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Potential for the speciation of Zn using fast protein liquid chromatography (FPLC) and convective interaction media (CIM) fast monolithic chromatography with FAAS and electrospray (ES)-MS-MS detection

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Analytical procedures were developed for the speciation of Zn using fast protein liquid chromatography (FPLC), flame atomic absorption spectrometry (FAAS) and convective interaction media (CIM) fast monolithic chromatography with FAAS and electrospray (ES)-MS-MS detection. The investigation was performed on synthetic solutions (2 μg cm^{-3} Zn) of hydrated Zn^{2+} species and Zn complexes with citrate, oxalate and EDTA (ligand-to-Zn molar ratio 100 : 1) over a pH range from 5.4 to 7.4. It was found that Zn interacts with various buffers and the careful adjustment of the pH with diluted solutions of KOH is, therefore, required. FPLC separations were carried out on a Mono Q HR 5/5 strong anion-exchange column, applying an aqueous 1 mol dm^{-3} NH_4NO_3 linear gradient elution over 15 min, at a flow rate of 1.0 cm^3 min^{-1}. The separated Zn species were determined in 1.0 cm^3 eluate fractions "off line" by FAAS. Speciation of Zn was also performed on a weak anion-exchange CIM DEAE fast monolithic disc by applying an aqueous 0.4 mol dm^{-3} NH_4NO_3 linear gradient elution over 7.5 min, at a flow rate of 2.0 cm^3 $min^{-1}$and determination of the separated Zn species in 1.0 cm^3 eluate fractions "off line" by FAAS. Zn-binding ligands in separated fractions were also characterized by electrospray (ES)-MS-MS analysis. The CIM DEAE disc was found to be more efficient in the separation of negatively charged Zn complexes than the Mono Q FPLC column. On the CIM DEAE disc Zn–citrate was separated from both Zn–oxalate and from Zn–EDTA. All these species were also separated from hydrated Zn^{2+}, which was eluted with the solvent front. This method has an advantage over commonly used analytical techniques for the speciation of Zn which are only able to distinguish between labile and strong Zn complexes. Good repeatability of the measurements (RSD 2–4%), tested for six parallel determinations (2 μg cm^{-3} Zn) of Zn–EDTA, Zn–citrate and Zn–oxalate was found at a pH of 6.4 on a CIM DAEA disc. The limit of detection (3*s*) for the separated Zn species was 10 ng cm^{-3}. The proposed analytical procedure was applied to the speciation of Zn in aqueous soil extracts and industrial waste water from a lead and zinc mining area.

Introduction

In investigations of bioavailability to plants and leachability to ground water it is necessary to know not only the total metal concentration, but also the chemical forms of the metals present in soil solutions.[1–5] Agricultural soils and sludge-amended soils often contain heavy metals[6] such as Zn and Cd. In industrially polluted soils[5] the pollutants frequently present are Zn, Pb and Cd. Zn in soil solutions may be present as different chemical species, *e.g.*, hydrated divalent cations, inorganic complexes and organic complexes. Among the various approaches to heavy metal speciation in soil solutions, computer simulations have often been used[7–9] and the chemical forms of Zn in soil solutions have been calculated with the aid of computer programs. These theoretical predictions have been successful for well-defined solutions with known total metal concentrations, ligand concentrations and known corresponding stability constants, but tend not to predict accurately the metal species in soil solutions due to their very complex matrix. It is therefore necessary to experimentally determine the Zn species in soil solutions and environmental water samples. This need is also highlighted in investigations of the uptake of Zn by plants in contaminated soils.[10] Among various experimental methods for determination of dissolved free Zn ion concentrations in soil solutions and environmental water samples is anodic stripping voltametry.[11–14] A gel-integrated Hg-plated Ir-based microelectrode array for measuring Zn species in natural waters enabled the quantitative discrimination between mobile and colloidal metal species.[15] Analytical procedures were also reported for the determination of free Zn^{2+} and the operationally defined complexed fractions (labile and stabile complexes) in soil solutions by the combination of Amberlite CG 120 and Chelex-100 in a batch-column-batch procedure.[6] The same analytical procedure was also applied in the speciation of Cd and Zn in soil solutions from contaminated soils.[16] Labile complexes of Al and Zn in river water and snow samples were determined by the Chelex-100 batch technique.[17] A study of Zn speciation in waters from the polluted Cochin estuary area was made by applying the Chelex-100 column separation procedure.[18] Column chelating ion-exchange Chelex-100 resin in combination with the FAAS technique was also applied by our group for the speciation of Zn, Pb and Cd in aqueous soil extracts from a mining polluted area.[5] The results indicated that Zn, Pb and Cd existed in the form of positively charged species or weak labile metal complexes. In estimation of environmental burden and the ways of metal cycling, especially in mining and

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industrial areas, it is very important to determine particular metal species in soil solutions. For more detailed information on the identity of these metal species, more powerful analytical procedures are required.

The aim of the present work was to examine the possibilities of FPLC and CIM fast monolithic chromatography with FAAS detection for the speciation of Zn. For this purpose the investigation was carried out using synthetic solutions of Zn^{2+}, ($Zn(NO_3)_2 \cdot 4H_2O$), Zn–citrate, Zn–oxalate and Zn–EDTA at various pHs. The identification of ligands in the separated Zn complexes was also performed with the electrospray (ES)-MS-MS technique. The applicability of the CIM-FAAS procedure was examined for the speciation of Zn in aqueous soil extracts and industrial waste water from a lead and zinc mining area.

Experimental

Apparatus

The separation of Zn species was performed on FPLC columns and CIM fast monolithic chromatographic discs. Strong anion and cation exchange FPLC columns (Pharmacia, Uppsala, Sweden) of Mono Q HR 5/5 and Mono S HR 5/5 (column dimensions 5 × 50 mm, 10 μm beaded hydrophilic polyether resin substituted with quaternary amine (Q) or methyl sulfonate (S) groups, pH stability 2–12) and strong and weak anion exchange CIM discs (Bia Separations, Ljubljana, Slovenia) (disc dimensions 12 × 3 mm, based on poly(glycidylmethacrylate-co-ethyleneglycol dimethacrylate) matrix support and substituted with quaternary amine (QA) or diethylamine (DEAE) groups, pH stability 1–13) were used. The columns and discs were connected to a Waters (Milford, MA, USA) Model 600E gradient high-pressure pump, equipped with a Rheodyne (Cotati, CA, USA) Model 7725i injector (0.5 cm^3 loop). Separated Zn species were determined by FAAS on a Varian (Mulgrave, Victoria, Australia) SpectrAA 110 atomic absorption spectrometer in an air–acetylene flame. A Micromass (Micromass UK Ltd., Manchester, UK) Quatro LC tandem quadrupole mass spectrometer equipped with a Z spray ion source as the LC-MS interface, employing negative electrospray ionisation, was used for the identification of ligands in the separated Zn fractions. A WTW (Weilheim, Germany) pH 330 pH meter was employed to determine the pH of the samples. A Heraeus (Osterode, Germany) Model 17S Sepatech Biofuge was used for centrifugation of the soil extracts.

Reagents

Merck (Darmstadt, Germany) suprapur acids and bases, and water doubly distilled in quartz were used for the preparation of samples and standard solutions. All other chemicals were of analytical reagent grade. Stock standard solutions of Zn (1000 mg dm^{-3} in 5% HNO_3) were obtained from Merck. Fresh working standard solutions were prepared by the dilution of particular stock solutions with water, and these were used for the determination of total and total water-soluble Zn concentrations in the soil samples. In order to study the separation of Zn species by FPLC columns and CIM discs synthetic standard solutions were prepared. A Zn^{2+} solution (1000 mg dm^{-3}) was prepared from an appropriate amount of $Zn(NO_3)_2 \cdot 4H_2O$ salt dissolved in water. From this solution Zn was prepared at a concentration of 2 μg cm^{-3}, and organic complexes were formed by the addition of an appropriate amount of citric acid ($C_6O_7H_6$), oxalic acid ($C_2H_2O_4 \cdot 2H_2O$) or EDTA sodium salt ($C_{10}H_{14}N_2Na_2O_8 \cdot 8H_2O$), so that the ligand to Zn molar ratio was 100 : 1. In order to investigate the influence of buffer solutions on Zn speciation, the following Merck buffer substances (0.05–0.1 mol dm^{-3}) were used: potassium hydrogenphthalate ($C_8H_5KO_4$), adjusted to a pH of 7.0 with potassium hydroxide; imidazole ($C_3H_4N_2$), adjusted to a pH of 7.0 with nitric acid; piperazine ($C_4H_{10}N_2$), adjusted to a pH of 7.0 with nitric acid; TRIS ($C_4H_{11}NO_3$), adjusted to a pH of 7.0 with nitric acid; and boric acid (H_3BO_3), adjusted to a pH of 7.0 with potassium hydroxide. In order to avoid the influence of buffer constituents on Zn speciation, a careful addition of potassium hydroxide (0.1–2.0 mol dm^{-3}) was also used for the adjustment of pH. Sartorius cellulose nitrate membrane filters of 25 mm diameter and 0.2 μm pore size were used in the filtration procedure.

Sample preparation and determination of Zn

The total concentration of Zn in the soil samples was determined after nitric, perchloric and hydrofluoric acid digestion[19] by FAAS under optimised measurement conditions.

For the determination of total water-soluble Zn and its species,[5] 2.00 g of moist soil samples were shaken on a classical shaker for 16 h with 20 cm^3 of distilled water, centrifuged (10 000 rpm, 20 min) and decanted. The soil extracts were then filtered through a 0.2 μm filter. Aliquots of these solutions were used for the determination of total water-soluble Zn and for speciation analysis. One aliquot of filtered (0.2 μm) soil extract was used for determination of pH.

Recommended FPLC-FAAS procedure

Sample (0.5 cm^3) was injected into the FPLC column. An aqueous 1 mol dm^{-3} NH_4NO_3 linear gradient elution was applied for 15 min at a flow rate of 1.0 cm^3 min^{-1}. The eluate was collected in 1.0 cm^3 fractions in Eppendorf polyethylene cups and the concentration of Zn determined "off line" by FAAS under optimum measurement conditions. After each separation the column was regenerated for 5 min with 1 mol dm^{-3} NH_4NO_3 at a flow rate of 2.0 cm^3 min^{-1} and equilibrated with water, first for 10 min at a flow rate of 2.0 cm^3 min^{-1} and then for 10 min at a flow rate of 1.0 cm^3 min^{-1}. The eluents used in the chromatographic separations were adjusted to the pH of samples being analysed with an appropriate amount of diluted KOH.

Recommended CIM fast monolithic chromatography, FAAS and ES-MS-MS procedure

Sample (0.5 cm^3) was injected into a CIM fast monolithic disc. An aqueous 0.4 mol dm^{-3} NH_4NO_3 linear gradient elution was applied for 7.5 min at a flow rate of 2.0 cm^3 min^{-1}. The eluate was collected in 1.0 cm^3 fractions in Eppendorf polyethylene cups and the concentration of Zn determined 'off line' by FAAS under optimum measurement conditions. After each separation the disc was regenerated for 5 min with 1 mol dm^{-3} NH_4NO_3 at a flow rate of 3.0 cm^3 min^{-1} and equilibrated with water for 5 min at a flow rate of 4.0 cm^3 min^{-1}. The eluents used in the chromatographic separations were adjusted to the pH of samples being analysed with an appropriate amount of diluted KOH.

Zn-binding ligands in fractions after weak anion-exchange DEAE CIM separation were also characterized by ES-MS-MS analysis. For the identification of low molecular weight (LMW) ligands eluted under the chromatographic peaks, fractions were diluted 1 : 1 with acetonitrile and analysed by the ES-MS-MS technique employing a Z spray ion source. Sample (20 mm^3) was injected into the Micromass Quatro LC mass spectrometer. The mobile phase was composed of acetonitrile, 0.005 mol dm^{-3} ammonium acetate and formic acid (600/399/1: v/v/v).

The electrospray probe voltage and sample cone voltage were set at 2.5 kV and 35 V, respectively. The source temperature of the mass spectrometer was held at 80 °C, while the desolvation temperature was 350 °C. The MS analyses were performed by scanning negative ions. The Q1-scan represented the pseudo-molecular ions $(M - H)^-$ in the mass range *m/z* 50–1000. MS-MS collision-induced dissociation (CID) experiments were performed by introducing argon gas (2.0×10^{-3} mbar) into the collision cell and setting a collision energy of 18 eV. The first quadrupole analyser was set to transmit only the user-selected precursor ion.

Results and discussion

Influence of different buffer solutions on Zn speciation

In order to study the influence of various buffers on Zn speciation, synthetic solutions of 2 μg cm^{-3} hydrated Zn^{2+} ($Zn(NO_3)_2 \cdot 4H_2O$) were prepared at pH 7.0 using different buffer solutions. A buffer solution (45 cm^3) adjusted to pH 7.0, was transferred to a 50 cm^3 volumetric flask. An appropriate amount of the synthetic standard solution of Zn^{2+} was added and made up to the mark with buffer. The following buffer solutions were examined: K hydrogen phthalate, piperazine, TRIS–HNO_3, K borate and imidazole. To avoid the influence of buffer constituents on Zn speciation, the pH was also adjusted by the careful addition of diluted KOH. Samples were injected into an anion-exchange FPLC column and Zn was determined in the separated fractions by FAAS under the recommended FPLC-FAAS procedure. The results are presented in Fig. 1. At a pH of 7.0 it is to be expected that Zn in a nitrate solution exists as a Zn^{2+} ion (*e.g.* $Zn(H_2O)_4^{2+}$) and partially as $Zn(OH)^+$, $Zn(OH)_2$, $Zn(OH)_3^-$ and $Zn(OH)_4^{2-}$. Positively charged Zn species should be eluted with the solvent front on an anion-exchange FPLC column. The data in Fig. 1 indicate that Zn species interact with all the buffer solutions investigated. Broad chromatographic peaks, which are eluted from 8 to 15 min, are observed. In the presence of the K hydrogen phthalate buffer solution, Zn is also partially eluted with the solvent front. The data in Fig. 1 also indicate that when the pH was adjusted with diluted KOH, 80% of Zn was eluted with the solvent front as positively charged Zn species, 10% was strongly adsorbed on the column resin and 10% was eluted from 9 to 13 min as a small broad peak. Experiments at pH 5.4 and 6.4 showed similar behaviour. These observations suggest that the use of buffer solutions should be avoided when adjusting the pH in studies of Zn speciation. In the following experiments, therefore, the pH was adjusted by the careful addition of an appropriate amount of diluted KOH.

Distribution of Zn^{2+}, Zn–citrate, Zn–oxalate and Zn–EDTA on a strong anion-exchange FPLC column at various pHs

The gradient elution with NaCl and NH_4NO_3 (0.25–2 mol dm^{-3}) was examined first. The most efficient separation of Zn species was obtained by using 1 mol dm^{-3} NH_4NO_3 and the recommended analytical procedure.

The applicability of a strong anion-exchange FPLC column for the speciation of Zn was investigated on synthetic solutions (2 μg cm^{-3} Zn) of hydrated Zn^{2+} species ($Zn(NO_3)_2 \cdot 4H_2O$) and Zn complexes with citrate, oxalate and EDTA (ligand-to-Zn molar ratio 100 : 1) over a pH range from 5.4 to 7.4. The pH range examined was similar to the pH of the majority of environmental water samples and aqueous soil extracts. Data of these measurements are presented in Fig. 2. It is evident that the majority of positively charged Zn species (Zn–nitrate) are eluted with the solvent front in the pH range 5.4–7.4. The percentage of positively charged Zn species eluted with the solvent front decreased with increasing pH. Small, broad peaks eluted from 9 to 13 min are observed at pH 5.4 and 6.4. Zn–citrate is eluted as negatively charged species with a maximum concentration at 9 min. The percentage of negatively charged species decreased with increasing pH. The remaining Zn was strongly adsorbed on the column resin and did not influence further separations. Similar behaviour was exhibited by the Zn–oxalate. It is obvious that Zn–citrate is not separated from Zn–

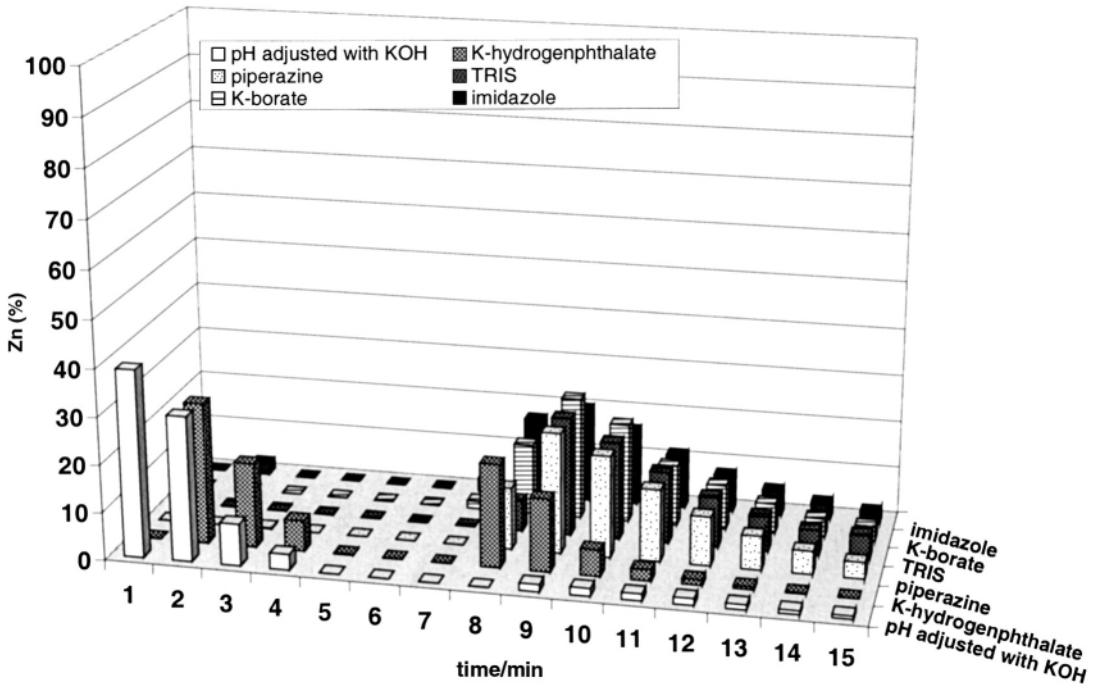

Fig. 1 Influence of various buffer solutions on the speciation of Zn ($Zn(NO_3)_2 \cdot 4H_2O$, 2 μg cm^{-3} Zn) at pH 7.0 employing strong anion-exchange FPLC with FAAS detection. Sample volume 0.5 cm^3, aqueous 1 mol dm^{-3} NH_4NO_3 linear gradient elution, flow rate 1.0 cm^3 min^{-1}, $n = 2$.

oxalate on a strong anion-exchange FPLC column. The data in Fig. 2 further indicate that Zn–EDTA is quantitatively eluted at pH 5.4–7.4 as negatively charged species in a narrow peak at 8 min.

Behaviour of Zn^{2+}, Zn–citrate, Zn–oxalate and Zn–EDTA on a strong cation-exchange FPLC column

A strong cation-exchange FPLC column was also tested for the speciation of Zn. The recommended FPLC-FAAS analytical procedure was applied at pH 5.4. Zn–EDTA was quantitatively eluted with a solvent front, while Zn^{2+} ($Zn(NO_3)_2{\cdot}4H_2O$), Zn–citrate and Zn–oxalate were quantitatively eluted in a narrow peak at 11–12 min. It can be concluded that the methyl sulfonate groups of the FPLC column broke the labile Zn–citrate and Zn–oxalate complexes, so that Zn was eluted as Zn^{2+}. For this reason the strong cation-exchange FPLC column was not appropriate for the speciation of Zn.

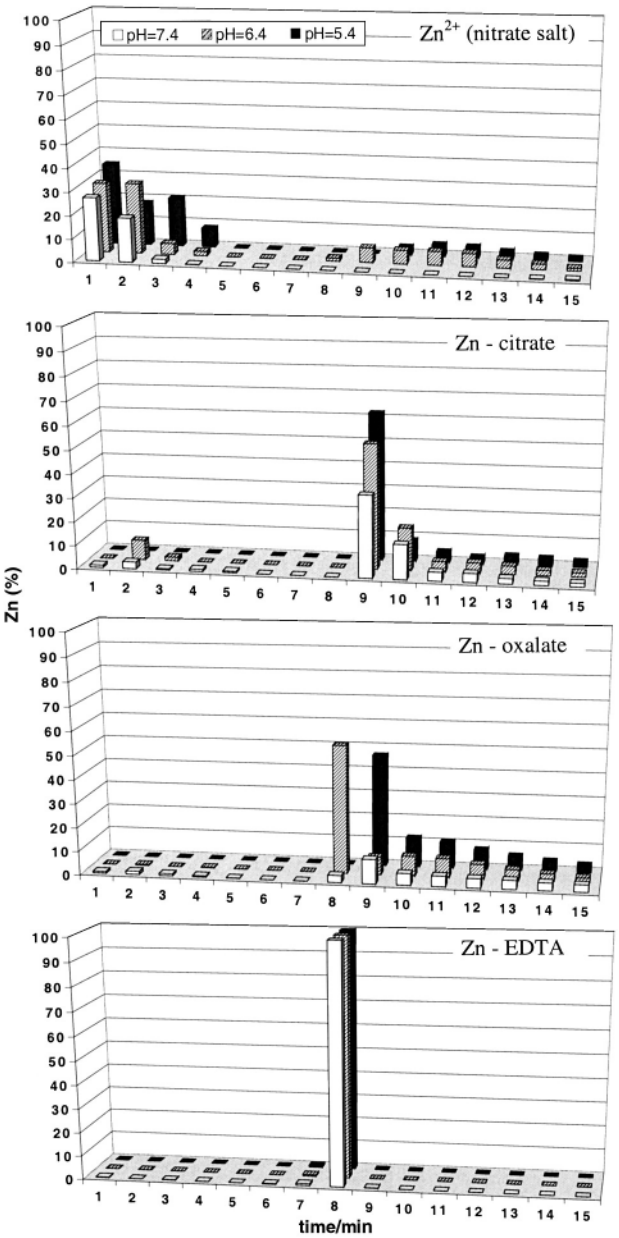

Fig. 2 Distribution of Zn^{2+} ($Zn(NO_3)_2{\cdot}4H_2O$), Zn–citrate, Zn–oxalate and Zn–EDTA (2 μg cm^{-3} Zn) at various pHs, employing strong anion-exchange FPLC with FAAS detection. Sample volume 0.5 cm^3, aqueous 1 mol dm^{-3} NH_4NO_3 linear gradient elution, flow rate 1.0 cm^3 min^{-1}, n = 2.

Distribution of Zn^{2+}, Zn–citrate, Zn–oxalate and Zn–EDTA on weak anion-exchange CIM DEAE fast monolithic disc at various pHs

The recently developed cation- and anion-exchange separation supports based on convective interaction media (CIM) offer fast separation of biomolecules.[20–22] In general, CIM are produced in disc units and are also used for fast separation of organic acids.[23] In order to examine the possibility of using CIM discs for the speciation of Zn, synthetic solutions (2 μg cm^{-3} Zn) of Zn^{2+}, $Zn(NO_3)_2{\cdot}4H_2O$ and Zn complexes with citrate, oxalate and EDTA (ligand-to-Zn molar ratio 100 : 1) were separated on strong anion-exchange QA and weak anion-exchange DEAE discs over a pH range from 5.4 to 7.4. The following eluents: NaCl, $NaNO_3$, $Mg(NO_3)_2$ and NH_4NO_3 in 0.25–1 mol dm^{-3} concentrations were examined first in gradient elution. The most efficient separation of Zn species was obtained with 0.4 mol dm^{-3} NH_4NO_3 using the recommended CIM fast monolithic chromatography FAAS procedure. The separations of Zn species on the strong anion-exchange QA disc were very similar to those obtained with the strong anion-exchange FPLC column. The speciation of Zn by the weak anion-exchange CIM DEAE-FAAS procedure is presented in Fig. 3. It is evident that Zn^{2+} ($Zn(NO_3)_2{\cdot}4H_2O$) is quantitatively eluted with the solvent front in the pH range 5.4–7.4, and that it is completely separated from the negatively charged species. Powell and Petit[24] provided a computer program for calculations of metal species over a wide pH range using stability constants from the IUPAC database. On the basis of these calculations the following Zn species with citrate (Zn-to-ligand molar ratio 1 : 100) were predicted: in the pH range 5.4–7.4 the $[Zn(Cit)]^-$ complex and $[Zn(Cit)_2]^{4-}$ complex coexist. At lower pH, free Zn^{2+} coexists with $[Zn(Cit)]^-$ complex. The percentage of free Zn^{2+} increases with decreasing pH. The data in Fig. 3 indicate that at pH 6.4 and 7.4 approximately 70% of Zn–citrate is eluted from 1.0 to 2.5 min and about 30% from 4.5 to 5.5 min. On the basis of the elution times the peak eluted from 1.0 to 2.5 min can be assumed to be $[Zn(Cit)]^-$ complex, and from 4.5 to 5.5 min $[Zn(Cit)_2]^{4-}$ complex. At pH 5.4 the percentage of Zn eluted from 4.5 to 5.5 min decreased to 8%, while 92% of Zn is eluted from 0.5 to 2.0 min, presumably as a mixture of $[Zn(Cit)]^-$ and free Zn^{2+} species. The distribution for Zn species with citrate from Fig. 3 is in agreement with the theoretically predicted species.[24] Calculations of the distribution of Zn species[24] for Zn–oxalate (Zn-to-ligand molar ratio 1 : 100) in the pH range 5.4 to 7.4 indicate that $[Zn(Ox)_2]^{2-}$ complex is the prevailing species and coexists with $[Zn(Ox)]$ neutral complex. It is evident from the data in Fig. 3 that Zn–oxalate is eluted from 4.5 to 5.5 min. On the basis of the elution time and theoretical predictions[24] the peak eluted from 4.5 to 5.5 min can be assumed to be $[Zn(Ox)_2]^{2-}$ complex. At a pH of 6.4, 95% and at a pH of 5.4, about 80% of Zn–oxalate was eluted as $[Zn(Ox)_2]^{2-}$ complex, the remaining Zn (presumably in the form of $[Zn(Ox)]$ neutral complex) was strongly adsorbed on the disc support and did not elute within 7.5 min. At a pH of 7.4 about 70% of Zn-oxalate was eluted as $[Zn(Ox)_2]^{2-}$ complex, the remaining Zn (presumably mixed Zn(Ox)–hydroxo species) was strongly adsorbed on the disc support and did not elute within 7.5 min. The calculated distribution of Zn species[24] for Zn–EDTA (Zn-to-ligand molar ratio 1 : 100) in the pH range 5.4 to 7.4 predicted the presence of $[Zn(EDTA)]^{2-}$ complex. The data in Fig. 3 indicate that from pH 5.4 to 7.4 Zn–EDTA is quantitatively eluted from 3.5 to 4.5 min. On the basis of the elution time and theoretical predictions,[24] the negatively charged species eluted corresponded to the $[Zn(EDTA)]^{2-}$ complex.

Identification of LMW–Zn ligands by the ES-MS-MS technique

In order to identify LMW–Zn binding ligands the ES-MS-MS technique using a Z spray ion source was applied. This ionisation source enabled the analysis of samples with a high salt content such as fractions eluted during the chromatographic separation. First, the mass spectra of standard solutions of Zn–citrate, Zn–oxalate and Zn–EDTA were recorded. The MS analysis was performed by scanning negative ions. Since ES is a "soft" ionisation technique, very little fragmentation was observed. The most intensive ion in the mass spectra of the studied Zn species was the deprotonated ligand ion $(M - H)^-$. This precursor (parent) ion was selected for a further collision-induced dissociation (CID) experiment and the resulting (daughter) ion mass spectra were recorded under optimum conditions. The mass spectra and the corresponding MS-MS spectra of Zn–citrate, Zn–oxalate and Zn–EDTA standard solutions (2 μg cm^{-3} Zn) are presented in Fig. 4. MS analysis of the blank indicated that *m*/*z* 81, 91, 108, 127, 137 and 154 corresponded to signals from the eluent used in the ES-MS procedure. It is evident (Fig. 4A) that in the mass spectrum of Zn–citrate the peak with *m*/*z* 191 is the most intense and corresponds to deprotonated citric acid. This peak was selected as a parent ion for the CID experiment. After fragmentation, masses of *m*/*z* 111, *m*/*z* 87 and *m*/*z* 85 were present in the resulting daughter ion mass spectra. In the mass spectra of Zn–oxalate (Fig. 4B) a peak of *m*/*z* 89 appeared and corresponded to deprotonated oxalic acid. This peak was selected as a parent ion for CID analysis, and in the daughter ion mass spectra two peaks were present (*m*/*z* 89 and *m*/*z* 61). Zn–EDTA mass spectra (Fig. 4C) resulted in mass *m*/*z* 291. In the daughter ion mass spectra of *m*/*z* 291 fragment, ions of *m*/*z* 88, 157, 159, 201, 215 and 273 appeared. The same standard solutions, prepared at pH 6.4, were also injected on the CIM DAEA disc and the ES-MS-MS spectra were recorded for the separated fractions eluted under the chromatographic peaks. It was found that in the ES-MS spectra additional peaks appeared (*m*/*z* 80, 103, 125, 142, 147, 171 and 188), all of which resulted from the eluent of the chromatographic run (NH_4NO_3). The ES-MS-MS spectra of fractions eluted under chromatographic peaks are presented in Fig. 5. It is evident from the data in Fig. 5A1 and A2, that in the mass spectra of the fractions eluted from 1.5 to 2.0 min and from 4.5 to 5.0 min, characteristic peaks with *m*/*z* 191 are present. The corresponding daughter ion spectra of *m*/*z* 191 resulted in characteristic masses of 111, 87 and 85. The same masses were also observed in fractions eluted from 1.0 to 1.5 and 2.0 to 2.5 as well as 5.0 to 5.5 min. These data confirmed the presence of the citrate binding ligand in these fractions. The data in Fig. 5B further indicate that in the mass spectra of the fraction eluted from 4.5 to 5.0 min the characteristic peak with *m*/*z* 89 is present, as are its daughter ions with *m*/*z* 89 and 61. The same masses were also observed in the fraction eluted from 5.0 to 5.5 min. These data confirmed the presence of oxalate binding ligand. From Fig. 5C it is also evident that in mass spectra of the fraction eluted from 3.5 to 4.0 min a characteristic peak with *m*/*z* 291 is present and its daughter ions with *m*/*z* 88, 157, 159, 201, 215 and 273. The same masses were observed also in fractions eluted from 3.0 to 3.5 and 4.0 to 4.5 min. These data confirmed the presence of the EDTA binding ligand.

ES-MS-MS analyses confirmed the presence of binding ligands in fractions eluted under the chromatographic peaks. This offers the additional identification of separated species, which is of great importance when the co-elution of Zn species appears *e.g.* partial co-elution of $[Zn(Cit)_2]^{4-}$ and $[Zn(Ox)_2]^{2-}$ at an elution time from 4.5 to 5.5 min.

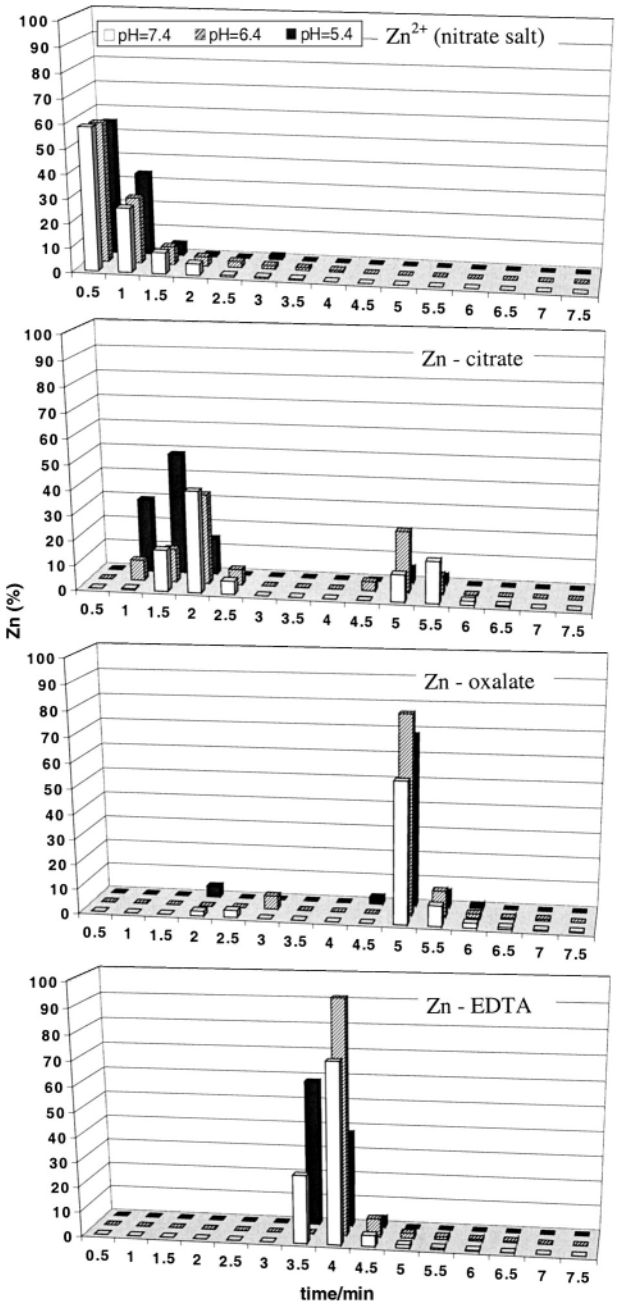

Fig. 3 Distribution of Zn^{2+} ($Zn(NO_3)_2{\cdot}4H_2O$), Zn–citrate, Zn–oxalate and Zn–EDTA (2 μg cm^{-3} Zn) at various pHs, employing weak anion-exchange CIM-DEAE with FAAS detection. Sample volume 0.5 cm^3, aqueous 0.4 mol dm^{-3} NH_4NO_3 linear gradient elution, flow rate 2.0 cm^3 min^{-1}, n = 2.

Linearity of measurement, repeatability of measurement and LOD for FPLC-FAAS and CIM DEAE-FAAS procedures

Linearity of measurement in synthetic solutions of Zn^{2+}, Zn–citrate, Zn–oxalate and Zn–EDTA for FPLC-FAAS and CIM DEAE-FAAS procedures was obtained in the range 0.02 to 2.0 μg cm^{-3} for particular separated Zn species with a correlation coefficient better than 0.998. The repeatability of the measurement was tested for six consecutive separations of synthetic solutions of various Zn species (2.0 μg cm^{-3} Zn, pH = 6.4) by applying FPLC-FAAS and CIM DEAE-FAAS procedures. The relative standard deviation (RSD) for Zn species applying the FPLC-FAAS procedure was found to be 4.2% for Zn^{2+}, 3.8% for Zn–citrate, 3.1% for Zn–oxalate and 2.5% for Zn–EDTA, while by applying the CIM DEAE-FAAS procedure the RSD was 3.3% for Zn^{2+}, 3.6% for Zn–citrate, 4.0% for Zn–oxalate and 1.9% for Zn–EDTA. The limit of detection (LOD) (3*s*) for

determination of separated Zn species on the FPLC column and CIM disc with FAAS detection was found to be 10 ng cm^{-3} (0.5 cm^3 sample).

Comparison of strong anion-exchange FPLC-FAAS and weak anion-exchange CIM DEAE-FAAS procedures for the speciation of Zn

The comparison of data from Figs. 2 and 3 indicates that the weak anion-exchange CIM DEAE disc is more effective in the speciation of various Zn species than the strong anion-exchange FPLC column. By applying the CIM DEAE-FAAS procedure Zn^{2+} is separated from the negatively charged Zn complexes. Separation of $[Zn(Cit)]^-$, $[Zn(EDTA)]^{2-}$ and $[Zn(Ox)_2]^{2-}$ species is also achieved on the disc support. Although $[Zn(Ox)_2]^{2-}$partially coeluted with $[Zn(Cit)_2]^{4-}$, these two species can be identified by applying ES-MS-MS analysis. The CIM DEAE-FAAS-ES-MS-MS procedure is therefore recommended for the speciation of Zn. Its selectivity is the main advantage in comparison with other previously reported analytical procedures that were only able to distinguish between strong Zn complexes and the sum of the labile Zn complexes and Zn^{2+} species. The developed analytical procedure for the determination of Zn species provides a promising basis for the speciation of Zn in environmental samples and in investigations of the uptake of various Zn species in plants.

Identification of Zn species in soil extracts and industrial waste waters by CIM DEAE FAAS ES-MS-MS procedure

In order to evaluate the capability of the method developed for the speciation of Zn in soil extracts and waste waters, soil samples and industrial waste water from a polluted lead and zinc mining area were selected. Soil sampling was performed on

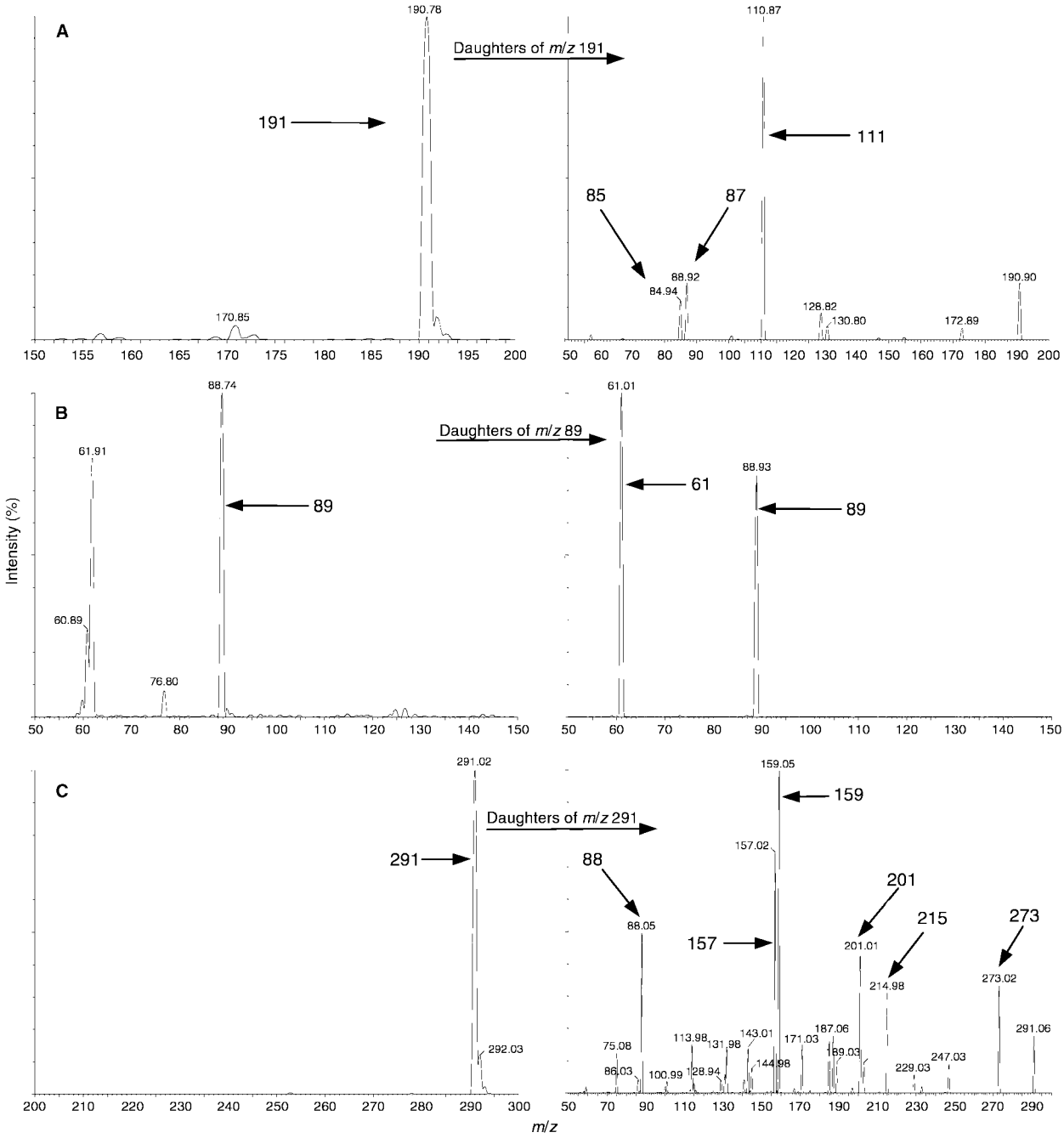

Fig. 4 ES mass spectra and corresponding daughter ion mass spectra for synthetic solutions of Zn–citrate (A), Zn–oxalate (B) and Zn–EDTA (C) (2 μg cm^{-3} Zn) at pH 6.4.

meadow soil from the top layer (0–10 cm). Sampling points were approximately 1 km apart. The total amount of Zn in the soil samples was determined first. The high concentrations of Zn in samples Nos.1–3: 5.9 ± 0.1 mg g^{-1}, 5.5 ± 0.1 mg g^{-1} and 5.9 ± 0.1 mg g^{-1} indicated that these soil samples were contaminated with Zn. For the determination of Zn species in the soil extracts, samples were prepared in duplicate as described in Experimental. The total water-soluble Zn was

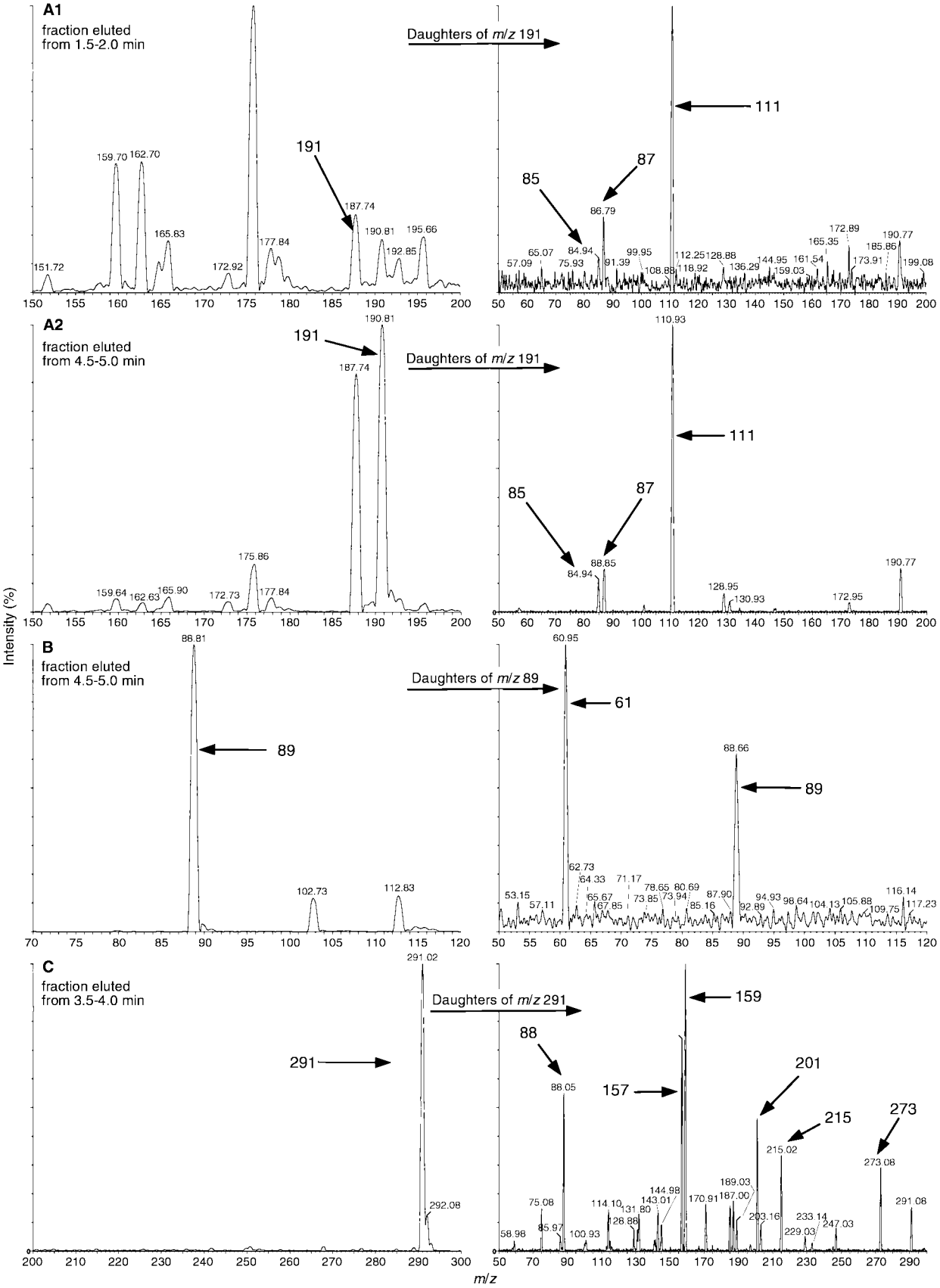

Fig. 5 ES mass spectra and corresponding daughter ion mass spectra for synthetic solutions of Zn–citrate (A1, A2), Zn–oxalate (B) and Zn–EDTA (C) (2 μg cm^{-3} Zn) at pH 6.4 in fractions eluted under chromatographic peaks on weak anion-exchange CIM-DEAE disc.

determined in the filtered (0.2 μm) soil extracts and in the filtered (0.2 μm) industrial waste water sample. These concentrations and pHs are presented in Table 1. The pH of the soil extracts and the industrial waste water ranged from 6.5 to 6.7. The concentration of Zn in the industrial waste water is appreciably higher than in soil extracts. The concentration of total water-soluble Zn in the soil extracts represented only 0.07% of the total Zn in sample No. 1, 0.09% of the total Zn in sample No. 2 and 0.11% in sample No. 3. Speciation of Zn in the filtered (0.2 μm) soil extracts and the filtered (0.2 μm) industrial waste water was then carried out with the CIM DEAE FAAS procedure. The results, see Table 1, indicate that in soil extracts Nos. 1–3 Zn is eluted with the solvent front from 2 to 3 min. In sample No. 1, 10% of Zn is eluted with the solvent front, 6% from 2.0 to 2.5 min, and the remaining Zn is adsorbed on the disc support. In sample No. 2, 31% of Zn is eluted with the solvent front, 15% from 2.0 to 3.0 min, and the remaining Zn is adsorbed on the disc support. In sample No. 3, 26% is eluted with the solvent front, 14% from 2.0 to 3.0 min, and the remaining Zn is adsorbed on the disc support. It can be presumed that at the pH of the soil extracts (6.5–6.7) the proportion of Zn species adsorbed on the disc support in samples Nos. 1–3 corresponded to the neutral Zn complexes and/or hydroxo Zn species, while the proportion eluted with the solvent front corresponded to the hydrated Zn^{2+} species. On the basis of the elution time and the pH of the samples the fraction of Zn in the soil extracts eluted from 2.0 to 3.0 min could be presumed to be $[Zn(Cit)]^-$ species. In order to confirm our prediction, ES-MS-MS analyses were performed on the fraction (samples Nos. 1–3) eluted from 2.0 to 2.5 min. The data for sample No. 3 are given in Fig. 6. In the mass spectrum the characteristic peak with *m*/*z* 191 is present. Additional peaks with *m*/*z* 160, 163, 176, 190, 196, 198 and 200 also appeared and corresponded, presumably, to fragments of decaying organic matter (*e.g.* humic acids) present in the soil solution. We were not able to identify these peaks. The corresponding daughter ion spectra of *m*/*z* 191 resulted in characteristic masses of 111, 87 and 85. These data confirmed the presence of the citrate binding ligand. The same mass spectra in the fraction eluted from 2.0 to 2.5 min were also observed in samples No. 1 and 2. The data in Table 1 further indicated that 53% of Zn in the industrial waste water sample No. 4 was eluted with the solvent front while the remaining Zn was adsorbed on the disc support. On the basis of the elution time and the pH of the sample it can be presumed that Zn eluted with the solvent front corresponded to the Zn^{2+} species and that the proportion of Zn adsorbed corresponded to the neutral complexes and/or hydroxo Zn species.

The speciation analyses of soil extracts from the contaminated mining area indicated that the percentage of particular Zn species varied between different locations, and that Zn species present in industrial waste water also differs from soil extracts. With the weak anion-exchange CIM-DEAE FAAS procedure we were able to determine the proportion of Zn^{2+} species present in the samples analysed. By applying ES-MS-MS analysis to the fractions eluted under the chromatographic peaks the citrate binding ligand was identified and on the basis of the elution time there is a strong indication of the presence of $[Zn(Cit)]^-$ complex in the soil extracts. With the weak anion-exchange CIM-DEAE FAAS procedure we were able to determine the proportion of the neutral and hydroxo Zn species in the soil extracts and the industrial waste water sample which were adsorbed on the disc support, but we were not able to identify them. Nevertheless, the developed analytical procedure gives more complete information on Zn species present in a complex matrix, *e.g.* soil solution and industrial waste water samples, than previously applied speciation techniques.

Table 1 Determination of Zn in filtered (0.2 μm) soil extracts and filtered (0.2 μm) industrial waste water by FAAS and the distribution of Zn species after CIM-DEAE FAAS procedure

			Zn eluted during the chromatographic run (%)						
			Time/min						
Sample No.[a]	pH	Total Zn concentration[b]/ μg cm^{-3}	0.0–0.5	0.5–1.0	1.0–1.5	1.5–2.0	2.0–2.5	2.5–3.0	3.0–7.5
1	6.7 ± 0.1	0.168 ± 0.002	5.3	5.0	[c]	[c]	6.2	[c]	[c]
2	6.5 ± 0.1	0.313 ± 0.004	14.3	16.7	[c]	[c]	11.6	4.2	[c]
3	6.6 ± 0.1	0.333 ± 0.003	15.2	11.1	[c]	[c]	8.8	5.3	[c]
4	6.7 ± 0.1	6.21 ± 0.05	32.5	20.4	1.6	1.8	1.0	[c]	[c]

[a] Sample Nos. 1–3: aqueous soil extract. Sample No. 4: industrial waste water. [b] Results are expressed as the mean of two parallel samples ± standard deviation of measurement. [c] Below instrumental LOD (10 ng Zn cm^{-3}).

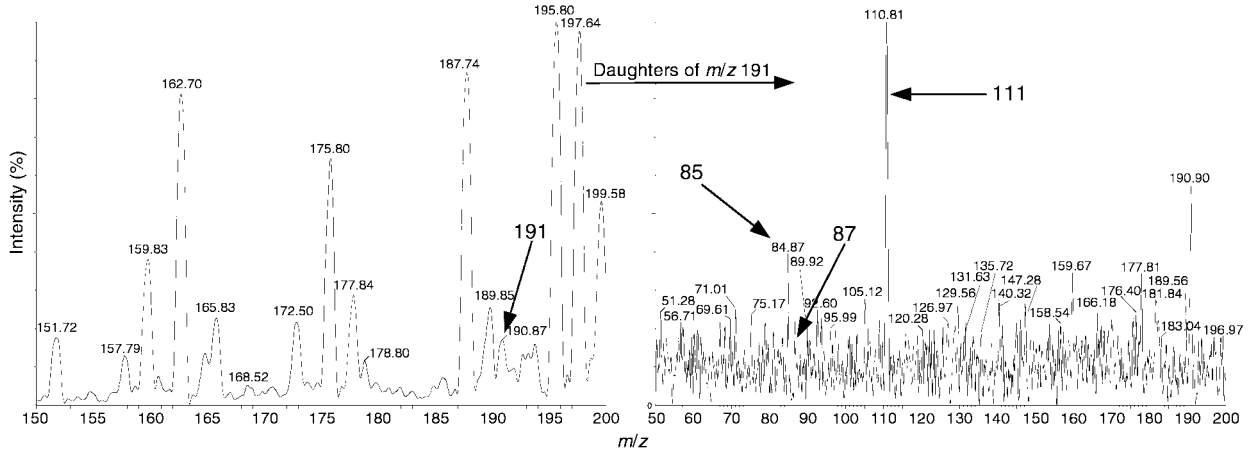

Fig. 6 ES mass spectra and corresponding daughter ion mass spectra of *m*/*z* 191 for eluted fraction from 2.0–2.5 min on a weak anion-exchange CIM-DEAE disc for soil extract sample No. 3, $n = 2$.

Conclusions

The developed anion-exchange CIM-DEAE FAAS procedure enables separation of Zn^{2+} and the negatively charged $[Zn(Cit)]^{-}$, $[Zn(EDTA)]^{2-}$ and $[Zn(Ox)_2]^{2-}$ species. Although $[Zn(Ox)_2]^{2-}$ partially coeluted with $[Zn(Cit)_2]^{4-}$, these two species can be identified by applying ES-MS-MS analysis. The selectivity of CIM-DEAE FAAS ES-MS-MS procedure is the main advantage in comparison with other previously reported analytical procedures which were only able to distinguish between strong Zn complexes and the sum of the labile Zn complexes and Zn^{2+} species. The developed procedure offers an opportunity for the speciation of Zn in soil extracts, waste waters and other environmental samples and will be applied in investigations of the uptake of various Zn species in plants.

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