Speciation of 2'-deoxymugineic acid-metal complexes in top soil extracts by multi-modal stationary phase LC-ICP-MS

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2'-deoxymugineic acid (DMA) is a principal representative of phytosiderophores exuded by graminaceous plants in response to iron limiting soil conditions. In the present work a method for selective quantification of metal-DMA complexes has been developed enabling to investigate the interplay of DMA with transition metals at the root-soil interface, i.e. the rhizosphere. Chromatographic separation was performed via gradient elution preserving complex integrity at pH 6.6 in 55 mM ammonium acetate and 10% methanol on a mixed-mode reversed phase/weak anion exchange stationary phase. Quantification of the DMA-complexes of Fe, Co, Cu, Ni, Mn and Zn in soil related matrices was performed via ICP-MS utilizing dynamic reaction cell technique with methane as reaction gas. Limits of detection and quantification were obtained in the range of 10 to 120 nM and 40 to 400 nM, respectively. Long term precision of the method was below <10% relative standard deviation (n = 10). The suitability of the method for the metal-DMA complex speciation was evaluated within three calcareous soil extracts exhibiting various metal abundances. Prior to this, the top soil solutions were spiked with 100 µM DMA to induce the complex formation. Metal complexes of DMA in a concentration range of 0.4 to 30 µM were detected.

Introduction

For nutritional values iron represents the most essential trace element for living organisms. Crop yields in areas with alkaline calcareous soils suffer from iron limitation as Fe(II) at pH 6 starts to precipitate into $Fe(OH)_2$ – ferric oxides/hydroxides. Moreover, solubility is reduced due to the oxidation of Fe(II) to Fe(III) as a result of soil aeration caused by low annual precipitation. Subsequently, a likewise transformation into $Fe(OH)_3$ takes place. The release of the major enclosed Fe(II) within primary and secondary minerals is largely dependent on soil equilibrium which is influenced by several factors such as temperature, CO_2/O_2/water/SOC (soil organic carbon contents), the pH, the coexistence of other organic molecules or soil micro-organisms.[1] Elemental iron is fixed to the solid soil phase and therefore only extreme low concentrations are present in soil solution. By the help of reductants-release (such as organic acids or phenols) strategy I plants[2] can easily acquire iron from soil minerals, whereas strategy II graminaceous plants do not reduce Fe(II) but increase the availability of Fe(III) by exuding complexing ligands.[3] A key substance in metal uptake is 2'-deoxymugineic acid (DMA) (Fig. 1 A), a low-molecular-weight organic acid belonging to the family of phytosiderophores (PS). DMA is released at the plant-soil interaction surface, i.e. the rhizosphere. In this soil region all major soil regulative processes for nutrition uptake by the plant roots take place.[1,4] Thus, iron bioavailability in soil is dependent on the interplay of mass flow and diffusion processes in opposition to the exudation and degradation rates. The hexadental DMA-ligand (Fig. 1 B) is a zwitterionic non-proteinogenic amino acid that complexes in accordance to the competitive equilibria of the soil trace elements either with bivalent or trivalent metals.[5]

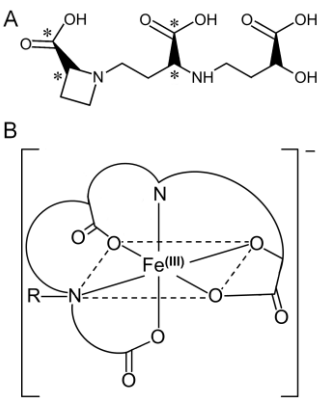

Fig. 1. 2'-deoxymugineic acid (DMA), the stars represent the labeling of the four C-atoms of the $^{13}C_4$-DMA used for internal standardisation (A); Proposed conformation of the negatively charged hexadental $[Fe(III)DMA]^-$ complex (B).[6]

After metal chelation (e.g. Fe(III)) the intact complexes are transferred into the plants via membrane transport systems.[7] The structural confirmation of the negatively charged metal complexes under acidic as well as neutral pH conditions has been postulated before.[6,8,9] In case of Me(II)-DMA complexes the α-hydroxyl-group remains protonated. Research in the context of DMA regulatory mechanisms aims to maximize the nutritional iron value and the yield in agricultural food production in connection with ecological and economical soil management, e.g. by reducing fertilization. Thereby, investigations have been made by transferring DMA production responsible genes into other plants.[10] These developments have been accompanied by a significant progress in analytical chemical methodology. In particular for the separation of metal PS-chelates, ion-pair HPLC and colorimetric procedures[11], followed by capillary electrophoresis[12,13] and hydrophilic interaction chromatography[9,14,15] have been applied. Calcareous soil that includes the free chelating ligand involved to metal mobilization reveals a high alkaline pH. Although within the soil medium the non-covalent metal-PS complexes manifest a high thermodynamic stability (10^{18} - 10^{25})[16], by the risk of changing the complex equilibrium during the analytical separation, method development in the context of metal-PS species has become extremely challenging. Complex dissociation, for example, may be induced by unwanted interaction with the stationary phase, by non-physiological elution conditions or by in-source fragmentation during electrospray ionisation (ESI). Several ESI-MS approaches[12,13,17,18,19] have been introduced for identification of the phytosiderophores and their metal complexes; however, the development of separation methods was confronted with stability problems, particularly in the context of iron complex dissociation.[9,14,20] In one case[19] isoelectric focusing was used as preparative enrichment technique prior to ZIC-HILIC hyphenated LC-ESI-MS^n. By the use of ZIC-HILIC coupled to ESI-MS[14,19], the metal-PS complexes (Fe, Cu, Ni, Zn) were separated by hydrophilic partitioning and weak electrostatic interaction, but resulted in broad chromatographic peaks. According to the chromatograms depicted in this work, the limit of detection (LOD) and the limit of quantification (LOQ) can be estimated as 3 µM and 10 µM, respectively. The micromolar concentration range is thoroughly suitable for the quantification purpose in hydroponic grown plant samples, but is not practicable to analyse root exudations in the rhizosphere soil, since typical phytosiderophore concentrations were found in the high nanomolar to low micromolar concentration range[21,22,23] and metal-DMA species are therefore expected to be present in a lower concentration range than the free ligand itself. Additionally, Fe(III)-DMA complex quantification was affected by dissociation problems. Nevertheless, the main disadvantage of the use of HILIC chromatography are long re-equilibration times (30 min) followed by column instabilities in relation to high salt levels present in real plant and soil related samples, thereby mitigating the method robustness and reproducibility. Thus, reproducibility in retention time by a <3% RSD has been reported.[14] Although co-elution of metal-DMA complexes was occurring, complex identification was possible by evaluation of characteristic isotope distribution patterns.[14,20] Another approach was developed by Tsednee *et al.*[18] which presented a LC-ESI-QTOF-MS method for the identification of Fe(III)-PS complexes in plant samples. In our opinion, the reliability of this method is questionable, as the shown chromatograms reveal that the putatively detected Fe(III)-PS complexes eluted within the void volume of the separation system which may lead to the formation of in-source artefacts. Due to the lack of appropriate standards, the ESI-method was not used for quantification.[18]

Compared to ESI-MS, ICP-MS methods offer a high compatibility with a broad range of buffer compositions and concentrations and ultimate selectivity regarding the speciation of metal containing compounds. In former approaches, investigations were made by anion-exchange chromatography[24,25] at alkaline pH, which was far away from the physiological conditions of real plant samples. Accordingly, the development of new separation methods closer to the physiological pH was necessary in order to maintain complex stability. Bakkaus *et al.*[9] have performed Fe-PS speciation at neutral condition by anion-exchange chromatography with gradient elution of ammonium nitrate combined with ICP-MS detection. The authors concluded that the separation method was not optimal for Fe(III)-DMA speciation as it resulted in broad peaks and incomplete baseline separation. An LOD of 110 µM was determined for the Fe(III)-DMA complex. In the same work a sodium hydroxide gradient was applied for Co and Ni speciation, respectively. The quantification of the metal-PS complexes was performed within hydroponically collected plant exudates.

As a matter of fact, separation mechanisms including ion exchange separation require high salt concentrations and a defined pH setting that are well tolerable when coupled to ICP-MS. These elution conditions however would affect the detection sensitivity considerably in the context of organic MS. Because of too strong electrostatic interactions, ion exchangers may lead to dissociation of the metal complexes during separation. Even much weaker interaction with the HILIC stationary phase[14] have provoked the dissociation of the Fe(III)-DMA complex. In this situation capillary electrophoresis (CE) offers a high degree of matrix independence. Due to the absence of a stationary phase, undesired interactions causing dissociation processes do not take place. Analytes are preserved in their original conformation by the help of the homogenous buffering background electrolyte and therefore stability problems are omitted. Thus, CE is not limited in pH as column materials are. The speciation of small metal chelates at physiological pH was emphasized to be well suitable by CE coupled methods.[13,17] For metal-PS complexes LOD of 10 to 60 µM were obtained by the use of ultraviolet visible spectroscopy in combination to diode array detection (200 nm) and conductivity detection; e.g. a concentration of 80 µM was shown in the case of Fe(III)-PS analysis.[12] Improvements of the LOD towards high nM to low µM range were achieved by the combination of CE to ESI-MS and ICP-MS in the context of hydroponic plant samples.[13] More recently, in our working group, investigations on a LC-ESI-MS/MS method with a multi-modal stationary phase revealed higher sensitivity and lower LOD compared to the CE method, but the drawback of in-source fragmentation and assignment problems of the competitive metal-DMA complexes hampered the accurate quantification of some analytes of interest.[17]

In the present work we selected the multi-modal interaction options of a RP-WAX stationary phase for combination with ICP-MS, as this material has the potential to separate neutral, acidic and basic compounds within one chromatographic run.[26] Depending on the analyte and mobile phase characteristic, combined orthogonal retention and selectivity principles can be implemented. Accordingly, we have combined retention and selectivity contributions given by the weak anion exchanger and the polar embedded functional groups. These separation conditions enabled the metal-PS species analysis without any stability problems. For the first time, the challenging Fe(III)-DMA complex was retained and preserved during an LC-based separation method. The sensitivity and selectivity of the ICP-MS detection enables the quantification of metal-DMA complexes in soil extracts in the sub-micromolar concentration range showing its high potential for rhizosphere research.

Materials and methods

Selection of soil samples

Soils from the top layer (0 - 20 cm) from different iron limited regions in Austria (Arnoldstein and Redlschlag) and in Spain (Santomera) were manually collected into bags. Selected soil parameters are summarized in Table 4. All soils are highly calcareous at physiological pH and contained significantly different fractions of soil organic matter (SOM). The Arnoldstein soil has been anthropogenically contaminated by metal smelter activity, the Redlschlag soil is a serpentinite soil with a naturally elevated Ni content. The Santomera soil is an uncontaminated calcareous clay soil. The soil samples were air-dried and sieved over 2 mm to remove root debris. A more detailed description of the soil properties is given in Schenkeveld *et al.*[23]

DMA-soil interaction experiment

For soil interaction experiments, the in-house synthesized DMA ammonium salt (see below) was used to prepare a 100 µM DMA solution by dissolving in ultra-pure water. $CaCl_2$ ($CaCl_2$*$2H_2O$ analytical grade, Merck KGaA, Darmstadt, Germany) was added as background electrolyte (10 mM). NaN_3 (NaN_3 extra pure, Merck KGaA, Darmstadt, Germany) was added as sterilant (2 g L^{-1}) to prevent DMA degradation by soil microbial activity. For investigation of DMA-soil interaction the 100 µM DMA solution was added to the soils in a soil-solution ratio of 1 (w/v, i.e. 30 g soil / 30 mL water) in 50 mL polypropylene tubes. The samples were placed in an end-over-end shaker rotating at 18 rpm in the dark. Sampling was done after 0.25 h, 1 h and 24 h of interaction (for Santomera soil, for Redlschlag soil, and for Arnoldstein soil), respectively. Samples were centrifuged for 5 minutes at 4500 rpm (4.0×10^3 g on a Jouan CR422 centrifuge, Thermo Electron Corporation, Beverly, USA) and filtered via 0.45 µM filters (cellulose acetate filter Aqua 30/0.45 CA from Whatman, GE Healthcare Europe GmbH, Vienna, Austria). The filtrates were immediately frozen and stored at –20 °C until analysis. All experiments were carried out in duplicates.**Chemicals**

Dilutions were carried out within ultrapure subboiled water. For this purpose, purified grade I water (according to ISO 3696 >10 MΩ cm^{-1} prepared via Ultra Clear basic ultrapure water system, SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany) was subboiled (Milestone-MLS GmbH, Leutkirch, Germany). In the same manner nitric acid (65%, Normapur, p.a., VWR International GmbH, Vienna, Austria) was double subboiled. Acetic acid 100% (v/v) (glacial) Suprapur® and ammonium hydroxide (ammonium solution 25% (v/v)) Suprapur® were obtained from Merck KGaA (Darmstadt, Germany). LC/MS grade Optima methanol was purchased from Fisher Scientific UK, Loughborough. In order to avoid contamination of the LC eluents the ammonium acetate buffer was prepared from 100 mM acetic acid solution and subsequent pH adjustment to 6.6 with ammonium hydroxide solution. DMA (ammonium salt, Mr = 321.33 g mol^{-1}) and $^{13}C_4$-labeled DMA (ammonium salt, Mr = 325.30 g mol^{-1}), respectively, was synthesized at the Division of Organic Chemistry at the Department of Chemistry at the University of Natural Resources and Life Sciences, Vienna.[27] For metal-DMA complexation the following metal chloride standards were obtained from Sigma-Aldrich Chemie, Steinheim, Germany: Iron(III) chloride (sublimed grade, 99.9% trace metals basis), Cobalt(II) chloride (anhydrous, 99.999% trace metals basis), Copper(II) chloride (anhydrous, 99.995% trace metals basis), Manganese(II) chloride (anhydrous, 99.99% trace metals basis), Nickel(II) chloride (anhydrous, 99.99% trace metals basis), Zinc(II) chloride (99.999% trace metals basis). Ferrioxamine E from Streptomyces antibioticus for microbiology, ≥95%, from Sigma-Aldrich Chemie, Steinheim, Germany was applied for internal standardisation. For the dynamic reaction cell placement methane cell gas (≥99.95) from Linde Gas GmbH, Stadl-Paura, Austria – at 0.8 mL min^{-1} gas flow, was used.

For the analysis of the total metal content via ICP-SFMS an ICP Multi Element Standard Solution VI CertiPUR® was purchased from Merck KGaA, Darmstadt, Germany. Indium 1000 mg L^{-1} from (Merck KGaA) was used as internal standard. TM-27.3 fortified lake water (Canadian National Research Lab., Ontario, Canada) was employed as certified reference material for the quality assessment of the aqueous external calibration.

Preparation of metal-DMA complex standards

In-house synthesized DMA was used for the preparation of metal-DMA complexes. To avoid precipitation of metal hydroxides, metal chloride standards were diluted in 0.25% HNO_3 (pH 1.2) to build 10 mM stock solutions while DMA was diluted in subboiled water. In terms of dilutions, all DMA-multi-element complex standards containing Mn, Fe, Co, Ni, Cu and Zn were carried out within 50 mM ammonium acetate. To assure complex stability[17] the DMA-multi-element complex standard was kept at a pH around 7. Fresh preparation of the external calibration standards was mandatory. To avoid the impact of freeze-thawing cycles, after instrument calibration, measurements were started the same day keeping the auto sampler tray at a temperature of 8 °C. Based on the knowledge of competition effects[28] metal-DMA complexes were generated by a stoichiometric 1:2 (metal:DMA) ratio. Within the multi metal-DMA complex stock all elements were added in equimolar abundance, except the Zn-DMA complex was doubled accordingly to the concentration range of the soil extracts. For quantification purpose by external calibration with internal standardization, calibration standards were established within a calibration range from 0.1 to 5 µM. A concentration of 0.46 µM of Ferrioxamine E from Sigma-Aldrich Chemie, Steinheim, Germany was added as internal standard to all samples.

Sample preparation

Metal-DMA complex quantification. 50 µL of sample was diluted 1:2 by 50 mM ammonium acetate (pH 7.3). No further sample preparation was applied. Internal standardisation was performed as described above: 30 µL of 2 µM of Ferrioxamine E from Sigma-Aldrich Chemie, Steinheim, Germany were added within the final volume of 130 µL.

Total DMA quantification. An aliquot of 100 µL soil extract was acidified and used for the total DMA quantification following the procedure described by Schindlegger *et al.*[21] Synthesized $^{13}C_4$-DMA was applied to assure the accuracy of quantification.

Total metal quantification. For the total trace metal analysis of soil extract solutions by ICP-SFMS (Element 2, Thermo Fisher, Bremen, Germany) the samples were diluted with 2% subboiled HNO_3 to fit into the calibration of 10 ng L^{-1} to 10 µg L^{-1} for the determination of Cu, Mn, Co, Ni and of 100 ng L^{-1} to 100 µg L^{-1} for the determination of Fe and Zn.^{115}In was added for internal standardisation (1 µg L^{-1}), also to the blanks. LOD and LOQ were calculated accordingthe Eurachem Guide[29], using the three- and tenfold standard deviation of low concentrated standards (containing 0.01 µg L^{-1} Cu, Mn, Co, Ni, and 0.1 µg L^{-1} Fe, Zn).

Instrumentation

LC-ICP-MS

Measurements were accomplished in a class 10000 cleanroom. To avoid iron-contamination by system parts HPLC was performed on a Rheos 2000 system (Flux Instruments AG, Basel, Switzerland) with an autosampler device from HTC Pal System (CTC Analytics AG, Zwingen, Switzerland) carrying a coated injection syringe (CTC Syringe gastight 100 µL, Serie X, gauge 22S, pointstyle 3 from CTC Analytics AG, Zwingen, Switzerland) and a column oven from Spark Holland B.V., Emmen, The Netherlands. Separation was achieved by a non-commercial[26] multi-modal RP-WAX stationary phase with inline-filter and column tempering at 40 °C. The stationary phase of 5 µm particle size was packed into a column of 50 x 2.0 mm ID dimension. The mixed-mode reversed-phase/weak anion-exchange type separation material consists on a hydrophobic alkyl strand attached to silica particles. The characteristic of the hydrophobic chain was given by inserted polar embedded groups (thioether and amide) and by a terminal weak anion-exchange-type quinuclidine functionality. The stationary phase is specified to be stable within a pH range from 2 to 8. Speciation was accomplished via coupled LC-ICP-QMS method on an ELAN 6100 DRC II system (PerkinElmerSCIEX™, Ontario, Canada) using Dynamic Reaction Cell™ (DRC™) technique. The operation parameters of the LC-ICP-MS system are summarized in Table 1. For the pH adjustment of the LC-eluent solution a pH electrode type Inlab® from Mettler Toledo in combination to a pH meter model PHM92 was used.
For data evaluation, obtained raw data from ELAN System (NetCDF-Files) was converted by Chromlink and Totalchrom (PerkinElmerSCIEX™, Ontario, Canada) into a format accessible for the Dionex Chromeleon Client (Version 6.8, Dionex, Sunnyvale, CA, USA), to allow integration of the chromatographic data.
LOD and LOQ were calculated accordingly to the 3- and 10-s criterion by using the standard deviation divided by the square root of n = 5 of a low concentrated (0.2 µM for Fe, Co, Cu Ni, Mn, 1 µM for Zn) single-point calibration (Eurachem[29]).

Table 1 LC-ICP-MS Operation parameters

HPLC	
Stationary phase	RP-WAX 50 x 2.0 mm, 5µm, with upstream inline-filter
Eluent A	H_2O
Eluent B	100 mM ammonium acetate pH 6.6
Eluent C	MeOH
Gradient	0 to 2 min: 2% v/v B, 2 to 3 min: 2% to 55% v/v B, 3 to 5.5 min: 55% v/v B, 5.6 min: 2% v/v B, 10% C (during the entire run)
Total analysis time (min)	10
Injection volume (µL)	5
Flow rate (µL min^{-1})	250
Column temperature (°C)	40
ICP-MS	
Nebulizer	PFA
Spray chamber	cyclonic
Rf power (W)	1350
Nebulizer gas flow (L min^{-1})	0.87 set for ≤3% oxides (CeO^+/Ce^+)
Auxiliary gas flow (L min^{-1})	1.2
Plasma gas flow (L min^{-1})	16
Monitored ions	^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{64}Zn, ^{65}Cu
DRC cell gas	methane
DRC cell gas flow (mL min^{-1})	1
RPq	0.6
RPa	0

ICP-SFMS

Total elemental concentrations in the soil extracts were determined by ICP-SFMS using an Element 2 system (Thermo Scientific, Bremen, Germany) in medium resolution ($R_{10\%}$ = 4500). Methodological details have been described elsewhere.[17]

LC-ESI-MS/MS

Quantification of the total DMA content of the investigated soil extracts was determined accordingly to our recently published method[21], thereby transferred to LC-ESI-Trap-MS (Agilent Technologies, 6340 LC/MSD Trap XCT Ultra). For quantification via internal standardization with ^{13}C-labeled DMA the *m/z* fragment-ion ratio 186/188 was used.

Results and discussion

Separation of metal-DMA complexes with a multi-modal stationary phase

Complex stability based on model equilibrium calculations has shown that the iron-DMA complex is stable within a pH range of 5.4-7.2.[17] Accordingly, the chromatographic separation of the metal-DMA complexes was performed at pH 6.6. The characteristics N-(10-undecenoyl)-3-aminoquinuclidine bonded to thiol-modified silica featuring both reversed phase and weak anion exchange properties as well as two polar embedded groups (amide and thioether) have been discussed elsewhere.[26] At the given pH ionic compounds (i.e. the negatively charged metal-DMA complexes) are predominantly retained due to weak electrostatic interaction which is principally controlled by the ionic strength, pH and organic solvent content of the mobile-phase. Separation is considered to be mainly achieved by the electrostatic interaction by the terminal weak-anion-exchange functionality, but it is most likely that the polar embedded groups are somehow involved in the retention mechanism too (by hydrogen bonding capacity[30,31]), e.g. especially in the case of PS-complexes containing bivalent metals, as the α- hydroxyl group remains protonated. Herein, by equilibrating the stationary phase at pH 6.6, the polar embedded amino group takes a positive charge which allows additional electrostatic interaction.
Isocratic elution with 55 mM ammonium acetate led to low retention of all complexes. To increase retention of the negatively charged complexes, equilibrium displacement is possible either by pH change or by change of the eluent concentration (ionic strength). Since the stationary phase as well as the stability of the complex is restricted to a limited pH range, retention was optimized by modifying the ionic strength via gradient elution via reducing the buffer concentration at starting condition. After this period, the ionic strength was gradually increased (Table 1). Applying the plasma conditions given in Table 1, the introduction of 10% MeOH via the LC eluent did not cause any carbon deposition or problems regarding long term stability. To lower the hydration of the ion exchange groups on the stationary phase as well as of the solute-ions 10% organic modifier (MeOH) was added to the mobile phase[32]. Thereby, peak symmetry was improved and peak width was reduced leading to an increase of chromatographic efficiency. Moreover, peak width and tailing could be further reduced by setting the column temperature to 40 °C. To a certain degree, the organic phase has an impact on the ionic strength of the mobile phase, whereby retention times are influenced. Thus, methanol minimizes the hydrophilic interaction between the stationary phase and the analyte as well as it masks the RP location[33]. Figure 2a and Figure 2b depict the

chromatograms obtained by the developed LC-ICP-MS speciation method for metal-DMA complexes in standard solution and a DMA-soil extract. It is noteworthy that the preparation of all standards and soil samples in the eluent buffer (50 mM ammonium acetate) was mandatory regarding complex stability and chromatographic performance of the developed method. To assure quantitative complex formation and long-term stability of metal-DMA calibration standards the free ligand was added in excess (2:1). The metal background in the separation and detection system was maintained lower than 3000 counts (^{56}Fe) by cleaning the HPLC system with 3% HNO_3 and injecting 200 µM of the free ligand (DMA) on a daily base during column equilibration.

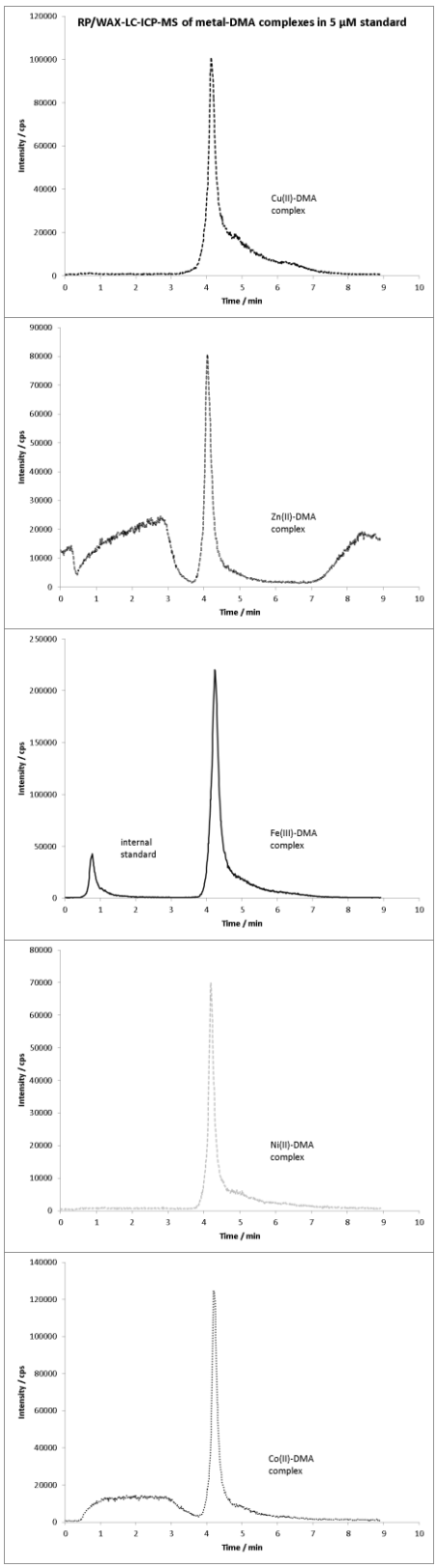

Fig. 2a Elemental speciation of metal-DMA complexes (5 µM Fe, Cu, Co, Ni; 10 µM Zn). The molar ratio of DMA and the total metal content was 2:1. For internal standardisation Ferrioxamine E, an iron chelating siderophore, was used.

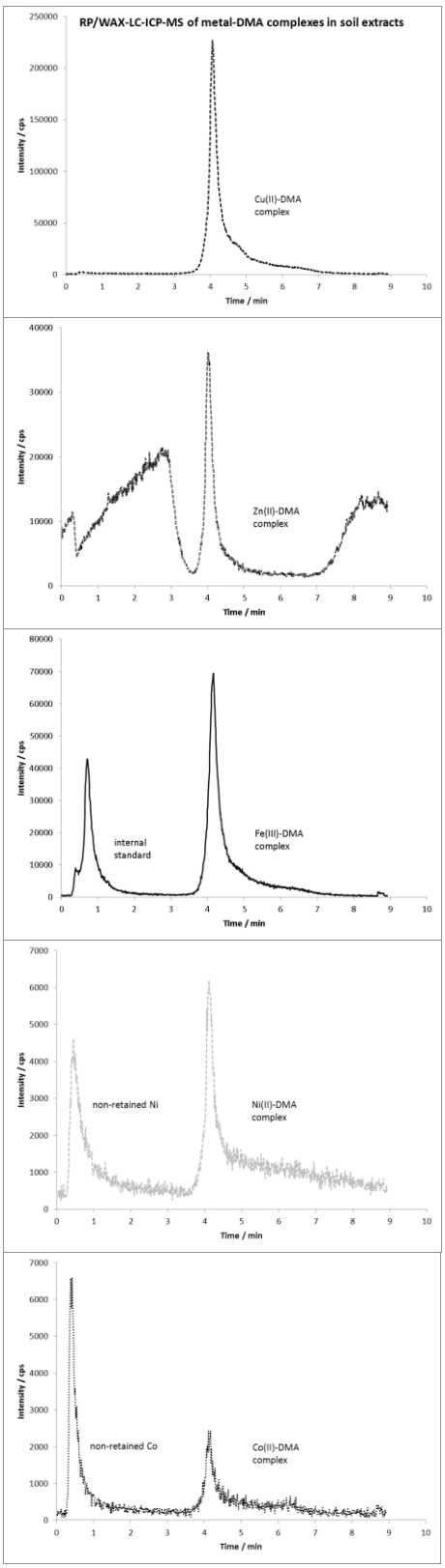

Fig. 2b Elemental speciation of metal-DMA complexes within a soil extract originating from the Arnoldstein site. The concentration of the metal-DMA complexes was quantified by external calibration revealing 2.8 µM, 0.9 µM, 0.4 µM, 8.0 µM, 20 µM of Fe(III)-, Co(II)-, Ni(II)-, Zn(II)- and Cu(II)-DMA, respectively.

Quantification of metal complexes in DMA-soil extracts

Detection of the elemental species was performed by online-combination of the developed separation method with ICP-MS employing dynamic reaction cell technique. In addition to the monoisotopic elements ^{55}Mn and ^{59}Co, the isotopes ^{65}Cu, ^{56}Fe, ^{60}Ni, ^{66}Zn were selected considering isotopic abundance and potential spectral interferences. In order to eliminate spectral interferences hampering the accurate quantification of the investigated transition metals and to improve LOD, measurements were performed utilizing methane as reaction gas in combination with appropriate quadrupole band-pass settings. Especially when determining the trace level of ^{56}Fe in the presence of argon- and calcium oxide (Ca is added in the form of $CaCl_2$ via the DMA extraction solution and eluted from the soil) occurring isotopes overlap can be inhibited by methane collision at RPq of 0.6 and RPa of 0.
LOD and LOQ in the range of 10 - 120 nM and 40 - 400 nM were obtained, depending on isotope specific sensitivity of the respective complexes. Element specific LOD and LOQ are listed in Table 2.

Table 2 Limits of detection (LOD) and limits of quantification (LOQ) obtained via LC-ICP-MS applying the conditions given in Table 1.

Metal-DMA complex	LOD /nM	LOQ /nM
Mn-DMA	50	160
Fe-DMA	10	40
Co-DMA	15	50
Ni-DMA	30	100
Zn-DMA	120	400
Cu-DMA	40	140

In the context of Fe(III)-DMA, the LC-ICP-MS based method revealed a LOD that was three times lower than that achieved with our complementary LC-ESI-MS/MS method[17] and 8 times lower compared to the work of Bakkaus *et al.*[9], who used strong anion-exchange chromatography combined with ICP-MS detection. In terms of sensitivity and LOQ the presented method is suitable to quantify metal-DMA complexes from soil and plant experiments: the free total DMA exudation concentrations in real plant samples as well as those concentrations in soil extracts have been determined within the low micromolar concentration range.[21,22,23]
The metal-DMA complex concentrations in the soil samples were determined by external calibration with internal standardization (Ferrioxamine E) within a calibration range from 0.1 to 5 µM (for Zn 0.5 to 10 µM), revealing determination coefficients of R^2 >0.9730.

Validation of metal-DMA quantification in soil extracts

In order to control the influence of matrix effects on measurement accuracy, standard addition experiments have been performed in a concentration range of 0.5 - 5 µM as described above. In brief, DMA-multi-element-complex standards were spiked to DMA extracts from Arnoldstein soil (n = 3). The slopes of the linear calibration functions calculated for external calibration were in good agreement with those obtained for the standard addition experiments (Table 3). The relative standard deviation (RSD) of <12% agrees well with the RSD obtained for long-term precision. It is noteworthy that all standards and samples were diluted in 50 mM ammonium acetate at pH 7.3. Under these conditions, the method showed a long term precision of <12% RSD (n = 10 samples measured over a time of 7 hours). Additionally, complexes were determined to be stable over a time range of 15 hours. Fig. 3 shows the quantitative complexation of three multi-element standards (1, 2 and 5 µM total metal concentration, respectively) by DMA applying the experimental conditions described above.

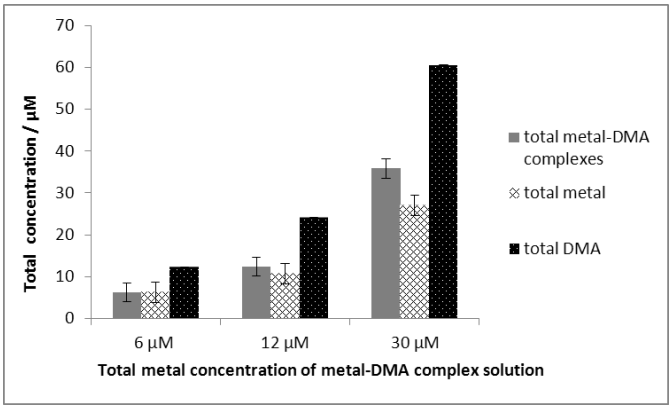

Fig. 3 Assessment of metal complexation by DMA in multi-element metal chloride solution applying the developed LC-ICP-MS method. Total metal concentrations were measured via ICP-SFMS. The error bars represent the standard deviation of n = 3 independently prepared solutions.

The integrity of the metal-DMA complexes during separation is assured by the data from the standard addition and stability experiments. Nevertheless, it is noteworthy that soluble components present in the soil extracts may shift complex equilibria leading to a non-retained metal fraction detected in the void volume of the chromatographic system (see Fig. 2b).

Table 3 Comparison of quantification results obtained by external calibration and standard addition in a soil extract from a soil from Arnoldstein, Austria

	External calibration				Standard addition			
Metal-DMA complex	Measured complex concentration in soil extract sample /µM	Determination coefficient	Slope	% RSD (n = 3)	Measured complex concentration in soil extract sample /µM	Determination coefficient	Slope	% RSD (n = 3)
Mn-DMA	-	0.9899	0.583	1.6	-	-	-	-
Fe-DMA	4.0	0.9880	1.72	4.7	3.5	0.9759	1.61	4.0
Co-DMA	0.67	0.9867	0.875	1.9	0.49	0.9747	0.839	6.6
Ni-DMA	0.56	0.9894	0.302	1.8	1.1	0.9901	0.425	3.2
Zn-DMA	11	0.9654	0.272	12	7.5	0.9881	0.342	9.7
Cu-DMA	16	0.9728	0.871	8.8	20	0.9451	1.21	6.6

Analysis of soil extracts

To test the suitability of the analytical method for the speciation of metal-DMA complexes, three different soil types with different heavy metal content were selected according to the results of a study determining diethylene triamine pentaacetic acid extractable (DTPA-extractable) trace elements shown in Table 4. Accordingly, mainly metal-DMA complexes formed with Fe(III), Cu(II), Ni(II), Co(II), Zn(II) are expected.[4] Orthogonal mass spectrometry techniques were applied to gain complementary information. By comparing the measured total DMA concentration, the measured total metal concentration and the measured metal-DMA complex concentration, different complexation behaviour within the soil matrixes was observed (Fig. 4-7). Results provide information about interaction and adsorption performance in soil, whereby geochemical processes are traceable.[28] The total DMA concentrations within the soil extracts were measured by ESI-LC-MS/MS as recently published.[21] The total metal concentrations were determined by ICP-SFMS, whereas metal-DMA complexes were quantified by external calibration (using multi-metal-DMA-complex standards described above).

Generally, complexes are formed in accordance to the mass law and their stability constants. Complexation behaviour and complex yield of a specific ligand is controlled by the competitive chemistry of all potential ligands in the soil. DMA has a high affinity to Fe(III) and Cu(II) with stability constants of $10^{33.35}$ and $10^{18.7}$ (Table 5), respectively, followed by Ni > Co > Zn > Mn.[13] Experiments were carried out to obtain more detailed understanding of DMA metal-mobilization in the soil. To get perceptions in the metal-mobilization processes and complexation behaviour, out of the quantitative LC-MS/MS analysis and the ICP-MS speciation, the total free metal concentrations and the total DMA concentrations[21] were compared to the metal-DMA complex concentrations. It was investigated, how metal-DMA complex formation is influenced and dependent on different soil parameters and characteristics such as pH, calcium content, content of soil organic carbon, water content and maximal water holding capacity (MWHC) and soil texture. General soil parameters are summarized in Table 4. Metal-DMA complex concentrations were in the range of 0.4 to 30 µM, thereby showing that the nanomolar concentration range of the LOQ made the method fit for purpose to investigate the interplay of DMA with transition metals.

Table 4 General soil parameters of the investigated soils

							DTPA-extractable trace elements /mg kg^{-1} soil					
Soil	country	pH ($CaCl_2$)	SOM content /g kg^{-1}	Clay content /g kg^{-1}	$CaCO_3$ content /g kg^{-1}	MWHC /g kg^{-1}	Cu	Ni	Zn	Mn	Co	Fe
Arnoldstein	Austria	7.2	66	235	323	453	8.3	0.70	120	8.0	0.10	18
Redlschlag	Austria	6.9	27	190	3	525	0.60	32	0.80	23	0.60	13
Santomera	Spain	7.5	15	300	499	504	1.6	0.30	0.50	3.1	<LOD	4.9

SOM…soil organic matter; MWHC…maximal water holding capacity

Table 5 Stability constants (logß) of metal-ions in complex with the DMA-ligand according to Murakami et al. (1989)[13, 34]

Metal-ions	Stability constant log ß
Fe(III)	33.35
Cu(II)	18.70
Ni(II)	14.78
Zn(II)	12.84
Mn(II)	8.29

Arnoldstein soil. Total metal concentration measurements revealed a dominant concentration of copper (35.8 ± 0.8 µM) and zinc (29.1 ± 1.2 µM) followed by a slightly lower manganese concentration (12.8 ± 0.2 µM). Other trace elements such as iron, cobalt, nickel appear in a concentration range <2 µM. A total DMA amount of 91.8% of the spiked 100 µM DMA could be recovered in the soil solution (Fig. 4). Nearly equal total DMA concentration and summarized metal concentrations were present in the soil extract. Single elemental metal-DMA complex concentration shown in Fig. 4, Fig. 5 and Fig. 6 is summed to a total metal-DMA complex concentration in Fig 7. It can be seen that around 40% of the investigated trace elements are detected as DMA-complexes. In the case of Cu(II)-DMA approximately 56% of Cu relative to the total metal concentration is present in the complexed form (shown in Fig. 4 and Fig. 2b). Due to the low complex stability constant[13] of $10^{8.29}$, Mn did not form a complex with DMA and was eluted as free metal within the void volume of the chromatographic system. Moreover, small fractions of Co and Ni were detected within the void volume.

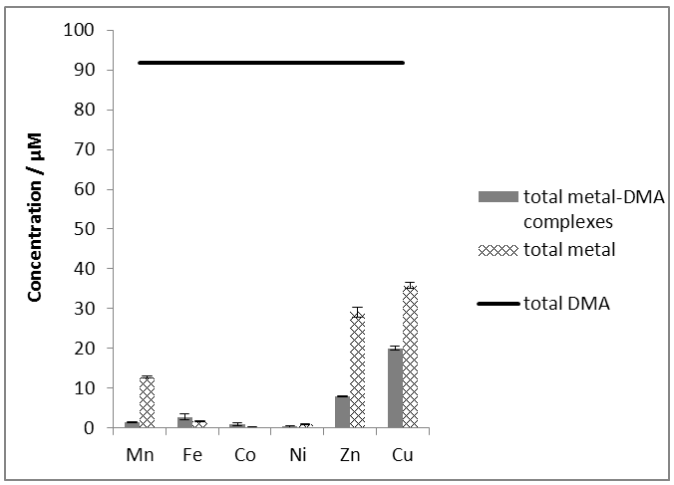

Fig. 4 Speciation of trace metals in the DMA-soil extract of Arnoldstein

Redlschlag soil. Total metal concentration measurements revealed a dominant concentration of nickel (45.8 ± <0.1 µM) and minor concentrations (<6 µM) of manganese, iron and copper (Fig. 5). A total DMA amount of 68.6% from the 100 µM spike was recovered in the soil extract. Originally 100 µM ligand was added to the soil solution. By soil interaction reactions (adsorption processes) and microbial degradation, approximately 30% of the free DMA was lost and no more available for the metal-DMA complex formation. Similar as in the example of Arnoldstein soil, by opposing the total metal and the total DMA concentrations, approximately equal concentrations are revealed (Fig. 7). Mainly Ni(II)-DMA complex is formed, whereby approximately 60% of Ni are complexed by the DMA. The void volume contained a fraction of Mn, Co, Ni and Cu.

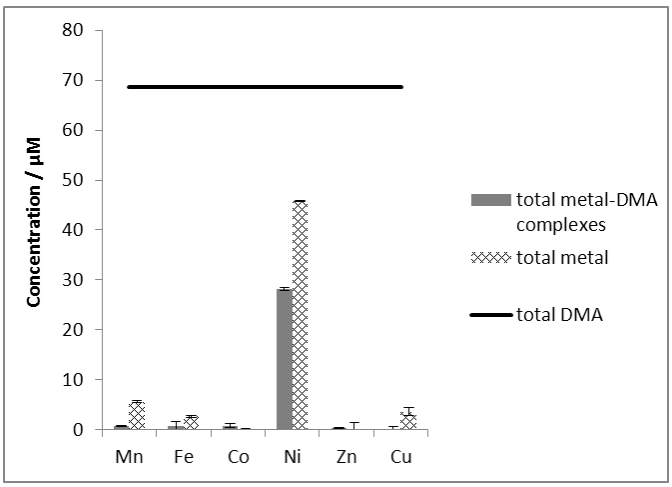

Fig. 5 Speciation of trace metals in the DMA-soil extract of a soil from Redlschlag which is contaminated with nickel.

Santomera soil. Santomera soil has very slow metal dissolution rates[23] which is shown by the determined low total metal concentrations <5 µM(Fig. 6). The trace elements Mn, Fe and Cu have approximately equal total metal concentrations (<4 µM) in soil, whereas the competitors Co, Ni, Zn are only low abundant (<1 µM). The total DMA concentrations (38.2 ± 0.1 µM) are approximately three times higher (Fig. 7) than the total metal concentrations in soil, and the DMA excess provoked the quantitative complexation of all investigated metals, besides manganese. In general, in Santomera soil, nearly 70% of the summarized metal amounts were present in complexed form. The void volume contained a non-retained fraction of free Mn, Co, Ni and Cu. By soil adsorption processes nearly 60% of the total free DMA was lost. The elevated clay content in Santomera soil (Table 4) induces DMA adsorption processes onto the soil solid phase. Therefore, only a total DMA amount of <40% of the spiked 100 µM DMA solution could be recovered after interaction with the soil.

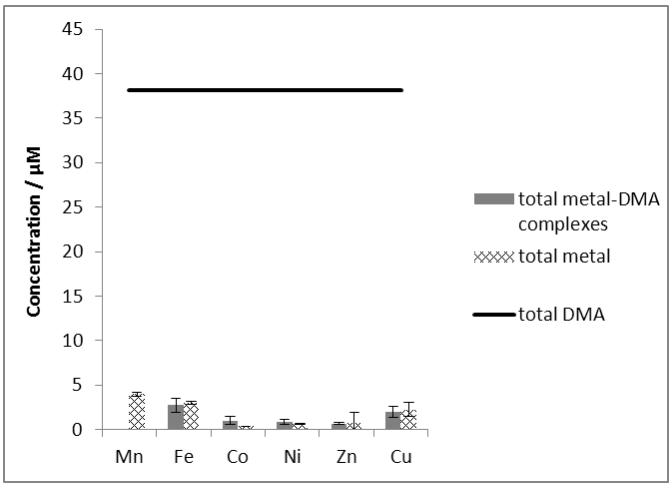

Fig. 6 Speciation of trace metals in the DMA-soil extract of a soil from Santomera containing non-elevated amounts of trace metals.

The comparison of the total metal concentration in solution and the total metal-DMA complex concentration (Fig. 7) revealed that not the entire metal fraction is recovered as DMA-complex in the case of the soils from Redlschlag and Arnoldstein which is not in full agreement with the data revealed from recently published equilibrium modeling.[23] The difference can be explained to some extend by the fact that Mn is not complexed by DMA and therefore only present in the total metal fraction of the data shown in the figures. On the other hand, the correlation of the concentration of SOM with the ratio of total metal to total metal-DMA complexes could indicate that additional competing ligands are present in the DMA-extracts. The elucidation of processes causing the difference to simulated data[23] will require further investigations.

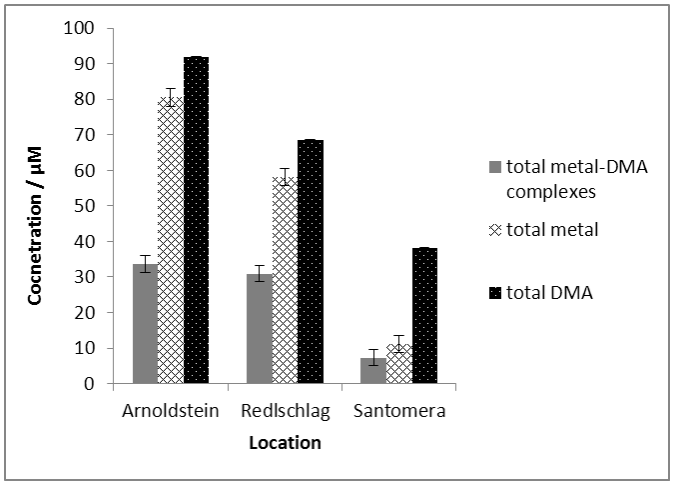

Fig. 7 Comparison of the sum of total element concentrations and concentrations of the different DMA-complexes in soil extract solutions from Fig. 4, Fig 5, and Fig 6.

Conclusion

For the first time speciation of different metal-DMA complexes in real soil extract samples obtained after incubation with DMA was investigated under physiological conditions at pH 6.6. A clear advantage compared to recent HPLC-based methods was the retention, separation and quantification of the intact Fe(III)-DMA species. During routine analysis the main drawback of the chromatographic phase was the need of frequent column cleaning and intermediate injection of acid cleaning solution (e.g. free DMA, formic acid) between the runs.

Nevertheless, the high sensitivity and robustness of the speciation method allows the quantification of metal complexes within the sub-micromolar concentration range in matrix rich samples and is therefore also fit-for-purpose for rhizosphere samples as the relevant concentration of Me-DMA complexes has been estimated within the micromolar concentration range.[21] Hence, future measurements of metal-DMA complexes will enable to investigate the contribution of DMA to Fe mobilization from soil in the context of Strategy II Fe acquisition, as well as to quantify the interference of other metals in this acquisition strategy. It may also contribute to clearing up the role of PS in the acquisition of other nutrients, e.g. Cu and Zn.

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Notes and references

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