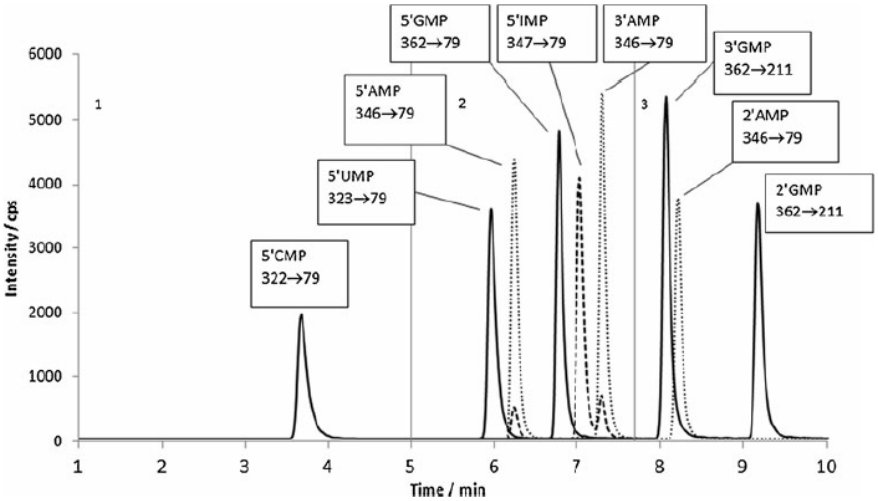

Fig. 2 Extracted chromatograms of six MRM transitions obtained by RPLC–MS–MS in negative-ionization mode from nine nucleotides in a 2 µmol L^{-1} aqueous standard mixture (except 3′GMP 1.26 µmol L^{-1} and 2′GMP 0.72 µmol L^{-1})

Reversed-phase chromatography and detection by ICP–MS

No reference materials are available for this type of bioanalytical application. Therefore, for a subset of target compounds a complementary measurement method was implemented. The method entailed quantification of nucleotides containing the hetero-element phosphorus by LC–ICP–MS. Figure 3 presents the LC–ICP–MS chromatogram of nine nucleotides in a 10 μmol L^{-1} aqueous standard mixture. The retention times, corresponding capacity factors, and theoretical number of plates (N) of the investigated nine nucleotide standards are in agreement with those obtained by LC–MS–MS (Table 2), taking into consideration the extra-column conditions of the different HPLC systems used (i.e. dead volume of HPLC, capillary length, and interface dead volume). For all peaks, resolution, calculated in accordance with the European Pharmacopeia, was >1.60.

Phosphorus detection was achieved by applying ICP–MS with the dynamic reaction cell technique. Thus, phosphorus was measured via the $^{31}P^{16}O^{+}$ molecular ion. As shown elsewhere, the sensitivity achieved in dynamic reaction cell mode is comparable with that of ^{31}P measurement in the standard mode of the quadrupole ICP–MS [27]. Detection by ICP–DRC–MS was preferred in this study to tackle spectral interferences inherent to N-containing analytes, for example $(^{15}N^{16}O)^{+}$ or $(^{14}N^{16}O^{1}H)^{+}$. The application of sector-field mass spectrometry would be beneficial in terms of sensitivity. Becker et al. reported off line LOD of 0.02 μg P L^{-1} when applying mass resolution R=4,000 [28]; this exceed the LODs routinely obtained by ICP–DRC–MS by a factor of 10–20. One disadvantage of combining sector-field MS with reversed-phase chromatography was lower robustness and more severe matrix suppression effects than for the combination of quadrupole ICP–MS and reversed-phase LC. In this work, as is apparent from Table 2, comparable signal-to-noise ratios were obtained for all the nucleotides investigated, despite the fact that gradient elution was used, because signal suppression was compensated by superior peak shape of the late eluting compounds. The limits of detection were approximately 100 nmol L^{-1}, 2–3 orders of magnitude higher than in LC–MS–MS. As a consequence, fundamental experiments were performed to address the question of whether sensitivity could be improved by down-scaling narrow-bore LC–ICP–DRC–MS to capillary LC–ICP–DRC–MS. A previous study showed that sensitivity loss because of matrix suppression effects exerted by organic solvents could be compensated to some extent by down-scaling LC, i.e. reducing the amount of HPLC eluent introduced to the plasma [29]. Hence, reversed-phase separation of two selected lead compounds was combined to ICP–DRC–MS using different column diameters, i.e. 4.6 mm, 2.1 mm, and 1 mm, with the respectively down-scaled LC flow rates and injection volumes. Table 3 summarizes the S/N ratios obtained and resulting limits of detection for 5′AMP and 5′CMP. No sensitivity improvement could be achieved for either substance eluting under different conditions.

Quantitative study in nucleotide enriched yeast

Finally, both methods LC–MS–MS and LC–ICP–MS were applied to a quantitative study of nucleotide-enriched yeast products. Because the aqueous solubility of nucleotides and related substances is high, sample treatment was straightforward and included dilution in saline buffer followed by

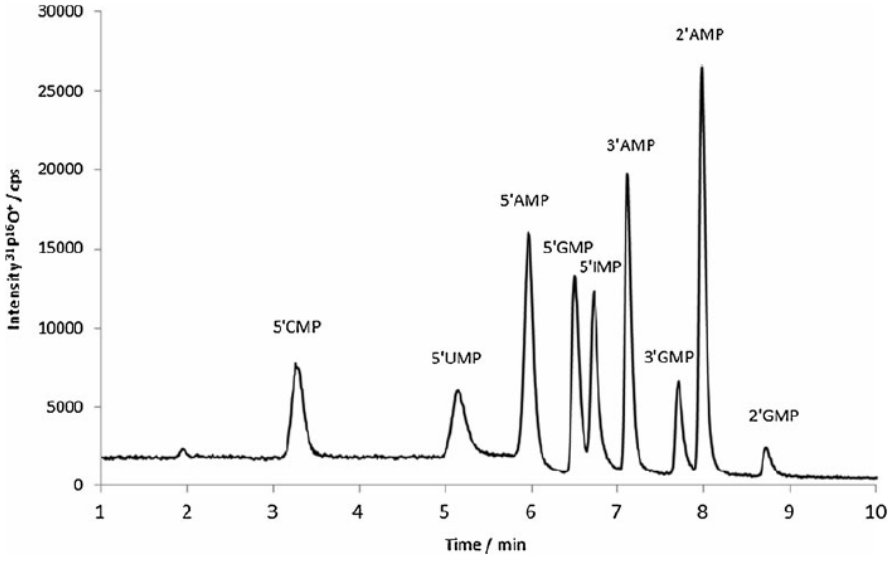

Fig. 3 Extracted chromatogram of PO^{+} at m/z=47 obtained by RPLC–ICP–DRC–MS of nine nucleotides in a 10 μmol L^{-1} aqueous standard mixture (except 3′GMP 6.3 μmol L^{-1} and 2′GMP 3.6 μmol L^{-1})

Table 4 Concentrations (μmol g_{DW}^{-1}) of nucleotides in one sample of feed supplement measured by LC–MS–MS and LC–ICP–MS via external calibration and standard addition, (standard uncertainty of external calibration calculated from n=3 determinations; standard uncertainty of standard addition experiments calculated according to DIN 32645)

Compound	External calibration LC–MS–MS	Standard addition LC–MS–MS	Standard addition LC–ICP–MS
5′CMP	132±4	131±1	131±3
5′UMP	162±5	161±2	144±7
5′AMP	2.44±0.04	3.76±0.54	4.50±2
3′AMP	2.78±0.04	3.43±0.43	n.a.[a]
2′AMP	0.77±0.02	0.83±0.45	n.a.[a]
5′IMP	249±5	232±3	240±12
5′GMP	198±6	180±2	185±4
3′GMP	4.41±0.15	4.88±0.32	n.a.[a]
2′GMP	1.62±0.07	1.67±0.11	n.a.[a]

[a]Not analysed

centrifugal filtration (10 kDa cut-off filtration) for removing high-molar-mass biomolecules (proteins, RNA) and particles. In the past, centrifugal ultrafiltration without further treatment steps was applied to infant formulas [11] and filtration through a 0.45 μm membrane was used for sample preparation of cell-free yeast autolysates [1].

For almost all the nucleoside and nucleobase compounds the approach implemented in this work resulted in satisfactory recovery (>90 %; determined by studying, by LC–MS–MS, the recovery of a sample spiked with 5 μmol L^{-1} standard). The exceptions were adenine and adenosine for which recovery was approximately 75 %. Corresponding experiments with nucleotide standards revealed average recovery was 95 %. Hence, internal standardization for accurate quantification by LC–MS–MS was implemented as given in Table S2 in the Electronic Supplementary Material. Using this approach, losses during the sample preparation could be corrected for and the standard uncertainty obtained for n=6 individually prepared standards by LC–MS–MS was approximately 4 %. Analysis of n=4 individually prepared samples revealed a repeatability was 10–15 %. Table 4 summarizes the comparative quantification of nucleotides by the two complementary MS-based methods using a nucleotide enriched yeast sample provided by our industry cooperation partner. The sample was prepared as described above, and the filtered aliquots were measured by use of different detection methods and quantification strategies. Because isotope dilution analysis could not be used for the monoisotopic phosphorus, standard addition by LC–ICP–MS was compared with external calibration and standard addition by LC–MS–MS, (both quantitative strategies using internal standardization as given in Table S2 in the Electronic Supplementary Material). As can be readily observed, the quantitative values obtained were in good agreement. Moreover, LC–MS–MS determinations revealed concentrations of nucleosides and nucleobases of approximately <15 μmol g_{DW}^{-1} for all the samples investigated (for methodological details see Electronic Supplementary Material Table S2).

Conclusion

Quantitative profiling of nucleotides, nucleobases, and nucleosides by different methods was investigated. RP–LC–MS–MS was the method of choice for accurate and sensitive quantification. The separation method was rapid and versatile, overcoming the technical restriction associated with ion-pair chromatography. Moreover, combination of the implemented reversed-phase separation with complementary ICP–MS detection was straightforward. In this way, a reference method for nucleotide quantification could be established and proved the validity of LC–MS–MS based quantification.

Acknowledgements This work was supported by the Biomin B.R.A.I.N program and by the Austrian Research Promotion Agency FFG (FHplus projekt METORGANIC).

References

1. Zhao J, Fleet GH (1996) Separation of twenty isomers of ribonucleotides and deoxyribonucleotides by reversed-phase ion-pairing high-performance liquid chromatography. J Chromatogr A 732:271–275
2. Yamaoka N, Kudo Y, Inazawa K, Inagawa S, Yasuda M, Mawatari K, Nakagomi K, Kaneko K (2010) Simultaneous determination of nucleosides and nucleotides in dietary foods and beverages using ion-pairing liquid chromatography-electrospray ionisation-mass spectrometry. J Chromatogr B 878:2054–2060
3. Aussenac J, Chassagne D, Claparols C, Charpentier M, Duteurtre B, Feuillat M, Charpentier C (2001) Purification method for the isolation of monophosphate nucleotides from Champagne wine and their identification by mass spectrometry. J Chromatogr A 907:155–164
4. Scholar EM, Brown PR, Parks RE, Calabresi P (1973) Nucleotide Profiles in the Formed Elements of Human Blood Determined by High-Pressure Liquid Chromatography. Blood 41:927–936
5. Zhao J, Fleet GH (2005) Degradation of RNA during the autolysis of *Saccharomyces cerevisiae* produces predominantly ribonucleotides. J Ind Microbiol Biot 32:415–423

6. Brovko LY, Romanova NA, Ugarova NN (1994) Bioluminescent Assay of Bacterial Intracellular AMP, ADP, and ATP with the Use of a Coimmobilized Three-Enzyme Reagent (Adenylate Kinase, Pyruvate Kinase, and Firefly Luciferase). Anal Biochem 220:410–414
7. Chen X, Yang F, Wang Y, Li S (2010) CE and CEC of nucleosides and nucleotides in food materials. Electrophoresis 31:2092–2105
8. Cohen S, Jordheim LP, Megherbi M, Dumontet C, Guitton J (2010) Liquid chromatographic methods for the determination of endogenous nucleotide analogs used in cancer therapy: a review. J Chromatogr B 878:1912–1928
9. Cichna M, Daxecker H, Raab M (2003) Determination of 18 nucleobases, nucleosides and nucleotides in human peripheral blood mononuclear cells by isocratic solvent-generated ion-pair chromatography. Anal Chim Acta 481:245–253
10. Gill BD, Indyk HE (2007) Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas. Int Dairy J 17:596–605
11. Inue K, Obara R, Akiba T, Hino T, Oka H (2008) Determination of Nucleotides in Infant Formula by Ion-Exchange Liquid Chromatography. J Agric Food Chem 56:6863–6867
12. Klawitter J, Schmitz V, Klawitter J, Leibfritz D, Christians U (2007) Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. Anal Biochem 365:230–239
13. Cordell RL, Hill SJ, Ortori CA, Barret DA (2008) Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry. J Chromatogr B 871:115–124
14. Seifar RM, van Dam JC, van Gulik WM, Heijnen JJ, van Winden WA (2009) Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry. Anal Biochem 388:213–219
15. la Marca G, Casetta B, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E (2006) Implementing tandem mass spectrometry as a routine tool for characterizing the complete purine and pyrimidine metabolic profile in urine samples. J Mass Spectrom 41:1442–1452
16. Preinerstorfer B, Schiesel S, Lämmerhofer M, Lindner W (2010) Metabolic profiling of intracellular metabolites in fermentation broths from β-lactam antibiotics production by liquid chromatography–tandem mass spectrometry methods. J Chromatogr A 1217:312–328
17. Xing J, Apedo A, Tymiak A, Zhao N (2004) Liquid chromatographic analysis of nucleosides and their mono-, di-, and triphosphates using porous graphitic carbon stationary phase coupled with electrospray mass spectrometry. Rapid Commun Mass Sp 18:1599–1606
18. Pabst M, Grass J, Fischl R, Léonhard R, Jin C, Hinterkörner G, Borth N, Altmann F (2010) Nucleotide and Nucleotide Sugar Analysis by Liquid Chromatography–Electrospray Ionization-Mass Spectrometry on Surface-Conditioned Porous Graphitic Carbon. Anal Chem 82:9782–9788
19. Fujii S, Inagaki K, Takatsu A, Yarita T, Chiba K (2009) Determination of phosphorus using capillary electrophoresis and micro-high-performance liquid chromatography hyphenated with inductively coupled plasma mass spectrometry for the quantification of nucleotides. J Chromatogr A 1216:7488–7492
20. Fujii S, Inagaki K, Chiba K, Takatsu A (2010) Quantification of phosphorus in DNA using capillary electrophoresis hyphenated with inductively coupled plasma mass spectrometry. J Chromatogr A 1217:7921–7925
21. Wrobel K, Landero Figueroa JA, Zaina S, Lund G, Wrobel K (2010) Phosphorus and osmium as elemental tags for the determination of global DNA methylation – A novel application of high performance liquid chromatography inductively coupled mass spectrometry in epigenetic studies. J Chromatogr B 878:609–614
22. Edler M, Jakubowski N, Linscheid M (2006) Quantitative determination of melphalan DNA adducts using HPLC – inductively coupled mass spectrometry. J Mass Spectrom 41:507–516
23. Edler M, Jakubowski N, Linscheid M (2005) Styrene oxide DNA adducts: quantitative determination using ^{31}P monitoring. Anal Bioanal Chem 381:205–211
24. Pröfrock D, Leonhard P, Prange A (2003) Determination of phosphorus in phosphorylated deoxyribonucleotides using capillary electrophoresis and high performance liquid chromatography hyphenated to inductively coupled plasma mass spectrometry with and octopole reaction cell. J Anal At Spectrom 18:708–713
25. Siethoff C, Feldmann I, Jakubowski N, Linscheid M (1999) Quantitative Determination of DNA Adducts Using Liquid Chromatography/Electrospray Ionization Mass Spectrometry and Liquid Chromatography/High-resolution Inductively Coupled Plasma Mass Spectrometry. J Mass Spectrom 34:421–426
26. Luo B, Groenke K, Takers R, Wandrey C, Oldiges M (2007) Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography—mass spectrometry. J Chromatogr A 1147:153–164
27. Popp M, Hann S, Mentler A, Fuerhacker M, Stingeder G, Koellensperger G (2008) Determination of glyphosate and AMPA in surface and waste water using ion chromatography coupled to inductively coupled plasma dynamic reaction cell mass spectrometry (HPIC–ICP–DRCMS). Anal Bioanal Chem 391:695–699
28. Becker JS, Boulyga SF, Pickhardt C, Becker J, Buddrus S, Przybylski M (2003) Determination of phosphorus in small amounts of protein samples by ICP–MS. Anal Bioanal Chem 375:561–566
29. Zs S, Koellensperger G, Stingeder G, Hann S (2006) A comparative study of different introduction systems. J Anal Atom Spectrom 21:86–89
30. Coulier L, Bas R, Jespersen S, Verheij E, van der Werf MJ, Hankemeier T (2006) Simultaneous Quantitative Analysis of Metabolites Using Ion-Pair Liquid Chromatography – Electrospray Ionization Mass Spectrometry. Anal Chem 78:6573–6582
31. Tuytten R, Lemière F, Van Dongen W, Esmans EL, Siegers H (2002) Short capillary ion-pair high-performance liquid chromatography coupled to electrospray (tandem) mass spectrometry for the simultaneous analysis of nucleoside mono-, di-, and triphosphates. Rapid Commun Mass Sp 16:1205–1215
32. Tuytten R, Lemière F, Van Dongen W, Witters E, Esmans EL, Newton RP, Dudley E (2008) Development of an On-Line SPE–LC–MS Method for Urinary Nucleosides: Hyphenation of Aprotic Boronic Acid Chromatography with Hydrophilic Interaction LC—ESI–MS. Anal Chem 80:1263–1271

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

S. Neubauer, A. Rugova, D. B. Chu, H. Drexler, A. Ganner, M. Sauer, D. Mattanovich, S. Hann, G.Koellensperger

Table S1 Selected publications on nucleotide analysis

Analytes	Detection Limits	Application, Samples	Chromatography	Analysis Time / min	Detection	Ref.	Year publ.
AMP, ADP, GDP, UTP, ATP		acid soluble extracts of human cell extracts	IC, solution of KH_2PO_4 and KCl	70	UV detection, full scale absorption	[1]	1973
20 isomers (5', 3' and 2'isomers) of NMPs and dNMPs		yeast autolysates	IP-RP-LC, 0.05 M tetrabutylammmonium-phosphate, phosphate buffer, pH 5.45	40	UV detection at 245 and 280 nm	[6]	1995
18 nucleobases, nucleosides and nucleotides	3 – 29 pmol on column	pharmacodynamic monitoring, human blood cells	isocratic IP-RP-LC, H_3PO_4/triethylamine/$MgSO_4$	55	UV detection at 254 nm	[7]	2003
Cd, Ud, As, Is, Gs, CMP, UMP, AMP, IMP, GMP	0.05 – 0.68 $mg*g^{-1}$	bovine and human milk, infant formulas	RP-LC, KH_2PO_4 buffer, pH 5.6	130	UV detection at 250, 260 and 270 nm	[3]	2006
CMP, UMP, AMP, IMP, GMP	0.01 – 0.05 $\mu g*mL^{-1}$	infant formulas	IC, 50 mM $(NH_4)_2HPO_3$ buffer, pH 4.0	30	UV detection at 254 nm	[2]	2008
16 nucleosides, NMPs, NDPs and NTPs		method development, standard solutions	adsorption chromatography PGC, H_2O/ACN, NH_4AC	18	ESI(+,-) LC-MS	[9]	2004
nucleotides, sugar nucleotides, sugar bisphosphates and coencyme A esters	0.01 – 0.06 $\mu m*mL^{-1}$	microbial metabolomics	IP-RP-LC, H_2O/MeOH, hexylamine, pH 6.3	51	ESI(-) LC-MS, linear ion trap	[20]	2006
AMP, CDP, UDP, ADP, GDP, CTP, UTP, ATP, GTP, NAD, FAD	0.25 – 4 pmol on column	cells and tissues of rats	IP-RP-LC, H_2O/MeOH, dibutylammonium formate	20	ESI(-) LC-MS	[17]	2007
23 nucleosides and nucleotides	LLOQ < 2.1 µM	dietary foods and beverages	IP-RP-LC, H_2O/ACN, dihexylammonium acetate, pH 5.0	50	ESI(+,-) LC-MS	[21]	2010
21 isomers (5',3', and 2'isomeres) of NMPs and dNMPs		Champagne wine	IP-RP-LC, H_2O/MeOH, tetrabutylamine, phosphate buffer, pH 4.45	90	ESI(-) LC-MS-MS, triple quadrupole MS	[16]	2001
mono-, di and triphosphorylated nucleotides of Cd, Ud, As, Gs	ca. 1 pmol on column	method development, standard solutions	capillary IP-RP-LC, H_2O/MeOH, N,N-dimethylhexylamine	60	ESI(+,-) LC-MS, MS-MS, triple quadrupole MS	[11]	2002
more than 30 nucleobases and nucleosides	0.1 – 10 µM	purine and pyimidine metabolic profile, urine	RP-LC, H_2O/MeOH, ammonium acetate	17	ESI(+) LC-MS-MS, triple quadrupole MS	[18]	2006
29 metabolites including AMP, cyclic GMP, ADP, ATP, NAD, NADH, NADP, NADPH	2.6 – 60 nM	cell extracts of Escherichia coli	IP-RP-LC, H_2O/MeOH, tributylammonium acetate	90	ESI(-) LC-MS-MS, quadrupole ion trap MS	[15]	2007
Cd, Ud, As, Is, Gs and a number of modified nucleosides		urine (cancer markers)	HILIC, ACN/H_2O, $HCOONH_4$, HCOOH	60	ESI (+) LC-MS-MS, quadrupole ion trap MS	[10]	2008
24 nucleotides and related phosphorylated species	100 – 250 nM	cultured chinese hamster ovary cells	IP-RP-LC, H_2O/MeOH, N,N-dimethylhexylamine	35	ESI(-) LC-MS-MS, tripple quadrupole MS	[12]	2008
16 nucleotides	0.03 – 0.62 pmol on column	intracellular nucleotides in microorganisms	IP-RP-LC, H_2O/ACN, dibutylamine, pH6.7	25	ESI(-) LC-MS-MS, triple quadrupole MS	[19]	2009

Table S2 MRM settings of the LC-MS-MS instrument

Compound	Polarity / time segment	Dwell time / ms	Precursor ion	Quantifier	Qualifier	FV[a] / V	CE[b] $_{Quantifier}$ / V	CE[b] $_{Qualifier}$ / V	IS for quantification
Cytosine	+ / 1	500	112.1	94.9	52.0	104	17	33	$^{15}N^{13}C_2$ Uracil
Uracil	+ / 2	100	113.0	43.0	-	90	21	-	$^{15}N^{13}C_2$ Uracil
$^{15}N^{13}C_2$ Uracil	+ / 2	100	116.0	70.9	97.9	100	13	13	-
Adenine	+ / 2	100	136.1	118.9	91.9	124	25	33	$^{15}N^{13}C_2$ Uracil
Guanine	+ / 2	100	152.1	134.9	109.9	104	17	21	$^{15}N^{13}C_2$ Uracil
Cytidine	+ / 2	100	244.1	111.9	94.9	70	9	40	$^{15}N^{13}C_2$ Uracil
Uridine	+ / 3	100	245.1	112.9	-	70	9	-	$^{15}N^{13}C_2$ Uracil
Adenosine	+ / 3	100	268.1	135.9	118.9	90	17	40	$^{15}N^{13}C_2$ Uracil
Inosine	+ / 3	100	269.1	136.9	-	80	9	-	$^{15}N^{13}C_2$ Uracil
Guanosine	+ / 3	100	284.1	151.9	134.9	80	13	40	$^{15}N^{13}C_2$ Uracil
5'CMP	- / 1	500	322.0	79.0	97.0	-124	-40	-21	$^{15}N_5$ 5'AMP
5'UMP	- / 2	100	323.0	78.9	96.9	-114	-40	-21	$^{15}N_5$ 5'AMP
5'AMP	- / 2	100	346.0	78.9	134.0	-114	-40	-40	$^{15}N_5$ 5'AMP
3'AMP	- / 2	100	346.0	211.0	79.1	-114	-13	-40	$^{15}N_5$ 5'AMP
2'AMP	- / 3	200	346.0	211.0	134.0	-114	-13	-40	$^{15}N_5$ 5'AMP
5'IMP	- / 2	100	347.0	79.1	-	-114	-40	-	$^{15}N_5$ 5'AMP
$^{15}N_5$ 5'AMP	- / 2	100	351.0	79.0	139.0	-104	-33	-37	-
5'GMP	- / 2	100	362.0	79.0	211.0	-124	-21	-13	$^{15}N_5$ 5'AMP
3'GMP	- / 3	200	362.0	211.0	79.0	-124	-13	-21	$^{15}N_5$ 5'AMP
2'GMP	- / 3	200	362.0	211.0	79.0	-124	-13	-21	$^{15}N_5$ 5'AMP

a) Fragmentor voltage

b) Collision energy

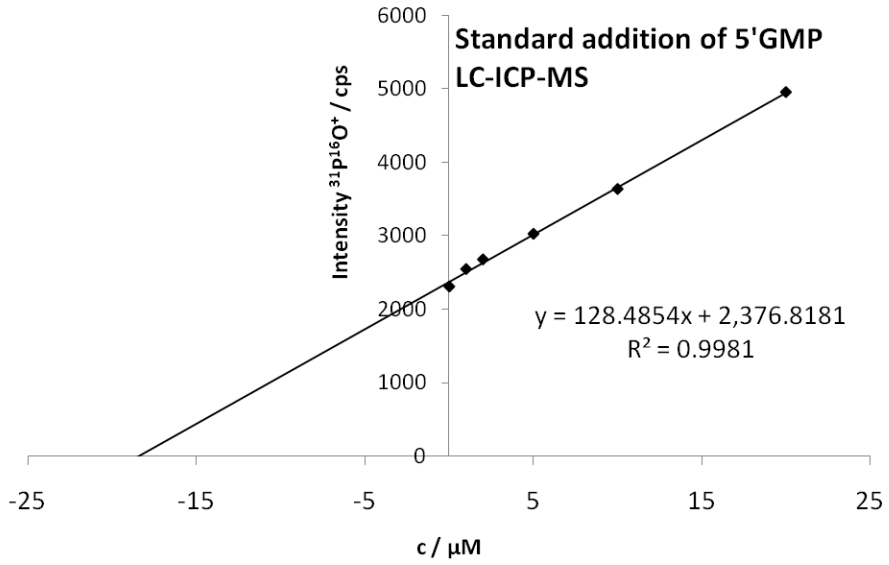

Figure S1. Calibration curve of standard addition experiment. The relative standard deviation for the procedure calculated according to DIN 32645 is 1.2 %.

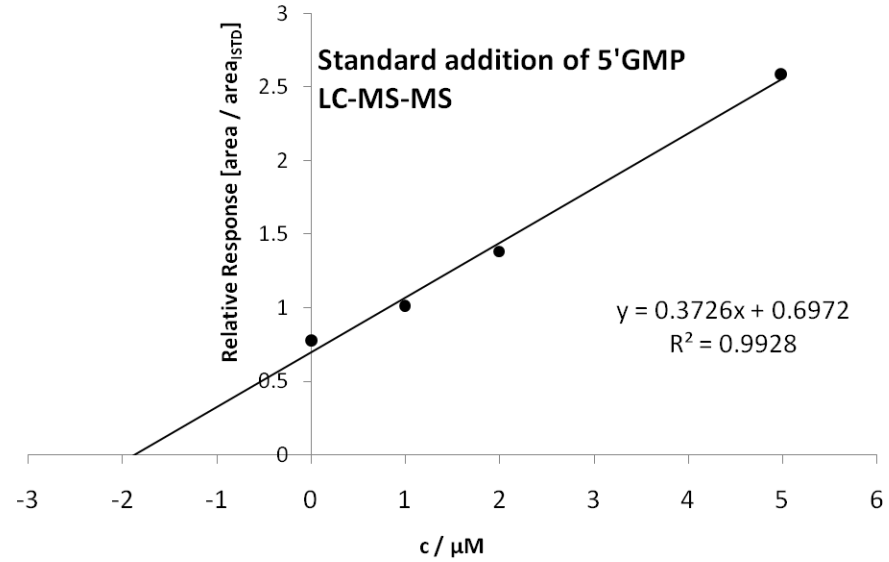

Figure S2. Calibration curve of standard addition experiment. The relative standard deviation for the procedure calculated according to DIN 32645 is 2 %.

CONCLUSION

Within this thesis rugged, sensitive, and selective methods for speciation and quantification of phosphorus-containing compounds employing ion exchange and reversed phase chromatography coupled to ICP-MS have been developed.

The first presented validated method based on strong anion exchange chromatography and ICP-DRC-MS detection is the first method to quantify simultaneously orthophosphate and *myo*-inositol hexakisphosphate (IP_6), both inorganic and organic phosphorus compounds in a single chromatographic run. This method is applied successfully in plant and soil related samples offering excellent reproducibility and limits of detection in the submicromol per liter range. NaOH/ EDTA Extraction of soil solution gave the highest extraction efficiency for IP_6 amongst other organic phosphorus compounds. Since phosphate is not amenable to LC-MS/MS, which is a clear drawback in the context of soil and rhizosphere research, ion chromatography employing acidic gradient elution coupled with ICP-DRCMS is a method of choice for quantification of orthophosphate and phytic acid in soil and plant related samples. This can be of great importance for studies aiming at the investigation of phosphorus mobilization and plant nutrition.

Another novel reversed-phase chromatography coupled to ICP-MS based method for quantitative profiling of nucleotides has been developed. The LC utilizing a stationary phase compatible with 100 % aqueous mobile phase was combined with both molecular and elemental mass spectrometry. Sensitivity of LC–ICP–MS was 1–2 orders of magnitude lower than that of LC–MS–MS. However, quantitative analysis of yeast products using complementary MS detection gave results in a good agreement. This is the first LC–ICP–MS reported method that has been used for quantification of free nucleotides in yeast products. This method can be established as a reference method for nucleotide quantification and proven the validity of LC–MS–MS based quantification due to the lack of reference materials for nucleotide quantification.

Moreover, within this PhD project a review discussing state-of-the-art mass spectrometric methods which have been developed and applied since the beginning of this century. for investigation of chemical processes in the rhizosphere is presented. This review covers both elemental and molecular mass spectrometry as well as their combination with different separation techniques.

Curriculum Vitae

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Conference Contributions

1. A. Rugova, J. Santner, M. Puschenreiter, G. Köllensperger, S. Hann. Speciation of organic phosphorus in soil and plant related samples by LC combined with ICP-MS, European Winter Conference on Plasma Spectrochemistry, 30.01-6.02.2011, Zaragoza, Spain.
2. A. Rugova, J. Santner, M. Puschenreiter, G. Köllensperger, S. Hann: Speciation of phosphorus compounds in soil and plant related samples by liquid chromatography combined with inductively plasma mass spectrometry, Symposium Massenspektrometrische Verfahren der Elementspurenanalyse und 22. ICP-MS Anwendertreffen, 06.09.-08.09, 2010 Berlin, Germany.
3. Koellensperger, G.; Werneth, M.; Falta, T.; Fischer, L.; Kretschy, D.; Puls, C.; Rugova, A. and Hann, S. (2011): Neue Entwicklungen zur Untersuchung von biologischen Proben mittels ICP-MS. In: ETH Zürich, Book of Abstracts [ANAKON 2011, Zürich, SWITZERLAND, MARCH 22-25, 2011

4. Hann, S.; Rampler, E.; Neubauer, S.; Rugova, A.; Pfeffer, M.; Mattanovich, D. and Köllensperger, G. (2011): LC-ICP-MS – in Biotechnological Applications. In: ETH Zürich, Book of Abstracts [ANAKON 2011, Zürich, SWITZERLAND, MARCH 22-25, 2011]
5. S. Neubauer, A.Rugova, H.Drexler, C. H.Troyer, S. Hann, G.Köllensperger, LC-MS and LC-ICP-MS Methods for Analysis of Nucleotide Enriched Yeast Products, 27th LC/MS Montreux Symposium, 10-12 November 2010, Montreux, Switzerland.
6. S. Neubauer, A.Rugova, H.Drexler, C. H.Troyer, M. Sauer, D. Mattanovich, S. Hann, G.Köllensperger, Metabolomics in the cell factory yeast-sample prep and LC-MS analysis, ASAC JunganalytikerInnen Forum, 11.-12.06.2010, Wien BOKU-VIBT.
7. N. Troni, M. Bacaj, F. Gashi, A. Rugova, & M. Sadiku. Elementet ekotoksike në gjurmë në burimet natyrore të ujërave të Kosovës, Takimi i Alb-Shkencës në Tiranë, Shtator 2008.
8. Sadiku, M.; Arbneshi, T.; Fuga, A.; Mele, A.; Rugova, A. Measurement of Gas Adsorption and Mercury Intrusion as Quantitative Characteristic of the Efficiency of an Acid Activation on Smectic Clay Minerals. Book of Abstracts, 8th European Meeting on Environmental Chemistry, 5-8 December, 2007 Inverness, Scotland.
9. Rugova, A.; Arbneshi, T.; Hashani, I. Paçarizi, M.; Sadiku, M.;Berisha, L. Levels of POPs in Soil and River Sediments of Kosova. 8th European Meeting on Environmental Chemistry, 5-8 December 2007, Inverness, Scotland.
10. A. Rugova, T. Arbneshi, M. Picer, N.Picer, V. Hodak Kobasić, V.Čalić. Pollution of soil and river sediment samples from Kosova with PCBs and Organochlorine insecticides. Environmental Quality Through Science, 10th Meeting of Society of Environmental Toxicology and Chemistry, 12-14 September 2007, Leipzig, Germany .
11. Paçarizi, M.; Shehdula, M.; Jusufi S.; Lajçi, A.; Faiku, F.; Rugova, A.. Complexation of Pb (II) ions with nitrile acetic acid in presence of carboxylic acids. Proceedings from the 1st Symposium of Chemistry and Environment, 12-15 June, 2007, Miločer-Budva, Montenegro.
12. Paçarizi, M.; Shehdula, M.; Jusufi, S.; Lajçi, A.; Faiku, F.; Rugova, A.. Polarographic investigation of interaction of Pb (II) with NTA and EDTA in

oxalate solutions. Proceedings from the 1st Symposium of Chemistry and Environment, June 12-15, 2007, Miločer-Budva, Montenegro.

13. M. Picer, N. Picer, T. Kovač, V. Čalić, N. Miošić, Z. Cencič Kodba, A. Rugova, War in some karstic area of former Yugoslavia, and PCB hazards to karst watersystem. The International Conference on “Environmental change and rational water use” ; Buenos Aires 29th August-1st September 2005.
14. Čalić, V.; Miošić, N.; Cencič Kodba, Z.; Picer, M.; Picer, N.; Rugova, A.; Arbneshi, T. 2005. Zagađenje riječnih sedimenata Bosne i Hercegovine i Kosova polikloriranim Bifenilima. Abstract Book-19: Hrvatski skup kemičara i kemijskih inženjera,´Opatija.
15. Čalić, V.; Miošić, N.; Cencič Kodba, Z.; Picer, M.; Picer, N.; Rugova, A.., Arbneshi, T.; Pollution of river sediments in Bosnia and Herzegovina and Kosovo with polychlorinated biphenyls. International Conference on “Environmental change and rational water use”; Buenos Aires 29th August-1st September 2005.
16. Čalić, V. ; Picer, N.; Picer, M.; Miošić, N.; Cencič Kodba, Z.; Rugova, Ariana. Levels of POPs in soil and sediment of Bosnia and Herzegovina and Kosovo. Workshop of Croatian-Norwegian project: Mitigation of environmental consequences of the war in Croatia – Risk assessment of hazardous chemical contamination, Zagreb, 26-27 September, 2005.
17. Picer, M.; Picer, N.; Kovač, T.; Hodak Kobasić, V.; Čalić, V.; Miošić, N.; Cencič Kodba, Z.; Rugova, A.; 2005. War in Croatia, Bosnia and Herzegovina, and Kosovo, and PCBs hazards to karst water system. Proceedings of the International conference and field seminar: Water resource and environmental problems in karst - CVIJIC.
18. Picer, M.; Picer, N.; Hodak Kobasić, V.; Kovač, T.; Čalić, V.; Miošić, N.; Cencič Kodba, Z.; Rugova, A.; 2005. War in Croatia, Bosnia and Herzegovina and Kosovo and PCBs hazards to water Ecosystem. Proceedings from the European conference on Industrial Pollution and Sustainable Development, Faculty of chemistry and chemical engineering, University of Maribor, Slovenia.
19. Picer, M., Kovač, T., Picer, N., Čalić, V., Miošić, N., Cencič K., Z., Rugova, A., War in Croatia, Bosnia and Herzegovina and Kosovo and PCBs hazards.

Organohalogen compounds 67 / E. Reiner and M. Alaee (ed.). Toronto Terrachem, (2005) 2048-2051

20. Čalić, V., Miošić, N., Cencič, K.Z., Picer, M., Picer, N., Rugova, A., Arbneshi, T., Pollution of river sediments in Bosnia and Hercegovina and Kosovo with polychlorinated biphenyls. Organohalogen compounds 67 / E. Reiner and M. Alaee (ed.). Toronto, Canada : Terrachem, (2005) 2052-2055.

Publications

1. A. Rugova, M. Puschenreiter, G.Koellensperger, S.Hann. Elucidating rhizosphere processes and root exudate dynamics based on mass spectrometric techniques, Analytica Chimica Acta, 2017, In press, accepted manuscript.
2. A. Rugova, M. Puschenreiter, J. Santner, L. Fischer, S.Neubauer, G. Koellensperger, S. Hann, Speciation of organic phosphorus compounds with Ion Chromatography coupled with Inductively Coupled Plasma Mass Spectrometry in soil and plant related samples, Journal of Separation Science, 2014, 30, 37 (14):1711-1719.
3. S. Neubauer, A. Rugova, D. B. Chu, H. Drexler, A. Ganner, M. Sauer, D. Mattanovich, S. Hann, G.Koellensperger. Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements. Analytical and Bioanalytical Chemistry, 2012, 404,3:799-808.