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Development of novel methods for the analysis of root exudates

This PhD thesis is presented for the fulfilment of the requirements for the degree of *Dr. nat. tech.* at the Vienna University of Natural Resources and Life Sciences – BOKU

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Summary

This work presents two novel methods for the analysis of two important plant metabolites – free proteinaceous amino acids and phytosiderophores – in root exudates.

Introductory, tasks and challenges of metabolomics in general and plant metabolomics in particular are described including definitions of relevant terms and the classification of these research areas within systems biology. In this context an overview of the analytical strategies and techniques is given comparing different sample preparation methodologies, various GC-MS and LC-MS instrumental set-ups and NMR as well as data evaluation and statistical approaches. Emphasis is devoted to hydrophilic interaction chromatography (HILIC) – focusing on zwitterionic stationary phases – and inductively coupled plasma mass spectrometry (ICP-MS), since the methods of choice of the presented publications rely partly on these special techniques. In order to characterize the nature of the investigated samples, an introduction to the topic of root exudates and its putative functions in the rhizosphere is presented highlighting three important classes of root exudates (organic acids, phytosiderophores and amino acids). In addition, several examples of "applied rhizosphere metabolomics" are chronologically summarized.

Publication I deals with the development of an LC-MS method for the analysis of underivatized, free proteinaceous amino acids. Prefatory, challenges and the state of the art of amino acid analysis in general are outlined. Subsequently the developed separation of 16 amino acids via HILIC and their detection using either a triple quadrupole mass analyzer in multiple reaction monitoring mode or an ion trap mass analyzer are described and various analytical figures of merit of the presented method are discussed. Moreover, an enrichment procedure based on solid phase extraction for amino acids in nutrient solutions is evaluated. Finally, the application of the proposed method to the analysis of tyrosine in samples obtained from soil adsorption experiments is illustrated.

Publication II presents a novel capillary electrophoretic separation of different phytosiderophores and their complexes with various metals employing a mass spectrometry-compatible background electrolyte. The detection via ESI-MS and ICP-MS for the free ligands and the metal complexes, respectively, is described and the applicability of the CE-ICP-MS method to quantitative analysis is discussed. In addition, the determination of relative complex stability constants using this method is explained based on incubation experiments under ligand deficient and therefore competitive conditions.

Zusammenfassung

Die vorliegende Arbeit präsentiert zwei neuartige Methoden für die Analyse von zwei bedeutenden Pflanzenmetaboliten – freie proteinogene Aminosäuren und Phytosiderophoren – in Wurzelexsudaten.

Die Einleitung setzt sich mit der Aufgabenstellung und den Herausforderungen der Metabolomik im Allgemeinen und der Pflanzenmetabolomik im Speziellen auseinander. In diesem Zusammenhang werden die Terminologie und die Klassifizierung dieser zwei Forschungsrichtungen innerhalb der Systembiologie behandelt. Ein Überblick der analytischen Strategien und Techniken dient dazu verschiedene Probenvorbereitungsmethoden, diverse GC-MS-und LC-MS-Konfigurationen, NMR sowie Herangehensweisen an die Datenauswertung und statistische Datenverarbeitung miteinander zu vergleichen. Ein Schwerpunkt ist der hydrophilen Interaktionschromatographie (HILIC) – mit besonderem Augenmerk auf zwitterionische stationäre Phasen – gewidmet sowie der ICP-MS (induktiv gekoppeltes Plasma – Massenspektrometrie), da die in den beiden Publikationen beschriebenen Methoden zum Teil auf diesen speziellen Verfahren beruhen. Eine kurze Einführung in die Thematik der Wurzelexsudate und deren möglichen Funktionen in der Rhizosphäre soll einen Einblick in die Beschaffenheit der untersuchten Proben gewähren, wobei drei wichtige Wurzelexsudat-Klassen (organische Säuren, Aminosäuren und Phytosiderophore) detaillierter beschrieben werden. Im Weiteren werden einige Bespiele für die „angewandte Rhizosphärenmetabolomik“ chronologisch zusammengefasst.

Publikation I beschreibt eine neue LC-MS Methode für die Analyse von underivatisierten, freien proteinogenen Aminosäuren. Einleitend werden die Anforderungen an die Aminosäureanalytik und deren neuesten Entwicklungen zusammengefasst. Im Anschluss daran werden die im Zuge der Dissertation entwickelte Trennung von 16 Aminosäuren via HILIC und Detektion mittels entweder einem Triple Quadrupole Massenanalysator im multiplen Reaktionsmonitoring Modus oder einer Ionenfalle dargestellt und verschiedene analytische Bewertungskriterien dieser Methode diskutiert. Ferner wird ein Anreicherungsverfahren mithilfe von Festphasenextraktion für Aminosäuren in Nährlösungen evaluiert und die Anwendung der beschriebenen LC-MS Methode für die Bestimmung von Tyrosin in Bodenadsorptionsproben gezeigt.

Publikation II präsentiert eine neue kapillarelektrophoretische Trennung verschiedener Phytosiderophore und deren Metall-Komplexe unter Verwendung eines MS-kompatiblen Hintergrundelektrolyten. Die Detektion mittels ESI-MS für die freien Liganden und mittels ICP-MS für die komplexierten Metalle wird beschrieben und die Anwendbarkeit der ICP-MS Methode für die quantitative Analyse diskutiert. Darüber hinaus wird die Ermittlung von relativen Komplexbildungskonstanten anhand der beschriebenen Methode gezeigt – basierend auf Inkubationsversuchen, die im Ligandenunterschuss und dadurch unter kompetitiven Bedingungen durchgeführt wurden

Introduction
Separation methods combined with ESI-MS and ICP-MS in Plant Metabolomics

1 Introduction to Metabolomics

Within this work metabolomics is considered as the entity of different analytical approaches and data evaluation (e.g. targeted and non-targeted, quantitative and non-quantitative strategies, as well as identification) with the objective to achieve information about the metabolites of any kind of biological system. However, terms and definitions concerning metabolomics as well as its role and its potential within the field of bioscience are equivocal in the literature [1-16]. Since the levels of metabolites in a living cell are regulated by the concentration and activity of enzymes that are itself on the other hand dependent on the transcription and translation of genes, metabolomics has been popularized as a tool of functional genomics (e.g. [1-7]). Fiehn described it as "the link between genotypes and phenotypes", Trethewey et al. compared metabolic profiling with "a rosetta stone for genomics" and Dettmer et al. depicted metabolomics as "the endpoint of the *omics cascade*" using the demonstrative scheme presented in Fig.1 [1,3,6]. Villas-Bôas et al. debilitated the feasibility of directly linking genes with metabolites mentioning that the concentration of a protein does not correspond to its enzymatic activity and hence the concentration of the metabolite, that the same metabolite might take part in many different pathways, and that in different organisms the number of metabolites does not correlate with the number of genes (e.g. plants feature more metabolites than genes, while it is the other way round for microorganisms, which are less complex) [5].

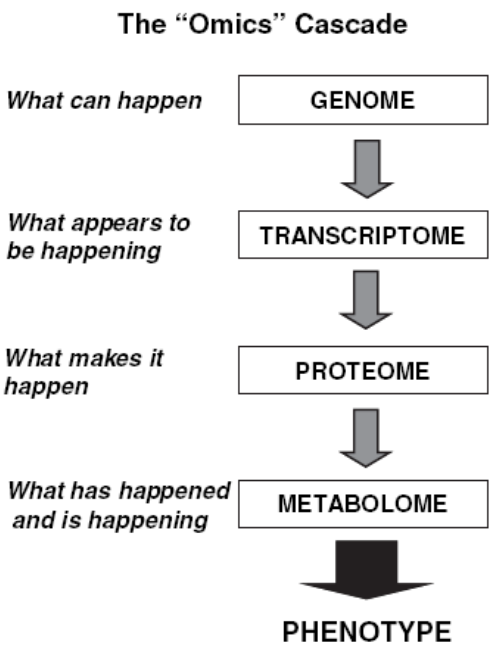

Fig.1. The "Omics" Cascade by Dettmer et al. [6] illustrating the powerful integration of the knowledge of all building blocks for the upcoming field of study of systems biology. Being the closest to phenotype, the metabolome is the most predictive of "what we can observe about the character of an organism" [3,6].

The emerging application of metabolomics apart from genetics is primarily related to ecology and environmental sciences, referred to as ecological metabolomics and environmental metabolomics, respectively [11]. E.g. Bundy et al.

highlighted the advantages provided by (non-targeted) metabolomics approaches in environmental questions at the molecular level, such as the feasibility to relate the actual functional status of an organism to its phenotype, the possibility to discover unexpected relationships and metabolite responses and the benefit of existing knowledge [8].

For profound understanding of the presented matter, several expressions that are frequently used in the context of metabolomics need to be defined. However, metabolomics related terminology is not consistent in the literature. Table 1 critically discusses definitions and discrepancies.

Genomics, transcriptomics and proteomics focus on the analysis of comparatively simply composed biopolymers (linear sequence of 4 different nucleotides or of 20 different amino acids). The chemical properties of these polymeric compounds are therefore homogenous and allow for optimization and speed-up of their analysis. Moreover, their linear structure of repeating monomers facilitates to read-out their sequence for identification. Metabolites, on the other hand, are characterized by substantial chemical, physical and structural diversity, ranging from volatile alcohols and hydrophobic lipids to polar organic acids and ionic inorganic compounds. In addition, the intricacy is increased due to dynamics in concentration, time and space. That complexity prevents the simultaneous extraction and determination of the complete metabolome so far and – instead of sequencing known monomers – requires *de novo* identification based on the elucidation of elemental composition, order of atoms and stereochemistry [3,5-6,14]. The number of analytes in metabolomic investigation is generally lower compared to the number of those in proteomics for example (100 000 – 1 000 000 human proteins and far more than 1 000 000 human peptides with molecular weights < 15 kDa [17]). Still, metabolites are considerably numerous, especially in higher organisms. According to genome-scale metabolic network reconstructions by Herrgård et al. there are 1,168 metabolites in *Saccharomyces cerevisiae* (microorganism (yeast)) [18]. Beecher estimated some 2,000 major metabolites for humans – disregarding secondary metabolites [19]. The plant kingdom is considered to be the metabolite richest with estimated numbers ranging from 100,000 to 200,000 metabolites [2-3,6,12,20-21].

In order to cope with this analytically troublesome situation, several strategies have been designed. Four approaches listed in Table 1 (targeted metabolite analysis, metabolite profiling, metabolite fingerprinting and metabolite footprinting) can be classified in two basic, complementary ideas: i) target specific studies and ii) global metabolomics, with the first strategy aiming at the quantification of a defined metabolite or group of metabolites and the latter approach intending to differentiate between relative samples by comparing patterns of as many metabolites as analytically possible (the necessity of quantification and identification or identity confirmation in this context is controversial in the literature) [5-6, 12,14]. Besides these “classical” metabolomic strategies, there is another approach called metabolic flux analysis (MFA) that incorporates enzymatic steady-state reaction rates striving for the characterization of large-scale metabolic flux networks [22].

Table 1. Definitions of metabolomics related terms.

Term	Definition	Comments
Metabolite	Small molecule (MW < 1000 Da) that takes part in metabolic reactions and that is required for cell functioning [5-6]	natural metabolite ≠ xenobiotic metabolite [6]
Metabolome	Complete set of metabolites synthesized by a biological system (organism, tissue, cell or cell compartment); time-resolved and substrate-dependent [3,5]	[11] discussed the problem of the term due to spatial and temporal chemical variation
"true metabolomics"	Comprehensive non-targeted and non-biased identification and quantification of the metabolome of a given system [2-3,5-6,12]	This is actually not possible because analytical instrumentation to date lacks global and non-biased applicability [5-6,12].
Metabolomics (within this text)	Umbrella term for several strategies aiming at the determination of the metabolome	
Metabonomics	"the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [13]	Although it has been reported that metabolomics and metabonomics may be used synonymously [14-15], both terms have also been confusingly described as subcategories of each other [2,15-16]. However, one can observe in the literature that the term metabonomics finds its principal application for the monitoring in biofluids of usually non-plant systems [12].
Targeted metabolite analysis	Quantitative analysis of a single metabolite or a selected group of metabolites [5]	This strategy is usually based on existing knowledge (e.g upon the metabolic pathway) and allows for the optimization of the extraction, separation and detection of the metabolite [12,14]
Metabolite profiling (or metabolic profiling)	"Identification and quantification of metabolites related through their metabolic pathway(s) or similarities in their chemistry" [12]	On the contrary [5] claimed that quantification is not necessary
Metabolite fingerprinting (or metabolic fingerprinting)	Rapid classification of sample types based on global metabolite profiles usually without quantification and identification [3,5-6,12]	[3] stated that chromatographic separation prior to detection (MS, NMR or IR) is not necessary
Metabolite footprinting (or metabolic footprinting)	Alternative approach to metabolic fingerprinting focusing on "direct, noninvasive, mass spectrometric monitoring of extracellular metabolites in spent culture medium" [23]	
Metabolic flux analysis (MFA)	Integration of enzymatic steady-state reaction rates in order to describe metabolic flux networks which is usually accomplished by the use of ^{13}C tracer experiments [22]	

Table 2. Advantages, limitations and applications of frequently used sampling and sample preparation techniques, separation modes, ionization methods, mass analyzing techniques, NMR, data processing tools and statistical approaches regarding metabolomic investigation.

Technique	Advantages	Limitations	Preferred application within metabolomics	Ref.
Sampling				
Quenching with cold methanol	immediate quenching of metabolism	provokes cell leakage causes cold shock (in particular to Gram-negative bacteria like *E. coli*)	quenching of cells and microbial samples	[24]
Quenching with 0.9% (w/v) NaCl at 0.5°C	slows down the conversion from ATP to ADP and AMP no cell damage		quenching of mammalian cells	[25]
Sampling by fast filtration and subsequent washing	less cell leakage when washing solution with optimum ionic strength is used	comparatively slow (< 30 s including washing) still cell leakage adsorption on filter material?	preparation of microbial samples	[24,26]
Extraction with organic solvents	possibility to mechanically enhance extraction efficiency by regulating temperature and pressure, by the use of microwaves or ultrasonic waves or by the execution of supercritical fluid extraction use of aqueous-organic mixtures → biphasic sample allows for fractionation and separate analysis	compatibility of the extraction solvent with the subsequent analytical step required no universal extraction solvent (loss of analytes)	tissue and cell extraction	[5-6]
Sample preparation (enrichment, purification)				
Liquid-liquid extraction	selectivity for non-polar analytes highly compatible with GC	low enrichment factor hardly automatable no on-line set up proper disposal of hazardous solvents required	sample preparation (of non-polar compounds) prior to GC-MS	

Solid-phase extraction	ideally permits both matrix separation and concentration not only offline, but also online set-ups coupled to LC-MS functionalized polymeric resins and mixed-mode sorbents provide better pH-stability and extend the range of diverse compounds (wide polarity range) that can be simultaneously extracted	possible loss of analytes compatibility of the elution solvent with the subsequent analytical step required	→ promising for global metabolomics	[5-6]
Direct injection	no loss of analytes	LC-ESI-MS: possible ion suppression and adduct formation due to high salt concentration LC-ESI-MS: possible negative effect on column performance and chromatographic separation	has been applied for metabolic fingerprinting in urine samples by LC-ESI-MS	[5-6]
Instrumental techniques				
Separation				
GC	high resolution high efficiency derivatization enhances stability easily combinable with EI-MS → reliable identity confirmation → no suppression of ionization efficiency →lower costs compared to HPLC-MS	analytes require thermal stability and volatility or … … appropriate derivatization → increases time, complexity and variance	targeted metabolomics (due to the rather small range of amenable analytes)	[5-6,12, 14]
HPLC	wide range of analytes amenable short sample runs with sub-2 µm phases various mechanisms of separation including mixed modes combinable with ESI- and APCI-$MS^{(n)}$ → structure confirmation	combination with ESI- and APCI-MS → potential suppression of ionization efficiency → eluent compatibility required	both targeted and global metabolomics investigation of disease markers	[5,12,14, 21]
CE	high resolution diminutive amounts of solvent and solvent waste range of analytes: charged, neutral, polar, hydrophobic reduced sample volumes	poor amount sensitivity need of time consuming preconditioning leading to long cycle times CE-MS: buffer compatibility required	both targeted and global metabolomics	[5,14]

Ionization				
EI	no suppression of ionization efficiency identity confirmation based on fragmentation (numerous databases)	no intact molecular ion produced	GC-MS	
CI including NICI (negative ion chemical ionization)	degree of fragmentation can be controlled by selection of reagent gas intact molecular ions are produced NICI: resonance capture ionization can be promoted by derivatization with groups of high electron affinity → sensitivity enhancement ($\geq 10^2$) → well suited for the analysis of complex mixtures	requires enhanced vacuum pump capacity and smaller orifices in order to maintain the pressure difference of 0.2 – 2 Torr in the ion source and $\leq 10^{-5}$ Torr in the mass analyzer	GC-MS (particularly GC-MS/MS)	[27]
ESI	suitable for both large and small molecules allows for ionization of polar (favored) and non-polar compounds	potential suppression of ionization efficiency adduct formation less suited for non-polar compounds when compared with APCI	LC-MS: both targeted and global metabolomics	[21]
APCI	well suited for ionization of non-polar compounds	potential suppression of ionization efficiency small range of amenable compounds	LC-MS: targeted metabolomics (e.g. lipids, sterols)	[21]
Mass analyzer				
Quadrupole	high repeatability large dynamic range sensitivity can be enhanced using selected ion monitoring (SIM) applicability to HPLC, GC and CE	only nominal mass information low S/N ratio no MS^n capability	quantitative profiling	[21]
Triple quadrupole	MS/MS capability → fragmentation information for identity confir-mation → higher selectivity applicability to HPLC, GC and CE	only nominal mass information	quantitative profiling (and also metabolite identity confirmation)	[21]

Ion trap	MS^n capability → highly detailed fragmentation information for identity confirmation → higher selectivity possibility to accumulate very low concentrated analytes (especially with linear ion traps (LITs)) applicability to HPLC, GC and CE	barely applicable to quantitative tasks (trapping time needs to be included in the calculation of the quantity) only nominal mass information scan speed insufficient for fast chromatography with narrow peaks space charge effects in 3D ion traps resulting in low accumulation ability	metabolite identity confirmation	[21]
TOF	high scan speed and S/N ratio (compared to quadrupole), high selectivity due to minimum extraction windows (extracted ion current, EIC) high level of mass accuracy allows for determination of molecular formula great applicability to HPLC, GC and CE	no MS^n capability	quantitative profiling	[21]
Q-TOF	MS/MS capability → CID for structural analysis → higher selectivity high level of mass accuracy great applicability to HPLC, GC and CE		quantitative profiling and metabolite identity confirmation	[21]
FT-ICR-MS	very high level of mass accuracy and resolution MS^n capability → highly detailed fragmentation information for identity confirmation → higher selectivity	scan speed insufficient for fast chromatography with narrow peaks	metabolite identity confirmation	[21]
LIT-Orbitrap	very high level of mass accuracy and resolution MS^n capability → highly detailed fragmentation information for identity confirmation → higher selectivity	scan speed insufficient for fast chromatography with narrow peaks	metabolite identity confirmation	[21]

NMR				
NMR	high reproducibility non-destructive minimal sample preparation, high through-put inexpensive in terms of consumables ^{1}H-NMR: almost non-biased since most metabolites contain hydrogen ^{31}P is NMR active and monosisotopic → analysis of phosphate bearing compounds (e.g. phospholipids or nucleotides) high magnetic field strengths enhance S/N and spectral dispersion optimized cryogenic probe technology and micro scale RF coils enhance S/N and mass sensitivity applicability to HPLC, GC and CE	low sensitivity water suppression (1-D ^{1}H-NMR spectroscopy) ^{13}C and ^{15}N nuclei not suitable due to low abundance	metabolite identification and quantification targeted metabolic profiling (1-D selective TOCSY) NMR is the method of choice for structure elucidation of high-abundant metabolites	[6,14, 28-29]
MS Data evaluation				
Data processing				
XCMS	works with universal netCDF file format → platform-independent software freely available under the GNU General Public license offers nonlinear retention time alignment, matched filtration, peak detection and peak matching without using internal standards additionally provides several statistical analyses (e.g. univariate *t*-test) additionally provides visualization of large raw data sets such as hundreds of superimposed peaks has been designed for LC-MS data, but also adaptable to other data, e.g. GC-MS data		metabolite quantification and identity confirmation	[14,30-31]

MZmine	works with universal netCDF file format → platform-independent software additionally supports for mzXML data format is freely available under the GNU General Public License offers noise reduction, smoothing and peak detection, peak alignment and normalization allows for the performance of batch processing for numerous data files additionally provides visualization methods that allow for data comparing of multiple samples designed for LC-MS and GC-MS data		differential profiling	[14,31-33]
FiatFlux	open source software works with universal netCDF file format → platform-independent software offers two modules: metabolic flux ratio analysis (RATIO) and ^{13}C-constrained flux balancing (NETTO) designed for non-expert users: fast computation, simple integrates MS data processing suitable for large-scale studies	public release limited to glucose substrates ([1-^{13}C], [U-^{13}C] and mixes thereof) and GC-MS data of TDBMS-derivatized amino acids	metabolic flux analysis	[34-35]
Statistical data analysis				
Principal component analysis (PCA)	multivariate statistical analysis exploratory analysis: no a priori bisection in dependent and independent variables required "principal components": linear combinations of the manifest variables → reduction in the number of variables/dimensions → simplification → minor influence of noise (new variables are weigh-ted averages)	non-dynamic metabolomic data analysis according to [36] statistical software required → loss of information	classification of unknown samples data visualization	[36-38]
Analysis of variance (ANOVA)	is capable of handling dynamic data readily feasible (e.g. MS Excel)	univariate statistical analysis → only 1 dependent vari-able confirmatory analysis: a priori knowledge and hypothesis required	discovery of characteristic biomarkers of known samples	[36,39]

For the realization of a complete analytical procedure, several methods and processes need to be well considered because every single component has great impact on the quality of analysis: i) sampling, ii) sample preparation, iii) instrumental techniques – subdivided into separation, ionization, mass analyzer and NMR – and iv) statistical data evaluation. Some of the methods most commonly used nowadays in metabolic investigation, i. e. i) quenching with cold methanol or NaCl, fast filtration and extraction with organic solvents, ii) liquid-liquid extraction, solid-phase extraction and direct injection, iii) GC-MS(^{n}), LC-MS(^{n}), CE-MS(^{n}) and NMR and iv) XCMS, MZmine and FiatFlux (three recent and popular software tools for data processing) as well as principal component analysis (PCA) and analysis of variance (ANOVA) are discussed in Table 2 regarding their advantages, applications and limitations in the context of metabolomics. For identity confirmation several online metabolite-focused libraries for mass spectra and retention time indices are available. While it is straightforward to compare GC retention time indices and fragmentation patterns of GC-EI-MS, for LC-MS$^{(n)}$ the realization of both a universal retention time index and universal libraries of fragmentation spectra is doubtful due to the variety of column stationary phases and instrument-dependent CID fragmentation behavior of the target compound [6,40]. However, some databases allow for MS or MS/MS based search: E.g. the Human Metabolome Database (www.hmdb.ca) comprises 840 compounds with MS spectra and offers ionization polarity- and mass analyzer type-specific MS, MS/MS and GC-MS search. MassBank (www.massbank.jp) provides spectra for several hundreds of compounds obtained using ESI-TOF, -TripleQuad, -IT, -QTOF, -Orbitrap or -IT-TOF. The largest metabolite database to date is METLIN (metlin.scribbs.edu) describing a total of 25,000 metabolites; thereof 2,600 metabolites are characterized by High Resolution MS/MS spectra (both ionization polarities, collision energy 40 V). Regarding GC-MS spectra of metabolites, the Golm Metabolome Database (gmd.mpimp-golm.mpg.de) offers 1,418 spectra linked to metabolites applying GC-quadrupole or GC-TOF. The database also includes spectra of trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (TBMS) derivatives as well as retention time indices [40]. Further approaches towards identity confirmation are e.g. interpretation of the measured spectra and chromatogram (based on assignment of logical losses, isotope pattern, retention time etc.) or *in silico* fragmentation for computer assisted interpretation [14,41].

In conclusion, metabolomics shows great promise for the contribution to the understanding of the impact of genetic alteration as well as nutrition, toxins, environment and pharmaceuticals on the phenotype of an organism [6]. It has proven to be a powerful tool to e.g. search for biomarkers or support biotechnological applications such as metabolic modeling. Two major issues need to be taken into account when it comes to interpretation of metabolic data: i) MS-based quantification is critical due to instrument-, molecule- and matrix dependent bias. ii) Classical metabolomic strategies usually give steady-state concentrations of temporal points disregarding dynamics; only MFA is capable of providing a complete and true dynamic reflection of the phenotype [6]. Advances have been made in instrumentation and data handling [14]. Still, several aspects such as vi-

sualization tools, libraries or the design of sets of isotopic-labeled reference standards need to be developed or improved. Mass spectrometry – providing high selectivity, low detection limits, compatibility with various separation techniques and rapidly developing technologies – is a promising key tool in metabolomic research [6].

2 Plant Metabolomics

Remarkable pioneering work on metabolite profiling was accomplished using plant systems [42]: In the late 90's GC-FID or GC-MS was applied for the analysis of broad range of metabolites after derivatization (e.g. sugars, sugar alcohol, organic acids) in plants such as eucalyptus, acacia, apricots, potato tuber or *Arabidopsis thaliana* [43-46]. The latter plant species, also known as thale cress, is frequently used as a model organism in various plant sciences. In 2000, its genome containing 25,498 genes was the first plant genome to be completely sequenced – marking a milestone in the development of plant functional genomics [47]. Other plants, in particular crops, that are of interest in plant metabolomics and have been subjected to metabolite profiling further include e.g. rice, wheat, tomato, lettuce, cucumber, strawberry and tobacco [48]. The motivation and driving forces for plant metabolite investigation are diverse: i) in the context of human nutrition: plant-breeding improvement based on qualitative trait loci identification, safety-testing of genetically modified food, optimization of food processing techniques, ii) regarding elucidation of plant biological functions and interactions: metabolic responses to herbicides (in order to understand their mode of action), pathogen infection, temperature- and nutrient-related stress and salinity, iii) in the perspective of functional genomics: gene annotation and classification of genotypes and iv) concerning drug discovery: search and identification of exotic plant compounds [42,48-50].

When the human genome and metabolome are compared with those of *Arabidopsis*, the quantity of genes is approximately equal, whereas the diversity of the metabolites is much greater in the plant. Fernie ascribed the evolution of the complex metabolism in plants to their lack of motility resulting in the need of metabolic communication and defense devices [7]. The plethora of metabolites in plants (estimated 200,000 within the kingdom, approx. 15,000 for a single species [7]) renders plant metabolomics particularly challenging.

2.1 HILIC in plant metabolomics

Hydrophilic interaction liquid chromatography (HILIC) combines three benefits for metabolomic studies: i) the possibility of separating polar compounds, ii) the use of volatile and therefore MS-compatible eluents and iii) the solubility of the majority of polar

metabolites in the mobile phase [51]. In addition, the usually high content of organic solvent in the mobile phase provides favorable ionization conditions. Naidong reported a compound-dependent enhancement of the signal intensity for various substances that was in the range of one order of magnitude compared to low organic solvent containing reversed phase separations. Moreover, he stated that HILIC is applicable to biological samples that have been subjected to sample preparation methods such as liquid/liquid extraction, solid-phase extraction or protein precipitation [52].

Stationary phases applied for HILIC require hydrophilic characteristics. Column materials commonly used include e.g. underivatized nonmodified silica, silica featuring functional groups such as aminopropyl, amide, diol, cyclodextrin, cyanopropyl or sulfoalkylbetaine groups, and nonsilica-based amino packings [53]. The mobile phase consists of water-miscible polar organic solvents (most commonly acetonitrile (ACN)) and an aqueous phase, with the aqueous phase being the stronger eluting component [6,53]. The preference of ACN to other HILIC-applicable organic solvents such as 2-propanol and methanol possibly relies on its aprotic nature. The strong ability of methanol to establish hydrogen bonds can cause perturbations of the water layer (see retention mechanism below) and can provoke the formation of a more hydrophobic stationary phase diminishing retention of polar compounds [54]. In the context of the analysis of polar compounds in biological samples using HILIC on silica columns combined with MS/MS, Naidong summarized various mobile phases chosen by different authors and showed that the aqueous solution was frequently acidified by the use of an acid (formic acid or trifluoroacetic acid (TFA)) and/or a buffer (ammonium salts of acetate and formate) [52]. In general, convenient buffer concentrations vary from 5 to 20 mM and pH values from 3 to 8 for silica based stationary phases [55-56]. For HILIC gradients, the content of organic solvent typically ranges from 95 to 50 % [55].

The retention mechanism of HILIC is nontrivial. The proper HILIC mechanism – based on the formation and semi-immobilization of an enriched layer of the more polar eluent (which is usually water) on the surface of the stationary phase and a liquid/liquid chromatography between the bulk mobile phase and that layer acting as the effective stationary phase – rarely occurs in its pure form. Most real HILIC retentions are multimodal involving coloumbic interactions (referred to ion-exchange) or hydrogen bonding with the primal stationary phase or the above mentioned water-enriched layer. Hence, the HILIC retention mechanism is not merely characterized by liquid/liquid partitioning phenomena but also by adsorption- and electrostatics-related aspects [53].

Spagou et al. classified three groups of HILIC stationary phases according to the strength of electrostatic interactions: neutral (no electrostatic interactions), zwitterionic (weak electrostatic interactions) and charged (strong electrostatic interactions) [56]. Recently, sulfoalkylbetaine zwitterionic polymers immobilized on silica surfaces (commercially available as ZIC-HILIC© from SeQuant) have experienced increased popularity in HILIC applications [53,57]. These materials hold equimolar presence of a quaternary ammonium

group and a sulfonic acid group resulting in a zero net charge, but still feature the property of strongly binding water to surfaces.

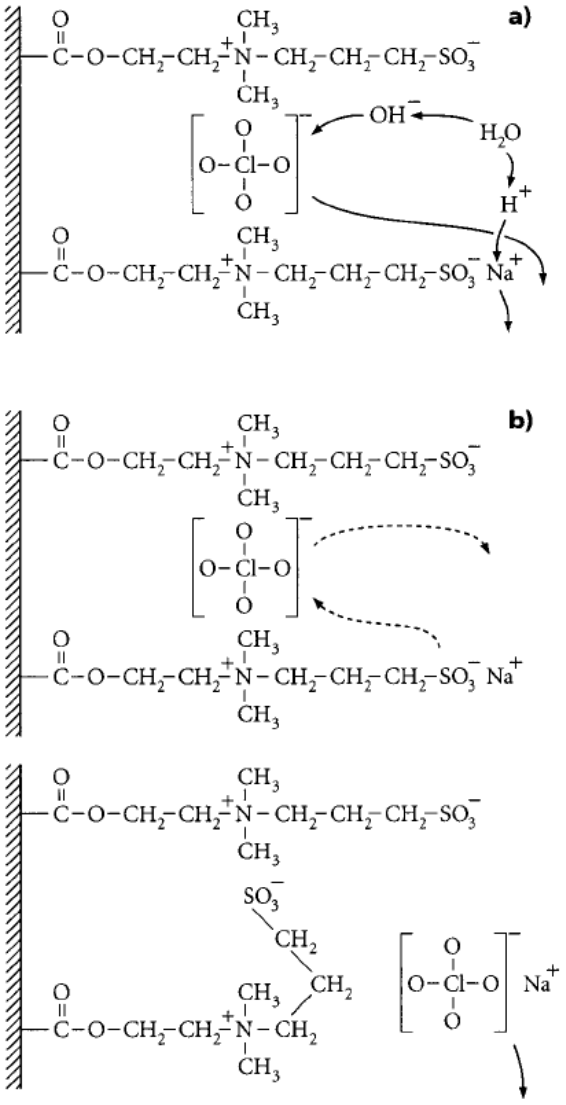

Fig.2. from Viklund et al. presenting a sketchy procedure of (a) water splitting and (b) zwitterion selfassociation as parts of the elution process for sodium perchlorate on a sulfoalkylbetaine zwitterionic phase [58].

That combination – the water-retaining capability and the low surface charge disadvantaging undesirable strong ion exchange interactions – offers beneficial use for HILIC separations [53,55]. Merely, the sulfonic group located closer to the mobile phase than the ammonium group slightly contributes to electrostatic interactions. It was found that this phenomenon was largely independent of pH and could be regulated using e.g. chaotropic/cosmotropic salts. Also, it was reported that the retention on the sulfoalkylbetaine zwitterionic phase was most robust against pH changes when compared to bare, aminopropyl and amide silica phases. It is worth mentioning that these materials were originally designed for chromatographic separation of inorganic salts [53]. The special mode of elution for ionic solutes on a sulfoalkylbetaine zwitterionics phase inte-grating water splitting and zwitterions selfassociation is shown in Fig. 2 reproduced from Viklund et al. [53,58].

It is obvious that HILIC promises great potential for metabolomic applications, since a substantial part of the metabolome comprises polar or highly polar compounds. Its nearly orthogonal selectivity to reversed phase chromatography awards HILIC an attractive approach to metabolic global analysis. The first implementation of HILIC in this perspective was in fact in the field of plant metabolomics [55-56], when in 2002 Tolstikov and Fiehn used a combination of HILIC and ESI-Ion Trap-MS for the analysis of various oligo-saccharides, glycosides, sugar nucleotides, amino sugars and amino acids. The selection of the reference compounds was based on a priori knowledge of metabolites present in dif-ferent plant species and on the purpose to cover a broad range of compounds with diverse physical and chemical properties. The authors employed the method for the analysis of phloem exudate samples from petioles of *Cucurbita maxima* (species of cultivated squash) leaves. Two

different HILIC columns, Polyhydroxylethyl A and TSK Gel Amide 80, were investigated and compared to RP obtaining the following findings: i) for both HILIC columns, the number of hydroxyl groups in oligosaccharides corresponded with the elution order, ii) also the presence of amino groups enhanced retention time on both columns presumably indicating that weak cationic interactions contributed to retention and iii) TSK Gel Amide 80 provided slightly higher separation efficiency, peak symmetry and retention time repeatability compared to Polyhydroxyethyl A [51].

Another interesting work was presented by t'Kindt et al. comparing the TSK Gel Amide 80 column with the Atlantis dC18 column (RP) for the analysis of plant extracts from *A. thaliana* leaves by LC-ESI-Q-TOF-MS. The latter stationary phase is designed to retain polar compounds in reversed phase mode; it is characterized by optimized silica particles (regarding pore size, ligand type, ligand density and endcapping) that prevent the repulsion of highly polar aqueous eluents from the silica pores and therefore allow for operation in 100% aqueous mobile phase. The suitability of both opposite stationary phases was assessed for metabolic analysis of plant extracts regarding repeatability of retention time and peak area, peak shape and matrix effect applying 20 standard compounds (amino acids, sugars, nucleotides, plant hormones, phenols etc.). In addition, the potential for unknown screenings was examined by subjecting the obtained LC-MS data of *Arabidopsis* leaf extracts to a software tool extracting mass-retention time pairs. It was found that the HILIC column helped to reveal a lot more mass-retention time pairs (503 compared to 321) and was capable to retain (capacity factor $k > 2$) more standard compounds (13 compared to 8) than the Atlantis column. On the other hand, concerning peak widths and repeatability of peak area the Atlantis column provided better performance. However, the authors did not discuss the influence of column length, particle size and flow rate in this context. Retentive repeatability and the grade of matrix effect were similar for both columns investigated [59].

A summary of several HILIC applications in the context of plant metabolomics is given in Table 3.

Table 3. Listing of several HILIC stationary phases used for plant metabolomic research. Description of functional groups, column parameters, mobile phase, flow rate, investigated sample and metabolites, retention time (RT) range of the analytes, applied detector and absolute LOD.

Ref.	HILIC stationary phase	Functional groups	Column parameters	Mobile phase	Flow rate
[51]	TSK Gel Amide 80	carbamoyl groups	250 x 2.0 mm, 5 µm d_p	ACN / 6.5 mM ammonium acetate pH 5.5	150 µL min^{-1}
	Polyhydroxylethyl A	poly(aspartamide)	150 x 1.0 mm, 5 µm d_p		150 µL min^{-1}
[59]	TSK Gel Amide 80	carbamoyl groups	250 x 2.0 mm, 5 µm d_p	ACN 0.1 % HCOOH / H_2O 0.1 % HCOOH pH 3	n.a.
[60]	Develosil Diol-100	bare silica	250 x 1.0 mm, 5 µm d_p	ACN 1% acetic acid / MeOH/H_2O 1% acetic acid	50 µL min^{-1}
[61]	Develosil Diol-100	bare silica	250 x 1.0 mm, 5 µm d_p	ACN 1% acetic acid / MeOH/H_2O 1% acetic acid	50 µL min^{-1}
[62]	Immobilized polyacrlyamide on monolithic silica	polyacrylamide	267 mm x 200 µm	ACN / 13 mM ammonium acetate pH 5.5	200 µL min^{-1}
[63]	ZIC©-HILIC	sulfoalkylbetaine	150 x 2.1 mm, 3.5 µm d_p	ACN 0.1 % HCOOH / 5 mM ammonium acetate 0.1% HCOOH pH 4)	200 µL min^{-1}
[64]	Zorbax RX-SIL	fully hydroxylated silica	50 x 4.6 mm, 1.8 µm d_p	ACN / H_2O 0.1% HCOOH pH 2.7	500 µL min^{-1}
[65]	Atlantis HILIC Silica	bare silica	150 x 2.1 mm, 3 µm d_p	ACN / 100 mM ammonium formate	250 µL min^{-1}
[66]	ZIC©-HILIC	sulfoalkylbetaine	150 x 1.0 mm	ACN 10 mM ammonium acetate / 30 mM ammonium acetate ACN	150 µL min^{-1}
	ZIC©-cHILIC	phosphorylcholine	150 x 1.0 mm		150 µL min^{-1}
[67]	Polyhydroxylethyl A	poly(aspartamide)	100 x 2.1 mm, 3 µm d_p	ACN / 5.5 mM ammonium acetate pH 5.5	180 - 450 µL min^{-1}

Table 3 continued.

Ref.	Sample	Target analytes	RT-range	Detector	LOD
[51]	*Cucurbata maxima*: phloem exudates from pietoles	N-acetyl-D-glucosamine, sucrose, D-raffinose, L-methionine, N-methyl-1-deoxynojirimycin, l-alanyl-L-alanine, 1,4-dideoxy-1,4-imino-D-arabinitol, stachyose, glucusaminic acid, 2-amino-2-deoxy-D-glucose, maltoheptaose	15-46 min	3D ion trap	stachyose: 0.5 ng
[59]	*Arabidopsis thaliana*: leaf extracts	L-lysine, L-leucine, L-serine, L-phenylalanine, γ-glutamyl-cystein, *S*-adenosyl-L-methionine, D-(+)-galactose, maltotriose, adenine, uracil, cytidine, IMP, UMP, zeatin, giberellic acid, (±)-jasmonic acid, (±)-*cis,trans*-abscisic acid, epibrassinolide, spermidine, chorismic acid, *p*-coumaric acid	run time: > 35 min	Q-TOF	n.a.
[60]	green tea (supermarket)	procyanidins, prodelphinidins, propelargonidins, phenolic acids, flavones, flavonols	run time: 70 min	Q-TOF	n.a.
[61]	Cocoa beans, apples (supermarket)	procyanidins	run time: 70 min	Q-TOF	n.a.
[62]	extracts from soybean seeds, corn kernels and leaves of *A. thaliana*	glucose, maltose, maltotriose, maltotetraose, maltoheptaose, maltohexaose, maltoheptaose, ribose, sedoheptulose, sucrose, trehalose, raffinose	1.3 – 12 min	LIT	sucrose, trehalose: 0.5 fmol
[63]	leaf extracts from *A. thaliana* (wild type and mutants)	*myo*-inositol, sugars: fructose, glucose, succrose, trehalose, raffinsoe, stachyose, verbascaose; sugar alcohols: sorbitol, mannitol, maltitol, galactinol; sugar phosphates: glucose-1-phosphate, fructose-6-phosphate, sucrose-6-phosphate, trehalose-6-phosphate, glucose-6-phosphate, fructose-1,6-biphosphate	7-14 min	3D ion trap	0.05 - 1.0 pmol
[64]	extracts from *Cyathula officinalis*	saponins	run time: > 30 min	TOF	n.a.
[65]	extracts from *Lonicera japonica*	uracil, uridine, pseudouridine, nicotine, propanolol, atenolol, palmatine, 4-hydroxybenzoic acid, 4-nitrobenzoic acid, N_2,N_2-methylguanosine, adenosine, hippuric acid, anisic acid, 4-chlorobenzoic acid, isonicotinic acid, Trp-Phe	2-23 min	UV	n.a.
[66]	*Arabidopsis thaliana*: xylem sap; wheat: press sap	phytosiderophores (epi-hydroxymugineic acid, deoxymugineic acid, mugineic acid, nicotianamine) and respective metal complexes wtih copper, nickel and zinc; amino acids (His, Glu, Asp, Arg, Lys); synthetic chelates (EDTA, EDDHA and Fe-complexes thereof); carbohydrates (glucose, inositol, glucuronic acid, sucrose)	1-23 min	3D ion trap	n.a.
[67]	extracts from potato tuber	glycoalkoloids (e.g. *α*-chaconine, *α*-solanine)	4-6 min	Triple Quad	< 0.06 ng

2.2 ICP-MS in plant metabolomics

The study of metal(loid)-binding metabolites represents a highly relevant research area within metabolomics, since many biochemical processes are metal-dependent. However, molecular mass spectrometry alone is barely capable of fundamental investigation of metal-metabolite complexes in biological samples, particularly at trace and ultra trace levels. This is partly due to the loss of information on complex behavior during the ionization process and formation of unwanted artificial metal-metabolite adducts, respectively. Moreover, analytical precision, accuracy, sensitivity and linear dynamic range are often insufficient. The implementation of element specific detection using inductively coupled plasma mass spectrometry (ICP-MS), on the other hand, promises great potential as it provides a number of benefits: versatility (the majority of elements are amenable), isotope specificity allowing for straightforward internal standardization or isotope dilution mass spectrometry (IDMS) and hence high precision and accuracy, high sensitivity and low LODs (down to the fg-range or even lower), large linear dynamic range ($10^5 – 10^6$) and a signal intensity being nearly independent of sample matrix and molecular environment. The hyphenation of ICP-MS with separation techniques enables molecule-specific detection and therefore speciation analysis. In this context, ion-exchange and reversed phase chromatography and sometimes capillary electrophoresis are most commonly applied in metabolomics, whereas size-exclusion chromatography – although very popular in metalloprotein research – is rarely used, since the poor selectivity of this separation mechanism is not able to cope with the diversity of the metabolome [68].

The target elements relevant to plant metabolomics comprise on the one hand nutrients (Fe, Cu, Mn, Zn,) and toxic metals (Al, Ag, Cd, Hg, Pb) complexed by metabolites and on the other hand heteroelements covalently bond to metabolites (Se, As, P, S). The latter group includes, e.g. i) phospholipids that can be found in the membranes of all types of cells and ii) organoselenium compounds like selenomethionine whose analysis in Se-accumulating plants potentially contributes to the understanding of the selenium metabolism in order to improve food crops and phytoremediation mechanisms of Se-contaminated soils. Metal-chelating metabolites play key roles in various processes such as heavy metal tolerance, metal homeostasis mechanisms in hyperaccumulating plants, solubilization of essential elements, nutrient uptake and metal transport. The respective organic ligands include phytochelatins, hydroxy acids and phytosiderophore [68]. Phytchelatins are oligopeptides structurally related to glutathione with the general formula $(GluCys)_nGly$ (Glu, glutamic acid; Cys, cysteine; Gly, glycine; n between 2 and 11) and participate in detoxification processes (primarily Cd) [69]. For information on hydroxy acids and phytosiderophores see section 3.2.

Phosphorus speciation, i.e. the identification and/or quantification of defined chemical species of phosphorus and the determination of their distribution in a system [68], within the intra- and extraorganismal biochemistry of plant roots including the rhizosphere (see section 3.2) is of special interest because organic phosphorus compounds as well as

inorganic phosphate constitute key components in the mechanisms of plant P nutrition [70-71]. These organic compounds comprehend mainly phosphate monoesters (such as inositol phosphates with its most dominant representative *myo*-inositol hexakisphosphate called phytic acid, sugar phosphates and mononucleotides), but also phosphate diesters (like nucleic acids and phospholipids), phosphonates and phosphoric acid anhydrides [70]. Several applications of HPLC-ICP-MS for the environmental analysis of organo-phosphorus pesticides and chemical warfare degradation products in soil and water have been summarized recently by Popp et al. [72]. In the context of plant metabolomics, speciation of phosphorus using LC-ICP-MS has not been reported yet, to the best of my knowledge. It is worth mentioning that the detection of ^{31}P by ICP-MS is nontrivial, since ^{31}P is monoisotopic, features a relatively high ionization potential and is interfered by $^{15}N^{16}O^+$, $^{14}N^{16}O^1H^+$ and $^{12}C^1H_3{}^{16}O^+$. However, using high resolution MS or reaction cell technology (monitoring of PO^+) eliminates these polyatomic isobaric interferences [68].

2.3 Metabolomics in rhizosphere research and the investigation of root exudates

The important functions of the root system of a plant – the „hidden half' – are not limited to anchorage and uptake of nutrients and water but also include the secretion of large quantities of highly diverse compounds into the soil. The volume of surrounding soil influenced by root activity is referred to as the rhizosphere (spatial extension in mm or sub-mm scale); the secreted compounds are termed root exudates. Root exudation comprises mainly the release of organic molecules but also comprehends the release of oxygen, water, inorganic acids and ions such as H^+. Those organic exudates include low-molecular weight compounds such as carbohydrates, amino acids, organic acids and phenols as well as high-molecular weight compounds like polysaccharides (mucilage) and proteins. As a matter of fact, almost every major type of compound that can be found in plants is released by the root, except for chlorophyll and certain photosynthesis-related substances. The mechanism of root exudation depends on the molecular weight, polarity, charge and exuded concentration of the respective compound and is either based on diffusion, ion channels, specific transporters or vesicles. Living root hairs, the root cap (see Fig.3) and actively growing primary and secondary roots are known to be involved in root exudation. The amount of root exudates is usually dependent on the age of the plant (decrease) and on soil stress like drought and lack of nutrients (increase). Generally, the proportion of carbon originating from photosynthesis products spent on root exudates is noticeable (e.g. approximately 30 – 40% during seedling stage). Root exudation drastically alters the chemical and biological characteristics (e.g. nutrient status) of the rhizosphere, which rigorously differs from the bulk soil and matches an „oasis in a desert' (according to [73]). Particular functions of root exudates are diverse and include but are not limited to the mobilization of nutrients, plant growth regulation, allelopathy (chemical interaction among plants), supply of energy for favorable soil microbes, promotion of bacterial movement towards the plant (chemotaxis), herbivore defense (e.g. via predator attraction),

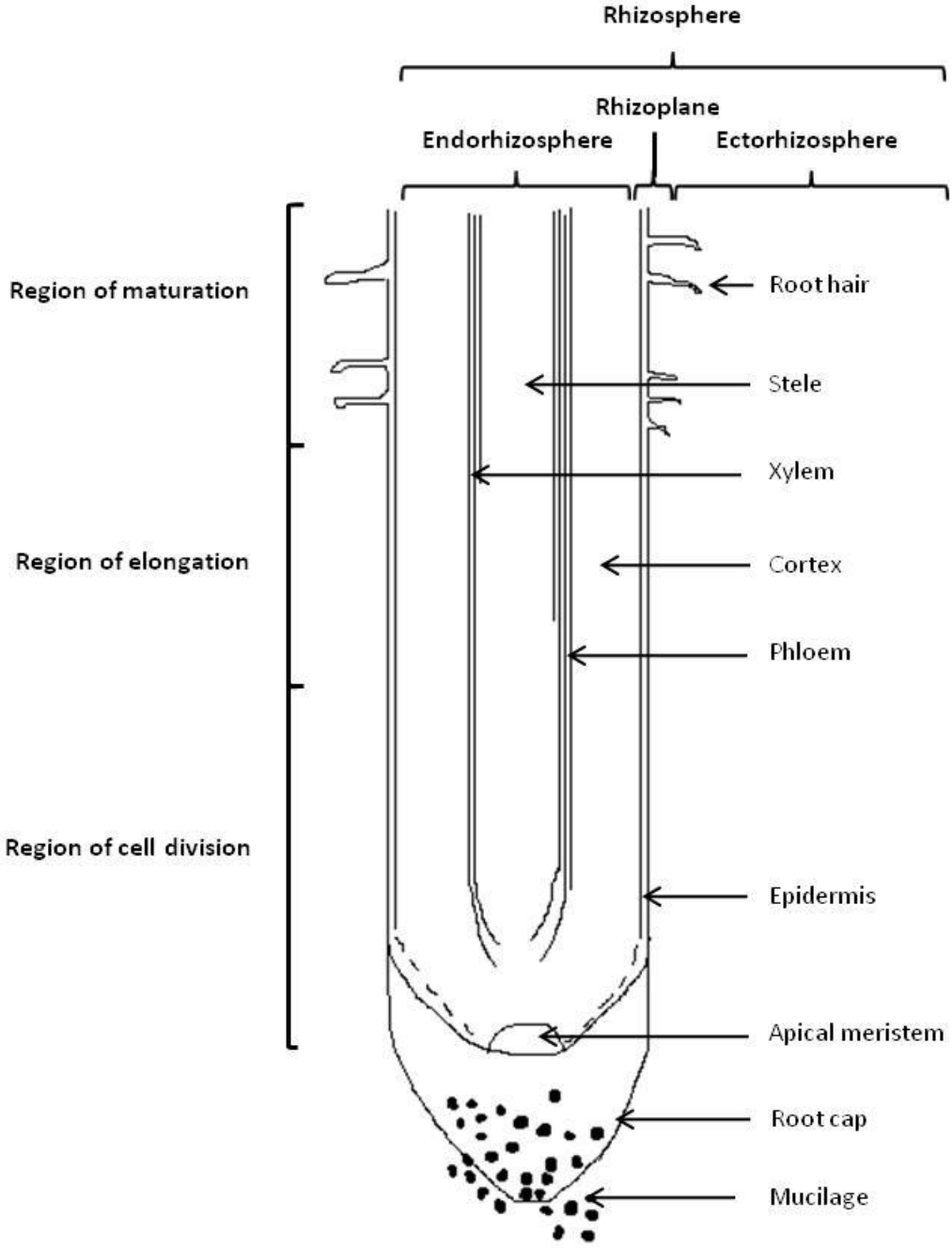

Fig 3. Longitudinal section of a root of a vascular plant in the soil, adapted from Bertin et al. [73] and modified. Caption: **endorizhosphere**, root tissue; **rhizoplane**; root surface including epidermis and mucilage; **ectorizhosphere**, soil altered by the presence and activity of the root (opposite to the bulk soil); **root hairs**, extensions of single epidermal cells comprising a substantial percentage of the total root surface area and constituting the major site of interaction between the plant and the rhizosphere; **stele**, entirety of vascular bundle related tissue; **xylem**, primarily dead cells transporting water and nutrients from the root throughout the plant; **cortex**, outer layer of the root located between endodermis and epidermis; **phloem**, living cells translocating photosynthates (e.g. sugars) from the photosynthetic active parts to the consuming parts of the plant; **epidermis**, cover of single-layered cells; **apical meristem**, growing tip; **root cap**, cells covering the apical meristem for protection, lubrication (decreasing frictional resistance) and graviperception; **mucilage**, polysaccharides secreted from the outer root cap cells and epidermal cells in the apical zone forming „mucigel' together with microbial cells and clay particles in order to enhance contact and interaction between root and soil [73-74,76].

engagement in tritrophic (plant-microbe-nematode) interactions and a possible involvement in the process of self/non-self discrimination in order to favor the growth of adjacent siblings and disadvantage strangers [73-75]. Three selected classes of compounds found in root exudates and their respective functions and roles in the rhizosphere are detailed in the following – with focus on metal chelate-based nutrient uptake.

Organic acids – primarily divalent and trivalent carboxylates such as malate, citrate and oxalate – have the ability to act as metal chelators. This capability is considered to have great impact on the availability of the macronutrient phosphorus which represents a major issue to plant development. Although phosphorus is usually abundant in soils, its bioavailability is low due to its common appearance as plant-inaccessible phosphate minerals incorporating various metals (Fe(III), Al(III) and Ca(II)). Organic acids may i) release plant-available phosphate by forming strong complexes with Fe^{3+}, Al^{3+} or Ca^{2+} of phosphate minerals, ii) enhance P availability by blocking adsorptions sites of P on soil particles or iii) influence the availability by stimulating microbial activity in the rhizosphere. However, research on the correlation between exudation of organic acids and P acquisition has not been completed yet and has only been reported for certain plant species. Further studies – particularly those carried out in live soils – are still required for in-depth elucidation of the proposed effects. Other suggested or reported functions of organic acids in the rhizosphere include potential influence on the availability of micronutrients (Fe^{3+}, Mn^{2+}, Cu^{2+} and Zn^{2+}), involvement in the mechanism of Al^{3+} tolerance, bacterial chemotaxis, soil structural improvement and alleviation of oxygen deficiency [75,77-79].

Phytosiderophores are nonproteinaceous amino acids that – as the greek name suggests (phýton, plant; síderos, iron; phorein, to bear) – are produced by plants in order to acquire iron (as Fe(III)). These compounds are of great importance, since the situation of Fe(III)-acquisition is similarly crucial to that of phosphorus: high Fe(III)-abundance in soils but low bioavailability, especially at high pH. The exudation of phytosiderophores is a strategy used by graminaceous plants like, e.g. rice or wheat in order to increase the solubility and mobility of Fe(III) and other micronutrients including Mn(II), Cu(II) and Zn(II) by complex formation of metal and phytosiderophore ligand. These complexes can be taken up by the plant via transport proteins [75]. The class of phytosiderophores comprises compounds like nicotianamine, mugineic acid, avenic acid, distichonic acid and various derivatives thereof. Biosynthesis of several phytosiderophores has been presented by Ueno et al. starting from the methionine cycle (see Fig. 4) [79].

Amino acids play a pivotal role in the soil nitrogen cycle [80]. They represent the prevalent organic N form in root exudates and in soil [81]. Symbiotic associations between plants and soil microbes are well-known phenomena, e.g. the fixation of atmospheric N in root nodules by rhizobia, or the increase of nutrient uptake by the presence of mycorrhizal fungi [75]. Together with carbohydrates and other low-molecular weight compounds amino acids constitute a major contribution to microbial nutrition. In temperate soils, where

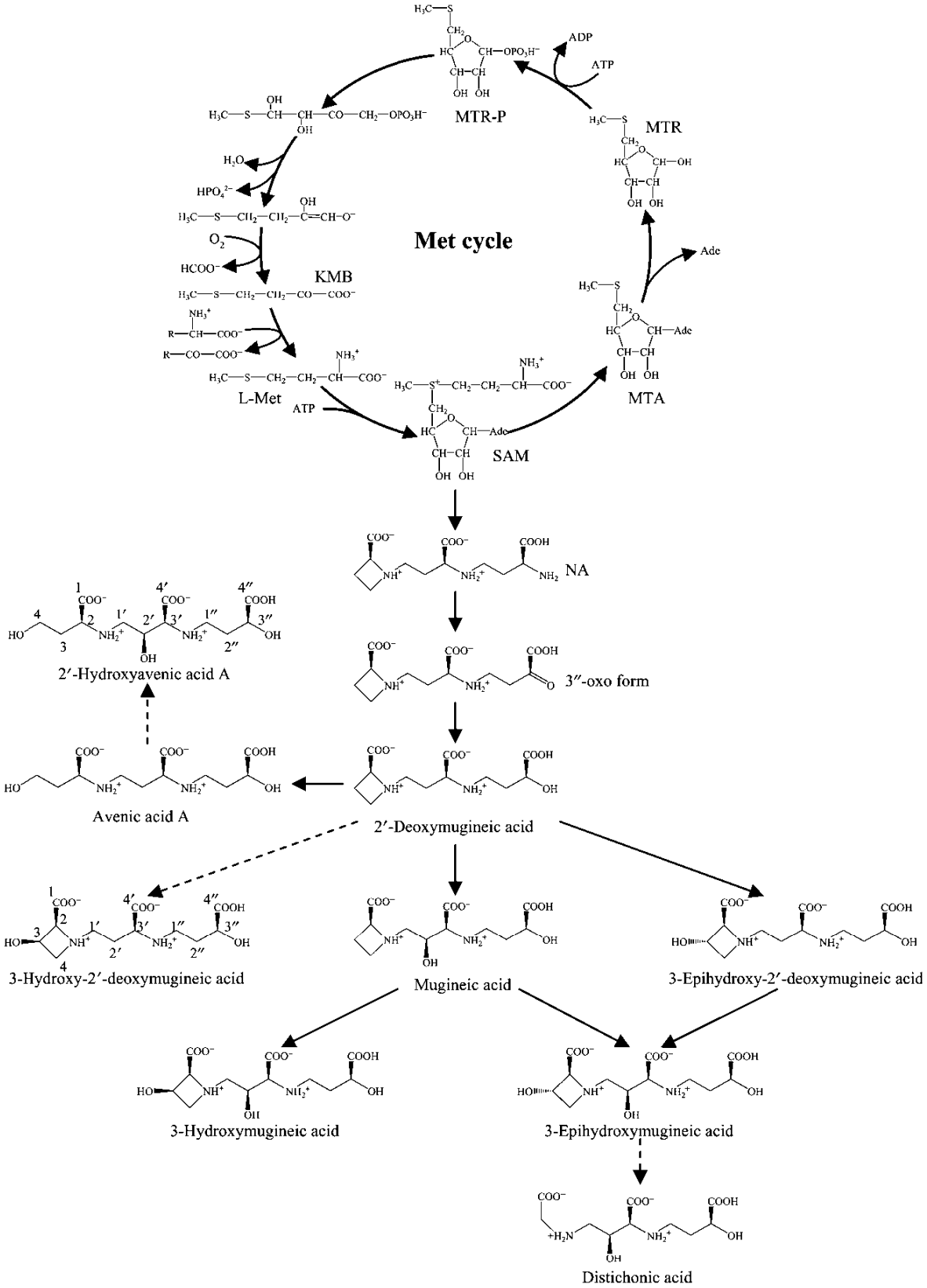

Fig.4. Reproduction from Ueno et al.: biosynthesis of different phytosiderophores (dashed arrows represent proposed pathways for two novel phytosiderophores). Caption: **MTA**, 5'-methylthioadenosine; **MTR**, 5-methylthioribose; **MTR-P**, 5-methylthioribose-1-phosphate; **KMB**, 2-keto-4-methyl-thiobutyric acid; L-Met, L-methionine; **SAM**, S-adenosylmethionine; **NA**, nicotianamine [79].

rhizosphere microbes are rather N- than C-limited, amino acids have been proposed to potentially play a decisive role in the nutrition of microbes [81]. Amino acids are also considered to stimulate chemotaxis of plant growth-promoting bacteria on root surfaces [75]. Regarding the mobilization of nutrients, however, (proteinaceous) amino acids show very low complexation abilities and therefore do not play a major role [82].

Investigation of root exudates is challenging regarding both sampling and analysis. Planting set-ups and sampling techniques aim at the optimum simulation of natural soil conditions on the one hand and at the slightest alteration of exudates concentration on the other hand, e.g. i) loss due to adsorption on the soil, ii) loss due to biodegradation by soil microorganisms or iii) increment due to microbial production. Concerning the analysis, complex matrices like soil or salts originating from nutrient solutions have to be taken into account as well as limited storage periods, low analyte concentrations and possibly very small sample volumes. When it comes to the investigation of root exudate-metal complexes, also complex stability has to be considered and analysis conditions and procedures need to be adjusted.

The work and technical advances in rhizosphere research accomplished in the recent years have yielded valuable information on root exudates-mediated communication below ground and have proven to be useful for phytoremediation and increased production of agricultural systems owing to improved defense mechanisms against soil-borne pathogens and enhanced plant-microbe interactions. However, the lack of detailed knowledge concerning root exudate transport systems, regulatory mechanisms, their behavior in soil and mode of action still impedes fundamental investigation of their relevance at the ecosystem level. There is an obvious demand for i) long-term studies under controlled conditions featuring high temporal and spatial resolution measurements, ii) sound validation of sampling and analytical methods and iii) reliable quantification strategies based on highly sensitive, rapid and robust analytical methods that provide information of a preferably broad array of target analytes [74-75,78].

Early implementation of hyphenated separation and mass spectrometric methods in rhizosphere research were presented by Tang et al. at the beginning of the 1980s. Benzyl isothiocyanate and twelve phenolic compounds were determined in root exudates using GC-MS [83]. Further pioneering work was realized by Fan et al. when they investigated amino acids, organic acids and phytosiderophores in root exudates of barley applying silylation GC-MS as well as by ^{1}H and ^{13}C multidimensional NMR [84].

More recently, Narasimhan et al. referred to the profiling of root exudates of different *Arabidopsis* strains as “rhizosphere metabolomics”. The identification via RPLC-MS of a total of 125 hydrophobic rhizosphere metabolites showed that the majority of these compounds were phenylpropanoids such as lignins, coumarins, flavonoids, aurones, sinapates and anthocyanins [85]. Although the identification strategy was not discussed in

depth and appeared to be objectionable, “rhizosphere metabolomics” seems an interesting and fertile approach.

Suzuki et al. also linked root exudates analysis to metabolomics presenting global metabolite profiling of rice root exudates. Samples were analyzed by GC-MS after methoxylation and trimethylsilylation. 71 compounds were identified according to retention index and mass spectrum: 53 sugars (xylose, tagatose, sorbose, fructose, mannose, galactose, talose, glucose, lactose, maltose, turanose, melibiose, isomaltose, gentiobiose, palatinose and several not specified mono-, di- and trisaccharides), three sugar alcohols (xylitol, ribitol and sorbitol), one sugar acid (gluconic acid), 11 α-amino acids (Val, Leu, Ile, Pro, Ser, Thr, Asp, Phe, Lys and Tyr), GABA (γ-aminobutyric acid), putrescine, *myo*-inositol and glycerol. Interestingly, no organic acids were detected [86].

In the course of this Ph.D. work two novel methods for the determination of low-molecular weight organic compounds in the context of root exudate analysis have been developed and published. Publication I (p. 36) deals with the analysis of underivatized amino acids by HILIC-MS/MS implementing a zwitterionic stationary phase for the separation of these polar analytes and a triple quad mass analyzer for highly sensitive and selective detection and quantification. Publication II (p. 49) describes the application of CE-ESI-MS and CE-ICP-MS to the investigation of phytosiderophores and their metal complexation behavior.

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Publications

I Hydrophilic interaction LC combined with electrospray MS for highly sensitive analysis of underivatized amino acids in rhizosphere research, *Dell'mour, M., Jaitz, L., Oburger, E., Puschenreiter, M., Koellensperger, G., Hann, S., Journal of Separation Science 2010, 33 (6-7), 911-922*

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Research Article

Hydrophilic interaction LC combined with electrospray MS for highly sensitive analysis of underivatized amino acids in rhizosphere research

Analysis of underivatized amino acids is challenging regarding the separation as well as the detection of these small polar analytes. Hydrophilic interaction LC using a 2.1 × 150 mm ZIC® (ZIC, zwitterionic)-hydrophilic interaction LC from SeQuant as stationary phase with 1% v/v formic acid in water and ACN as eluents was combined with MS/MS in multiple reaction monitoring mode for the separation and the detection of 16 underivatized amino acids. Regression coefficients of eight or seven point calibrations varied from 0.9454 to 0.9993. Absolute LODs and LOQ (on column) were in the fmol range (0.1–12 and 0.4–41 fmol, respectively). A fast screening method of 19 min total runtime has been developed offering applicability to samples from rhizosphere studies – characterized by low analyte concentrations and complex matrices. A successful application to the analysis of tyrosine in samples from soil adsorption experiments is presented as well as an evaluated enrichment procedure for amino acids derived from plant culture in nutrient solution.

Keywords: Amino acid analysis / Hydrophilic interaction LC / Rhizosphere / Triple Quad MS
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1 Introduction

Amino acid analysis has been for decades a big issue in the investigation of clinical, food and environmental samples. The importance of reliable quantification of amino acid traces in various matrices is without controversy. However, solutions to overcome its difficulties – affecting both chromatography and detection of these polar and low-molecular weight compounds – still partly remain to be optimized in a satisfactory way. Many different promising methodologies have been presented in the literature so far. Its beginnings and improvements as well as ongoing developments are summarized in the following.

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Abbreviations: FMOC, 9-fluorenylmethyl chloroformate; **HILIC,** hydrophilic interaction LC; **MRM,** multiple reaction monitoring; **OPA,** *o*-phthaldialdehyde; **TAHS,** p-*N,N,N*-trimethylammonioanilyl *N*-hydroxysuccinimidyl carbamate iodide; **ZIC,** zwitterionic

1.1 Amino acid analysis involving derivatization

In the 1950s automated amino acid analysis was born: Moore and Stein developed a separation of amino acids using ion exchange chromatography followed by post-column derivatization with ninhydrin and VIS detection [1]. This technique was commercially introduced by Beckman, remained the gold standard for decades and is still used nowadays. However, since the method suffers from long analysis time (110 min), low sensitivity and co-eluting compounds, it has been more and more substituted by methods implementing pre-column derivatization.

Pre-column derivatization strategies allow controlling both chromatographic and detection properties of the analytes but are still not free from drawbacks. *E.g.* pre-column derivatization with *o*-phthaldialdehyde (OPA) – first published in 1971 – offers favorable conditions for high-sensitivity fluorescence detection since the derivatives are highly fluorescent while OPA itself is nonfluorescent. However, derivatives are rather unstable and only primary amino acids are detected [2]. 9-Fluorenylmethyl chloroformate (FMOC) derivatization on the other hand provides universal derivatization and stable derivatives but requires removal of the excess FMOC due to its strong UV absorbance and its high fluorescence. This approach was introduced and optimized in the 1980s [3–5]; in this period also Hewlett Packard/Agilent presented an automated sequential combination of an optimized OPA and a

FMOC derivatization step prior to DAD and FLD detection offering absolute LODs (on column) of about 2–5 pmol and 20–50 fmol, respectively [6, 7]. Further development reduced analysis time from about 35 to 13.5 min [8].

Selected current studies on pre-column derivatizing agents, including reagents used for GC, are listed in Table 1 together with analysis times, detection mechanisms, absolute LODs, LOQs and applications of the corresponding methods. As can be readily observed, Shimbo *et al.* achieve the by far lowest LODs in the subfemtomol to attomol range using p-*N,N,N*-trimethylammonioanilyl *N*-hydroxysuccinimidyl carbamate iodide (TAHS). Their method also shows good linearity for 20 amino acids as well as applicability to comparative analysis of amino acids between TAHS and TAHS-d_3 samples but no studies on recovery, reproducibility or derivative stability are presented. Additionally, the reagent is not commercially available [13].

1.2 Analysis of underivatized amino acids

In times of LC-MS, the major advantages of amino acid determination techniques involving pre-column derivatization are (i) the enabling in most cases of reversed phase chromatography characterized by good reproducibility and symmetric peak shapes and (ii) shift of the molecular ion masses to higher *m/z* ranges usually showing lower background ion concentrations. However, for most of these approaches restrictions have been mentioned in the literature. *E.g.* the derivatization process of amino acids with FMOC or butanol is critical and strongly depends on concentration, pH and time. Furthermore, the formation of some multiply derivatized species complicates quantification [9, 16]. Uutela *et al.* studied memory effects and derivatization yield decreases upon high salt concentrations for some derivatizing agents [10]. Regarding GC-MS, a number of amino acids are not amenable due to secondary interactions with the liner, thermal instabilities or low vapor pressure [12]. Hence, when comparable sensitivity, reproducibility and linearity are given, measurement of underivatized analytes is always preferable to analysis of derivatized compounds in order to avoid additional sample preparation time and alteration of the sample and its analytes. Therefore, we consider appropriate LC separation and MS detection of underivatized amino acids to be of advantage for reliable and straightforward amino acid quantification. It is a matter of choice of the retention mechanism as well as of the compatibility with and the optimization of the MS detection to reach the qualities provided by the analysis of derivatized amino acids.

Considerable work on this field has been done since 1999, when Chaimbault *et al.* first published an LC-MS analysis of underivatized amino acids [17]. Preferred chromatographic techniques are ion pair reversed phase and hydrophilic interaction LC (see Table 2). Particularly in metabolomic research including metabolic flux investigation, the analysis of underivatized target compounds is favored. *E.g.* Rabinowitz and his group reported several different separation methods – thereunder different hydrophilic interaction LC (HILIC) columns – of more than hundred underivatized analytes including amino acids [32, 33]. In the biotechnological context, comprehensive analytical work was very recently realized by Preinerstorfer *et al.* [29]. Regarding the use of zwitterionic stationary phases, apart from the implementation of the ZIC® (ZIC, zwitterionic)-HILIC column listed in Table 2, Hoffmann *et al.* recently presented the investigation of quinine-based zwitterionic chiral stationary phases using chiral analytes including amino acids and amino acid derivatives [34].

1.3 Triple Quad detection of underivatized amino acids

A frequently used MS tool for the detection of underivatized amino acids is a triple quadrupole instrument in multiple

Table 1. Selected recent publications on amino acid analysis involving precolumn derivatizaion

Derivatizing agent	Detection	Analysis time/min	$LOD_{on\ column}$/fmol	$LOQ_{on\ column}$/pmol	Application	Ref.	Year publ.
OPA/FMOC	DAD - FLD[a)]	13.5	2000–5000 - 20–50	n.s.	Beers	[8]	2007
FMOC	DAD - FLD	40	n.s.	1–10	–	[9]	2009
FMOC	Triple Quad MS	20	7.5–75	n.s.	Rat brain microdialysates	[10]	2009
Butanol	Triple Quad MS	20	15	n.s.	Rat brain microdialysates	[10]	2009
Propyl chloroformate	Triple Quad MS	20	7.5–15	n.s.	Rat brain microdialysates	[10]	2009
Propyl chloroformate	GC-MS	14	5–2000	0.05–6	Human urine, mice urine	[11, 12]	2008, 2009
TAHS	Triple Quad MS	30	0.05–0.34	n.s.	Deproteinized rat blood plasma	[13]	2009
APDS[b)]	Triple Quad MS	12	n.s.	0.9–19.2	Deproteinized rat blood plasma	[14]	2009
AQC[c)]	Ion trap MS	50	2000–6000	n.s.	Soil hydrosylates	[15]	2009
iTRAQ®[d)]	Triple Quad MS	16+equilibration	n.s.	1–20	Urine	[12]	2009

a) Diode array detection–fluorescence detection.
b) APDS: 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate.
c) AQC: 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.
d) iTRAQ®: amine-specific isobaric tagging.

Table 2. Several retention mechanisms, columns and eluents chosen for the analysis of underivatized amino acids[a)]

	Stationary phases	Eluents	Detection	AT[b)]/min	aa[c)]	LOD[d)]/fmol	LOQ[d)]/fmol	Ref.	Year publ.
Ion pair	Purospher RP-18e 125 × 4 mm	0.5 mM PDFOA[e)]/ACN	Triple Quad MS	27	20	3500–7000	–	[17]	1999
	Supelcosil ABZ+Plus 150 × 4.6 mm	1 mM TDFHA[f)]/ACN	Triple Quad MS	40	20	3500–7000	–	[17]	1999
	Purospher RP-18e 125 × 4 mm	0.5 mM PDFOA isocr.	Triple Quad MS	20	20	0.2–2	–	[18]	2002
	QS UptiSphere 50 × 2 mm	0.5 mM TDFHA/ACN	Triple Quad MS	31	76	250–40 000	–	[19]	2005
	Discovery C18 50 × 2.1 mm	0.1% TDFHA/ACN	Triple Quad MS	15	40	1000–40 000	–	[20]	2006
	XDB-C18 50 × 2.1 mm	0.5 mM TDFHA/ACN	TOF MS	29	25	–	–	[21]	2007
	Purospher STAR RP-18e 125 × 2.1 mm	7 mM HFBA[g)]/ACN	Triple Quad MS	31	20	3–90	–	[22]	2008
	UPLC BEH C18 100 × 2.1 mm	0.5 mM TDFHA/ACN	Triple Quad MS	30	52	10–75	–	[23]	2009
HILIC	TSK-Gel Amide 80 100 × 2.1 mm	2.6 mM NH_4Ac/ACN	LCQ Ion Trap MS	90	6	–	–	[24]	2003
	TSK-Gel Amide 80 150 × 2.1 mm	2.5 mM NH_4Ac/ACN	Triple Quad MS	50	20	< 50 000	–	[25]	2006
	ZIC-HILIC 100 × 4.6 mm	5 mM NH_4Ac/ACN	Triple Quad MS	29	2	–	2.2, 154	[26]	2007
	ZIC-HILIC 100 × 4.6 mm	5 mM NH_4Ac/ACN	Triple Quad MS	30	4	1–20	–	[27]	2007
	ZIC-HILIC 250 × 2.1 mm	10 mM acetic acid/ACN	Single Quad MS	88	12	6–80	20–200	[28]	2009
	ZIC-HILIC 150 × 4.6 mm	20 mM NH_4Ac/ACN	Triple Quad MS	45	44[j)]	–	30–15 000	[29]	2010
SCX[h)]	Luna SCX 150 × 2 mm	30 mM NH_4Ac/5% CH_3COOH	Triple Quad MS	75	20	2000–60 000	–	[30]	2008
ANP[i)]	Cogent diamond hydride 150 × 2.1 mm	0.1% formic or acetic acid/ACN	TOF MS	15–19	19	–	–	[31]	2009

a) Selected validation parameters of the corresponding methods.
b) AT: analysis time.
c) aa: number of amino acids analyzed.
d) Absolute LODs and LOQs (on column).
e) PDFOA: pentadecafluorooctanoic acid.
f) TDFHA: tridecafluoroheptanoic acid.
g) HFBA: heptafluorobutyric acid.
h) SCX: strong cation exchange.
i) ANP: aqueous normal phase.
j) Including small peptides.

reaction monitoring (MRM) mode [17–20, 22, 23, 25–27, 29, 30, 35], not only for LC-MS but also for CE-MS methods [36, 37]. The major benefit of using a triple quad mass instrument in the MRM mode is high selectivity. A number of MRM transitions have been reported for underivatized amino acids. However, to the best of our knowledge, only single transitions for quantification purpose ($[M+H]^+ \rightarrow$ Quantifier) are mentioned in the literature for underivatized amino acids lacking a second transition ($[M+H]^+ \rightarrow$ Qualifier) for confirmation of the identity of the target compound. As a matter of fact, the simultaneous measurement of quantifier and qualifier transitions enhances reliability of results, which is especially important for the investigation of complex biological and environmental matrices, *e.g.* soil solutions.

1.4 Functions of amino acids in the rhizosphere

The present work aimed at the development of an LC-MS methodology, which is suitable for rapid analysis of amino acids in rhizosphere soil solutions. Moreover, we have adapted a strategy for selective pre-concentration of amino acids from samples derived from plant culture in nutrient solution (hydroponic) to subsequent LC-MS/MS analysis. Amino acids are part of the so-called root exudates together with other compounds such as organic acids, sugars, phenols, purines or vitamins [38]. Root exudates are actively released into the soil by the roots of plants in order to biochemically modify the rhizosphere, which is defined as the volume of soil affected by the presence of roots of a growing plant. Interactions between root exudates and rhizosphere include the mobilization of nutrients and the stimulation of microbial activities. Amino acids are known to take an active part in these processes but their exact role still needs to be clarified [38–40].

2 Materials and methods

2.1 Chemicals

ACN and methanol LC-MS grade were purchased from Fisher Scientific UK, Loughborough, UK. An Ultra Clear

basic system (SG Wasseraufbereitung und Regenerierstation, Barsbüttel, Germany) was used for purifying reverse osmosis water. Formic acid 98–100% v/v Suprapur® and hydrochloric acid fuming 37% v/v p.a. were obtained from Merck KGaA, Darmstadt, Germany. Aqueous ammonium hydroxide 25% v/v p.a. from Carl Roth, Karlsruhe, Germany, was used. Amino acid standard mix containing 1000 μM of each L-aspartic acid, glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-tyrosine, L-proline, L-arginine, L-histidine, L-glutamic acid, L-cystine, L-phenylalanine, L-lysine and L-methionine in 0.1 M hydrochloric acid was purchased from Agilent Technologies, Waldbronn, Germany. As internal standard L-4-hydroxyphenyl-$^{13}C_6$-alanine (termed ^{13}C-tyrosine in the text, min. 99 atom% ^{13}C) from Isotec, Miamisburg, OH, USA, was used. The components of the root exudate standard solution described in Section 2.3 are listed in the following: Sucrose (>99.0 %), tyrosine (>99.0%) and malate (puriss.>99%) were purchased from Fluka, Neu-Ulm, Germany. Oxalate (p.a.) and citrate (>99%) were obtained from Merck. Raffinose (>99%), histidine (>99%), caffeic acid (purum>95%) and *p*-coumaric acid (purum>98%) were purchased from Fluka.

2.2 Soil extracts for matrix effects evaluation

Extraction of amino acids from soil was performed after [41]: 4 g of an air dried Tschernosem soil (Hollabrunn, Lower Austria) were shaken with 20 mL of water on an end-over-end shaker for 15 min. After the shaking procedure, the soil suspension was centrifuged for 10 min at 6000 g at 4°C and the supernatant solution was transferred into polyethylene vials (20 cm^3). In total, 500 μL of methanol were added for analyte stabilization (storage at 4°C). For standard addition experiments samples were diluted 1:10 in 1% v/v HCOOH in ACN/H_2O 50:50 v/v and spiked with 7.5 μM of internal standard as well as with different concentrations of the Agilent amino acid standard mix (0, 0.1, 0.5, 2 and 5 μM).

2.3 Adsorption experiments

In total, 6 g of oven dried soil (105°C, 24 h) were equilibrated with 1.2 mL of water for 72 h at 20°C in order to adjust the water content to 20% w/w. The moist soil was then incubated for 2 h with 0.8 mL of a freshly prepared root exudate standard solution containing raffinose, sucrose, histidine, tyrosine, citrate, malate, oxalate, caffeic acid and *p*-coumaric acid in water at different concentrations ranging from 125 to 2500 μM resulting in a soil to solution ratio of 1:0.33 w/w. Incubation was either carried out at 20°C or at 4°C. Final concentrations of the analytes in the soil pore water – considering the soil water content – were 0, 50, 100, 250, 500, 750 and 1000 μM. Subsequent centrifugation at 3500 rpm for 15 min was performed as well at either 20 or 4°C. The obtained solution was spiked with 100 μL of methanol for stabilization of the analytes in the sample.

2.4 Enrichment procedure

In hydroponic samples, amino acids are expected to be present at very low concentrations. Hence, an enrichment procedure was adapted to our methodology following a procedure that had been proposed for cleanup of amino acids in plasma [21]. Enrichment was realized by implementing SPE. Strata-X-C cartridges from Phenomenex, Torrance, CA, USA, providing a mixed-mode stationary phase (strong cation exchange and reversed phase, 30 mg), were placed on a vacuum SPE manifold. The cartridges were conditioned with 1 mL of methanol, subsequently equilibrated with 1 mL of 0.1 M hydrochloric acid in water and loaded with sample volumes of 5 or 10 mL, while vacuum was applied. The SPE cartridges were then washed with 1 mL of methanol and afterwards, amino acids were eluted into test tubes using 1 mL of 5% v/v ammonium hydroxide in methanol. The solvent was evaporated by speed evacuation at 40°C and the samples were subsequently reconstituted with 100 μL of 1% v/v formic acid in a mixture of water and ACN (50:50 v/v).

2.5 HILIC-ESI-MS methods

2.5.1 LC-Triple Quad system

An Agilent G1312A Binary Pump 1200 series from Agilent Technologies, together with an Agilent G1367B high performance autosampler and an Agilent G1316A column compartment thermostated at 40°C was employed for HPLC using a 2.1 × 20 mm ZIC®-HILIC guard column (5 μm particle size) and a 2.1 × 150 mm ZIC®-HILIC separation column (3.5 μm particle size) from SeQuant, Umeå, Sweden, as stationary phase. In order to decrease gradient delay, a splitter unit (IDEX Health and Science, Oak Harbour, WA, USA) was installed in front of an inlet filter (polyether ether ketone frits, 0.2 μm pore size, IDEX Health and Science) and the columns reducing the initial flow rate of 800 μL/min to an effective flow rate of 100 μL/min, which is the recommended flow rate for 2.1 mm id ZIC®-HILIC columns. This set-up required an injection volume eight times higher than the volume effectively entering the chromatographic and MS system (40 compared with 5 μL, see Section 2.5.2). The applied gradient involved Eluent A (98% v/v water, 1% v/v ACN, 1% v/v formic acid) and Eluent B (98% v/v ACN, 1% v/v water, 1% v/v formic acid) according to the following timetable: 90% B was constant for 1 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 19 min.

For MS detection an Agilent 6410 Triple Quad LC/MS from Agilent Technologies was used featuring an ESI interface. Source parameters in positive ionization mode were set as follows: drying gas temperature 300°C, drying gas flow 8 L/min, nebulizer pressure 25 psi and capillary voltage 4000 V. MRM transitions of 16 amino acids have been determined *via* flow injection of a 20 μM amino acid standard mix (isocratic conditions: 1% v/v formic acid in ACN/H_2O 50:50 v/v). For optimization Mass Hunter Optimizer Software from Agilent was applied processing the following four steps in this order: (i) optimization of the isolation of the selected precursor ion by varying the fragmentor voltage, (ii) determination of the four most abundant product ions, (iii) optimization of the collision energies for each of these product ions and (iv) determination of the exact *m/z* value of the product ions. Out of the four proposed transitions, the two transitions featuring the highest signal to noise ratios were chosen for the individual amino acid detection with the quantifier showing the highest signal to noise ratio. Dynamic MRM was performed setting the retention times of the analytes, a time window of 2 min and a cycle time of 1000 ms. These settings resulted in a total of 29 transitions with a minimum dwell time of 59.00 ms (16 concurrent MRMs) and a maximum dwell time of 496.50 ms. Precursor and product ions as well as specific values for fragmentor voltage and collision energy are listed in Table 3.

2.5.2 LC-ion trap system

An Agilent Capillary Pump 1100 series, an Agilent μ-wellplate sampler, and an Agilent column oven from Agilent Technologies were used to realize the chromatographic separation described in Section 2.5.1 based on a ZIC®-HILIC column from SeQuant. Installation of a splitter unit was not required, since a built-in splitter was provided by the capillary pump facilitating fast gradients as well as lower injection volumes. Accordingly, flow rate and injection volume were 100 μL/min and 5 μL, respectively. For eluent and gradient conditions, see Section 2.5.1.

An Agilent 6430 Ion Trap LC/MS together with an ESI source from Agilent Technologies was used for MS detection. Monitoring was carried out in positive ionization mode and full scan mode ranging from *m/z* 50 to 1000. Further source parameters were nebulizer pressure at 25 psi, drying gas flow at 10 L/min, capillary voltage at 4000 V and drying gas temperature at 300°C. Data interpretation and peak integration was based on extracted ion chromatograms with $[M+H]^+ \pm 0.7$ *m/z* peak width.

3 Results and discussion

3.1 Evaluation of the HILIC separation and the Triple Quad detection in MRM mode

The objective of the present work was to develop a fast screening method of underivatized amino acids offering absolute LODs in the subpicomol to fmol range. Short chromatographic cycle times are important since the stability of the analytes is compromised; low LODs are necessary due to the low concentrations of amino acids in the samples to be analyzed. Additionally, the method has to deal with complex matrices, *e.g.* soil solutions. The resulting chromatograms

Table 3. MRM settings of the Triple Quad instrument

Amino acid	MW	Precursor ion	Quantifier	Qualifier	FV[a)]/V	$CE^{b)}_{Quantifier}$/V	$CE^{b)}_{Qualifier}$/V
Alanine	89.1	90.1	90.1[c)]	–	50	0	–
Serine	105.1	106.1	60.0	–	80	4	–
Proline	115.1	116.1	70.0	68.1	80	12	32
Valine	117.2	118.1	72.0	55.1	80	8	20
Threonine	119.1	120.1	101.9	74.1	140	4	8
Leucine	131.2	86.0	43.0	–	130	20	–
Isoleucine	131.2	86.0	57.0	–	130	20	–
Aspartic acid	133.1	134	115.9	–	80	4	–
Lysine	146.2	147.1	84.0	130.0	80	16	4
Glutamic acid	147.1	148.1	130.0	102.0	80	4	8
Methionine	149.2	150.1	132.9	104.0	80	4	4
Histidine	155.2	156.1	110.0	93.0	80	12	24
Phenylalanine	165.2	166.1	120.0	103.0	80	8	28
Arginine	174.2	175.1	116.0	130.0	110	12	12
Tyrosine	181.2	182.1	164.9	135.9	80	4	8
Ring-$^{13}C_6$-tyrosine	187.2	188.1	170.9	141.9	80	4	8
Cystine	240.3	241.0	151.9	119.9	80	8	16

a) Fragmentor voltage.
b) Collision energy.
c) Pseudo-MRM.

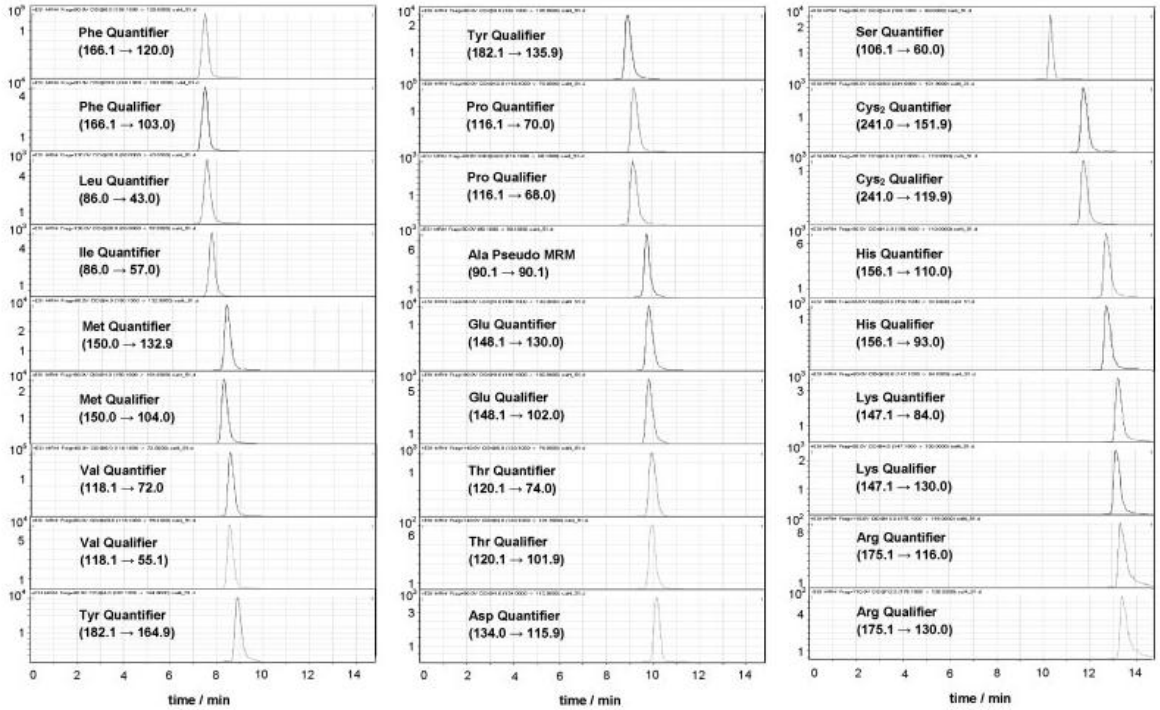

Figure 1. Extracted chromatograms of 27 MRM transitions for 16 amino acids (2.5 μM in aqueous standard solution) obtained by HILIC-Triple Quad MS.

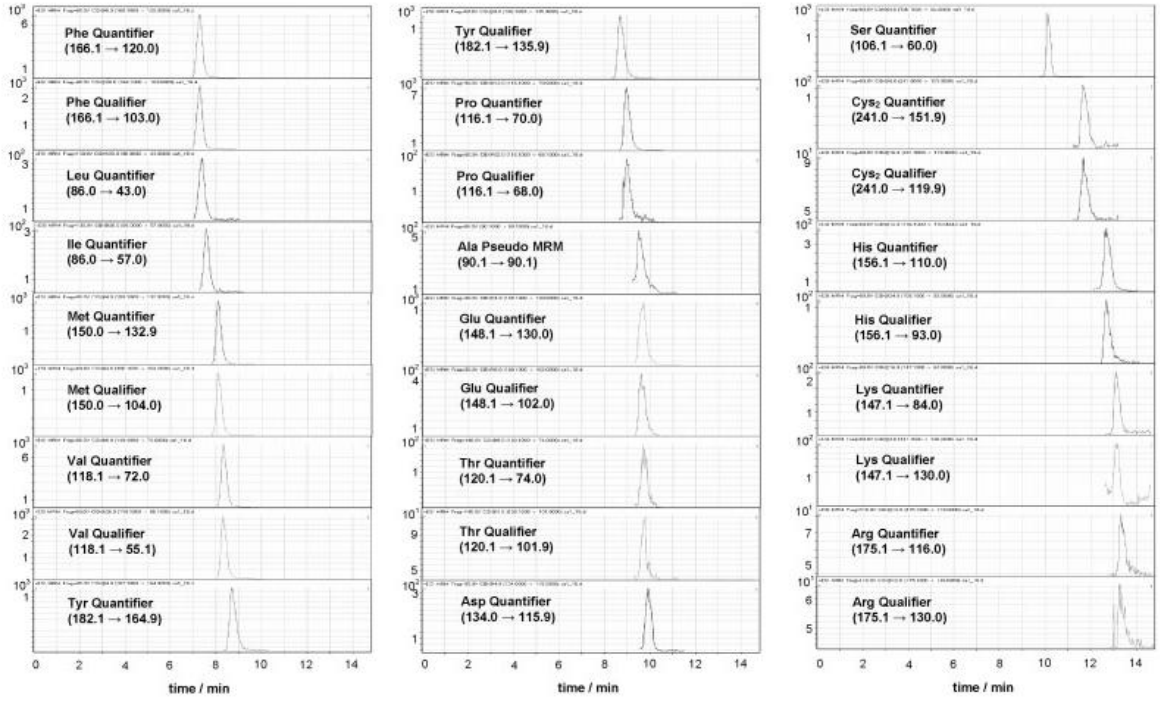

Figure 2. Extracted chromatograms of 27 MRM transitions for 16 amino acids obtained from a soil extract sample with analytes spiked at a low concentration level (0.1 μM) applying the proposed HILIC-Triple Quad MS method.

Table 4. Analytical figures of merit of the HILIC-Triple Quad MS method developed for separation and quantification of underivatized amino acids in aqueous standard solution (five repetitive measurements within approx. 18 h) as well as in matrix (soil extracts; three repetitive measurements within approx. 10 h)

Amino acid	Retention time/min (RSD%; $n = 40$)	Calibration range/µM	No. of calibration points	Regression type[a)]	Correlation coeff. r^2	Slope
Alanine	10.2 (1.6)	0.5–10	7	1/x weighted linear	0.9953	0.4475
Serine	10.7 (1.5)	0.5–10	7	LINEAR	0.9954	1.0146
Proline	9.6 (1.4)	0.05–10	8	Quadratic	0.9981	8.7316
Valine	9.0 (1.5)	0.05–10	8	1/x weighted linear	0.9977	9.5765
Threonine	10.3 (1.5)	0.5–10	7	Quadratic	0.9636	0.0749
Leucine	8.0 (1.6)	0.05–10	8	Quadratic	0.9987	0.3046
Isoleucine	8.2 (1.6)	0.05–10	8	Quadratic	0.9987	0.2726
Aspartic acid	10.5 (1.4)	0.05–10	8	Linear	0.9918	0.1649
Lysine	13.6 (1.1)	0.05–10	8	Quadratic	0.9955	0.1717
Glutamic acid	10.3 (1.7)	0.05–10	8	Linear	0.9917	0.6862
Methionine	8.7 (1.6)	0.05–10	8	Quadratic	0.9989	1.7107
Histidine	13.1 (1.2)	0.05–10	8	1/x weighted linear	0.9884	0.5103
Phenylalanine	7.9 (1.6)	0.05–10	8	1/x weighted linear	0.9993	7.563
Arginine	13.8 (1.2)	0.5–10	7	Linear	0.9454	0.0683
Tyrosine	9.3 (1.4)	0.05–10	8	Linear	0.9982	1.3892
Cystine	12.2 (1.2)	0.5–10	7	Quadratic	0.9957	0.1068

Table 4. Continued

Amino acid	Repeatability of the ratio peakarea$_{qualifier}$/peakarea$_{quantifier}$/%RSD ($n = 40$ injections)	$LOD_{conc.\ based}$/nM	$LOQ_{conc.\ based}$/nM	$LOD_{on\ column}$/fmol	$LOQ_{on\ column}$/fmol	Correlation coeff. r^2 in matrix[a)]	Slope in matrix
Alanine	–	12	41	62	206	0.9967	0.4641
Serine	–	0.6	2.1	3.1	10	0.995	1.036
Proline	10	0.4	1.4	2.1	7.0	0.9989	10.4784
Valine	3.7	0.9	2.9	4.4	15	0.9989	9.6478
Threonine	4.0	8.3	28	41	138	0.9956	0.0991
Leucine	–	1.4	4.5	6.8	23	0.999	0.3372
Isoleucine	–	1.7	5.6	8.4	27	0.9996	0.2899
Aspartic acid	–	8.6	29	43	144	0.993	0.1957
Lysine	16	2.8	9.2	14	46	0.9947	0.2139
Glutamic acid	8.1	3.1	10	16	52	0.9956	0.8314
Methionine	2.4	0.4	1.4	2.0	6.8	0.9979	1.9476
Histidine	9.2	3.2	11	16	53	0.9953	0.5324
Phenylalanine	1.6	0.1	0.4	0.6	2.1	0.9989	8.4418
Arginine	20	5.6	18	28	93	0.9777	0.0543
Tyrosine	6.1	1.4	4.6	6.9	23	0.998	1.4238
Cystine	7.3	3.2	11	16	54	0.9954	0.1012

Amino acid	Calibration range in matrix/µM	No. of calibration points in matrix	Measured conc. in matrix (theoret. conc. = 0.1 µM)	Precision/%RSD	Measured conc. in matrix (theoret. conc. = 2 µM)	Precision/%RSD	Measured conc. in matrix (theoret. conc. = 5 µM)	Precision/%RSD
Alanine	0.1–5	4	0.120	17	2.084	6.9	5.221	4.8
Serine	0.1–5	4	0.116	4.4	1.887	6.1	5.102	5.5
Proline	0.1–5	4	0.069	9.6	2.264	4	5.411	2.5
Valine	0.1–5	4	0.086	2.1	1.978	2.8	5.051	3.8
Threonine	0.1–5	4	<LOQ	n.a.	2.346	6.4	5.539	6.6
Leucine	0.1–5	4	0.122	3.2	2.242	2.5	5.627	2.6
Isoleucine	0.1–5	4	0.113	4.9	2.151	4.8	5.425	0.4
Aspartic acid	0.1–5	4	<LOQ	n.a.	2.495	4.8	5.721	4.4
Lysine	0.1–5	4	0.094	18	2.349	3.3	5.666	5.4
Glutamic acid	0.1–5	4	<LOQ	n.a.	2.582	4.8	6.016	4.4
Methionine	0.1–5	4	0.097	4.5	2.225	4.4	5.440	3.9
Histidine	0.1–5	4	0.085	7.8	1.948	3.9	5.327	6.2
Phenylalanine	0.1–5	4	0.116	1.8	2.264	3.9	5.551	3.6
Arginine	0.1–5	4	0.090	36	1.665	12	3.987	12
Tyrosine	0.1–5	4	0.094	7.3	1.993	3.7	5.105	3.9
Cystine	0.1–5	4	0.129	5.4	1.937	3.5	4.807	5.3

a) Selection of the type of regression curve is based on results in Table 5.

and analytical figures of merit proving that the proposed method is able to cope with the mentioned challenges are presented in Figs. 1 and 2 and Table 4, respectively.

The extracted MRM chromatograms of the quantifier and – when monitored – qualifier transitions feature excellent symmetric peak shapes with an average peak width of 0.3 min at 50% peak height. Chromatographic separation of isobaric leucine and isoleucine was not in the focus of our work but could be partly achieved. Distinction of the two isomers was enabled *via* monitoring of selective transitions proposed by Bishop *et al.* (*m/z* 86.0→43.0 and *m/z* 86.0→57.0 for leucine and isoleucine, respectively) [42]. Since 86.0 is no $[M+H]^+$-ion but an in-source fragment, loss in sensitivity was observed when compared with transitions emanating from the respective $[M+H]^+$-ion. Regarding the order of elution of the 16 target compounds under the given HILIC conditions, amino acids with nonpolar side chains eluted first followed by those with polar side chains (except for tyrosine that in spite of its polar moiety eluted earlier than nonpolar proline and alanine). The three alkaline amino acids histidine, lysine and arginine eluted last in order of their basicity (His < Lys < Arg). Concerning the fragmentation of the amino acids, a neutral loss of *m/z* 46 can be readily deduced from the majority of the mass differences between precursor and product ions. Langrock *et al.* attributed this mass difference to the loss of formic acid [25]. The simultaneous monitoring of quantifier and qualifier transitions improved the reliability of our results and hence robustness against complex matrices such as soil and has not been published for underivatized amino acids in this context so far, to the best of our knowledge.

Validation results of the quantification properties of the presented method are listed in Table 4. It is remarkable that the repeatabilities of retention times were excellent within 40 repetitive injections, ranging from 1.1 to 1.7% RSD. This is noteworthy considering (i) the comparatively short reconditioning times of 10 min between the runs (*e.g.* the method proposed by Kato *et al.* requires an re-equilibration for 33 min resulting in a total run time of 88 min [28]), and (ii) the installation of an inactive splitter without flow control. High repeatability of retention times is relevant to allow for narrow time segments for the individual MRM transitions in order to reduce the number of overlapping time segments and hence enhancing sensitivity. For each of the individual calibration curves within the given ranges (either 0.05–10 μM or 0.5–10 μM) obtained after correction using ^{13}C-enriched tyrosine as internal standard, the goodness-of-fit of four curve fit types (linear, $1/x$ weighted linear, $1/x^2$ weighted linear and quadratic) were compared based on the maximum % relative residual error as described by Almeida *et al.* (see Table 5) [43]. The curve fit type providing the lowest value for the maximum % relative residual error was selected for calibration. The resulting regression coefficients varied from 0.9454 to 0.9993. The LOD and LOQ were calculated according to the 3σ and 10σ criterion, *i.e.* the three- and tenfold standard deviation of the noise quantified *via* single point calibration (after DIN 32465:2008-11) [44]. The lowest absolute LOD and LOQ (on column) were achieved for phenylalanine (0.6 and 2.1 fmol), while alanine featured the highest LOD and LOQ (62 and 206 fmol). Concerning the ratio of the peak areas qualifier/quantifier satisfying repeatability was accomplished ranging from 2.4 (Met) to 19.7 (Arg) %RSD.

Table 5. Calibration curve fit: maximum % relative residual error for different regression types for each individual amino acid[a)]

Amino acid	Linear regression	$1/x$ Weighted linear regression	$1/x^2$ Weighted linear regression	Quadratic regression
Alanine	12.4	**6.9**	7.9	14.6
Serine	**5.9**	6.4	7.0	7.7
Proline	17.2	17.1	17.8	**15.0**
Valine	8.6	**6.9**	9.8	10.2
Threonine	20.2	24.9	34.3	**20.1**
Leucine	5.3	6.8	9.1	**5.2**
Isoleucine	9.9	8.7	8.2	**8.1**
Aspartic acid	**9.0**	13.4	24.5	11.9
Lysine	29.5	13.7	22.6	**12.3**
Glutamic acid	**13.3**	15.3	21.7	13.3
Methionine	4.7	4.6	4.9	**3.2**
Histidine	32.6	**11.6**	20.0	20.4
Phenylalanine	5.7	**3.6**	5.5	7.1
Arginine	**20.3**	22.1	24.1	27.5
Tyrosine	**3.2**	4.8	8.6	3.4
Cystine	22.3	22.3	11.4	**8.1**

a) Selection of the curve fit type was based on the lowest values (enlarged numbers in bold).

The influence of the sample matrix was assessed *via* measurement of a standard addition experiment of soil extracts described in Section 2.2. For fitting of the calibration, the same curve fit types as for the calibration curves in aqueous solution were selected. Method accuracy for soil matrices was determined by calculating the concentrations of three repetitive soil extract samples with spiked analytes at theoretical concentrations of 0.1, 2 and 5 μM (low, middle and high concentration range, respectively) using external calibration. The average values of the respective measured concentrations for each of the investigated amino acids are presented in Table 4. As can be seen, we observed signal enhancement caused by the matrix in most of the cases indicating that the implementation of multiple internal ^{13}C-labelled standards for the individual amino acids instead of a single internal standard would be beneficial. However, regarding future routine analysis the rise in costs caused by employment of multiple ^{13}C-enriched internal standards is considerable. Precision in matrix is given as the relative standard deviation obtained for three independent measurements ranging from 0.4 (Ile, 5 μM) to 18% RSD (Lys, 0.1 μM). Figure 2 presents chromatograms obtained from a soil extract sample spiked with 16 amino acids at a concentration of 0.1 μM and demonstrates the applicability of the method to real samples.

3.2 Application to samples of adsorption studies

The proposed HILIC separation has been successfully applied to the analysis of adsorption experiments of the amino acid tyrosine with two different soils. Adsorption studies were carried out as described in Section 2.3. The centrifuged solutions were diluted as necessary, spiked with L-4-hydroxyphenyl-$^{13}C_6$-alanine as internal standard and injected into the LC-MS system. Adsorption isotherms for tyrosine on agricultural soil and grassland shown in Fig. 3a and b, respectively, were determined based on the generated LC-MS data. For fitting of adsorption curves Freundlich equation $q = A \times c^{(1/N)}$ was used with q corresponding to the concentration adsorbed at equilibrium (μmol/g), A to adsorption capacity (L/g), c to the equilibrium concentration of the adsorbate (μM) and $1/N$ corresponding to the bond energy to the mineral. As can be observed, the different mineral composition of the two experimental soils (different geographical and geological origin) clearly affects the sorption behavior of tyrosine to the soil matrix. Furthermore, the difference between the two sorption curves for each soil at the two experimental temperatures demonstrates the impact of microorganisms – that are active at 20°C – on adsorption only (4°C). Results were obtained implementing the HILIC-Ion Trap MS set-up, featuring LODs ranging from 0.05 to 2 pmol. Standard additions to selected samples proved that the method was robust against the investigated matrices (data not shown).

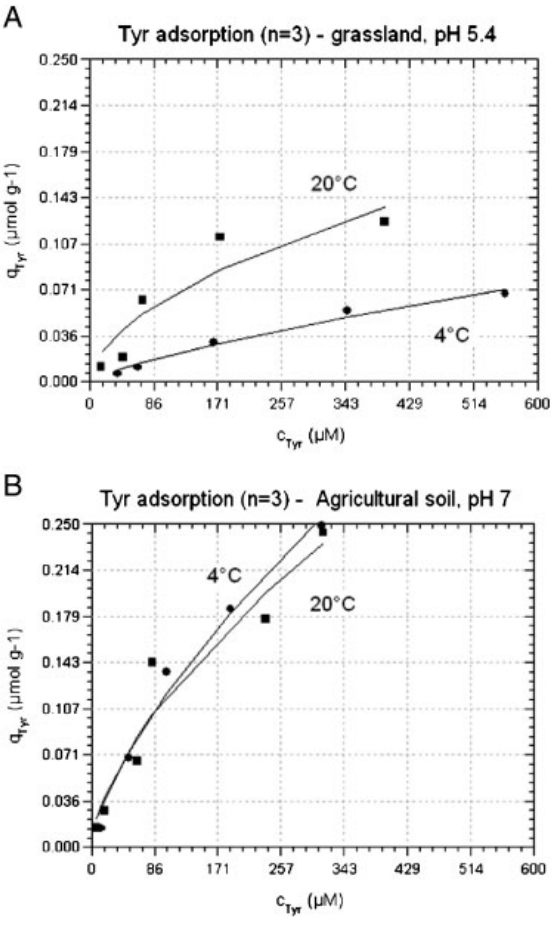

Figure 3. Adsorption isotherms. Concentration adsorbed at equilibrium (q_{Tyr}/μmol/g) against the equilibrium concentration of the adsorbate (c_{Tyr}/μM) for adsorption of tyrosine after 1 h of equilibration at 4 or 20°C on two different soils (6 g dried soil + 2 mL solution): grassland (A) and agricultural soil (B).

3.3 Evaluation of the enrichment procedure for hydroponic samples

Hydroponic samples are characterized by large sample volumes (approximately 50–100 mL) and low analyte concentrations and require therefore an enrichment step prior to analysis. The SPE procedure described in Section 2.4 was evaluated regarding two different figures of merit: (i) enrichment factors and (ii) linearity of a standard addition experiment with a hydroponic sample of *H. distichon*, cv. Bodega (spring barley). Enrichment factors were determined as follows: After measuring a standard solution containing each of the studied amino acids at a concentration of 2 μM, peak areas (normalized to the internal standard) were compared with the ones obtained from a 0.02 μM standard solution that had been enriched prior to analysis. Since the enrichment procedure reduced sample volume from 10 mL to 100 μL resulting in an enrichment of 1:100, the recovery would be 100% for the case both measurements yielded equal normalized peak areas. Enrichment factors were calculated based on these recoveries and are presented in Table 6. The enrichment process was carried out in triplicates in order to validate

Table 6. Results of the evaluation of the enrichment procedure for 12 amino acids: enrichment factors for a loading volume of 10 mL with uncertainties determined out of three independently enriched samples, linearity of a standard addition experiment performed on a hydroponic sample of spring barley using $^{13}C_6$- tyrosine as internal standard (four calibrants ranging from 50 to 1000 nM; for details see Section 3.3)

	Enrichment factor ($n = 3$)	Linearity (r^2)
Alanine	59 ± 8	0.9981
Proline	58 ± 5	0.9970
Valine	70 ± 10	0.9579
Threonine	13 ± 3	0.9593
(Iso)Leucine	43 ± 5	0.9938
Lysine	55 ± 6	0.8972
Methionine	33 ± 2	0.9973
Histidine	70 ± 9	0.9403
Phenylalanine	49 ± 6	0.9938
Tyrosine	63 ± 7	0.9997
Cystine	66 ± 8	0.9314

repeatability. Twelve out of 16 amino acids could be successfully enriched with enrichment factors of approximately 50. As a drawback, the acidic amino acids glutamic and aspartic acid as well as arginine could not be enriched using the proposed method. The amino acid serine was not included in this preliminary study, since the sensitivity of the full-scan mode of the ion trap MS system used for detection was compromised.

Next, standard additions were performed in order to validate the linear working range of enrichment procedure for hydroponic samples: prior to the enrichment step five 5 mL sample aliquots were spiked with different concentrations of an amino acid standard mix (0, 50, 400, 700 and 1000 nM of each amino acid). Again, the final sample volume after enrichment was 100 µL resulting in a volumetric enrichment factor of 50. The calibration curves obtained *via* HILIC-Ion Trap MS measurement (see Table 6) showed linearities between 0.8972 (lysine) and 0.9997 (tyrosine) revealing the benefit of the ^{13}C-enriched internal standard tyrosine, which was added prior to SPE in order to correct enrichment variations.

4 Concluding remarks

A fit-for-purpose method for investigation of amino acid composition in rhizosphere samples has been developed. Future work will include (i) further optimization of the solid phase extraction procedure by implementing a SPE-automatization device and (ii) the application to several different types of samples, such as hydroponics and further soil adsorption studies using various amino acids as well as root exudate samples derived from rhizobox studies [45].

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The authors have declared no conflict of interest.

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Research Article

Complexation of metals by phytosiderophores revealed by CE-ESI-MS and CE-ICP-MS

CE-ESI-MS and CE-ICP-MS were implemented for studying three phytosiderophores (mugineic acid, epi-mugineic acid and deoxymugineic acid) and their metal complexes. Free ligands and ferric complexes were analyzed using the first methodology, while six free metals (Co(II), Cu(II), Fe(III), Mn(II), Ni(II) and Zn(II)) together with the corresponding complexes were investigated by the latter technique. CE separation was realized at a voltage of +25 kV employing a BGE containing 20 mM ammonium bicarbonate at pH 7.2. Both techniques revealed limits of detection in the high nM to low μM range. Standard additions to hydroponic samples of *H. distichon*, cv. Bodega (spring barley) showed regression coefficients for the metal–ligand complexes ranging from 0.984 to 0.999. Additionally, results of a competitivity study allowed the determination of relative metal–phytosiderophore complex stability constants of deoxymugineic/mugineic acid.

Keywords:
CE-ESI-MS / CE-ICP-MS / Metal complexation / Phytosiderophores / Rhizosphere
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1 Introduction

1.1 Phytosiderophores in the rhizosphere

Phytosiderophores (PS) are organic ligands with similar structure to amino acids forming stable complexes with metals [1]. They are synthesized by graminaceous plants as a tool to control mineral concentrations in the rhizosphere by forming soluble metal–PS complexes. The rhizosphere is defined as the volume of soil affected by the presence of roots of a growing plant [2]. Intact metal–PS complexes of different metals are subsequently taken up by means of a transport protein, which implies that the role of PS to nutrition and toxicology is of great importance to plant physiology and human health [1, 3]. We are interested in understanding the interactions of PS with essential trace metal nutrients, in particular Fe(III). Iron is abundant in the lithosphere; however, its bioavailability is substantially compromised by the low solubility of iron oxides that is the largest pool of iron in most soils. In addition, iron oxides form in alkaline soils, which cover about one-third of the world's land surface area [3].

Mugineic acids (MAs) are the most common PS, including MA, hydroxymugineic acid (HMA) and deoxymugineic acid (DMA). They have three carboxyl and two amino functional groups in common but differ in their hydroxylation pattern. Under neutral conditions employed in our experiments, the MAs are zwitterions and singly negatively charged in total, as can be derived from the pK_a values in Fig. 1. The complexation of both trivalent and bivalent metals involves six functional groups forming hexadental complexes with octahedral shape. The difference between the complexation of trivalent (M^{3+}) and bivalent (M^{2+}) metals is the protonation state of the α-hydroxyl group. This functional group is deprotonated or protonated in the singly negatively charged metal–PS complexes of M^{3+} or M^{2+}, respectively [4].

1.2 Analysis of PS

Several different analytical methodologies have been applied for PS investigation, so far. LC was first implemented by Kawai *et al.* and Hiradate *et al.* [5, 6]. Kawai separated and determined MA and analogues by HPLC using fluorescence detection after post-column *o*-phthaldialdehyde-derivatization; the latter used ion pairing HPLC and colorimetric procedures for the analysis of MA, HMA and DMA and their iron complexes. UV detection after phenylisothiocyanate-derivatization and reversed phase LC of DMA, MA and epi-HMA and the corresponding Fe(III)-complexes was

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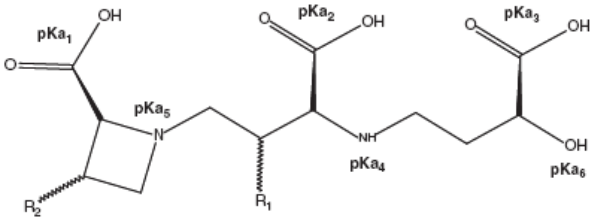
Abbreviations: **DMA**, deoxymugineic acid; **HMA**, hydroxymugineic acid; **MA**, mugineic acid; **PEEK**, polyether ether ketone; **PS**, phytosiderophores; **SFMS**, sector field MS



	MA	DMA
pKa_1	2.39	2.35
pKa_2	2.76	2.74
pKa_3	3.40	3.20
pKa_4	7.78	8.25
pKa_5	9.55	10.00
pKa_6	17.1	17.1

Figure 1. Chemical structures and pK_a values of MA derivatives [4, 31]. R_1 = α-OH, R_2 = H: MA; R_1 = β-OH, R_2 = H: 2′-epi-MA; $R_1 = R_2$ = H: 2′-DMA; $R_1 = R_2$ = α-OH: HMA; R_1 = β-OH, R_2 = α-OH: 3-epi-HMA.

published by Howe *et al.* [7]. Weber *et al.* studied free ligands of MA analogues in wheat and barley root washing samples using anion-exchange LC with pulsed amperometric detection [8]. Early MS measurements of free PS ligands were presented by Sugiura and Nomoto in 1984 (field desorption and electron impact mass spectra) and by Kenny and Nomoto (1994) applying FAB-MS/MS [9, 10]. In 1995 Kenny *et al.* also studied metal–PS complexes using the very same technique. More recently, Weber *et al.* (2006) proposed the application of nano-ESI FT ion cyclotron resonance MS for the analysis of iron(II) and iron(III) complexes of MA and nicotianamine (NA) [11]. The implementation of ICP-MS for speciation of metal–PS complexes was first realized by Schaumlöffel *et al.* and Vacchina *et al.* in 2003. Both suggested two different separation mechanisms – SEC and CE – combined with parallel ICP-MS and ESI-MS-MS detection for nickel speciation in plant extracts [12, 13]. More recently, Ouerdane *et al.* (2006) proposed a laborious method involving isolation of Ni-complexes in plant extracts by 2-D SEC-hydrophilic interaction liquid chromatography-ICP-MS and a subsequent identification using ESI-Q-TOF-MS/MS [14]. In 2006 a completely different approach was suggested by Bakkaus *et al.* hyphenating anion exchange LC with ICP-MS for the analysis of iron(III), nickel(II), cobalt(II) and copper(II) complexes of MA, DMA and EDTA [15]. Most recent published strategies for PS investigation comprise the combination of zwitterionic hydrophilic interaction chromatography (ZIC®-hydrophilic interaction liquid chromatography) with ESI-MS (Xuan *et al.*, 2006 and Weber *et al.*, 2008) and CE with UV or conductivity detection (Xuan *et al.*, 2007) [16–18].

1.3 Metal complexes stability constant determination by CE-ICP-MS

It has been shown very recently that the hyphenation of CE with ICP-MS is a viable tool for the determination of 1:1 lanthanum-oxalate and uranyl–oxalate complexes stability constants by applying affinity CE [19]. CE-ICP-MS has also been successfully implemented in order to study interactions of Np(V) and Pu(V) with sulfate, chloride and carbonate [20, 21] as well as for elucidation of lanthanide(III) carbonate complex formations [22]. Yin *et al.* summarized applications of CE-ICP-MS for the investigation of the kinetics and thermodynamics of metals-humic acids and metals-fulvic acids interactions aiming for the understanding of metal mobilization in natural soils and aquatic systems [23]: Sonke and Salters *et al.* and Kautenburger *et al.* studied the complexation of lanthanides with humic or fulvic acid [24–28]. Kautenburger concluded that CE-ICP-MS is a reliable and highly reproducible method for the determination of stability constants of heavy metal–humic acid complexes [27]. Therefore, we considered this technique an ideal approach that is able to meet the demands of the present analytical task, *i.e.* revealing mobilization properties of PS and detecting metal–PS complexes in hydroponic samples.

2 Materials and methods

2.1 Chemicals

Ammonium acetate puriss. p.a. and ammonium bicarbonate ReagentPlus™ ≥99.0% were obtained from Fluka Chemie AG, Buchs, Switzerland and Sigma Aldrich Chemie GmbH Steinheim, Germany, respectively. Sodium hydroxide-Monohydrate Suprapur®, acetic acid p.a. and nitric acid p.a. were purchased from Merck KGaA, Darmstadt, Germany (nitric acid was double sub-boiled before use). Ammonium hydroxide p.a. was from Carl Roth GmbH, Karlsruhe, Germany. ACN and Methanol LC-MS Grade were obtained from Fisher Scientific UK, Loughborough, UK. Reverse osmosis water was purified using an Ultra Clear basic system (SG Wasseraufbereitung und Regenierstation GmbH, Barsbüttel, Germany) and subsequently sub-boiled for CE-ICP-MS experiments.

Iron(III) chloride hexahydrate, copper(II) chloride dihydrate, nickel(II) chloride, cobalt(II) chloride hexahydrate and manganese(II) chloride tetrahydrate were purchased from Merck KGaA in p.a. quality. The mixed metal stock solution (1 mM) was prepared by dissolving the metal chlorides in sub-boiled water. The resulting solution featured a pH of 3.0 and was stable for several hours. The 1:1 metal–MAs complexes were produced – if not stated differently in the text – by mixing the freshly prepared metal solution with an aqueous solution of either DMA or the diastereomeric mixture of MA and epi-MA. For LOD measurements and for competitivity experiments the stoichiometric ratio of total metal to ligand in the solution was 1:0.6 and for standard additions to a hydroponic sample of

Hordeum distichon, cv. Bodega (spring barley) the ratio was equimolar (1:1.2).

2.2 Synthesis of MA, epi-MA and DMA

MA, epi-MA and DMA ammonium salts were synthesized based on the literature [29] and stored at −20°C. Standards were characterized by LC-TOF-MS (Agilent Time-of-Flight LC/MS 6210 system) based on accurate mass determination (2 ppm mass accuracy) that allows identification *via* sum formula; the purity was > 95%. The diastereomers MA and epi-MA resulted in a diastereomeric mixture upon synthesis with a ratio of 1:2.9 (determined by NMR).

2.3 Instrumental

2.3.1 CE-ESI-MS

An Agilent 3-D capillary electrophoresis system was used operated by Agilent ChemStation Software (Waldbronn, Germany). Electrophoresis experiments were carried out in fused silica capillaries 50 μm id and 375 μm od purchased from Polymicro Technologies (Pheonix, AZ, USA). The capillaries were 100 cm long and thermostated at 25°C in the CE-MS-cassette.

For initialization, the capillaries were conditioned with 0.1 M sodium hydroxide (30 min) and water (30 min) using a syringe pump at a flow rate of 100 μL/min. Prior to each run conditioning was performed with 1% ammonium hydroxide (1 min), water (1 min) and BGE (6 min) by flushing at 1 bar. An injection time of 5 s was applied at 50 mbar. Separation voltage at positive polarity was ramped from 0 up to 25 kV within 0.30 min and was kept constant until the end of the measurement time of 40 min. In addition to the voltage a marginal pressure of 5 mbar was applied throughout the whole electrophoretic separation process. As BGE a 20 mM ammonium bicarbonate buffer (pH 7.2) with 30% ACN was used; pH was adjusted by adding diluted acetic acid. For each run a fresh buffer solution in a new inlet vial was supplied.

MS detection was carried out on an Agilent 6320 ion trap MS system in combination with an Agilent G1607A ESI interface. An Agilent 1200 pump together with a built-in active 1:100 flow-splitter provided a constant sheath-liquid flow rate of 4.5 μL/min. The sheath-liquid consisted of methanol and purified water (50/50 v/v). Detection was performed in negative ionization mode, scanning from *m/z* 200–500. Further ESI parameters were set as follows: electrospray voltage +3.0 kV, drying gas temperature 325°C, nebulizer pressure 7.0 psi, dry gas flow rate 5 L/min.

2.3.2 CE-ICP-MS

The CE apparatus and conditions were applied as described above. However, pressurization and addition of ACN to the BGE were omitted (see Section 3). The employed CE-ICP-MS interface has been described earlier [30]. Briefly, it consisted of a four-way cross made of polyether ether ketone (PEEK) purchased from IDEX Health and Science (Oak Harbor, WA, USA), a microconcentric MicroMist nebulizer (Glass Expansion, Sarl, Switzerland) and a cyclonic spray chamber (Glass Expansion). The CE capillary was passed through the PEEK cross into the nebulizer and was locked in the cross using two finger-tight PEEK fittings and a PEEK sleeve (IDEX Health and Science). Another fitting was used to attach a PFA tubing (IDEX Health and Science, 0.75 mm id) to the PEEK cross transporting the makeup liquid containing 1 μg/L gallium in 2% nitric acid by self-aspiration of the nebulizer. The makeup liquid was mixed with the CE effluent at the end of the CE capillary and was grounded using a gold wire (0.5 mm od) that was also connected to the PEEK cross. Either an Element 2 high resolution sectorfield ICP-MS instrument (ICP-SFMS) from Thermo Fischer, Bremen Germany or an Elan 6100 dynamic reaction cell II quadrupole ICP-MS from Perkin-Elmer-Sciex, Ontario, Canada equipped with a dynamic reaction cell was used as MS detector. For elimination of spectral interferences hampering the accurate analysis of the investigated transition metals the ICP-SFMS was operated in high resolution mode ($m/\Delta m = 10\,000$). In the case of ICP-quadrupole MS, interferences were minimized employing dynamic reaction cell technique using ammonia as reaction gas. ICP-MS operation conditions are listed in Table 1.

3 Results and discussion

3.1 CE-ESI-MS: Separation of free MA, epi-MA and DMA and their metal complexes

To our knowledge, no CE separation of free MA, epi-MA and DMA based on volatile and ESI-MS compatible BGEs has been reported so far. In this work, for the first time a

Table 1. ICP-MS operating conditions

	ICP-SFMS	ICP-dynamic reaction cell -MS
Nebulizer	Micromist	Micromist
Spray chamber	Cyclonic	Cyclonic
Rf power	1300 W	1300 W
Nebulizer gas flow	1.16 L/min	1.00 L/min
Auxiliary gas flow	1.15 L/min	1.20 L/min
Plasma gas flow	16.0 L/min	15.0 L/min
Cell gas flow (NH_3)		0.8 mL
Rpq		0.60
Resolution (m/Dm)	10 000	Nominal
Datapoints (s^{-1})	0.65	2.6
Monitored ions	^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{64}Zn, ^{69}Ga	^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{64}Zn, ^{69}Ga

separation was optimized using ammonium acetate as BGE at different concentrations and different pH (concentration range from 5 to 100 mmol/L, pH range from 3.0 to 6.7). Moreover, ACN content was varied from 0 to 90%; 20 mM ammonium acetate buffer, pH 6.5, containing 30% ACN revealed to be the optimum conditions. Due to the low buffer capacity at this pH the ammonium acetate buffer system was replaced by ammonium bicarbonate. Further optimization of the bicarbonate buffer system revealed optimum separation efficiency at a pH of 7.2.

As can be readily observed in Figs. 2A and B baseline separation of the three free ligands was achieved at the optimized BGE conditions. A voltage of +25 kV was applied, transporting the singly negatively charged MAs towards the anode by means of the EOF resulting in migration times of 23.3, 24.3 and 27.2 min for DMA and the MA-diastereomers, respectively. The repeatability of the migration times was ranging from 0.9 to 1.2% relative standard deviation.

Next, the metal complexation of the three selected MAs was addressed for the first time by CE-ESI-MS. MAs standard solutions were stoichiometrically incubated with an $FeCl_3$ solution producing 1:1 ferric complexes. Using the CE separation conditions optimized for the free MAs, these complexes were successfully separated from the free ligands. Extracted ion electropherograms of $[DMA\text{-}4H+Fe(III)]^-$ and $[MA\text{-}4H+Fe(III)]^-$ diastereomers are shown in Figs. 2C and D, visualizing the separation of the ferric complexes from the free ligands. Extraction of DMA-H and MA-H ions in the same electropherogram did not show any signal at the retention time of the complexes demonstrating that the Fe–MAs complexes were not fragmented during ESI at the given conditions.

The technique revealed estimated LODs (calculated by peak height calibration of the base line noise corresponding to 6 sigma) in the range of 0.8–1.4 μM and 0.9–2.2 μM for free ligands and ferric complexes, respectively, clearly demonstrating its potential for rhizosphere research.

3.2 CE-ICP-MS: Analysis of metal–MA complexes

In a next step, complementary CE-ICP-MS studies on metal complexation of the three investigated MAs were carried

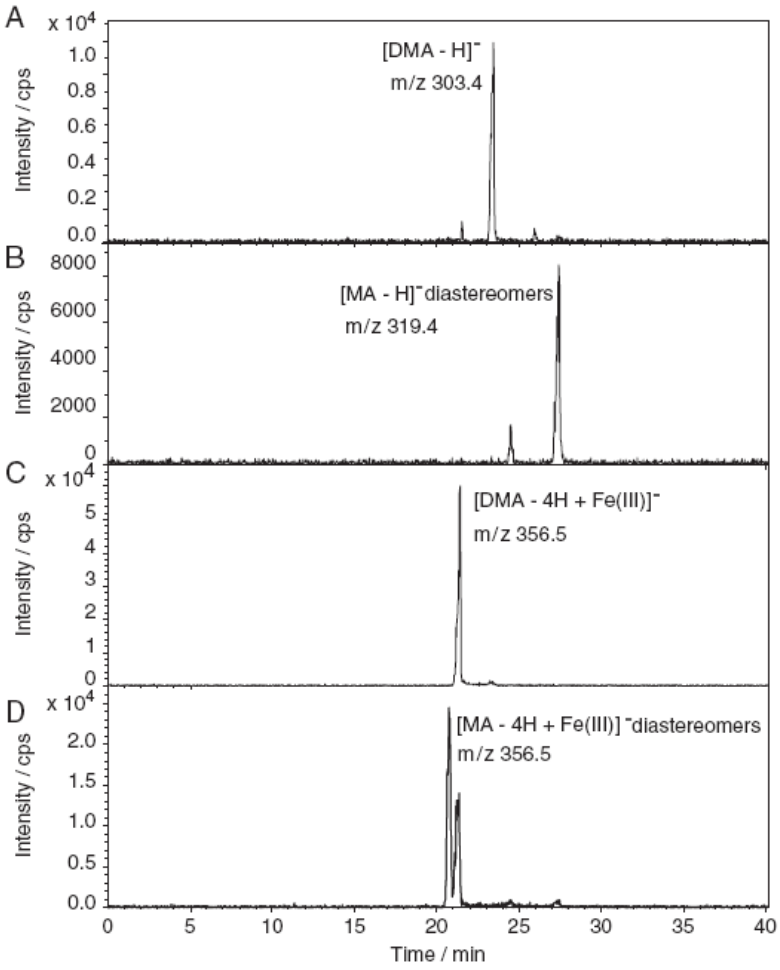


Figure 2. Extracted CE-ESI-MS ion electropherograms of MA diastereomers, DMA and the corresponding iron complexes: (A) $[DMA\text{-}H]^-$ (B) $[MA\text{-}H]^-$ diastereomers, (C) $[DMA\text{-}4H+Fe(III)]^-$ and (D) $[MA\text{-}4H+Fe(III)]^-$ diastereomers. All four extracted ion tracks originate from a single TIC-electropherogram obtained after injection of an aqueous solution of 1 mM $FeCl_3$, 0.5 mM MA diastereomers and 0.5 mM DMA.

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out. 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. This makes the technique a fit-for-purpose detector for simultaneously studying mobilization properties of MAs with different metals. Accordingly, the developed CE separation was transferred to the ICP-MS systems using identical CE settings except pressurization and ACN addition to the BGE, as both settings would compromise the stability of the CE-ICP-MS system. The omission of ACN in the BGE did not affect the separation of the complexes among themselves, but only decreased the resolution regarding the separation of the complexes from the free ligands.

Figures 3A and B prove the ability of the presented combination of CE with ICP-SFMS to separate and simultaneously detect 1:1 Fe(III)-, Cu(II)-, Co(II)- and Ni(II-)-complexes with DMA and MA diastereomers, respectively. The separation was obtained for a metal standard mixture, which was incubated in excess with the three investigated MAs, monitoring ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{64}Zn and ^{65}Cu. Migration times were around 9 min for the different free metals and around 17 min for the different metal–MA complexes. The electropherograms featured excellent peak shapes. However, despite the fact that it was possible to separate the free diastereomer ligands, it was not possible to base-line separate the similarly sphere-shaped complexed forms. Therefore, combined peak integration was used as sum parameter for method evaluation and competitivity studies. As can be observed, under these experimental conditions Mn(II) did not form complexes with the studied MAs, and no free metal was detected for Fe and Cu; Zn(II) was not added to the metal stock solution but was present as a contamination in the synthesized MAs ammonium salt standards.

The LODs of the Co(II), Cu(II), Fe(III) and Ni(II) complexes – calculated as described above – obtained with the CE-ICP-SFMS system ranged from 0.1 to 1.2 μmol/L depending on the isotope specific response of the instrument. Again, these LODs meet the requests of rhizosphere related research as the average PS concentrations in the samples are expected to be in the lower micromolar range [3]. 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 (*e.g.* Fe(III)-DMA: an LOD of 0.87 μM was realized with the CE-ESI-MS system compared with an LOD of 0.35 μM achieved using CE-ICP-SFMS). This is due to dilution of the capillary effluent in the particular interface: within the CE-ESI-MS set-up the sample in the

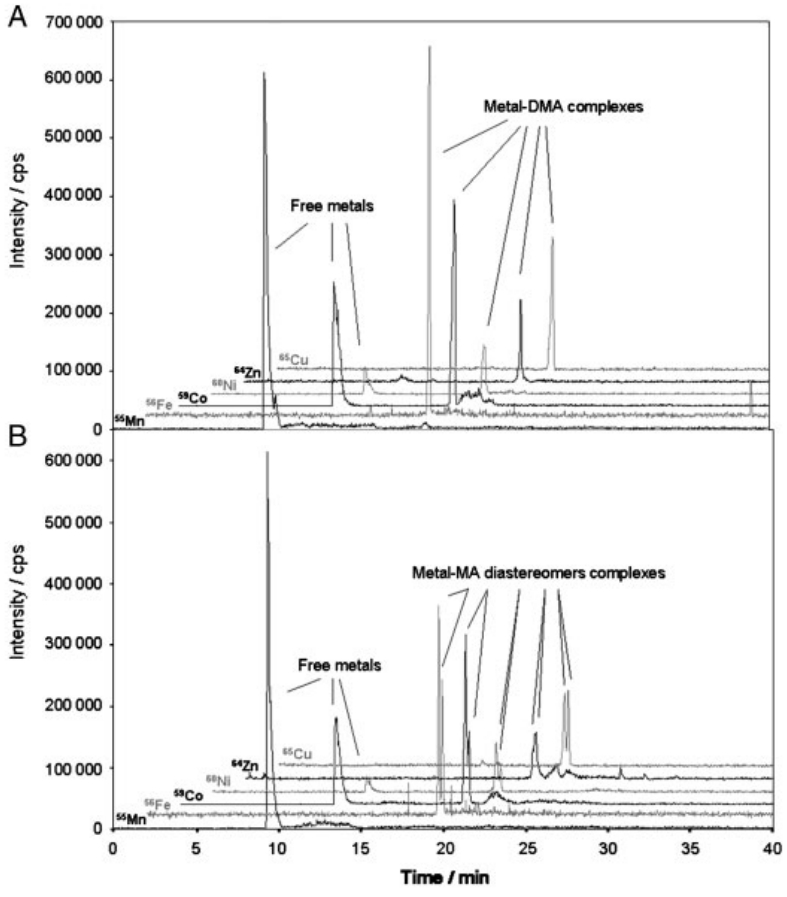

Figure 3. Electropherograms detected with a high-resolution ICP-SFMS. (A) Injection of a metal solution incubated with DMA (100 μM of each metal, 300 μM DMA); (B) injection of a metal solution incubated with MA diasteriomers (100 μM of each metal, 300 μM MA diastereomers).

Table 2. Regression coefficients, literature complex stability constants, relative complex stability constants

	r^2 [a]	Slope[a]	$\log K_{\text{metal-PS}}$ Lit.[b]	$\log K_{\text{metal-DMA}}/\log K_{\text{metal-MA}}$ Lit.[b]	$\log K_{\text{metal-DMA}}/\log K_{\text{metal-MA}}$
Mn(II)-DMA	–	–	8.29 ± 0.02	1.00 ± 0.04	–
Co(II)-DMA	0.992	49.998	–	–	1.53 ± 0.90[c]
Ni(II)-DMA	0.984	14.980	14.78 ± 0.01	0.99 ± 0.01	0.97 ± 0.17[c]
Cu(II)-DMA	0.993	34.245	18.70 ± 0.01	1.03 ± 0.04	1.14 ± 0.20[c]
Fe(III)-DMA	0.992	165.870	33.35 ± 0.01[d]	1.03 ± 0.01	1.25 ± 0.18[c]
Mn(II)-MA	–	–	8.30 ± 0.04		
Co(II)-MA	0.993[c]	36.212[c]	–		
Ni(II)-MA	0.999[c]	16.084[c]	14.92 ± 0.01		
Cu(II)-MA	0.999[c]	32.199[c]	18.10 ± 0.04		
Fe(III)-MA	0.999[c]	139.800[c]	32.49 ± 0.01[d]		

a) Obtained from standard additions to a hydroponic sample of barley.
b) Values from [31]: equilibrium constants were determined using a non-linear least-squares computational program based on potentiometric titration data.
c) Peak areas of metal-MA and metal-epi-MA complexes were summed up for calculations.
d) Stability constants $\log K_{\text{Fe-PS(H-1)}}$ estimated considering the deprotonation of the a-hydroxy group (pK_a is estimated to be 17.1, see Fig. 1).

BGE was mixed with 4.5 μLmin of sheath liquid, while it was mixed with approximately 90 μL/min of makeup liquid in the CE-ICP-MS interface.

In order to evaluate the quantification potential of the proposed method regarding working range and linearity as well as matrix effects, standard additions were carried out by spiking hydroponic samples of *H. distichon*, cv. Bodega (spring barley) with different concentrations of metal-ligand solutions (with the ligand being present in excess). Calibration curves were calculated based on peak areas of metal–MA complexes. The resulting linear regression coefficients presented in Table 2 range from 0.984 to 0.999 proving the applicability of the method to matrices from hydroponic experiments.

3.3 CE-ICP-MS: Competitivity study

The developed CE-ICP-MS methodology was ideally suited for gaining a more fundamental understanding in the MA–metal interaction. Hence, the competitivity experiments involving the incubation of the metal solution with deficient ligand concentration (300 μM ligand + 100 μM of each of the five metals) were repeated five times over a period of 16 h. Measurements were carried out with CE-ICP-SFMS system; peak areas (combined peak areas in case of the metal–MA diastereomers) were normalized according to isotope intensities and plotted against time, as shown in Figs. 4A and B. The following conclusions can be drawn from these data: (i) all complexes were immediately formed after incubation, (ii) Fe(III)-, Cu(II) and Ni(II)-complexes were stable for almost 20 h within a common estimated CE uncertainty of 10%, (iii) the distribution of these three metals between the given ligand remained constant for the mentioned time period.

Concerning the relative complex stability of DMA with different M^{2+} (Fig. 4B) our results were consistent with the

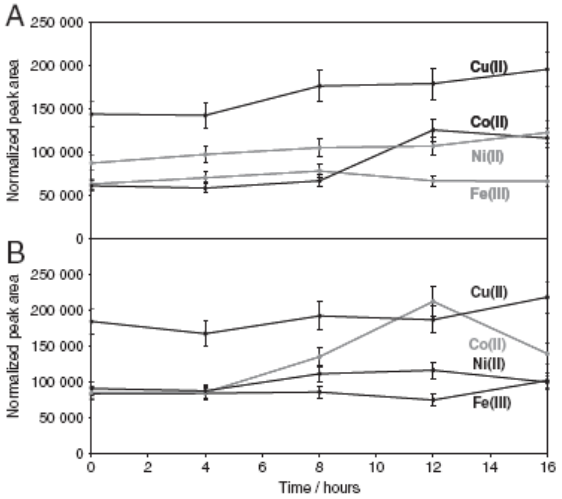

Figure 4. Time-dependent complex formations: Combined peak areas of (A) metal–MA and metal–epi-MA complexes and (B) peak areas of metal–DMA complexes normalized according to isotope intensities after five repetitive injections of metal solutions incubated with deficient ligand concentrations (300 μM ligand + 100 μM of each of the five metals) into the presented CE-SF-ICP-MS system. The error bars represent the uncertainty of the CE-ICP-MS system (10%).

Irving-Williams series as well as with the literature [31]; the same finding held for the complex stability of the MA diastereomers with different M^{2+} (Fig. 4A). However, regarding the complexation of the trivalent ferric ion, our results were not in agreement with the literature. Due to the protonation state of the α-hydroxy group Murakami *et al.* stated that complex stability constants epi-HMA, MA and DMA with Fe(III) were about $10^{32.5}$–$10^{33.3}$ and therefore considerably higher than the stability constants of these ligands with M^{2+} such as Mn(II), Ni(II) and Cu(II) ($K = 10^{8.3}$–$10^{18.7}$) [32].

According to Murakami's stability constant estimation, under competitive conditions the normalized signal of Fe(III)-PS is expected to be significantly more abundant than the normalized signals of Mn(II)-PS, Ni(II)-PS and Cu(II)-PS. However, this was not the case as can be observed in Figs. 4A and B. Possible explanations for the disagreement were coupled equilibria taking place in the metal-ligand solution as well as precipitation of $Fe(OH)_3$ in the CE capillary during the electrophoretic separation process due to the high pH of the BGE.

Based on the data of the competitivity experiment relative complex stability constants $K_{\text{metal-DMA}}/K_{\text{metal-MA}}$ could be determined by calculating the ratio of the average normalized peak areas of the metal–DMA complexes and the metal–MA complexes shown in Fig. 4. The uncertainties of the ratios $K_{\text{metal-DMA}}/K_{\text{metal-MA}}$ have been calculated *via* error propagation. The resulting values are listed in Table 2 and show good agreement with the literature, emphasizing the promising potential of the proposed method to elucidate complexation properties of small organic molecules such as PS.

4 Concluding remarks

A fit-for-purpose method for investigation of phytosiderophore chemistry in rhizosphere related samples has been developed. Future work will include affinity CE experiments with ligand present in the BGE and injections of different metal concentrations in order to determine complex stability constants based on the shift of the electrophoretic mobility. We also plan to quantify metal–MAs complexes in batches of hydroponic samples and root exudates collected by microsuction-cups investigating plants such as maize, barley and wheat.

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The authors have declared no conflict of interest.

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Conclusion

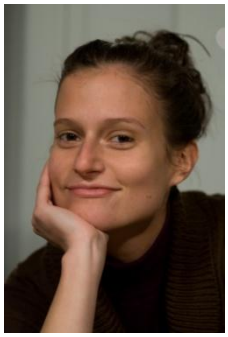
Fundamental investigation of the rhizosphere and the complex mechanisms and modes of action of root exudates is highly relevant for the understanding of plant metabolism and, as a consequence, for potential use in agricultural science and research at ecosystem scale. Analytical strategies addressing questions in the context of rhizosphere research are nontrivial and highly challenging.

For the identification and quantification of root exudates in hydroponic or soil solution, according to the literature, rhizosphere scientists most usually apply GC-MS after derivatization. E. g. for the analysis of free amino acids in rhizosphere-related samples, to date no method devoid of derivatization has been reported in the respective literature. This is surprising, because a derivatization step in many cases comes along with loss of information and with an enhancement of measurement uncertainty. The presented work comprised the development of novel strategies that i) implemented high-end analytical techniques involving the hyphenation of separation methods and mass spectrometry , ii) constituted attractive usage in metabolomics in general and in the field of rhizosphere research in particular, and iii) provided high sensitivity, accurate and precise quantification, matrix robustness, simple sample preparation and straightforward performance. These advantages were beneficial for investigation of free proteinaceous amino acids in samples from soil adsorption experiments as well as for the examination of phytosiderophores and their complexes with micronutrients.

Both methods are highly promising for further applications in rhziosphere research. The first method is currently used for the analysis of low concentrated amino acids in samples from root exudate collection, hydroponic plant growth and microsuction cups in order to assess and compare these three different sampling techniques. This method also shows potential for applications beyond the field of rhizosphere research as the target analytes are of high relevance in many areas. The latter method has proven to be applicable to quantitative analysis and is therefore planned to be used for the quantification of metal complexes with phytosiderophores in root exudate samples.

Curriculum Vitae

Personal Data



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Conference Contributions and Publications

Oral Presentations

Dell'mour M., Jaitz L., Oburger E., Puschenreiter M., Koellensperger G., Hann S., *LC-MS Analytik von Pflanzenmetaboliten in der Rhizosphären-Forschung.* Agilent Anwendertreffen Massenspektrometrie 2009, December 7 – 8, 2009, Würzburg, Germany.

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