

Experimental determination of zinc isotope fractionation in complexes with the phytosiderophore 2'-deoxymugeneic acid (DMA) and its structural analogues, and implications for plant uptake mechanisms

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ABSTRACT

The stable isotope signatures of zinc are increasingly used to study plant and soil processes. Complexation with phytosiderophores is a key process and understanding the controls of isotope fractionation is central to such studies. Here, we investigated isotope fractionation during complexation of Zn^{2+} with the phytosiderophore 2'-deoxymugeneic acid (DMA) - which we synthesised - and with three commercially-available structural analogues of DMA: EDTA, TmDTA and CyDTA. We used ion exchange chromatography to separate free and complexed zinc, and identified appropriate cation exchange resins for the individual systems. These were Chelex-100 for EDTA and CyDTA, Amberlite CG50 for TmDTA and Amberlite IR120 for DMA. With all the ligands we found preferential partitioning of isotopically heavy zinc in the complexed form, and the extent of fractionation was independent of the Zn:ligand ratio used, indicating isotopic equilibrium and that the results were not significantly affected by artefacts during separation. The fractionations (in ‰) were $+0.33 \pm 0.07$ (1σ , $n=3$), $+0.45 \pm 0.02$ (1σ , $n=2$), $+0.62 \pm 0.05$ (1σ , $n=3$) and $+0.30 \pm 0.07$ (1σ , $n=4$) for EDTA, TmDTA, CyDTA and DMA, respectively. Despite the similarity in Zn-coordinating donor groups, the fractionation factors are significantly different and extent of fractionation seems proportional to the complexation stability constant. The extent of fractionation with DMA agreed with observed fractionations in zinc uptake by paddy rice in field experiments, supporting the possible involvement of DMA in zinc uptake by rice.

INTRODUCTION

With the introduction of multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS), it has become possible to measure stable-isotope fractionation of metals in natural systems in the way that is routinely done for light elements such as C, O, N, and S¹. Isotope systems are now available to study biogeochemical processes controlling trace element cycling in the natural environment. Of special interest are applications to study metal cycling in soil environments and during plant uptake, as mediated by rhizosphere processes. To date, complex root-soil interactions have only been studied indirectly using experiments in artificial laboratory systems or using mathematical modelling. The lack of direct techniques without artificial manipulations has hampered progress. Isotope fractionation at natural abundance has much to offer in this. Recent work has shown significant isotope fractionations in trace element uptake by plants, as well as differences between plant species, likely reflecting different uptake mechanisms².

In previous work on zinc uptake in rice, we found a light isotope bias in experiments conducted with solution cultures³ but a neutral or heavy isotope bias in zinc uptake by rice grown in soils under aerobic and anaerobic conditions and with different zinc status^{4, 5}. This is suggesting that uptake mechanisms in rice are controlled by environmental factors. Indeed, studies with other plant types (hyper-accumulators and non-accumulators, grasses, trees) showed equally a neutral or heavy isotope bias during zinc uptake when grown in soils⁶⁻⁹ and a light isotope bias in studies when grown in hydroponic solutions¹⁰⁻¹².

Different processes have been proposed to explain the observed isotope patterns including zinc uptake from different soil pools⁶ and the involvement of Zn-chelating phytosiderophores. The latter mechanism has been invoked because a heavy bias is expected in equilibrium fractionation during

ligand formation¹³. Indeed, Guelke and von Blankenburg (2007) found a heavy isotope bias in iron uptake by grass species, which are known to secrete phytosiderophores to facilitate iron uptake; but a light isotope bias in iron uptake by non-grass species, which do not secrete phytosiderophores¹⁴.

It has been speculated that phytosiderophores are involved in the solubilisation and uptake of soil zinc by rice, as well as in its transport within the plant^{4, 15-17}. To assess if observed isotope patterns in rice are possibly linked to Zn-chelating phytosiderophores, there is a need to constrain the equilibrium isotope fractionation during the complexation of zinc with phytosiderophores. However, there are significant experimental and analytical challenges to this. First, the phytosiderophore studied needs to be in a very pure state to avoid interferences during complexation. Isolates from plants and root secretions are prone to impurities¹⁸. It is preferable to synthesise the phytosiderophore. Protocols for the multi-step synthesis of the phytosiderophore DMA have been reported^{19, 20}, making DMA a suitable model phytosiderophore to study zinc fractionation. Second, there is the considerable challenge of separating free and complexed species from aqueous solutions without inducing artificial isotope fractionation^{21, 22}. The only previous attempt to do this for isotope fractionation studies of Zn-organic ligand complexation used a Donnan membrane²³. Use of Donnan membranes, however, is time consuming, prone to blank contributions due to the numerous steps involved, and there are possible implication of slow dissociation of metals²⁴. Ion exchange chromatography can avoid these problems if suitable resins can be found as successfully demonstrated for iron²². The ion-exchange properties of potential resins can be predicted from the protonation and complexation constants of the resin's hydro-soluble active groups in aqueous solution. However, sorption of divalent metal ions on resins does not take place through simple ion exchange, and so the separation of free and complexed species is not easily predictable from the resin's ion-exchange properties alone²⁵. To determine equilibrium isotope fractionation, there should be no exchange of zinc between the complex and exchange resin. One widely used approach to test this is to determine the isotope fractionation between reactants and products using a range of metal:ligand ratios^{21, 26, 27}. The net isotope fractionation must be independent of the metal:ligand ratio within analytical precision. Other methods include the use of isotope spikes^{21, 22} but these are prone to issues such as equilibration rates.

Given these challenges, experimental studies of isotope fractionation between metal cations and organic ligands are limited. Jouvin and colleagues²³ investigated the isotopic fractionation during adsorption onto purified humic acid (PHA) and found that zinc bound to PHA was isotopically heavier than free Zn²⁺ ($\Delta^{66}\text{Zn}_{\text{ZnPHA-freeZn}^{2+}} = 0.24 \pm 0.06$). The fractionation factor depended on the affinity of the sites and on the pH of the solution. Using humic acids to improve our understanding of the underlying physical-chemical controls of isotope fractionation, however, has the disadvantage that they are structurally poorly constrained and hence a systematic investigation of structural controls (i.e. numbers of donors such as nitrogen, oxygen, the effect of the denticity, ligand affinity etc.) is not possible. Experimental studies involving other transition metals were conducted with iron and desferrioxamine B (DFOB)^{21, 22}, EDTA and oxalate²² and with copper and insolubilized humic acid (IHA)²⁸, ethylenediaminetetraacetic acid (EDTA), nitrotriacetic acid (NTA), iminodiacetic acid (IDA) and DFOB²⁹. These experimental studies found all a preference for the heavy isotope during complexation and structural controls including complexation strength and bond distances were put forward as possible controls.

The goal of the present study was to determine for the first time isotopic fractionation factors for zinc complexation by a natural phytosiderophore, i.e., DMA, and structurally similar polydentate ligands. We synthesised DMA using recently published methods, and we identified the best resins to separate free and complexed Zn^{2+} for the ligands under study. We then determined the direction and extent of isotopic fractionation during complexation at different Zn:ligand ratios, and tested for possible controls such as ligand affinity and bonding environment.

MATERIALS AND METHODS

Choice of ligands. We chose DMA since it has been proposed to play a major role in zinc uptake in rice. There is a good synthetic protocol to prepare pure samples of DMA in good yields, therefore from a practical point of view it is possible to get pure sample material. We chose ethylenediaminetetraacetic acid (EDTA), trimethylenedinitrilotetraacetic acid (TmDTA) and cyclohexanediaminetetraacetic acid (CyDTA) as additional ligands because they are commercially available, hexadentate ligands (like DMA) that bind zinc with high affinities giving complexes with the same overall geometry and coordination sphere as DMA – i.e. all the complexes are octahedral and they all use the same donor atoms to coordinate zinc: 4 oxygens and 2 nitrogens (Figure 1).

Synthesis of DMA. We used synthesis protocols previously published^{19,20}. Details are given in the Supporting Information. All starting materials and reagents were purchased from commercial sources and used without further purification. The progress of the synthesis was monitored by ^1H NMR spectroscopy at 297 K in the solvent indicated, using a Bruker AC300 spectrometer. The spectra were calibrated with respect to tetra-methylsilane and the residual solvent peaks indicated in the relevant spectrum.

Choice of resins. Three resins were assessed based on their suggested abilities to sequester free Zn^{2+} without interacting with the Zn-ligand complex. The resins were Chelex-100 (BioRad, Na^+ form, 100–200 mesh, containing carboxyl functional groups) for the complexes with EDTA and CyDTA³⁰, Amberlite CG50 (Dow, H^+ form, 100–200 mesh, also containing carboxyl functional groups) for the complexes with TmDTA^{25,30}, and Amberlite IR120 (Alfa Aesar, H^+ form, containing sulfonic acid functional groups) for the complexes with DMA¹⁸.

Preparation of solutions. All solutions were prepared in Teflon Savillex vials (Savillex, MN, USA). Acid solutions were prepared using 18 M Ω -grade Millipore water (Bedford, MA, USA) and AnalaR grade HCl (6 M) and HNO_3 (15.4 M), both sub-distilled. Stock solutions were prepared as follows: 1 mM $\text{Zn}(\text{OAc})_2$ at pH 6.2 by dissolving $\text{Zn}(\text{OAc})_2$ dihydrate (0.11 g) in 500 ml of MQ H_2O ; and 1 mM Na_4EDTA by dissolving Na_4EDTA dihydrate (0.095 g) in 250 ml of MQ H_2O and heating at 60°C until complete dissolution. Similarly, 250 ml of 1 mM stock solutions of TmDTA (0.077 g) and CyDTA monohydrate (0.091 g) were prepared. Potassium 2-(*N*-morpholino)ethanesulfonic acid (KMES) buffer solution (0.5 M) was prepared by dissolving MES monohydrate (26.66 g) in 250 ml of Millipore H_2O and stirring at 60 °C until complete dissolution was achieved. The pH of the solution was adjusted to 6.2 by the addition of 3 M KOH aqueous solution. $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$, CyDTA monohydrate and TMDTA were purchased from Sigma-Aldrich, Na_4EDTA from Fisher Scientific and MES monohydrate from VWR.

Different ratios (mol:mol) of free Zn^{2+} to complexed ZnL^{2-} were prepared by adding 10 ml of 1 mM $\text{Zn}(\text{OAc})_2$ to a corresponding volumes of 1 mM ligand solution. All reagents were prepared using

18.2 mΩ cm Millipore water. The solutions were buffered to pH 6.2 using 0.5 M KMES and equilibrated overnight before proceeding to the ion exchange separation. Although all weighing was done gravimetrically, some error in molar quantities is possible for the ligand compounds due to their hygroscopic character. We confirmed that complete complexation was reached upon mixing equimolar solutions of Zn(OAc)₂ and L⁴⁻ where L⁴⁻ refers to the deprotonated ligand at pH 6.2 using GEOCHEM-EZ software³¹.

Commercial solutions of Cu (ROMIL Ltd, Cambridge, UK) and Zn (ROMIL Ltd, Cambridge, UK) were used as dopant solution for instrumental mass bias correction and for quality control of the isotope measurement on the MC-ICP-MS, respectively³².

Ion exchange procedures. We adapted two previously published ion exchange protocols: a cation exchange procedure for the separation of free and complexed zinc³³ and an anion exchange procedure for the separation of zinc fractions from the Na-rich solution matrix for subsequent isotope ratio measurements³⁴. The protocol of these procedures is shown in Table 1. All resins were prepared and cleaned according to the manufacturers' recommendations and loaded onto BioRad PolyPrep columns. In general, the resin was soaked in 100 ml of Millipore H₂O per 5 g of resin, and then pipetted into BioRad Poly-Prep (Bio-Rad Laboratories, CA, USA) columns (i.d. 8 mm). The resin was cleaned with 2 M HCl and equilibrated with 0.5 M KMES buffer (pH 6.2). The buffered samples were loaded on to the column. The Zn-ligand complex was collected straight away as the samples ran down the column. The resin was further equilibrated with the buffer to elute any remaining complexed zinc. Washing the column with 1M HCl eluted all free Zn²⁺ initially ex-changed with the resin matrix. After collecting both free and complexed zinc, the fractions were evaporated to dryness and refluxed in 15.6 M HNO₃ at 100 °C for 3 h prior to drying at 120 °C to remove the easily oxidisable organic ligand material. After final drying, the samples were re-dissolved in 0.3 M HNO₃ for concentration measurements.

All collected samples, containing free Zn²⁺ or digested Zn-ligand complex, were evaporated to dryness, refluxed in 5.8 M HCl, diluted in 1 ml of 5.8 M HCl and passed through PolyPrep columns containing 0.7 ml AG-MP1 resin (Bio-Rad, Cl⁻ form, 100–200 mesh) anion-exchange resin, before evaporation and reflux in 15.6 M HNO₃. Evaporated samples were re-dissolved in 0.5 M HNO₃. The fractions containing Zn-ligand complexes were dissolved in a mixture of 5 ml 15.6 M HNO₃ and 3 ml 30% (v/v) H₂O₂, and digested using a microwave oven (210 °C, 1.7 kPa, 90 min) to break down the organic matrix³⁵. All experimental work associated with preparation of samples and ion exchange chromatography was carried out in Class 10 laminar flow hoods in a Class 1000 Clean Laboratory.

Zinc concentration and isotopic composition measurements. Zinc concentrations were determined using ICP-AES (Thermo iCap 6500 Duo, Thermo Scientific, UK). Zinc isotope ratios were measured using multi collector ICP-MS (Nu Plasma, Nu Instruments, UK) and are expressed using the conventional δ⁶⁶Zn notation (‰):

$$\delta^{66}\text{Zn} = ((^{66}\text{Zn}/^{64}\text{Zn})_{\text{sample}} / (^{66}\text{Zn}/^{64}\text{Zn})_{\text{standard}} - 1) \times 1000 \quad (1)$$

The empirical external normalisation method³² was used to correct for instrumental mass bias and the measurements were bracketed with the in-house standard London Zn. Accuracy and precision of the isotope measurements were assessed by analysing two single element solutions during each measurement session: IRMM 0072 and Romil Zn³⁶. The results were δ⁶⁶Zn_{IRMM} – δ⁶⁶Zn_{London} = -0.25

184 ± 0.07 ‰ (2 SD, $n = 6$) and $\delta^{66}\text{Zn}_{\text{Romil}} - \delta^{66}\text{Zn}_{\text{London}} = -9.00 \pm 0.06$ ‰ (2 SD, $n = 6$). These $\delta^{66}\text{Zn}$ values
 185 agree well with previously published values³⁶.

186 For every ligand system tested, the $\delta^{66}\text{Zn}$ values of the initial solution (i.e. $\text{Zn}(\text{OAc})_2$) and of the
 187 free and complexed zinc fractions were determined. To quantify the isotope effect caused by
 188 complexation of zinc with the test ligands, the isotopic fractionation was calculated as:

$$189 \quad \Delta^{66}\text{Zn}_{\text{ZnL}^{2-} - \text{Zn}^{2+}} = \delta^{66}\text{Zn}_{\text{ZnL}^{2-}} - \delta^{66}\text{Zn}_{\text{Zn}^{2+}} \quad (2)$$

190 , where L refers to the tested ligand.

191 The isotope value for the complexed zinc fraction was also calculated using mass balance
 192 constraints to test the integrity of the data as organic containing samples are well known to be difficult
 193 for precise and accurate isotope ratio measurements:

$$194 \quad \delta^{66}\text{Zn}_{\text{system}} = (\delta^{66}\text{Zn}_{\text{Zn}^{2+}} f_{\text{Zn}^{2+}}) + (\delta^{66}\text{Zn}_{\text{ZnL}^{2-}} f_{\text{ZnL}^{2-}}) \quad (3)$$

195 , where $\delta^{66}\text{Zn}_{\text{system}}$ is the isotope composition of the initial solution, $\delta^{66}\text{Zn}_{\text{Zn}^{2+}}$ and $\delta^{66}\text{Zn}_{\text{ZnL}^{2-}}$ are the
 196 isotope values of the free and of the complexed Zn fraction, respectively, and $f_{\text{Zn}^{2+}}$ and $f_{\text{ZnL}^{2-}}$ are the
 197 mol fractions of free and of complexed Zn fractions calculated as $f_x = m_{\text{fraction}} / m_{\text{total}}$.

198 RESULTS AND DISCUSSION

199 **Separation of free and complexed zinc using cation exchange chromatography.** We
 200 confirmed using GEOCHEM-EZ³¹ that complete complexation was reached upon mixing equimolar
 201 solutions of $\text{Zn}(\text{OAc})_2$ and L^{4-} , where L^{4-} refers to the deprotonated ligand at pH 6.2, and that no other
 202 complexes were formed. Table 2 shows the separation performance of the resins with respect to the
 203 different $\text{Zn}^{2+}/\text{ZnL}^{2-}$ systems studied in this work. Chelex-100 shows quantitative recovery and
 204 separation within 5% of the prepared mol fractions of free Zn^{2+} and ZnEDTA^{2-} and ZnCyDTA^{2-}
 205 complexes. EDTA and CyDTA were the ligands used with the highest affinity for zinc(II), i.e. with
 206 $\log K = 16.4$ and $\log K = 18.5$, respectively³⁷. In contrast, Chelex-100 is too strong for the
 207 ZnTmDTA^{2-} complex ($\log K = 15.6$) and we observe partial dissociation of the complex leading to an
 208 increased mol fraction of $\text{Zn}^{2+}/\text{Zn}_{\text{total}}$ in the eluent (Table 2). However, we found good separation in
 209 line with the mol fractions prepared for free Zn^{2+} and ZnTmDTA^{2-} using Amberlite CG50. With
 210 respect to DMA ($\log K = 12.8$), we found a slight difference between the initial molar fraction and the
 211 measured one (Table 2). Although all weighing was done gravimetrically, there is inevitably some
 212 variability in the molar quantities of DMA due to its hygroscopic character. Other possible processes
 213 which could affect the molar fractions for the $\text{Zn}^{2+}/\text{ZnDMA}^{2-}$ in the starting solution are shifts in pH,
 214 complexation with the resin or effects of the matrix²⁵. The differences between targeted and real mol
 215 fractions, however, did not affect the isotope fractionation (see discussion below), suggesting that
 216 dissociation from the resin was not the controlling process.

217 Figure 2 shows the elution sequence of the $\text{Zn}^{2+}/\text{ZnCyDTA}^{2-}$ system. The zinc complexes are
 218 eluted from the corresponding resin during the sample loading process in H_2O and the subsequent
 219 matrix elution step using KMES buffer, whereas free Zn^{2+} is retained and only eluted on addition of 1
 220 M HCl. Figures 2a to 2c show the elution profiles for three samples of the $\text{Zn}^{2+}/\text{ZnCyDTA}^{2-}$ system
 221 with different molar ratios of free Zn^{2+} to total Zn. With no free Zn^{2+} , the ZnL^{2-} complex is eluted
 222 instantly and no further zinc is recovered upon elution with 1 M HCl. For the 0.5 mol fraction of free
 223 Zn^{2+} , the complexed ZnL^{2-} fraction is eluted during the sample loading and buffer elution steps, while
 224 the free Zn^{2+} is eluted with 1 M HCl. Finally, for the solution with only free Zn^{2+} , no Zn^{2+} is eluted

during the initial two steps (sample loading and matrix elution with the buffer solution, Table 2), whereas upon addition of 1 M HCl, elution of free Zn^{2+} was instantaneous, explaining the sharp peak after 24 ml following the change to the 1M HCl solution (Figure 2a). Between 96 and 105 % of the zinc was recovered in all test conducted and shown in Table 2.

Isotope fractionation during complexation reactions. Table 3 shows the isotope ratios (expressed using the $\delta^{66}\text{Zn}$ notation) of the free Zn^{2+} fraction (experimentally determined) and of the complexed zinc fraction (experimentally determined and calculated based on mass balance, see equation 3) for the four different ligands (DMA, EDTA, CyDTA, TmDTA) systems and for different mol fractions. Also shown is the recovery of zinc, i.e. zinc loaded onto the column vs zinc eluted. In general, we obtained a very good recovery in all of them. Only experiments where measured and calculated values for $\delta^{66}\text{Zn}_{\text{ZnL}^{2-}}$ agreed within the reproducibility of the isotope ratio determinations were considered for further evaluation, guaranteeing an internally consistent data set.

As seen in Table 3, we found that the heavier isotope is preferred in the complexed zinc in all four ligand systems investigated during the course of this study (Table 3). The preferential accumulation of the heavy isotope in the ZnL^{2-} complexes is in agreement with equilibrium reaction dynamics on formation of strong bonds between metals and ligands¹³. The magnitude of isotope fractionation between free and complexed zinc (expressed as $\Delta^{66}\text{Zn}_{\text{ZnL}^{2-} - \text{Zn}^{2+}}$, Equation 2) are within error for the different mol fractions studied in each system. Theory predicts that if a closed system is at isotopic equilibrium, then the Δ -value will be independent of the mol fraction³⁸. The fractionation factors determined in this study are therefore at thermodynamic equilibrium. Dissociation of the ZnL^{2-} complex on the resins, including for the $\text{Zn}^{2+}/\text{ZnDMA}^{2-}$ system, is thus unlikely or at least insignificant as discussed above. The average values for $\Delta^{66}\text{Zn}_{\text{ZnL}^{2-} - \text{Zn}^{2+}}$ are $+0.33 \pm 0.07 \text{ ‰}$ (1σ , $n = 3$) for ZnEDTA^{2-} , $+0.45 \pm 0.02 \text{ ‰}$ (1σ , $n = 2$) for ZnTmDTA^{2-} , $+0.62 \pm 0.05 \text{ ‰}$ (1σ , $n = 3$) for ZnCyDTA^{2-} , and $+0.30 \pm 0.07 \text{ ‰}$ (1σ , $n = 4$) for ZnDMA^{2-} .

Table 4 gives a compilation of selected fractionation factors normalised per atomic mass unit for the complexation of transition row metals (Fe, Zn, Ni and Co) with organic ligands derived from experimental and theoretical studies alike. We find that the experimentally determined fractionation factor for zinc complexation with humic acid²³ is smaller than that for zinc complexation with DMA and the other synthetic ligands studied in this study. Computationally determined fractionation factors for zinc complexation with citrate and malate, thus organic molecules smaller than the ligands studied in this study, show less positive or even negative fractionation³⁹⁻⁴¹. Negative fractionation is also observed in computational studies of the complexation of citrate with Ni and Fe⁴¹. A recently published experimental study of copper complexation with natural and synthetic ligands²⁹ showed fractionation factors of similar magnitudes for EDTA and for CyDTA, in line with our present work with zinc (Table 3). It is also noteworthy that the isotope fractionation of copper is (i) larger for the complexation with CyDTA than with EDTA and (ii) lower for the complexation with fulvic acid than with synthetic ligands. Both observations seem to hold for Zn (see Table 3 and Jouvin et al., 2009). For Fe, experimental and theoretical studies showed larger fractionation factors during complexation with phytosiderophores and synthetic ligands than with smaller organic ligands such as oxalate or citrate^{21, 22, 42}. Table 4 also highlights the disagreement between previous experimental²¹ and theoretical⁴³ studies on the sense of fractionation between Fe-desferrioxamine B (Fe-DFBO) and $\text{Fe}(\text{H}_2\text{O})_6^{3+}$. Finally, the range of zinc isotope variation observed to date in the terrestrial environment

is approximately $\Delta^{66}\text{Zn} \sim 1.8\%$ ⁴⁴ and therefore our data suggests that the extent of fractionation for zinc observed during complexation with phytosiderophores is significant and likely plays a major control of the global biogeochemical cycle of Zn isotopes ⁴⁵.

Controls of isotope fractionation. The results for the four hexadentate ligands allow us to explore the link between isotope signatures, reactivity and structure. Despite the similarity in Zn-coordinating donor groups, the differences in the exact geometries of the ZnL^{2-} complexes result in a range of affinity constants ($\log K$) between 12.8 and 18.5 ^{37,46} and lead to significantly different isotope fractionation. Figure 3 shows the relationship between $\log K$ and the isotopic fractionation found in our study. There is strong evidence for an increase in heavy bias with increasing complexation strength. This trend has been inferred before by computational studies of organic and inorganic zinc complexes ³⁹. Similar conclusions were drawn in a theoretical study of organic and inorganic ligands using transition metals including iron, nickel, zinc and copper ⁴¹.

We obtain the relationship $\Delta^{66}\text{Zn} = (0.049 \pm 0.02) \times \log K - (0.366 \pm 0.390)$ ($r^2 = 0.67$, $p = 0.035$). A strong relationship between isotopic fractionation and $\log K$ with organic ligands has been suggested experimentally also for iron ^{22, 47} and copper ^{28, 29}. We note that the slope of the linear regression determined for zinc (0.049, this study) and for copper (0.036, ²⁹) are very similar. While the assessed linear relationship obtained in Figure 3 is affected by the lower value for the EDTA, it is worthwhile to note that the empirical equation predicts negative fractionations for smaller organic molecules such as oxalate, malate and citrate as predicted using calculation before ⁴¹ (Table 4).

The positive correlation between complexation constant and isotope fractionation observed here may provide a simple empirical tool that may be used to predict fractionation factors for Zn-ligand complexes not yet studied experimentally but relevant to a wide range of biological, medical and environmental relevant ligands.

Comparison with observed isotope fractionation of zinc during plant uptake. Significant positive isotope fractionation has been observed for zinc uptake by rice grown in paddy soil ^{4, 5}. The authors tentatively ascribed the heavy isotope bias to uptake of zinc complexed to DMA, consistent with a mathematical modelling exercise ⁴⁸. The extent of the heavy isotope fractionation we have determined during zinc complexation by DMA matches the fractionation measure for soil-grown rice ⁴ as shown in Figure 3. Further work is needed to confirm that rates of DMA secretion by rice under relevant conditions are sufficient to account for enhanced zinc uptake. Further evidence of heavy isotope discrimination during uptake of complexed metals by plants is provided for zinc uptake by tomatoes growing in zinc deficient soil ⁴⁹, by hyperaccumulators ^{8, 50}, and for iron uptake by phytosiderophore-secreting grasses ¹⁴. Whereas in field and hydroponic studies, Jouvin and co-worker found a light isotopic fractionation of between 0 and -1 ‰ in copper uptake by graminaceous and non-graminaceous plants, suggesting that uptake was not mediated by complexation ¹².

The findings presented here should make an important contribution to the emerging picture of isotopes as novel technique to study the cycling of zinc and other trace element in the plant-soil environment and to resolve key questions such as mechanisms of zinc uptake in plants.

ASSOCIATED CONTENT

Supporting Information

308 Further details on the synthesis and characterization of DMA ligand material is available free of
309 charge via the Internet at <http://pubs.acs.org>.

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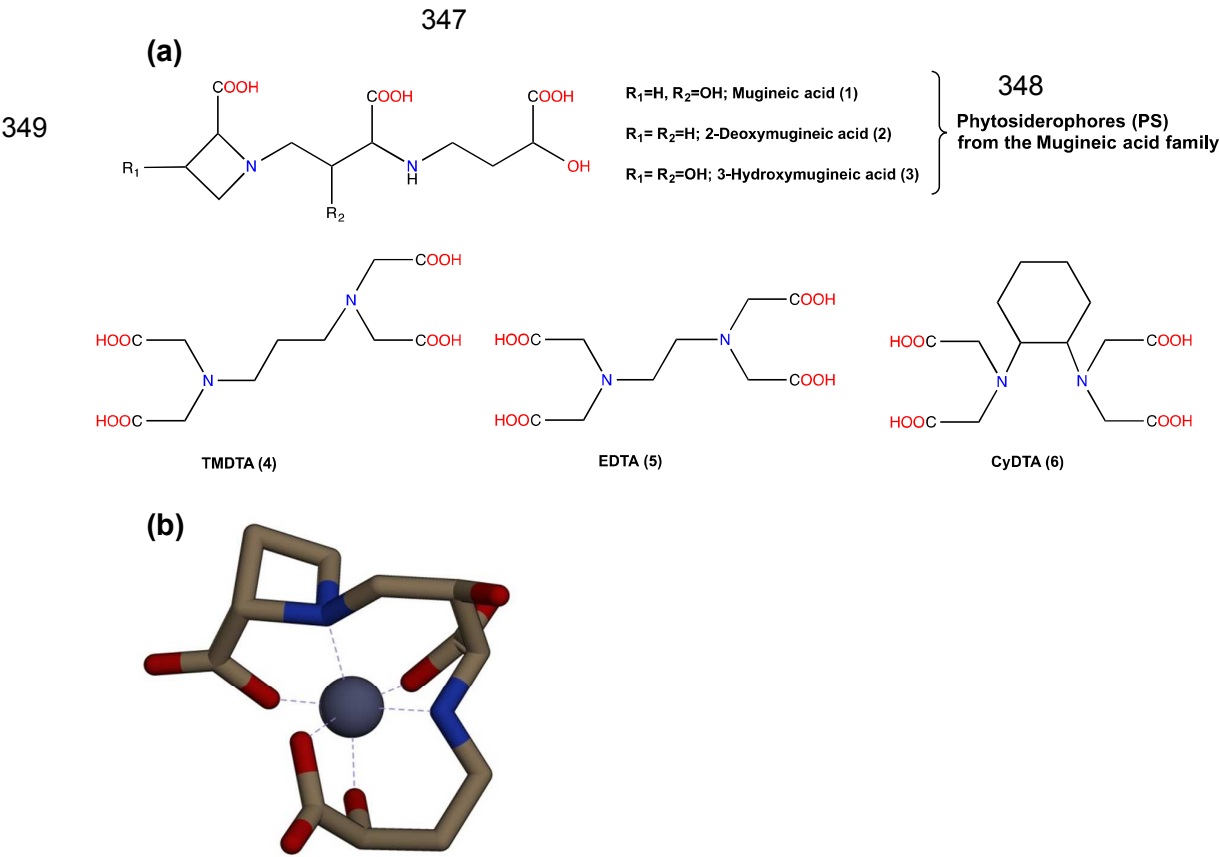
322

323 **Figures**

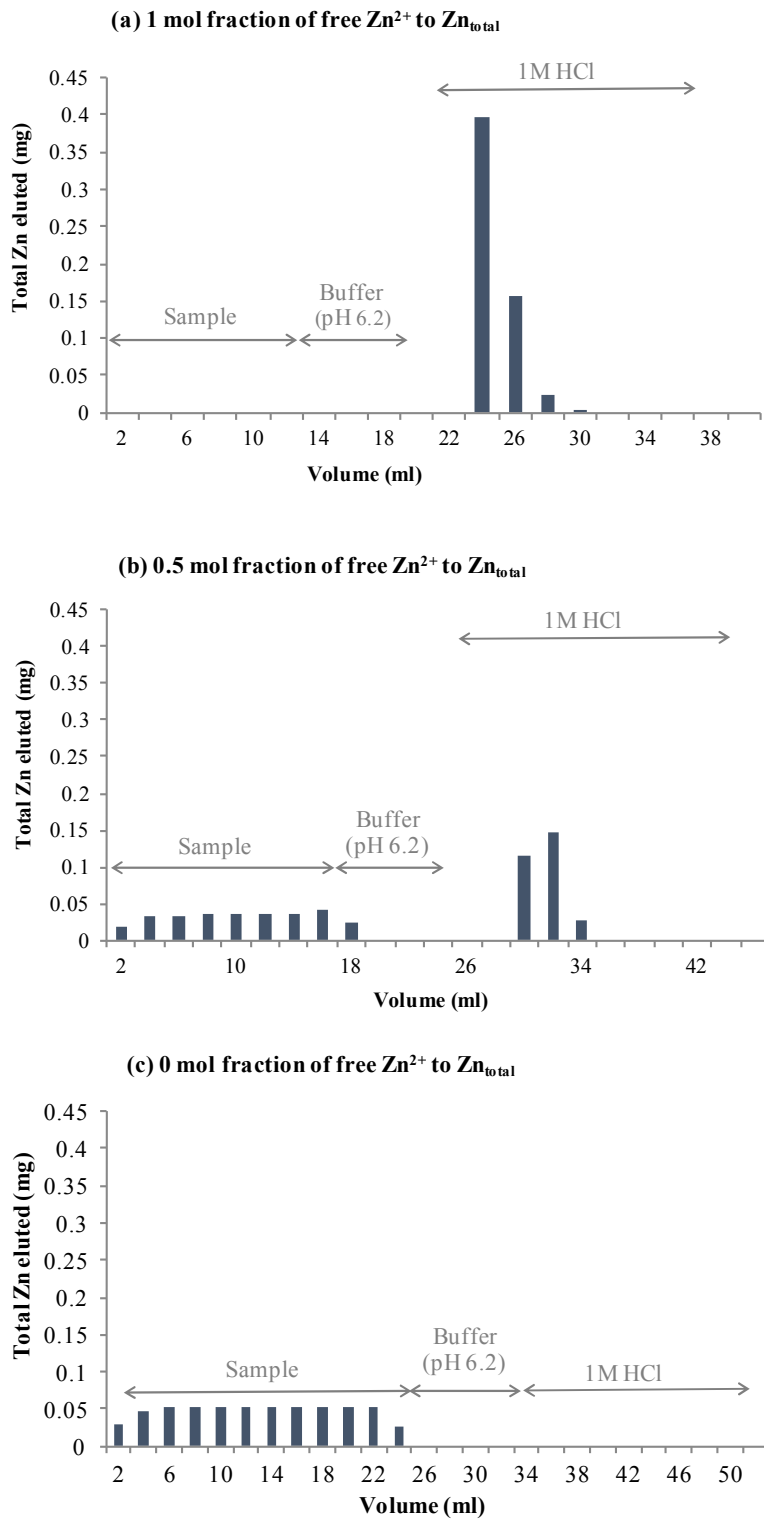
- 324 1. (a) Chemical structures of the four organic ligands tested in this study (2, 4-6), including
325 natural phytosiderophore-ligand from the family of mugineic acids. (b) Molecular
326 structure of the Zn-MA complex modelled with molecular mechanics using ChemBio3D.
327 Note that the colour structures refer to: Zn (iris, central atom), O (red) and N (blue).
328 TmDTA, EDTA and CyDTA coordinate to Zn(II) in an analogous fashion: via the two
329 nitrogen atoms and the four carboxylate groups to give an octahedral complex.
- 330 2. Elution profiles of solutions containing different mol fractions (i.e., 1, 0.5, 0) of free Zn^{2+}
331 and complexed ZnCyDTA^{2-} at pH 6.2 (buffered with 0.5 M KMES buffer). (a) 1 mol
332 fraction of free Zn^{2+} to total Zn in the solution shows complete elution of Zn^{2+} in
333 presence of 1 M HCl. (b) 0.5 mole fraction of ZnCyDTA^{2-} is eluted instantly with 0.5 M
334 KMES whereas for eluting free Zn^{2+} fraction 1 M HCl is needed. (c) In 0 mol fraction
335 sample all zinc is eluted instantly in the complexed form. No free Zn^{2+} is present, as
336 visible from the elution profile after addition of 1 M HCl to the columns.
- 337 3. Measured and calculated isotopic fractionation of zinc upon complexation by the four
338 organic ligands studied (EDTA, CyDTA, TmDTA and DMA) as a function of the
339 stability constants ($\log K$) of the complex formation^{37, 46}. The linear regression is given
340 as $y = 0.049 \pm 0.02 x - 0.366 \pm 0.390$, $R^2 = 0.6766$, $p < 0.35$). Diamonds symbolise $\Delta^{66}\text{Zn}$
341 values calculated using measured $\delta^{66}\text{Zn}$ for the ZnL^{2-} fraction, crosses symbolise $\Delta^{66}\text{Zn}$
342 values calculated using calculated $\delta^{66}\text{Zn}$ for the ZnL^{2-} fraction using mass balance
343 considerations. The triangle symbolise the $\Delta^{66}\text{Zn}$ values between rice stem and soil
344 solution determined in field experiments in paddy field soils⁴

345

346 Figure 1

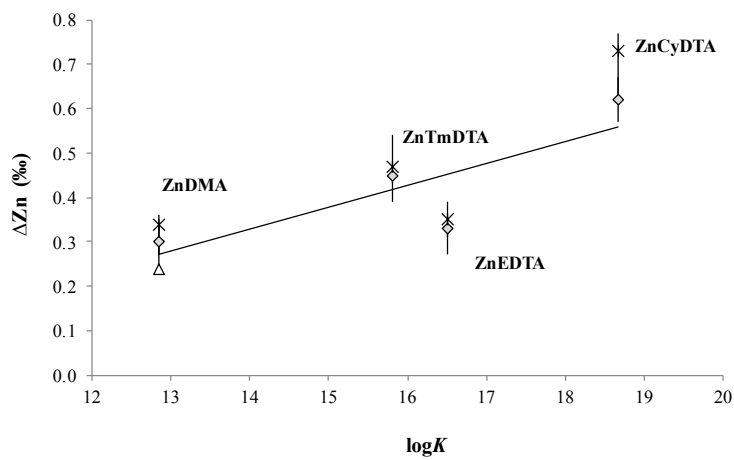


350 Figure 2



351

Figure 3



356 **Tables**

- 357 1. Protocol for the two different ion exchange procedures used during this study. The
 358 cation exchange procedure for the separation of free from complexed zinc used
 359 Chelex-100, Amberlite CG50 and Amberlite IR120. The anion exchange
 360 chromatography for the removal of spectral and non-spectral interferences derived
 361 from the Na-rich matrix for subsequent high precision isotope ratio measurements
 362 used AG-MP1
- 363 2. Separation of free (Zn^{2+}) from complexed (ZnL^{2-}) zinc using the three different resins
 364 Chelex-100, Amberlite CG50 and Amberlite IR120. Shown are the affinity constant
 365 ($\log K$) for the formation of the relevant complex, the mol fraction of free Zn/total Zn
 366 in solutions before and after the passage through the resin, the total amount of zinc
 367 loaded onto the resin and the amount of zinc eluted from the resin after passage
 368 through column
- 369 3. Experimentally determined $\delta^{66}\text{Zn}$ -value for free Zn^{2+} and for complexed ZnL^{2-}
 370 fractions in solutions with different mol fractions (expressed as $f_{\text{Zn}} = \text{Zn}^{2+}/\text{Zn}_{\text{total}}$). The
 371 $\delta^{66}\text{Zn}$ -value of the complexed ZnL^{2-} fraction was also calculated using mass balance
 372 constraints. (See text for details). The experimental fraction factor for the
 373 complexation of Zn^{2+} with ZnL^{2-} was calculated for each solution using $\Delta^{66}\text{Zn} =$
 374 $\delta^{66}\text{Zn}_{\text{ZnL}^{2-}} - \delta^{66}\text{Zn}_{\text{Zn}^{2+}}$ and then averaged for each $\text{Zn}^{2+}/\text{ZnL}^{2-}$ system using the
 375 available $\Delta^{66}\text{Zn}$ values (the mean is shown in bold, n indicates the number of $\Delta^{66}\text{Zn}$
 376 values used, $\pm 1\text{SD}$ indicates the standard deviation). Also shown are recoveries of the
 377 ion exchange procedure and the amount of zinc loaded upon the resin and collected
 378 afterwards.
- 379 4. Published fractionation factors of transition row metals during complexation with
 380 organic ligands using laboratory experiments and theoretical calculations. The
 381 fractionation is expressed using Δ -values in per mill per atomic mass unit, i.e., $\Delta^{x/y}\text{M}$
 382 $= (\delta^{x/y}\text{ML}^{2-} - \delta^{x/y}\text{M}^{2+}) / (x-y)$, where x and y are two different isotopes (x = heavy and
 383 y = light), M is the metal studied and δ is the small delta value for free (M^{2+}) and
 384 complexed (ML^{2-}) species. DFBO = desferrioxamine B, PHA = purified humic acid,
 385 IHA = insolubilized humic acid, EDTA = ethylenediaminetetraacetic acid, TmDTA =
 386 trimethylenedinitrilotetraacetic acid, CyDTA = cyclohexanediaminetetraacetic acid

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388 Table 1

Ion exchange procedure	Objective	Resin	System studied	Step	Medium	Volume ml
Cation Exchange	To separate free Zn ²⁺ from complexed ZnL ²⁻	Chelex-100, Na ⁺ form, 200-400 mesh	Zn/ZnEDTA	Resin Loading	H ₂ O	1 - 2
			Zn/ZnCyDTA	Cleaning	2M HCl	5 x 2
				Conditioning	H ₂ O	3 x 2
		Amberlite CG50, H ⁺ form, 100-200 mesh	Zn/ZnTmDTA	Equilibration	KMES buffer (pH 6.2)	3 x 2
				Sample loading	H ₂ O (pH 6.3)	5 x 2 up to 10 x 2
				Matrix elution	KMES buffer (pH 6.2)	2 x 2
		Amberlite IR120, H ⁺ form	Zn/ZnDMA		H ₂ O	3 x 2
				Zn2+ fraction	1M HCl	5 x 2
				Cleaning	1M NaOH	2 x 2
					H ₂ O	3 x 2
Anion Exchange	To remove isobaric and non isobaric interferences	AG MP1, BioRad, Cfrom, 100-200 mesh		Resin Loading	0.5M HNQ	1 - 2
				Cleaning	0.5M HNQ	5 x 6
				Conditioning	H ₂ O	5 x 3
					6M HCl	4 x 1
				Sample loading	6M HCl	1 x 1
				Matrix elution	6M HCl	3 x 3
					2M HCl	2 x 3.5
				Zn elution	0.1M HCl	2 x 3.5
				Cleaning	0.5M HNQ	5 x 2
					H ₂ O	5 x 1

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391 Table 2

Ligand	logK	Resin	Before column	After column				Dissociation of complex
			Mol fraction targeted Zn^{2+}/Zn_{total}	Total Zn added mg	Zn^{2+} - fraction mg	Zn^{2+} - fraction mg	Mol fraction effective Zn^{2+}/Zn_{total}	
			-				-	
CyDTA	18.5	Chelex-100	1.00	0.580	0.000	0.580	1.00	
			0.50	0.580	0.289	0.291	0.50	No
			0.00	0.580	0.580	0.000	0.00	No
EDTA	16.4	Chelex-100	1.00	0.463	0.000	0.463	1.00	
			0.50	0.555	0.258	0.297	0.53	No
			0.00	0.530	0.518	0.013	0.02	No
TmDTA	15.6	Chelex-100	1.00	0.610	0.000	0.610	1.00	
			0.50	0.622	0.131	0.492	0.79	Partial
			0.00	0.662	0.235	0.426	0.64	Partial
		Amberlite CG50	0.50	0.380	0.168	0.212	0.56	No
			0.00	0.371	0.328	0.044	0.12	No
DMA	12.8	Amberlite CG50	0.50	0.266	0.010	0.256	0.96	Full
			0.00	0.267	0.019	0.248	0.93	Full
		Amberlite IR120						
			0.50	0.190	0.127	0.062	0.33	Possible
			0.00	0.204	0.175	0.029	0.14	Possible

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394 Table 3

Ligand	Sample ID	Fractions		ZnL ²⁺						Δ-value			Mass balance			
		Zn ²⁺ mass	mol fraction	δ ⁶⁶ Zn ±2SD		mass	mol fraction	δ ⁶⁶ Zn		±2SD	δ ⁶⁶ Zn		n	Zn added	Zn eluted	Recovery
				measured	calculated			measured	calculated		per mill	per mill				
		mg		per mill		mg		per mill					mg	mg	%	
EDTA	Stock Solution								-0.06							
	1	0.114	0.20	-0.33	0.07	0.47	0.80	n.d.	n.d.	0.01	n.d.	0.34		0.583	0.582	100
	2	0.488	1.00	0.04	0.03	0.000	0.00	n.d.	n.d.	n.d.	n.d.	n.d.		0.583	0.488	84
	3	0.157	0.27	-0.33	0.08	0.421	0.73	0.07	0.00	0.04	0.40	0.37		0.583	0.579	99
	4	0.249	0.43	-0.27	0.07	0.327	0.57	0.07	0.03	0.10	0.34	0.38		0.583	0.576	99
	5	0.398	0.70	-0.15	0.06	0.174	0.30	0.10	0.04	0.16	0.26	0.31		0.583	0.572	98
	6	0.564	1.00	-0.04	0.01	0.00	0.00	n.d.	n.d.	n.d.	n.d.	n.d.		0.583	0.564	97
	mean										0.33	0.35	3			
	± 1SD										0.07	0.04				
	TmDTA	Stock Solution								0.09						
7		0.026	0.03	-0.31	0.05	0.774	0.97	0.12	0.07	0.10	0.43	0.41		0.739	0.800	108
8		0.270	0.35	-0.25	0.11	0.505	0.65	0.21	0.18	0.27	0.46	0.53		0.739	0.775	105
mean											0.45	0.47	2			
± 1SD										0.02	0.08					
CyDTA	Stock Solution								0.01							
	9	0.003	0.01	n.d.	n.d.	0.545	0.99	n.d.	n.d.	n.d.	n.d.	n.d.		0.583	0.548	94
	10	0.086	0.16	-0.50	0.16	0.453	0.84	0.06	0.01	0.11	0.57	0.61		0.583	0.539	92
	11	0.456	1.00	-0.01	0.1	0.000	0.00	n.d.	n.d.	n.d.	n.d.	n.d.		0.583	0.456	78
	12	0.002	0.00	n.d.	n.d.	0.613	1.00	-0.02	0.00	n.d.	n.d.	n.d.		0.583	0.615	105
	13	0.109	0.20	-0.62	0.02	0.437	0.80	0.04	0.02	0.17	0.66	0.79		0.583	0.546	94
	14	0.266	0.48	-0.40	0.02	0.283	0.52	0.23	0.00	0.39	0.62	0.79		0.583	0.550	94
	15	0.528	1.00	0.05	0.01	0.000	0.00	n.d.	n.d.	n.d.	n.d.	n.d.		0.583	0.528	91
	mean										0.62	0.73	3			
	± 1SD										0.05	0.10				
DMA	Stock Solution								0.01							
	16	0.133	0.31	-0.23	0.07	0.300	0.69	0.13	0.01	0.12	0.36	0.34		0.414	0.434	105
	17	0.203	0.53	-0.16	0.06	0.179	0.47	0.07	0.03	0.21	0.24	0.37		0.414	0.382	92
	18	0.142	0.35	-0.21	0.01	0.258	0.65	0.15	0.06	0.13	0.36	0.34		0.414	0.400	97
	19	0.216	0.57	-0.12	0.09	0.165	0.43	0.13	0.03	0.18	0.26	0.31		0.414	0.381	92
	mean										0.30	0.34	4			
± 1SD										0.07	0.03					

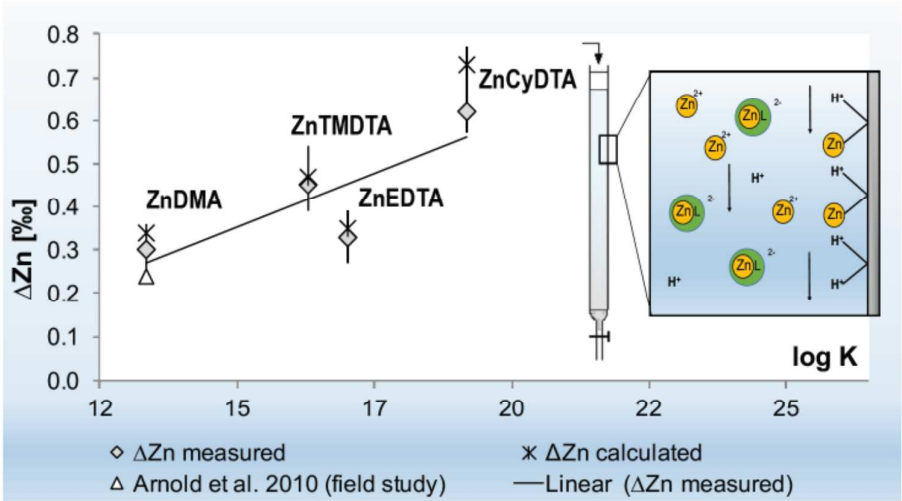
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396 Table 4

Element	Complexation reaction	Isotope Fractionation per mill per atomic mass unit	Comment		Reference
Iron	$\text{Fe}^{3+} + \text{DFOB}^+ = [\text{Fe}(\text{DFOB})]^+$	0.3	Experimental	Phase separation	Dideriksen et al., 2008
	$\text{Fe}^{3+} + \text{DFOB}^+ = [\text{Fe}(\text{DFOB})]^+$	>0	Experimental	Membrane separation	Morgan et al., 2010
	$\text{Fe}^{3+} + \text{DFOB}^+ = [\text{Fe}(\text{DFOB})]^+$	-0.2	Ab initio calculations	DFT theory	Domagal-Goldman et al., 2009
	$\text{Fe}^{3+} + (\text{citrate})_2^{\text{6-}} = [\text{Fe}(\text{citrate})_2]^+$	-0.4	Ab initio calculations	DFT theory	Fujii et al. 2014
	$\text{Fe}^{2+} + (\text{citrate})_2^{\text{6-}} = [\text{Fe}(\text{citrate})_2]^+$	-0.6	Ab initio calculations	DFT theory	Fujii et al. 2014
	$\text{Fe}^{2+} + \text{Nicotinamine}^+ = [\text{Fe}(\text{Nicotinamine})]^{2+}$	-0.03	Ab initio calculations	DFT theory	Moynier et al., 2013
	$\text{Fe}^{3+} + \text{Phytosiderophore}^{3-} = [\text{Fe}(\text{Phytosiderophore})]^0$	0.5	Ab initio calculations	DFT theory	Moynier et al., 2013
Zinc	$\text{Zn}^{2+} + \text{PHA}^{\text{6-}} = [\text{Zn}(\text{PHA})]^{\text{m-}}$	0.1	Experimental	Membrane separation	Jouvin et al., 2009
	$\text{Zn}^{2+} + \text{citrate}^{3-} = [\text{Zn}(\text{citrate})]^+$	0.07 to 0.25	Ab initio calculations	DFT theory	Black et al., 2011
	$\text{Zn}^{2+} + [\text{citrate}(\text{H}_2\text{O})_3]^{3-} = [\text{Zn}(\text{citrate}(\text{H}_2\text{O})_3)]^+$	0.1	Ab initio calculations	DFT theory	Fujii and Albareda, 2012
	$\text{Zn}^{2+} + (\text{citrate})_2^{\text{6-}} = [\text{Zn}(\text{citrate})_2]^+$	-0.4	Ab initio calculations	DFT theory	Fujii and Albareda, 2012
	$\text{Zn}^{2+} + [\text{malate}(\text{H}_2\text{O})_4]^{4-} = [\text{Zn}(\text{malate}(\text{H}_2\text{O})_4)]^0$	0.1	Ab initio calculations	DFT theory	Fujii and Albareda, 2012
	$\text{Zn}^{2+} + [(\text{malate})_2(\text{H}_2\text{O})_2]^{4-} = [\text{Zn}(\text{malate})_2(\text{H}_2\text{O})_2]^{\text{m-}}$	-0.2	Ab initio calculations	DFT theory	Fujii and Albareda, 2012
Nickel	$\text{Ni}^{2+} + (\text{citrate})_2^{\text{6-}} = [\text{Ni}(\text{citrate})_2]^+$	-0.6	Ab initio calculations	DFT theory	Fujii et al. 2014
Copper	$\text{Cu}^{2+} + \text{IHA}^{\text{6-}} = [\text{Cu}(\text{IHA})]^{\text{m-}}$	0.1	Experimental	Membrane separation	Bigalke et al, 2010
	$\text{Cu}^{2+} + \text{DFOB}^+ = [\text{Cu}(\text{DFOB})]^{2+}$	0.42	Experimental	Membrane separation	Ryan et al., 2014
	$\text{Cu}^{2+} + \text{CyDTA}^{4-} = [\text{Cu}(\text{CyDTA})]^{2+}$	0.31	Experimental	Membrane separation	Ryan et al., 2014
	$\text{Cu}^{2+} + \text{EDTA}^{4-} = [\text{Cu}(\text{EDTA})]^{2+}$	0.25	Experimental	Membrane separation	Ryan et al., 2014
	$\text{Cu}^{2+} + \text{Nitrilotriacetic acid}^{3-} = [\text{Cu}(\text{Nitrilotriacetic acid})]^+$	0.22	Experimental	Membrane separation	Ryan et al., 2014
	$\text{Cu}^{2+} + \text{Fulvic acid}^{\text{6-}} = [\text{Cu}(\text{Fulvic acid})]^{\text{m-}}$	0.07	Experimental	Membrane separation	Ryan et al., 2014

398 TOC/ Abstract art

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