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Review

Separation of low-molecular mass organic acid–metal complexes by high-performance liquid chromatography

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Abstract

The solution speciation of metals is a critical parameter controlling the bioavailability, solution-solid phase distribution and transport of metals in soils. The natural metal-complexing ligands that exist in soil solution include inorganic anions, inorganic colloids, organic humic substances, amino acids (notably phytosiderophores and bacterial siderophores) and low-molecular mass organic acids. The latter two groups are of particular significance in the soil surrounding plant roots (the rhizosphere). A number of analytical methodologies, encompassing computational, spectroscopic, physico-chemical and separation techniques, have been applied to the measurement of the solution speciation of metals in the environment. However, perhaps with the exception of the determination of the free metal cation, the majority of these techniques rarely provide species specific information. High-performance liquid chromatography (HPLC) coupled to a sensitive detection system, such as inductively coupled plasma mass spectrometry (ICP–MS) or electrospray ionisation mass spectrometry (ESI–MS), offers the possibility of separating and detecting metal–organic acid complexes at the very low concentrations normally found in the soil environment. This review, therefore, critically examines the literature reporting the HPLC separation of metal–organic acid complexes with reference to thermodynamic equilibrium and kinetic considerations. The limitations of HPLC techniques (and the use of thermodynamic equilibrium calculations to validate analytical results) are discussed and the metal complex characteristics necessary for chromatographic separation are described.

Keywords: Solution speciation; Metal complexes; Organic acids; Soil

Contents

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1. Introduction

Low-molecular mass organic acids are ubiquitous in the soil environment [1,2]. Soil solution concentrations of aliphatic mono-carboxylic acids (formic, acetic, propionic, etc.) are commonly found in the range 0–1 mmol l^{-1}, whereas the di- and tri-carboxylic acids, including oxalic, citric, malonic, malic, succinic, and tartaric, are usually detected between 0 and 50 μmol l^{-1} [2]. However, it is not uncommon to find higher concentrations of the di- and tricarboxylic acids in the rhizosphere [1]. In soils, organic acids may be derived from vegetal, fungal or microbial sources [1,2] and are generally characterized as having weak metal complexing properties.

Organic acids have been implicated in a number of soil processes, including mineral weathering/dissolution [1,3], podzolisation [2,4], metal leaching [2,5], the phytoavailability of metals [6–8], phosphate desorption and dissolution [9] and metal desorption and solubilization reactions [10–12]. In many cases, these causal relationships have been attributed to complexation reactions between metals and organic acids. However, organic acids may stimulate the desorption/solubilization of metals through a number of processes other than simple aqueous metal complexation reactions [6,10,11]. For example, organic acids may promote the desorption of metals by dissolving minerals which adsorb the metal [13–16]. Furthermore, as organic acids may induce changes in soil pH, solubilization of solid bound metal may occur solely because of a decrease in pH [17,18].

In light of the myriad mechanisms that organic acids may influence metal behavior in soils, it is apparent that the development of analytical methodology to quantify metal–organic acid complexes in soil solution is paramount to further our current understanding of many processes occurring in soils. Moreover, such analytical methodology would significantly aid the resolution of the ‘misconceptions and knowledge gaps’ recently outlined by Jones [19] on the importance of organic acids in soils.

A number of analytical techniques have been reported for identifying, but much less frequently quantifying, metal–organic acid complexes in a range of environmental matrices. After complete characterization of plant xylem exudate, computer modeling programs have often been employed to estimate the solution speciation of metals [20–22]. Generally, these results indicate that metals are complexed by organic acids. Unfortunately, there is some difficulty in applying these speciation programs to soil solutions as assumptions must be made regarding the complexing characteristics of humic substances and inorganic colloidal material. Although significant progress has been made in modeling the reactions between these materials and metals, there is still some uncertainty to their accuracy when applied to real soil solutions [23,24].

Two powerful spectroscopic techniques that have been used to detect metal–organic acid complexes in solution are nuclear magnetic resonance (NMR) and extended X-ray absorption fine structure (EXAFS) spectroscopy. Nuclear magnetic resonance spectroscopy has been used to investigate the citrate and oxalate complexes of Al [25–27] and the acetate and salicylate complexes of Cd [28] in standard solutions as well as Al–oxalate complexes in plant material [29]. However, conventional NMR suffers from extremely high detection limits (>1 mmol l^{-1}) and many metals do not have diamagnetic isotopes necessary for utilizing NMR. Nevertheless, the development of NMR microcoil-based probes has significantly decreased absolute detection limits [30,31] and, therefore, has future potential for the determination of metal–organic acid complexes.

Similar to conventional NMR, EXAFS also suffers from high detection limits and has, to date, only been used for the detection of the organic acid complexes of Ni and Zn in some hyperaccumulator plants at mmol l^{-1} concentrations [32–35]. Needless to say, these concentrations, particularly of Cd, Co, Ni and Pb, in soil solution are rarely observed—even in many polluted soils. In addition, the significance of the results obtained from EXAFS analyses are highly dependent upon the completeness of the reference database and, when numerous complexes exist, such as in soil solution, the use of advanced statistical programs to ‘estimate’ the concentration of the complexes present [34,36,37].

Mass spectrometry detection after ‘complete’ [inductively coupled plasma mass spectrometry (ICP–MS)] or ‘soft’ [electrospray ionisation mass spectrometry (ESI–MS)] ionization can routinely quantify metals and some metal complexes at μmol l^{-1} and, sometimes, even sub-μmol l^{-1} concentrations. Therefore, when mass spectrometry is preceded by an efficient separation procedure, such as high-performance liquid chromatography (HPLC) or capillary zone electrophoresis (CZE), the resulting system can furnish specific solution speciation information at very low metal concentrations. The coupling of separation techniques to mass spectrometry detection for the determination of solution metal speciation in environmental samples has been well reviewed in recent years [38–46]. However, only has Szpunar [43] and Timerbaev [42] made passing reference to metal–organic acid complexes, each citing only one reference.

This review, therefore, critically examines the literature reporting the HPLC and CZE separation of metal–organic acid complexes on the basis of kinetic and thermodynamic equilibrium considerations. The limitations of separation techniques for measuring these complexes are described and, hence, the necessary complex characteristics and experimental conditions under which they can be analyzed are summarized.

2. Chromatographic techniques for separating aqueous metal complexes

Many separation procedures have been exploited to examine the aqueous speciation of metals in biological and environmental matrices. Although these chromatographic techniques have been amply reviewed, it is judicious to briefly describe these techniques before discussing their application to the separation of metal–organic acid complexes.

Four chromatographic techniques have principally been used for separating metals that have different oxidation states (e.g. As, Cr, Fe and Se), metal–inorganic anion complexes (e.g. Al–F complexes), metals covalently bound to organic molecules (e.g. As, Hg, Pb and Se) and those metal–organic molecule complexes formed by chelation reactions (e.g. metal–EDTA complexes). These techniques are reversed-phase liquid chromatography (RP-HPLC), reversed-phase ion-pair chromatography (RP-IPC), size-exclusion chromatography (SEC) and ion-exchange chromatography (IEC) [40,43,44].

Separation using reversed-phase chromatography is achieved through the partitioning of a metal-complex between a non-polar stationary phase and a polar liquid mobile phase. Continuous partitioning between the mobile and stationary phase, which depends upon the polarity of the metal complex, results in differential migration through the analytical column. As a result, metal complexes of higher polarity elute earlier than those with lower polarity.

The technique of RP-IPC is suitable for separating ionic or non-ionic analytes. In this type of chromatography, it is generally believed that an ion-pair is formed between the analyte and a relatively hydrophobic ion of opposite charge (a counter ion) present in the mobile phase. Separation is achieved through continuous partitioning of the hydrophobic ion-pair between the mobile phase and non-polar stationary phase under conditions similar to those of RP-HPLC.

As the name implies, SEC, ideally, separates metal complexes according to their effective size in solution. The stationary phase consists of pores with a particular average size and complexes larger than this pore size are not retained and rapidly elute from the column. On the contrary, smaller complexes enter the pores of the stationary phase and move through the column at speeds relative to their molecular size. However, all SEC columns exhibit ionic properties as a result of the residual organic functional groups (primarily COOH and OH) remaining after manufacture of the stationary phase. Although this interaction may occasionally be minimized with the use of high ionic strength mobile phases, the process of separation in SEC is actually a combination of size, ion exclusion and ion exchange effects.

As many metal complexes are charged, IEC has formed the basis of many HPLC separation procedures. Basically, charged complexes compete with ions of the same charge in the mobile phase for ionic sites on the stationary phase that have an opposite charge. The affinity of the metal complex for the stationary phase and the extent of competition with the other charged molecules in the mobile phase primarily determines retention times.

Although not a HPLC technique, CZE will also be discussed in this review due to its unique separation mechanism and applicability to aqueous metal speciation. As opposed to HPLC, where retention times are primarily based upon the distribution of a metal complex between a mobile and stationary phase, separation in CZE is a result of the electrophoretic mobility of the complex in an electric field. In CZE, samples are first injected into a fused-silica capillary tube and the outlets of the tube are then immersed into different reservoirs that contain the identical mobile phase. A large potential difference is initiated by placing a cathode in the reservoir containing the outlet of the tube and an anode in the reservoir containing the inlet of the tube (where the sample was injected). As such, positively-charged metals and metal complexes move towards the negative electrode (e.g. Al^{3+} and AlF^{2+} [42]). Normally, as a result of electroosmotic flow (EOF), neutral and negatively charged metal-complexes also move towards the same electrode. However, under conditions of low EOF, highly mobile negatively-charged complexes may not migrate towards the negative electrode. Further, in depth, descriptions of the CZE separation mechanism in relation to aqueous metal speciation are widely available [40,42,47].

Other existing chromatographic techniques for separating metal complexes include gas chromatography (GC), micellar liquid chromatography and supercritical fluid chromatography [40]. However, GC is not well suited for many aqueous metal complexes and the development and application of the other two techniques have not been extensively developed nor applied to the examination of metal–organic acid complexes.

3. Chromatographic separation of metal–organic acid complexes

3.1. Reversed-phase liquid chromatography

There has only been one paper describing the use of reversed-phase liquid chromatography to separate metal–organic acid complexes [48]. In this study, a standard solution containing a two-fold molar excess of citrate, presumably, over Fe^{2+} and Fe^{3+} was examined using a silica-based Spherisorb S5 ODS 2 column and a 50 mmol l^{-1} ammonium acetate/methanol (70:30, v/v, pH 4) mobile phase. Although Fe^{2+} and Fe^{3+} could be separated in the absence

of citrate, they could not after its addition (i.e. the retention time of Fe^{3+} was reduced). Therefore, electrochemical detection was used to quantify Fe^{2+}, FAAS for total Fe and the difference was taken to represent Fe^{3+}. However, the effect imparted by citrate is difficult to resolve when thermodynamic equilibrium calculations, using GEOCHEM PC [47], estimate that only 2% of the Fe^{3+} is complexed to citrate. As standard solutions were prepared in the mobile phase, it is appropriate to assume thermodynamic equilibrium. Therefore, citrate should have little influence on the retention time of Fe^{3+}.

Weber [48] further describes the separation of standard solutions containing Fe^{2+}tartrate, malate and citrate complexes using another mobile phase (100 mmol l^{-1} ammonium sulfate, pH 2.5). The low pH of the mobile phase was not considered problematic as the method was to be used for the analysis of apple juice and white wine, both of which have a similar pH. However, if the thermodynamic equilibrium speciation of these solutions (1 g organic acid l^{-1}, 1 mg Fe^{2+} l^{-1}) are calculated, under the conditions of chromatography (100 mmol l^{-1} ammonium sulfate at pH 2.5), it is observed that only 0.5% of the Fe^{2+} is estimated to be complexed to citrate, with even less complexation in the solutions containing the other two organic acids. In all cases, it is predicted that approximately 50% of the Fe^{2+} is complexed to $SO_4{}^{2-}$ with the remainder predominately found as the free cation. Discordance between thermodynamic equilibrium calculations and HPLC or CZE analytical results are not uncommon [50,51]. Therefore, these significant differences may possibly be reconciled through kinetic limitations to the dissociation of these complexes during chromatography (if the standards were not prepared in the mobile phase) or through experimental verification of the stability constants of these metal–organic acid complexes.

3.2. Size-exclusion chromatography

Size-exclusion chromatography has been used to separate metal–organic acid complexes in standard solutions [52], breast milk and commercial infant formulas [53]. In the latter study, metal complexes were separated with two Shodex analytical columns (GS 620 HQ, 300 mm × 7.6 mm and GS 520 HQ, 300 mm × 7.6 mm, Showa Denko KK, Tokyo, Japan) using 100 mmol l^{-1} tris(hydroxymethyl)aminomethane (Tris) buffered to pH 7.1 as the mobile phase. These two columns, combined together, have a relative molecular mass (M_r) separation range of 1×10^3–2×10^6. This separation range is significantly higher than the M_r of either $Zn(H_2O)_6{}^{2+}$ (173) or $(Zn–citrate)^-$ (254) implying that any chromatographic separation of the metal–organic acid complex from the aqueous metal cation, when using these columns, will not be a result of a size-exclusion mechanism. Furthermore, there must be a typographical error in the manuscript of Brätter et al. [53] as a M_r of 1.1×10^4 has been assigned to the Zn–citrate complex (Fig. 1). Nevertheless, despite a significant lack of information regarding the methodology for Zn–citrate quantification, this complex was apparently separated from five other Zn complexes and detected at concentrations <2 μg Zn l^{-1} by online coupling to ICP-AES [53].

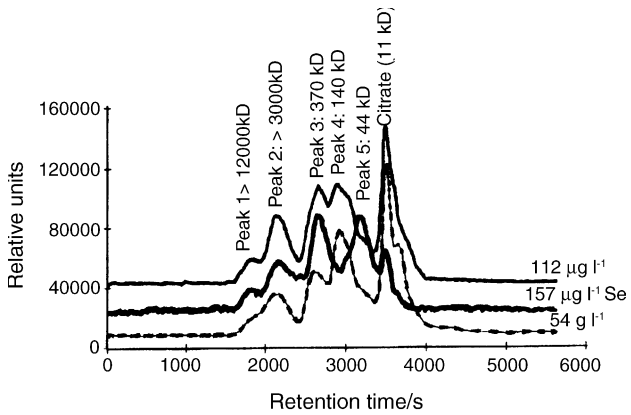

Fig. 1. Size-exclusion chromatography elution profiles of Zn in breast milk (as a function of dietary selenium intake). ICP-AES was used as the detection system. From [53] with permission.

The SEC of standard solutions containing Al, Cu and citrate by SEC was tested by Kerven et al. [52] using columns slurry packed with Fractogel TSK HW-40(S) (Merck). The technical specifications of this column are not provided in the manuscript. However, as noted in the study of Brätter et al. [53], the M_r separation range for this slurry-packed column is also certainly to be greater than the M_r of the Al and Cu–citrate complexes examined. As such, any separation of these complexes is also unlikely to be effectuated by a size-exclusion mechanism. Indeed, the use of a 20 mmol l^{-1} KCl (pH 4.2) mobile phase resulted in the co-elution of Al and Cu-citrate, nevertheless, both were completely separated from Cu^{2+}. However, it may be incorrect to state that this separation was due to a size-exclusion mechanism due to the fact that these molecules are smaller than the M_r separation range of the column. Therefore, as a result of the residual functional groups (COOH and OH) of the stationary phase, a cation exchange mechanism is the most plausible mode for the separation of the copper cation from the metal–citrate complexes.

Upon ICP–MS analysis, after separation on a Superdex peptide HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden), of a water extract of the latex of *Sebertia acuminata*, a Ni-hyperaccumulating tree, Ni has been found to elute as six discrete peaks [54]. Unlike the two proceeding studies, the contribution of a size-exclusion mechanism to the chromatographic process, in addition to ion-exchange or ion-exclusion mechanisms, is more probable since this column has a M_r separation range of 1×10^2 to 7×10^3.

In a set of comprehensive follow-up experiments one of the six peaks was later identified by ESI–MS as a Ni complex of nicotianamine [Ni(nicotianamine)$_2$]. Nicotianamine is a non-proteinaceous aminocarboxylic acid that is chemically similar to the mugineic acid family of phytosiderophores [55]. However, this complex only comprised 0.6% of the total Ni content in the water extract. The remaining 99.4% of Ni in the

sample was retained to the column when using a 5 mmol l^{-1} ammonium acetate (pH 6.8) mobile phase. As mentioned previously, the residual functional groups (COOH and OH) of the stationary phase of size-exclusion columns signifies that a cation exchange mode of separation is also observed in SEC which, in this study, resulted in the adsorption of uncomplexed and/or weakly complexed Ni. This is a significant problem for SEC and its adverse affects on accurately determining metal speciation have been widely reported [43,56–60]. Although attempts have been made to chemically modify the residual functional groups of SEC columns, the results have been somewhat disappointing [56,57]. In some cases, metal retention was actually enhanced while, in other cases, the stationary phase was completely destroyed [56]. By the lack of subsequent publications, it appears that these techniques are not popular for minimizing the cation exchange properties of SEC columns.

Further experiments by Schaumlöffel et al. [54] revealed that citrate was a major component of the organic acid content of the latex (Fig. 2). However, presumably as a result of the process stated above, Ni–citrate complexes were not observed during chromatography. By loading the column with Ni, akin to immobilized-metal affinity chromatography [61], injection of the tree latex resulted in one major peak that was attributable to Ni–citrate. However, further CZE experiments were necessary to verify the presence of Ni–citrate in the sample, which, apparently, did not dissociate during CZE analyses. Quantification by the method of standard addition indicated that the 99.4% of the Ni retained by the SEC column was, in fact, Ni dissociated from citrate complexes during the chromatographic process [54].

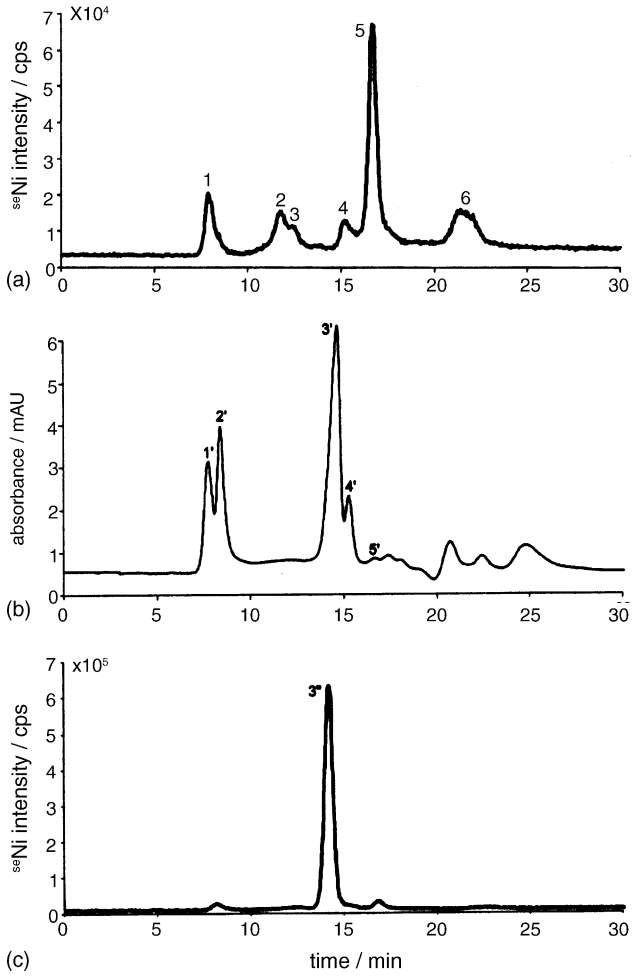

Fig. 2. SEC separation of the latex water extract from *S. acuminate*: (a) with ICP–MS detection of ^{58}Ni after separation on a Ni-free column; (b) with UV detection at 213 nm using the same column as in (a); (c) with ICP–MS detection of ^{58}Ni after separation on the column used in (a) loaded with Ni^{2+}. Peak 3′ was identified as citric acid. From [54] with permission.

3.3. Ion-exchange chromatography

Ion-exchange chromatography has been a popular method of choice for determining the solution speciation of Al [62–75]. Bertsch and Anderson [63] examined the separation of Al^{3+} from its oxalate and citrate complexes at pH values ranging from 2 to 4.2 using a Dionex CS5 cation-exchange column and a mobile phase of 700 mmol l^{-1} NH_4Cl. Acetate, propionate, benzoate, SO_4^{2-} and Cl^- were also examined but these ligands did not form complexes which remained intact during chromatography. Good agreement between their analytical results and the predicted thermodynamic equilibrium species distribution, using GEOCHEM (the precursor to GEOCHEM-PC), was observed when the standards were prepared in the same mobile phase used for chromatography (Fig. 3). However, less complexation than predicted was noted when the Al–citrate standards were made in a solution of low ionic strength (0.2 mmol l^{-1}). The authors attributed this result to the standard reequilibrating to the higher ionic strength of the mobile phase (700 mmol l^{-1} NH_4Cl). In addition, the peaks attributed to the $(AlF_2)^+$, $(Al–oxalate)^+$, $(Al–citrate)^0$ and $(Al–Hcitrate)^+$ complexes had the same retention time, therefore, limiting the application of this method to more complex environmental solutions.

Sutheimer and Cabaniss [67] later conducted similar experiments using another cation exchange column (Synchropak CAT-15, SynChrom Inc., Lafayette, IN, USA) and gradient elution with 400 mmol l^{-1} $CaCl_2$ (pH 4). When standard solutions containing an organic acid and Al were examined, they also found that their results corresponded well to thermodynamic equilibrium calculations. However, as observed by Bertsch and Anderson [63], the Al complexes presumed to have a negative, neutral or +1 positive charge coeluted. Michalas et al. [76] obtained identical results when using reversed-phase ion-pairing chromatography and also noted that recovery of the Al–organic acid complexes varied from 26 to 91%. An earlier study, that tested nine different HPLC columns, also found that the maximum recovery of Al–citrate was only 65% [65].

Unlike the preceding studies, Borrmann and Seubert [68] were able to separate the Al complexes of citrate and oxalate through a combination of cation exchange and size-exclusion processes on a self-prepared column at pH

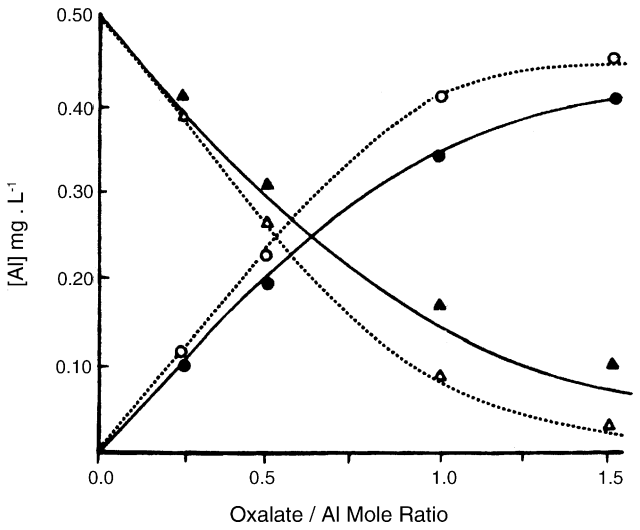

Fig. 3. Measured and predicted concentrations of $Al(H_2O)^{3+}$ and Al complexed with oxalic acid. Solutions contained 0.5 mg l^{-1} of Al and varying concentrations of oxalic acid. Open symbols with dashed lines and closed symbols with solid lines represent, respectively, solutions having an ionic strength of 2×10^{-4} and 7×10^{-1} M. Triangular symbols indicate concentrations of Al and circular symbols correspond to concentrations of oxalate-complexed Al (as mg l^{-1} of Al). From [63] with permission.

3 (Fig. 4). Although a large improvement on the previous studies, all these methodologies are somewhat limited by their restrictive pH range and their sole application to Al complexes. Many metal–organic acid complexes do not form at such low pH values (due to competition with H^+). Nevertheless, there is still some application of this methodology to the separation of $Al(H_2O)^{3+}$ in acidic solutions from some of its inorganic and organic complexed forms.

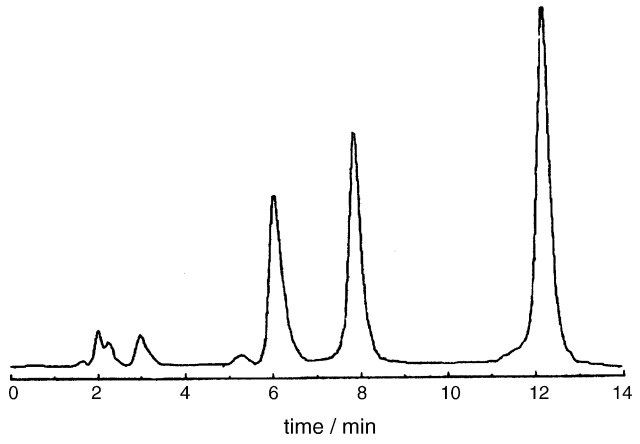

Fig. 4. Ion chromatogram of a solution containing $Al(H_2O)^{3+}$, fluoride, oxalic acid and citric acid at a mole ratio of 3:1:1:1 (pH of 3). Detection was performed by UV (310 nm) following post-column reaction with Tiron. The peaks were assigned to the following complexes: retention time 1.7 min = $(Al–citrate)^-$, 1.8 min = $(Al–oxalate_2)^-$, 2.2 min = $(Al–F–oxalate)^0$, 3.0 min = $(Al–citrate)^0$, 5.2 min = $(Al–F_2)^+$, 6.2 min = $(Al–oxalate)^+$, 7.9 min = $(Al–F)^{2+}$, 12.4 min = $Al(H_2O)^{3+}$. From [68] with permission.

Mitrovic and co-workers [69,71] have tested cation-exchange chromatography for separating the citrate and oxalate complexes of Al up to pH values of 6.5. However, similar to the results obtained by Bertsch and Anderson [63] and Sutheimer and Cabaniss [67], it was observed that these complexes co-eluted and, furthermore, eluted at the solvent front (void volume) with a number of other complexes that the authors proposed to exist in other standard solutions (AlF_2^+, $Al(SO_4)^+$, $Al(OH)_2^+$). The authors attributed this result to the non-retention of negatively charged Al–citrate and oxalate complexes to the cation exchange column. As these complexes co-eluted with the positively charged inorganic complexes of Al, this indicates, rather, that the eluting cation (Na^+) had a stronger affinity than the single positively charged complexes for the functional groups of the column. This is in despite of the fact that a gradient elution from 0 to 800 mmol l^{-1} $NaNO_3$ was used in these experiments (i.e. the column is still saturated with Na^+ at 0 mmol l^{-1} $NaNO_3$ and only those complexes having a stronger affinity than Na^+ for the functional groups of the column will be retained). Although the method was further applied to measuring the speciation of Al in soil solutions, it is not possible to make any presumption as to the identity of the Al complexes, contrary to the statements made by the authors, when there are potentially five complexes having the same retention time.

The same group also observed the co-elution of the malate, tartrate, aconitate, gluconate and citrate complexes of Al when using the same technique at pH 5 [74]. In the same study, anion exchange chromatography (Mono Q HR 5/5 column, Pharmacia, Uppsala, Sweden) was attempted to separate these complexes. However, at two different Al concentrations (0.1 and 4 mg l^{-1}) these complexes could not be separately distinguished and, in many cases, the recovery of Al was incomplete. The latter result was attributed to the strong adsorption of positively charged and neutral Al complexes to the column [74]. However, it is unclear as to which process of adsorption would result in the retention of positively and neutrally charged species to a positively-charged anion exchange column.

Nevertheless, upon application of these methods to determine the speciation of Al in solutions (pH not given) extracted from plant material (*Sempervivum tectorum* and *Sansevieria trifasciata*), it was concluded that Al existed as negatively charged complexes [74]. Analysis of the fractions eluting at the retention time of these peaks, by Z-spray ESI–MS, indicated the presence of citrate and aconitate. However, measurement of the complexes could not be performed. The combination of anion exchange chromatography and electrospray mass spectrometry was subsequently applied to eluted fractions containing Al, citrate and phosphate in Al-spiked serum samples [75].

Similar anion exchange techniques have been attempted to separate the citrate, oxalate and EDTA complexes of Zn [77]. In addition, a convective interaction media (CIM) disk with diethylamine (DEAE) functional groups (Bia Separations, Ljubljana, Slovenia) was also tested in this study. Buffer

Table 1
Predicted thermodynamic equilibrium solution speciation of Zn in the experiments of Svete et al. [77], as stated by the authors, compared to values calculated in this manuscript using GEOCHEM PC and the stability constants of the NIST 2001 selected stability constants of metal complexes database [78]

	pH	% Species distribution of Zn			
		Zn^{2+}	ZnHCit	$(ZnCit)^-$	$Zn(Cit_2)^{4-}$
Svete et al. [77]	5.4	(92)		(92)	8
(NIST 2001)		1	3	96	
Svete et al. [77]	6.4			70	30
(NIST 2001)				98	2
Svete et al. [77]	7.4			70	30
(NIST 2001)				97	3

solutions were initially examined to determine the effect imparted on the retention time of Zn. It was observed that Tris, imidazole, borate, piperazine and hydrogenphthalate influenced the retention time of Zn almost certainly as a result of Zn^{2+} complexation. As such, these buffers were not used as mobile phases during further experiments.

Standard solutions, made at a Zn-to-ligand ratio of 1:100, were analyzed with the DEAE CIM disk using gradient elution from 0 to 400 mmol l^{-1} NH_4NO_3 at pH values ranging from 5.4 to 7.4. It was observed that Zn^{2+} eluted at the solvent front while Zn–citrate standards had two retention times. It was postulated that the $(Zn–citrate)^-$ complex co-eluted with Zn^{2+}, while the $(Zn–citrate_2)^{4-}$ complex had a longer retention time. Although the authors state that the analytical results were in accordance with theoretically-predicted thermodynamic equilibrium speciation calculations, using the 1997 IUPAC Stability Constants Database, results obtained using the 2001 NIST selected stability constants of metal complexes database [78] predict a somewhat different theoretical species distribution at thermodynamic equilibrium (Table 1). The question, therefore, arises as to which one of these recent compilations of stability constants is authoritative, and, furthermore, whether the verification of analytical results by a comparison to thermodynamic equilibrium speciation calculations does, in fact, validate chromatographic experimental data.

It was observed that Zn had the same retention time as the presumed $(Zn–citrate_2)^{4-}$ complex when a standard solution containing Zn and oxalate was analyzed. The recovery of Zn was 95 % at pH 5.4, but decreased to 70 % at pH 7.4. Similarly, when this method was applied to soil extracts and industrial waste waters, 53–84% of the Zn in the samples was retained by the column. The remaining Zn eluted from the disk was defined as Zn^{2+} and, after Z-spray electrospray mass spectrometry detection of citrate, a mixture of $(Zn–citrate)^-$ and other complexes composed of decaying organic matter. However, considering that the $(Zn–citrate)^-$ complex could not be directly identified and that Zn eluted with a number of other possible complexing ligands, it cannot be unequivocally stated that Zn was complexed to citrate. As a result, this methodology does not necessarily provide more complete information on the solution speciation of Zn when compared to other speciation techniques [77].

Ammann [79] has published the most recent report on the separation of metal–citrate complexes by anion-exchange chromatography. Using ICP–MS as the detection system, a Dionex AS11 column was used with varying concentrations of NH_4NO_3 as the mobile phase at pH 6–8. It was observed, under these experimental conditions, that 100% of the Cd, Cu and Pb from metal–citrate standard solutions, prepared at a 1:2 metal-to-citrate ratio, were retained by the column. In contrast, the metal–citrate standards of Co and Ni could be detected, despite their stability constants being less than that of Cu–citrate. The recovery of Co and Ni was 78 and 55%, respectively, and it was, therefore, postulated that the slower dissociation kinetics of these two complexes resulted in their ability to be detected [79].

3.4. Capillary zone electrophoresis

Despite its novel separation technique, CZE has had little application to the analysis of metal–organic acid complexes. In an early study, Wu et al. [80] examined the complexation of Al to oxalate under similar experimental conditions, albeit by CZE, to the HPLC study of Bertsch and Anderson [63]. As did Bertsch and Anderson [63], these authors also observed good agreement between their experimental results and the solution speciation predicted at thermodynamic equilibrium by SOILCHEM (a computer program based on GEOCHEM) (Fig. 5). However, it is interesting to compare the results of these two studies as the concentrations of Al complexed by oxalate (at the same Al:oxalate ratio) in the study of Bertsch and Anderson [63] are exceptionally, at times approximately two-fold, higher than those obtained by Wu et al. [80]. It may be argued that these differences were a result of the analysis of sample solutions having a different pH (pH 3.2 versus pH 3.5) or that the solutions had different Al concentrations (18.5 μmol l^{-1} versus 250 μmol l^{-1}) or even that the ionic strength was different between the two studies. Although these factors can all contribute to altering solution speciation, it must be noted that Bertsch and Anderson [63] did not observe a significant deviation in Al speciation when the pH of their samples was increased to 4.2 nor when their analyses were conducted with a high ionic strength mobile phase (700 mmol l^{-1} NH_4Cl).

When the thermodynamic equilibrium speciation of Al in the solutions used by Bertsch and Anderson [63] are recalculated, using the stability constants given in their manuscript, identical results are obtained (Table 2). These authors used the $(Al–oxalate)^+$ complex in their calculations. As noted previously by Kerven et al. [52], this complex is not present in the GEOCHEM PC, version 2 database and, therefore, its inclusion is required to recalculate the data of Bertsch and Anderson [63].

In contrast, when these calculations are performed with the data of Wu et al. [80] a large discrepancy is observed

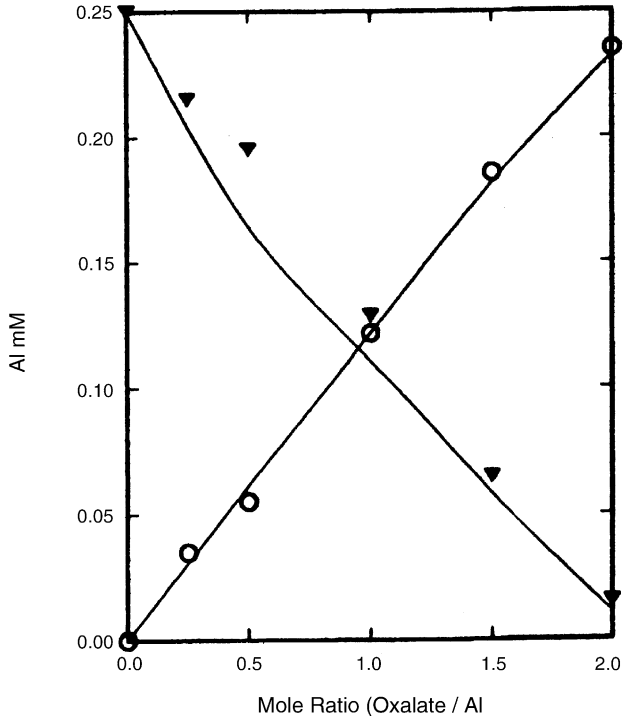

Fig. 5. Measured (triangular symbols = $Al(H_2O)^{3+}$, circular symbols = Al complexed with oxalic acid) and predicted (solid lines) speciation of solutions containing 0.25 mM of Al and varying concentrations of oxalic acid at pH 3.5. From [80] with permission.

between their experimental results and the predicted species distribution [i.e. if the $(Al–oxalate)^+$ complex is included in the calculations]. In fact, the results are similar to the predicted solution speciation of Bertsch and Anderson [63]. Indeed, agreement between the results of Wu et al. [80] with the predicted thermodynamic equilibrium speciation is only observed when the $(Al–oxalate)^+$ complex is not included in the calculations. It, therefore, appears that agreement between the experimental results and the calculated solution speciation at thermodynamic equilibrium in these two studies is not dependent upon the experimental system, but, rather, on the stability constant database used to calculate the solution speciation.

Table 2
Calculated thermodynamic equilibrium solution speciation in the studies of Bertsch and Anderson [63] and Wu et al. [80]

	Oxalate:aluminum ratio[a]				
	0.25	0.5	1.0	1.5	2.0
Bertsch and Anderson [63]	24	44	84	92	nd[b]
With $(Al–oxalate)^+$	24	47	84	94	–
Wu et al. [80]	14	24	50	74	94
With $(Al–oxalate)^+$	25	49	91	99	100
Without $(Al–oxalate)^+$	12	24	49	72	94

Recalculated values were conducted, as described in the text, using GEOCHEM PC.

[a] % complexation of Al.

[b] Not determined in the study of Bertsch and Anderson [63].

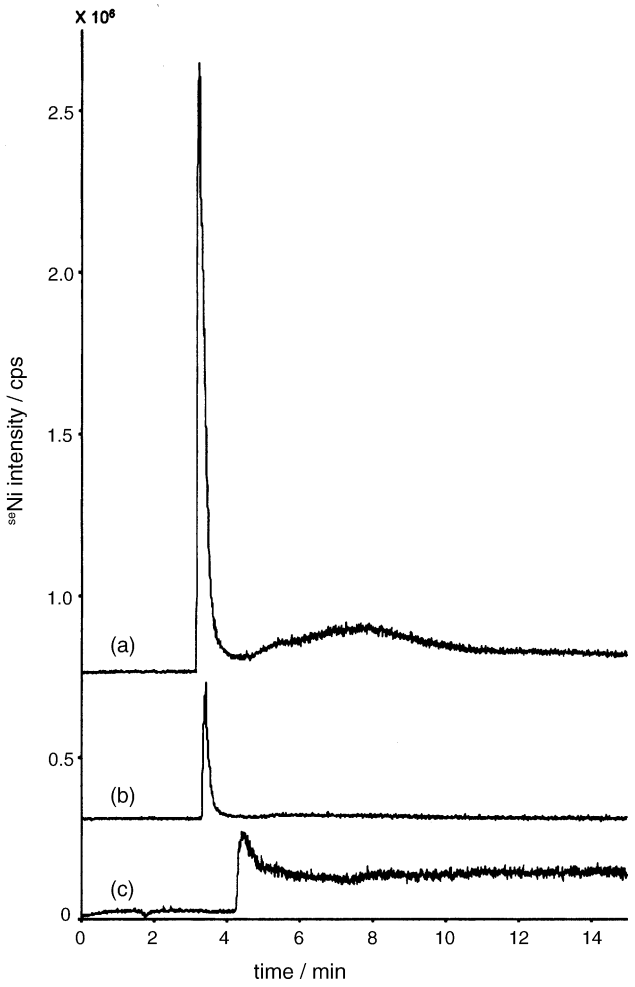

Fig. 6. CZE-ICPMS electropherograms of: (a) a 1:2000 diluted water extract of the latex from *S. acuminate*; (b) standard solution containing citric acid and 1 mg l^{-1} of Ni; (c) standard solution containing 1 mg l^{-1} of Ni. From [54] with permission.

As mentioned previously, CZE has also been applied to the analysis of Ni–citrate complexes in water extracts from the latex of *S. acuminata* [54]. In this study, a standard solution of Ni–citrate was prepared by dissolving equimolar concentrations of Ni and citrate to produce a final Ni concentration of 1 g l^{-1}. If it is assumed that this stock solution was buffered to pH 7.4 (the pH of the CZE conditions), and at equilibrium, then thermodynamic equilibrium calculations predict that 99% of the Ni and citrate are complexed to form $(Ni–citrate)^-$. However, this solution was diluted 1000-fold to produce standards used in the CZE experiments. At this concentration only 87% of the Ni is predicted to be complexed by citrate (if, of course, the diluted standard is at thermodynamic equilibrium). However, the electropherogram of the Ni–citrate standard has only one peak, which was not at the retention time of Ni^{2+} (Fig. 6). This implies, therefore, that the standard solution was not, in fact, at thermodynamic equilibrium before injection into the capillary nor during electrophoresis.

4. Dissociation kinetics of metal–organic acid complexes

The separation of thermodynamically stable metal complexes by HPLC and CZE has been amply applied and well reviewed in recent years. The criterion of stability has been defined as an aqueous species which does not undergo a change during its migration through the separation medium [43]. As metals complexed by organic acids are in constant exchange with the solvated metal in solution, metal–organic acid complexes, and, indeed, any ionic metal complex, will only share this characteristic of 'stability' when this exchange (or dissociation) is insignificant during the time of separation.

This can be illustrated by taking the following example of a 10 mM $NaNO_3$ solution, at pH 7, containing 1 μM Cd and 100 μM citrate. At thermodynamic equilibrium 69% of the Cd is complexed with citrate as $(Cd–citrate)^-$ and the remainder is predominately found as Cd^{2+} (30%). If the same $NaNO_3$ solution, without Cd and citrate, is used as the mobile phase, then it may be considered that the complex is thermodynamically stable in the mobile phase (e.g. Cd does not precipitate). However, after the initial moments of separation, using a hypothetical size-exclusion column which, for simplicity, contains no functional groups, Cd^{2+}, citrate and the $(Cd–citrate)^-$ complex are separated by their effective size in solution. As a result, the $(Cd–citrate)^-$ complex is now in solution at equimolar Cd and citrate concentrations and is no longer at thermodynamic equilibrium (i.e. the Cd-to-citrate ratio is no longer 1:100). Thus, the complex will dissociate to Cd^{2+} and citrate in an attempt to re-establish thermodynamic equilibrium. However, the dissociated Cd^{2+} and citrate do not move at the same velocity as the complex through the column and the complex will, therefore, continue to dissociate to achieve thermodynamic equilibrium. In this fashion, depending upon the kinetics of dissociation, complete dissociation of the complex will occur during the separation process.

On the contrary, a complex that is not thermodynamically stable in the mobile phase used for HPLC or CZE may also be analyzed if the dissociation kinetics of the complex is extremely slow. For example, the Al complex of EDTA is not thermodynamically stable at pH 9.9 in a mobile phase consisting of 2.5 mM $(NH_4)_2CO_3$, 9.7 mM NH_4OH and 4% (v/v) methanol. However, due to slow dissociation kinetics, this complex was successfully separated by anion chromatography and subsequently detected by electrospray mass spectrometry [51].

At thermodynamic equilibrium the dissociation rate constant (k_d) of a complex (ML) is related to the formation rate constant (k_f) and the stability constant of the complex (K_{ML}):

$$K_{ML} = \frac{[ML]}{[M]+[L]} = \frac{k_f}{k_d} \quad (1)$$

It, therefore, follows that the dissociation rate of a complex decreases when the formation rate decreases or as the stability of the complex increases. While the stability constants for many metal–organic acid complexes are widely available, the kinetics of their formation and dissociation are less well reported. However, k_f can often be predicted based on the Eigen–Wilkins mechanism:

$$k_f = k_{M-H_2O} \times K_{OS} \quad (2)$$

where K_{OS} is the outer-sphere stability constant of the metal and ligand:

$$K_{OS} = \frac{[M(H_2O)_xL^{n-m}]}{[M(H_2O)_x^{n+}][L^{m-}]} \quad (3)$$

and k_{M-H_2O} is the rate constant of water exchange between the inner- and outer-coordination spheres of the metal:

$$M(H_2O)_x{}^{n+} + H_2O^* \xrightarrow{k_{M-H_2O}} M(H_2O)_{x-1}(H_2O^*) + H_2O \quad (4)$$

combined, these two reactions can be written as:

$$M(H_2O)_x^{n+} + L^{m-} \xrightarrow{k_f} M(H_2O)_{x-1}L^{n-m} + (H_2O) \quad (5)$$

Although outer-sphere stability constants may be experimentally determined for some metals and ligands, these reactions are normally too rapid to be measured [81]. Therefore, K_{OS} is often calculated based on electrostatic considerations (e.g. the equation postulated by Fuoss [82]) and for Cd, Pb and the first-row divalent transition metals these values are, for all practical purposes, approximately equal for the same ligand [83]. Consequently, the rate-limiting step for complexation reactions involving these metals and a large number of monodendate and multidendate ligands is the exchange of a water molecule, coordinated to the inner-sphere of the metal, and the ligand [83,84]. In fact, for many complexation reactions involving monodendate ligands, such as acetate, the substitution rate of a ligand for an inner-sphere water molecule coordinated to a metal is indistinguishable from the rate of water exchange between the inner- and outer-coordination spheres of the metal [83]. As a first approximate, it is, therefore, appropriate to compare the reactivity of these metals based solely on their rate constant of water exchange (Fig. 7).

Calculated values of K_{OS} for Al^{3+} are one-to-two orders of magnitude greater, depending largely upon the charge of

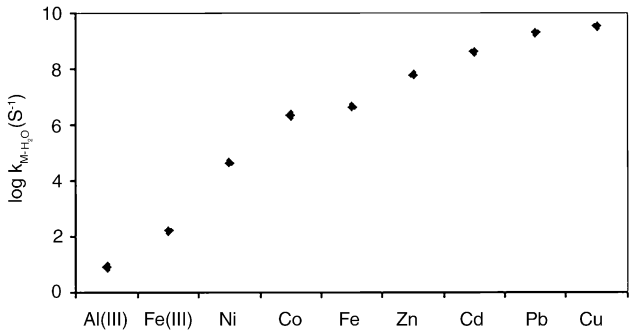

Fig. 7. Rate constant of water exchange (k_{M-H_2O}) for a range of divalent and trivalent metals. Data points represent the average of values from the reviews of Marjerum et al. [83] and Helm and Merbach [85]. Unless stated otherwise, the valence of the metals is (M^{2+}).

the ligand, than those of the divalent metal cations in Fig. 7. However, some comparison based on k_{M-H_2O} is still possible if this is taken into account. On the contrary, the k_f for Fe^{3+} is as sensitive to the entering ligand as to the leaving water molecule (i.e. the exchange is associative) and this, therefore, makes comparisons based solely on values of k_{M-H_2O} difficult [84,85].

It is immediately apparent from Fig. 7 that the rate of water exchange between the inner- and outer-coordination spheres of Al^{3+} is much slower than that for the divalent cations. Even if the influence of K_{OS} is considered, the k_f of Al^{3+} with the di- and tri-carboxylic organic acids would still be slower than the respective rates for the Ni^{2+} complexes. However, this comparison must be considered with some caution as it is only valid for those reactions where displacement of the first inner-coordination sphere water molecule is the rate-limiting step in metal complexation. Although the available evidence tends to suggest that this is the case for aliphatic organic acids [83,84], if complexation also involves chelate ring enclosure of the metal then the overall formation kinetics, in addition to being much slower, will also depend on the metal [83]. Nevertheless, it would be predicted that the organic acid complexes of Al would be the most suitable for HPLC and CZE applications and, as evidenced by the number of publications, this appears to be the case.

The data in Fig. 7 also indicates that many organic acid complexes of Fe^{3+} could be amenable to the separation techniques of HPLC and CZE. However, as the formation rates of Fe^{3+} complexes are sensitive to the nature of the incoming ligand, it is not feasible to make accurate predictions of the dissociation rate of these complexes using Eq. (2). In fact, other than the report of Weber [48], only the ferric complexes of mugineic acid, 3-*epi*-hydroxymugineic acid, numerous hydroxamate siderophores, such as desferri-ferroxamine-B (DFOB), EDTA and also a range of other synthetic chelates have been successfully separated using HPLC [50,79,86–90]. These complexes all have exceptionally high stability constants ($>10^{18}$ M) and, as complexation by many of these ligands is also likely to involve chelation processes, their dissociation rates are extremely slow. As such, further experiments are required to confirm the organic acid complexes of Fe^{3+} that can be separated by HPLC.

Of the divalent metals, Ni^{2+} has the slowest rate of water exchange and, as mentioned previously, the CZE separation of the Ni–citrate complex $(Ni–citrate)^-$ has been published [54]. If it can be assumed that displacement of the first inner-sphere coordinated water molecule is the rate-limiting step in complexation, then it can be predicted that the stability constants of the other divalent metal–citrate complexes need to be larger, in the same proportion as their k_{M-H_2O} is faster, than the $(Ni–citrate)^-$ complex if they are to be successfully separated by this technique. For example, if the stability constant of the $(Ni–citrate)^-$ complex is taken as $10^{6.6}$ M [47], then the stability constant of the $(Co–citrate)^-$ complex would need to be approximately $10^{8.6}$ M. This is two orders of magnitude higher than the stability constant compiled for this complex ($10^{6.3}$ M) [49]. However, if the uncertainty inherent in the range of values reported for K_{ML} and k_{M-H_2O}, as well as in calculating K_{OS}, are taken into account, it may only conservatively be concluded that of the divalent metal complexes formed with citric acid, only those of Co^{2+} and Fe^{2+} may potentially be suitable for separation by the CZE method of Schaumlöffel et al. [54].

It should also be possible to use Eqs. (1) and (2) to calculate the dissociation rate of $(Ni–citrate)^-$ and, thereby, obtain a rate constant that can be used to identify other metal complexes potentially suitable for the same CZE method. Taking the stability constant from [49], the k_{M-H_2O} of Ni^{2+} from [85] and using the modified Fuoss equation for determining K_{OS} in [83], the k_d of $(Ni–citrate)^-$ is calculated to be approximately $12\,s^{-1}$. This first-order rate constant results in a somewhat surprisingly short half-life of 0.06 s, considering that CZE separation of the $(Ni–citrate)^-$ complex requires 240 s [54]. This apparent contradiction between theoretical calculations and reported results may possibly be due to the rate-limiting step of $(Ni–citrate)^-$ formation being chelation or ring enclosure of the metal. However, as mentioned previously, the data compiled on the complexes of Ni, including those with organic acids, tends to suggest that this is not the case [83,84]. Recent experiments examining the citrate complex of Cu [91] and the nitrilotriacetic acid (NTA) complex of Ni [92] with diffusive gradients in thin films (DGT) also supports the earlier work reviewed by Margerum et al. [83] and Burgess [84].

The influence of various concentrations of citrate on the complexation of Ni by dimethlglyoxime (DMG) has been experimentally examined using adsorptive cathodic stripping voltammetry and the competing ligand-exchange method [93]. Although it was observed that the formation rate of the $Ni(DMG)_2$ complex decreased with increasing citrate concentrations, this does not necessarily indicate that the dissociation of Ni–citrate complexes was the rate limiting step. In fact, the formation rate of $Ni(DMG)_2$ continued to decrease at citrate concentrations above which Ni would be expected to be 100% complexed by citrate. In this case, it is the competition between citrate and DMG to complex the dissociated Ni^{2+} cations that causes the decrease in the rate of $Ni(DMG)_2$ formation. Therefore, when using these experimental conditions, the rate limiting step is not the dissociation of Ni–citrate complexes but, rather, the formation of these complexes. Although the pseudo first-order kinetics of the reaction:

$$(Ni–citrate)^- + 2DMG^- \Rightarrow Ni(DMG)_2 + citrate^{3-} \qquad (6)$$

were reported to be between 0.12 and $0.01\,s^{-1}$ [93], depending upon the concentration of both citrate and DMG, it is, unfortunately, not possible to determine the formation or dissociation rate constants of Ni–citrate complexes from this data.

In conclusion, the uncertainty involved in the use of Eq. (2) to predict the formation rate of metal–organic acid complexes, and the lack of experimental data describing the dissociation and/or formation rate of many of these com-

plexes, limits the complete identification of the metal–organic acid complexes amenable to HPLC and CZE analyses. Nevertheless, the characteristic rate of water exchange is an important factor in determining the formation rate of metal–organic acid complexes and, in combination with the stability constant of the complex, provides an initial indication of those complexes potentially suited to these separative techniques.

5. Conclusion

The coupling of an efficient separation technique to a sensitive detection system is a popular method for determining the solution speciation of metals in the environment. However, the characteristics of the metal complexes amenable to these techniques need to be defined and the limitations of these techniques must be identified. The application of HPLC and CZE to separating metal–organic acids complexes, with the exception of Al complexes, has not been widely reported. However, even in the literature reporting the separation of Al–organic acid complexes the results are sometimes contradictory and agreement with calculated thermodynamic equilibrium species distribution has often depended on which species (and the value of their stability constant) are included in the calculations. As such, the verification of analytical methodology by computer speciation programs should be considered with caution.

Only those metal–organic acid complexes whose dissociation rate is insignificant during the separation process will be amenable for analysis by HPLC and CZE (i.e. only those complexes which are not at thermodynamic equilibrium during separation). Where the dissociation rates of these complexes have not been determined, these rates can be predicted considering the stability constant of the complex (K_{ML}), the stability constant of the outer-sphere complex (K_{OS}) and the rate constant of water exchange (k_{M-H_2O}). However, this relationship is only valid for metal complexes where the rate limiting step of formation is the exchange of the ligand with a water molecule coordinated to the inner-sphere of a metal. This is likely to be the case for many organic acids, however, chelation and steric effects as well as the protonation of organic acids may decrease metal–organic acid complex formation rates. Nevertheless, these processes would only serve to decrease the dissociation rate of the complex. Therefore, on the basis of thermodynamic equilibrium and complex dissociation kinetics it would appear that the Fe, Ni and Co complexes of some di- and tricarboxylic acids may have the necessary characteristics to be analyzed by these separation techniques.

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