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Evaluation of immobilized metal-ion affinity chromatography for the fractionation of natural Cu complexing ligands

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Abstract

An immobilized metal-ion affinity chromatography (IMAC) method has been developed and validated for the separation of copper complexing ligands from soil solution. We first investigated the retention behavior of simple model ligands on the IMAC column and found that the ability to form ternary complexes of the structure Cu–IDA–ligand was the dominant factor influencing ligand retention on the IMAC column. The log *K* value of the Cu-complex was found to have only a minor influence on the retention. Legends containing only carboxylic acid functional groups were not retained on the column. To optimize reproducibility and quantitative recovery of copper ligands from soil solution, different composition and pH values of eluting buffer were tested. Soil solution chromatograms exhibited one non-retained fraction and two retained fractions. The elution times of the retained fractions were characteristic of peptides and proteins (first peak) and for compounds containing aromatic amines (second peak). The results show that IMAC is an effective tool for the fractionation of copper complexing ligands that are capable of forming ternary complexes.

Keywords: IMAC; Copper; Ligands; Soil solution

1. Introduction

Immobilized metal ion affinity chromatography (IMAC) was developed originally for the fractionation of proteins from biological samples [1]. Since then the method has gained broad popularity and has been extensively used for the efficient purification of a variety of biomolecules. Only the main concepts of IMAC will be summarized here, since several reviews have been published [2–4]. IMAC is based on the interaction between molecules in solution and metal ions immobilized on a solid support. The molecules are separated according to their affinity for the chelated metal ions, which depends on the coordination between the chelated metal ion and electron donor groups from the ligand. The ligands are assumed to bind to the metal through ligand exchange. Two characteristics of the metal–ligand bond can be used for the successful separation of different ligands. Firstly, the strength of the metal–ligand bond varies from ligand to ligand, and secondly, binding between the immobilized metal ions and the ligand is reversible. Hence elution can be carried out by changing the conditions and therefore breaking the metal–ligand bonds. Three different elution principles are used in IMAC: competitive elution, stripping elution, and pH adjustment.

In recent years, IMAC has experienced a rapid expansion into a variety of environmental applications. IMAC has been employed in combination with hyphenated techniques to isolate and characterize natural organic ligands from aquatic environments and other natural macromolecules [5–16]. Ligand retention using Fe, Co, Cd and Ni as immobilized metals has been investigated [10,17–19], but most studies have examined Cu-binding ligands. Fractions separated by IMAC have been further characterized and evaluated. In most of these studies the retained fraction was assumed to consist of strong metal complexing ligands [11,15,16]. Other studies showed that very strong ligands may not be retained [20] or that IMAC isolates only weak ligands from natural waters [7]. Most authors assumed that the value of the conditional stability constant with Cu is the dominant factor influencing the retention of ligand on the IMAC column [5,7,9,11–13,16,18]. Despite significant information about the nature of the separated ligand fractions, previous studies failed to precisely define the critical factors determining separation and selectivity of the IMAC column. The nature of the

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binding ligands has important implications for the design of an efficient separation method as well as for analyzing separated fractions.

This study had two aims. The first aim was to investigate the selectivity of the IMAC for Cu-binding ligands. For this purpose we investigated the behavior of different model copper complexing ligands since currently little information is available about the interactions of non-peptidic and non-proteinous ligands with the Cu-IMAC column.

The second objective was to develop an IMAC method for the separation and isolation of copper ligands from natural soil solution. To this point IMAC methods were mainly used for ligand separation from aquatic environments. Requirements of the new method were the optimization of binding and elution of the complex soil solution ligands and avoidance of alteration of the solubility of the humic acid fraction during IMAC separation. Therefore, effects of composition and pH value of the eluting buffers on the ligand recovery were examined.

2. Materials and methods

2.1. Materials

The following analytical grade reagents, all purchased from Merck, were used in the study: $CuSO_4$, HCl, 3-morpholino-2-hydroxypropanesulphonic acid (MOPSO), H_3PO_4, H_3BO_3, KCl, NaCl, Na_2EDTA, Na_3NTA, Na_2HPO_4, glycine, and oxalic acid. The ligands 2-methylimidazole, tryptophane, phthalic acid, 8-aminoquinoline, 2-picolinic acid, salicylhydroxamic acid, oxamide, and citric acid were obtained from Aldrich, 2,2-bipyridyl and salicylic acid from Fluka and sodium oxamate, histidine, cysteine, γ-glutathione and human serum from Sigma. Na_3-*S*,*S*-ethylenediaminedisuccinic acid (EDDS) was obtained from Proctor & Gamble.

Soil solution samples were collected from neutral top soil (pH 6.8) at a depth of 10 cm in parallel with another project [21]. Humic and fulvic acid were extracted from soils [22]. All soil solution samples were filtered through a 0.45 μm filter before use.

Model solutions of individual ligands were prepared with nano-pure water at concentrations of 10–100 μM, except for the column capacity measurements in which the ligand concentration was 10 mM. Ligand mixtures were prepared by mixing 100 μM ligand solutions at ratio of 1:1. Similarly, ligand mixtures with copper were prepared by mixing l00 μM ligand mixture with 100 μM $CuSO_4$ at a ratio of 1:1. For method validation experiments spiking of soil solution samples with 10 μM of model ligands to at ratio of 1:1 was performed.

2.2. IMAC procedure

We used HiTrap Chelating HP 5 ml columns purchased from Amersham Pharmacia Biotec throughout this study. Chelating Sepharose™ High Performance, the chelating gel supplied in the column, consists of highly cross-linked agarose beads to which iminodiacetate (IDA) moieties are covalently bound via hydrophilic spacer arms. This type of column matrix provides low specificity, excessive capacity and high adsorption affinity for all copper ligands.

The IMAC column was connected to a HPLC pump (Jasco PU-980), and a UV (Jasco UV-970) or fluorescence detector (Jasco FP-2020 plus). For each model ligand, the UV spectrum (Cary 1E-UV–vis) was recorded and IMAC detection was carried out at the wavelength of maximum UV absorbance for the specific model ligand. Fluorescence detection was used for humic and fulvic acids and soil solutions. The excitation wavelength was 370 nm; the emission wavelength was set to 460 nm. For compounds that did not display UV or florescence absorbance (EDTA, EDDS, NTA, oxalic and citric acid), fractions were collected and analyzed by ion or liquid chromatography. EDDS was analyzed after derivatization [23].

Prior to use, the columns were washed with 25 ml of distilled water to remove any trace of storage solution (20% ethanol) [24] and were charged with copper by adding 1 ml of 0.1 M $CuSO_4$ solution. This quantity of copper only incompletely saturated the chelating groups on the column, as indicated by the fact that only three quarters of the column length turned blue. The function of free chelating groups at the end of the column was to prevent copper leaching from the column. Loosely bound copper ions were removed by an equilibration step in which 20 ml of distilled water was followed by binding buffer (2 mM MOPSO, 0.5 M NaCl, pH 6.9) until a stable base line was obtained by UV detection.

2.3. IMAC measurements

The standard IMAC procedure consists of the following five steps: equilibrating the column, loading the sample, washing with buffer, elution and re-equilibration of the column. All experiments were performed at a volumetric flow rate of 2 ml/min. For equilibration the column was washed with binding buffer (2 mM MOPSO, 0.5 M NaCl, pH 6.9). The sample was loaded onto the column by switching from binding buffer to sample for a specified time and acquisition of data was started. Sample volumes applied to the column were 0.5–4 ml. After sample loading the column was washed for 20 min with binding buffer to remove the non-retained compounds. The ligands retained on the column were removed from the column with 20 ml elution buffer (2 mM phosphate buffer, pH 3.5, 0.5 M NaCl). Prior to running the next experiment, the column was thoroughly washed and re-equilibrated with binding buffer. An additional step was introduced after each experimental section in which 25 ml of 0.25 M EDTA was used to strip Cu ions from the column matrix to regenerate the column. IMAC chromatograms peaks areas were measured in duplicate with a maximum error of 5%.

To select an appropriate buffer for eluting complex natural samples, four different eluting buffers were tested. Buffers were: 10 mM HCl, 1 mM HCl, 0.32 mM HCl, and 2 mM sodium phosphate buffer (pH 3.5). All binding and eluting buffer solutions were prepared with 0.5 M NaCl because it has been reported that salts promote complexation of ligands in IMAC column [18,25–27].

After each sample run two additional runs were performed: (1) To assess possible column contamination, blank runs were

performed with no sample addition using 25 ml of binding buffer followed by 25 ml of elution buffer, and finishing with 20 ml of binding buffer. (2) To assess the recovery, the ligands were also measured on-line without passing through the IMAC column. The recovery of a given ligand is expressed as the ratio between the sums of all peak areas for all IMAC fractions with the peak area of measurements without the IMAC column passage.

To investigate metal leaching, a blank run was performed and then 2 ml samples of different model ligands (phthalic acid, 8-aminoquinoline, EDDS) were loaded onto the column and measured using standard IMAC procedure. 1 ml fractions were collected and analyzed for Cu by Atomic Adsorption Spectrometer at a wavelength of 324.5 nm (Varian Spectr.AA300).

Chemical speciation calculations were performed with the program CHEMEQL [28]. Values for stability constants were obtained from the NIST Metal Complexes Database [29].

3. Results

3.1. Ligand retention on the IMAC column

The model ligands were selected to represent a wide range of log K values and functional groups of copper ligands occurring in nature. The tested model ligands can be divided into two groups on the basis of the observed IMAC behavior. The first group consists of ligands that did not interact with the column and that were eluted during application of the binding buffer at the void volume of the column at 6.0 ± 0.5 min (e.g. phthalic acid peak A, Fig. 1). Table 1 lists the ligands that were not retained on the column, their structure, recovery rates and log K values of the Cu-complexes. The range of log K values of these ligands was from 2.8 to 18.8.

As could be seen from Table 1 many copper complexing ligands did not interact with Cu charged IMAC. None of the investigated carboxylic acids were retained regardless of the number of carboxylic groups. Also strong chelating agents with amino-carboxylic groups such as EDTA, NTA, and EDDS were not retained. Compounds containing a non-aromatic primary, secondary, tertiary amine or thiol group were also not retained.

The second group consists of ligands that were retained on the IMAC column and were eluted only after applying the eluting buffer and changing the pH value in the column. Three chromatograms of retained ligands are shown in Fig. 1 (see peaks B–D representing γ-Glu-Cys, 2,2′-bipyridyl and 8-aminoquinoline, respectively). The elution times of most retained compounds ranged between 19 and 20.1 min. Only 8-aminoquinoline had a longer elution time of 23.4 min, and γ-Glu-Cys a shorter elution time of 17.9 min. It is known that polypeptides and proteins are binding to the IMAC column through the formation of complexes with cysteine [30]. γ-Glu-Cys, biologically important for copper complexation [31], was retained although a solitary thiol group was not sufficient to result in the retention of a cysteine by our IMAC column. Table 2 lists the ligands that were retained on the column, their structures, recovery rates, elution times and log K values of the Cu-complexes. The range of log K values of the retained ligands was between 3.7 and 13.3. As could be seen from Table 2 almost all tested compounds retained on the column have N-heterocyclic aromatic structure.

In order to verify that the retention of ligands did result from interaction of the ligand with Cu, we conducted measurements with 2,2′-bipyridyl on Cu-charged and Cu-free columns. As Fig. 2 shows only one not retained peak was detected with Cu-free column (elution time of 5.7 min). This means that the entire sample passed through the column without binding (Fig. 2, peak A). No peaks were observed when copper charged column was eluted which had not been loaded with sample. In the presence of Cu in the column 2,2′-bipyridyl was completely retained and eluted at 19.7 min (Fig. 2, peak B). 2,2′-Bipyridyl was therefore interacting with the immobilized Cu on the column, and no selective adsorption of 2,2′-bipyridyl onto the solid matrix of the IMAC gels had taken place.

In natural samples, Cu-ligands will be present as a mixture and mainly in copper complexed form. We therefore also investigated the retention of ligands applied in mixture as well as the retention of ligands already complexed with Cu. Mixtures

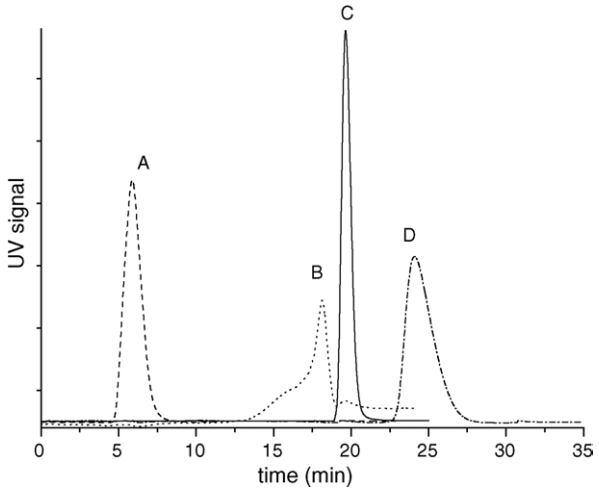


Fig. 1. IMAC chromatograms of a non-retained ligand: phthalic acid (peak A, elution time 6.3 min) and retained ligands: γ-Glu-Cys (peak B, elution time 17.9 min), 2, 2′-bipyridyl (peak C, elution time 19.7 min) and 8-aminoquinoline (peak D, elution time 23.4 min).

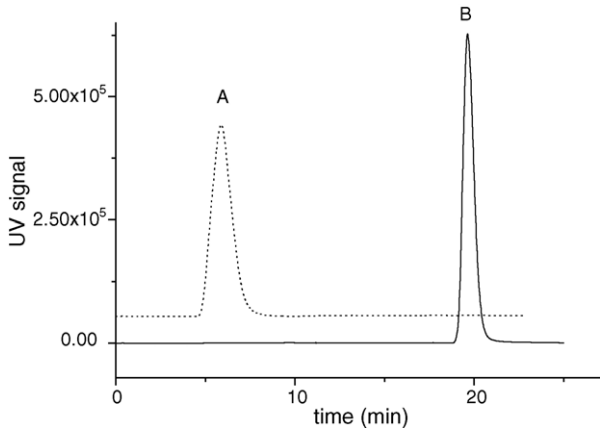
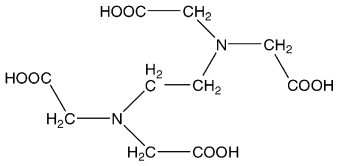
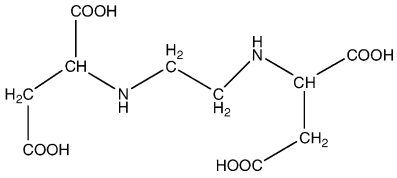
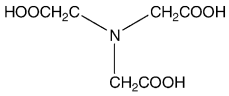
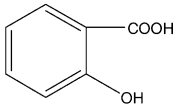
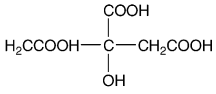
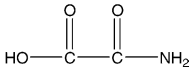
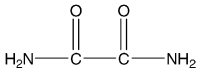
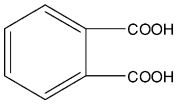
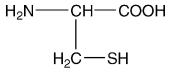


Fig. 2. IMAC chromatograms of 2,2′-bipyridyl with Cu-charged (peak B) and Cu-free IMAC column (peak A).

Table 1
Ligands not retained by Cu–IMAC, chemical characteristics (log K values of the Cu-complex, structure) and experimental data (recovery rates)

Ligand	log K [29]	Structure	Recovery (%)
EDTA	18.8		nd
EDDS	16.9		nd
NTA	13.3		nd
Salicylic acid	10.6		91
Glycine	8.1	$H_2N{-}CH_2{-}COOH$	nd
Citric acid	7.2		92
Oxamic acid	na		87
Oxamide	na		80
Oxalic acid	4.8	$HOOC{-}COOH$	87
Phthalic acid	2.8		98
Cysteine	na		nd

nd: not determined; na: not available.

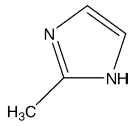
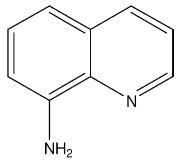
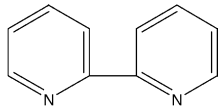
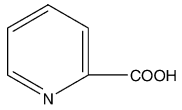
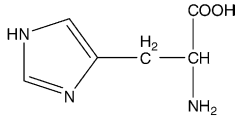
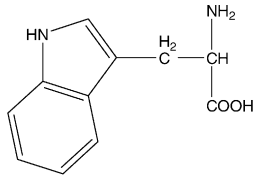
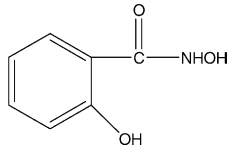
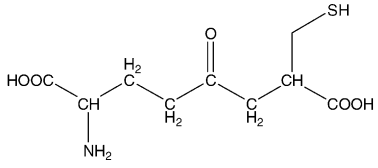
of oxalic acid, phthalic acid and 8-aminoquinoline with and without copper were separated on the IMAC column using the standard procedure. Chromatograms of the ligand mixture and ligands in presence of copper showed the same peaks as chromatograms of single ligands. Ligand behavior, elution time, and peak area did not depend on the presence of other ligands or Cu in the sample. The additional Cu in the sample did not elute and thus was bound to the column.

Separation of proteins is the most commonly used IMAC application. To validate our IMAC method we have also analyzed human serum, a mixture of 15 proteins with 5 of them constituting more than 90% of the total (Albumin, Immunoglobulin G, Immunoglobulin A, Transferrin, α_1 Antitrypsyn). Contrary to the chromatograms of model ligands which displayed only one retained peak, the chromatograms of human serum showed one non-retained peak and two retained peaks with elution times of 17.5 and 19.5 min. 66.4% of the serum proteins were recovered as retained fraction. These results correspond well with previous studies of human serum IMAC fractionation [32].

3.2. Cu leaching from the column

Regardless of the retention behavior (retained or non-retained), a ligand eluted from the column can be present in the eluent as a free ligand or as a Cu-complex if it is able to strip Cu from the column. Fig. 3 shows that both phthalic, which was non-retained, and 8-aminoquinoline acid which was retained, were primarily eluted as free ligands. Only traces of copper were found in the eluent (maximum amount was 1% of ligand concentration). Strong chelating agents, such as EDDS

Table 2
Ligands retained by Cu-IMAC, chemical characteristics (log *K* values of the Cu-complex, structure) and experimental data (recovery rates and elution times)

Ligand	log *K* [29]	Structure	Recovery (%)	Elution time (min)
2-Methylimidazole	3.7[a]		nd	19.0
8-Aminoquinoline	6.1		93	23.4
2,2-Bipyridyl	8.1		98	19.7
2-Picolinic acid	8.2		nd	20.1
Histidine	10.6		98	19.7
Tryptophan	10.6		87	19.1
Salicylhydroxamic acid	13.3		85	19.2
γ-Glu-Cys	na		nd	17.9

nd: not determined, na: not available.
[a] Value is for 1,2-methylimidazole.

and EDTA, were not retained but were able to strip a significant amount of Cu from the column (24% of loaded EDDS and 96% of EDTA). This shows that very strong ligands are able to remove non-negligible amounts of Cu from the column. According to a speciation calculation almost 100% of EDDS and EDTA should have been complexed by Cu at pH 6.9 in equilibrium with Cu adsorbed to the column. The non-retention of EDTA could therefore be due to formation of dissolved EDTA-Cu complexes that are no longer able to interact with the column matrix. Seventy-six percent of EDDS was eluted in uncomplexed form, however, it might be that in the bottom Cu-free part of the column the Cu-EDDS complex was dissociated again (log *K* CuEDDS 18.4 versus 18.8 for CuEDTA) or that kinetic issues played a role.

3.3. Column capacity

The adsorption capacity of the IMAC column was examined by loading various amounts of high concentrations (10 mM) 2,2′-bipyridyl onto the column. When the IMAC column was not saturated with 2,2′-bipyridyl, it was totally retained on the column with an elution time of 19.7 min. With increasing sample volume a second peak, non-retained (elution time around 5.5 min), appeared indicating that the column was over-

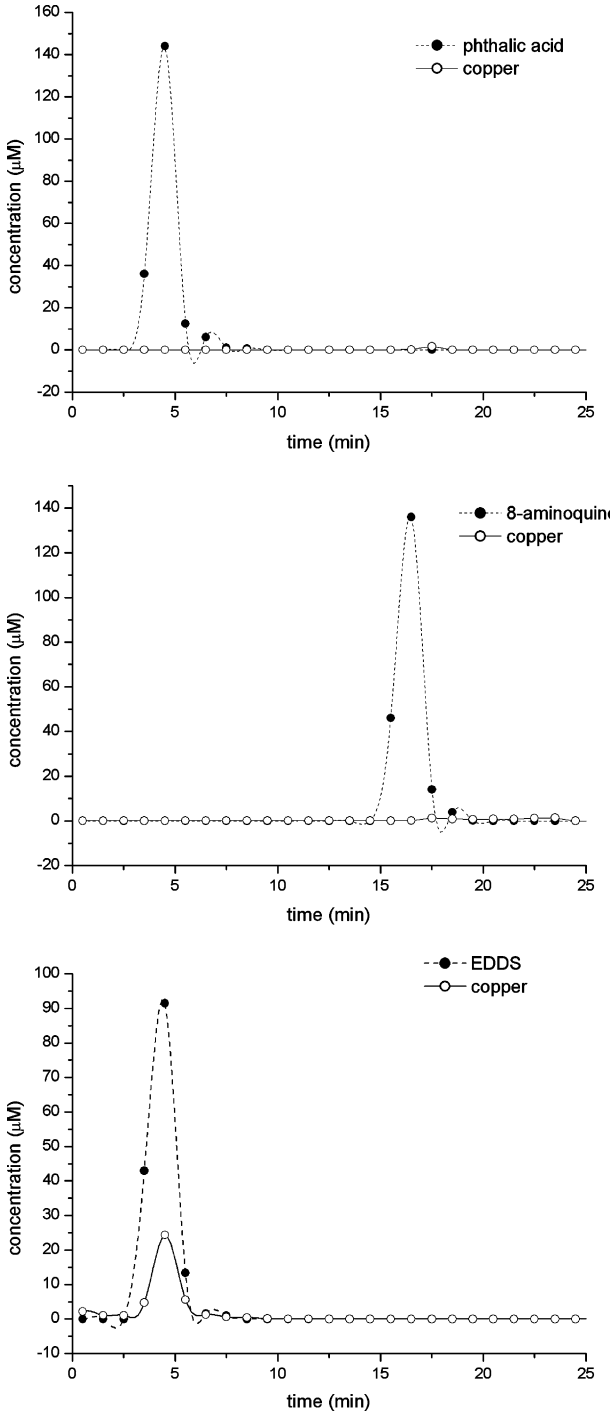


Fig. 3. Chromatograms of phthalic acid, 8-aminoquinoline and EDDS together with co-eluted Cu. All compounds were injected as uncomplexed ligand.

saturated. As the sample volume further increased, the peak area of the retained peak remained constant, while the non-retained peak started to increase linearly (Fig. 4). Extrapolation of this linear relationship revealed that the maximum volume of 10 mM ligand retained on the column was 0.73 ml. Maximum concentrations of organic ligands in soil solution have been reported not to exceed several 100 μM [33]. The binding capacity of the column is thus at least an order of magnitude larger than required

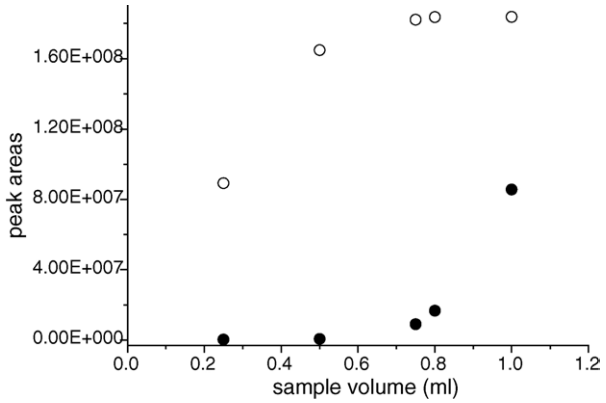


Fig. 4. Adsorption capacity of the IMAC column measured with different volumes of 10 mM of 2,2′-bipyridyl. The peak area of the retained peak (◯) remained constant, while the non-retained peak (●) started to increase linearly in column over-saturation conditions.

to bind all ligands applied with soil solution samples of a volume used in this study.

3.4. Eluting buffer for natural ligands

The effects of various compositions and pH values of eluting buffers on humic acid IMAC chromatography were examined. Humic acid was selected as an important component of the soil solution with highly pH dependent solubility.

The percentage of retained humic acid fraction was used as an overall guide for the selection of the eluting buffer. The suitability of the eluting medium was also assessed on the basis of measurements of additional important parameters including total recovery of the loaded sample, elution time reproducibility and correlation between applied sample volume and peak area. The following four eluting buffers were compared: HCl with pH value of 2, 3, and 3.5 and sodium phosphate buffer (pH 3.5). The results of comparison are presented in Table 3.

As could been seen the percentage of the retained peak fraction, the elution time and the recovery were strongly dependent on the elution buffer pH and the concentration. With increasing pH the percentage of the retained fraction also increased. This increase could have been due to two occurrences at low pH: (1) precipitation of humic acids leading to irreversible retention on the column or (2) destruction of some ligand. These results are in agreement with previous IMAC studies on organic ligands and the effects of different eluting buffers pH [11,12,18]. The strong correlation between eluting buffer composition and elution time can be explained by considering the pH of eluent. Regardless

Table 3
Effect of eluting buffers on humic acid IMAC behavior

Eluting medium	Retained fraction (% of total peak area)	Recovery (%)	Elution time (min)
HCl, pH 2	5.2	78	19.3
HCl, pH 3	7.8	85	28.2
HCl, pH 3.5	14.5	98	29.4
H_3PO_4, pH 3.5	17.2	100	19.7

of the type of buffer, elution of the retained humic acid fraction started when the pH dropped from 6.5 to 5.5. A higher pH value of the elution buffer with low concentration (e.g. HCl pH 3.5) led to a slower pH change in the column and resulted in longer elution time.

As can be seen from Table 3, the highest percentage of the retained fraction and complete recovery off all loaded humic acid from the IMAC column was obtained when the two eluting buffers used were HCl pH 3.5 and 0.02 M phosphate (pH 3.5) buffer. However, with HCl pH 3.5 longer elution times and peak broadening reduced the method reproducibility. The suitability of the phosphate buffer was further demonstrated by a linear correlation between the peak area and the sample volume. We therefore chose the phosphate buffer as the most suitable buffer for eluting soil solution samples.

3.5. Soil solution separation

All IMAC chromatograms of soil solution, humic and fulvic acid samples revealed three peaks. A representative chromatogram is shown in Fig. 5. The elution times of the separated soil solution fractions matched the elution times of the model ligands. About 80% of the organic matter was not retained and was eluted after 6.3 min. The remaining 20% were retained on the IMAC column and eluted upon applying an eluting buffer. The retained peak fraction was always portioned into two peaks. The elution time of the first retained peak of the soil solution sample, humic and fulvic acid agreed well with that of γ-Glu-Cys and some proteins, which had elution times of about 17.5 min. The second retained peak of the soil solution sample, humic and fulvic acid with an elution time of 19.3 min corresponded well to the average elution time of most model ligands. All soil solution samples were completely recovered, all elution times were reproducible and sample volumes and peak areas were closely correlated.

To validate the IMAC separation procedure for natural samples, soil solution samples were spiked with one non-retained (oxalic acid) and one retained model ligand (2,2′-bipyridyl). Resulting IMAC chromatograms of soil solution samples and spiked samples were compared. Addition of oxalic acid increased the non-retained peak area and did not affect recovery of the retained fraction. In the case of addition of 2,2′-bipyridyl an increase in the peak area of the retained fraction with an elution time of 19.6 was measured. Peak areas of non-retained and retained fractions with an elution time of 17.3 min were not affected by addition of 2,2′-bipyridyl. The recovery of added models ligands was 97% for oxalic acid and 93% for 2,2′-bipyridyl.

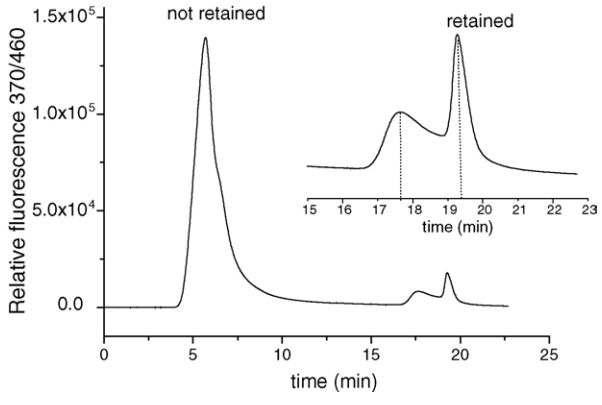


Fig. 5. IMAC chromatogram a soil solution sample collected from a neutral top soil at a depth of 10 cm. The inset shows an enlargement of the retained peak.

4. Discussion

4.1. Ligand retention on the IMAC column

The IMAC retention of the tested model ligands could not be predicted from of their Cu-complexing stability constants. This observation is in sharp contrast to previously reported IMAC environmental studies where the stability constant was considered to be the determining factor for the retention of a ligand by the IMAC column. Our results show that the log K value can only play a secondary role in the overall retention behavior of model ligands. The results provide clear evidence that another decisive factor governs ligand behavior on the IMAC column.

The ability of a compound to form a complex with Cu in solution does therefore not necessarily translate into the ability to form a complex with bound Cu in the IMAC column. In the column, the coordinative positions of Cu are partially occupied by IDA. To be retained, ligands must be able to form a stable complex with the remaining coordinative positions in to the form of a ternary IDA–Cu–ligand complex.

Almost all retained compounds have N-heterocyclic aromatic structure. Consequently that ligand structure is recognized to be of primary importance for the retention on to the column. It is well known that the N-heterocyclic aromatic structure has a strong ability to form “ligand 1–Cu–ligand 2” ternary copper complexes [34]. The formation of π-bonds between copper ion and N-heterocyclic aromatics is considered to be the most effective factor for the stability of ternary complexes [35].

This agrees well with our own view that ligands that are retained on the IMAC column form a ternary complex with the IDA–Cu group. Compounds that are able to form such ternary complexes in solution are apparently also able to form such complexes with immobilized IDA–Cu on the IMAC column system. This hypothesis is further supported by our result with salicylhydroxamic acid (SHAM) IMAC measurements. SHAM has a structure lacking an N-heterocyclic aromatic structure, but is able to form a stable ternary complex with IDA and copper in solution [36]. In accordance with our hypothesis it was also retained on the IMAC column.

The knowledge of the stability constant of the ternary IDA–Cu–SHAM complex in solution [36], the pK_a values of immobilized IDA and the log K values for Cu binding to the IMAC column [37], allows us to estimate the binding strength of that ligand in the IMAC column. The log K value for the formation of the Cu–IDA complex in the column was 8.05 compared to 10.57 in solution [37]. The difference of 2.52 between log K for solution and surface complexes indicates a much weaker binding of Cu to immobilized IDA. The IDA–Cu–SHAM ternary

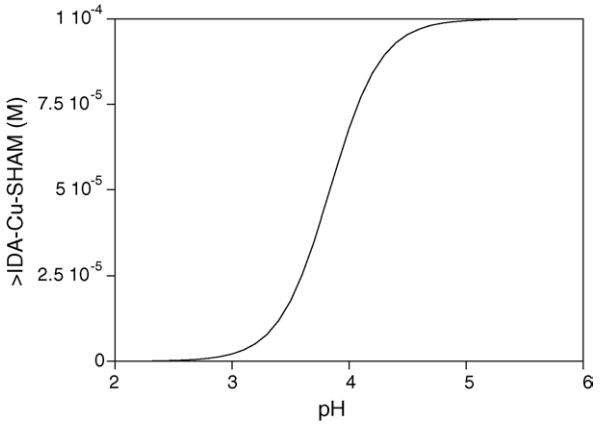


Fig. 6. Formation of ternary >IDA–Cu–SHAM complexes in the IMAC column calculated with the Cu–IDA equilibrium in the column reported by Zachariou et al. [37] and the Cu–IDA–SHAM equilibrium reported by Khairy et al. [36]. Conditions: 0.02 M Cu, 22 M IDA (23 mmol/ml IMAC gel), 0.1 mM SHAM.

complex in solution has a log K of 21.62 [36]. The ternary complex with immobilized Cu would therefore have a log K value of 19.1, assuming that the difference between solution and surface complex remains the same, $\Delta \log K$ of 2.52.

We calculated the interactions between SHAM, Cu and IDA under the assumption that the IMAC column is locally a mixed system in equilibrium, i.e. that any kinetically limited process can be neglected. Fig. 6 shows that at pH above 5 the ternary complex prevails, while at pH below 3 the ligand is present in solution in the free form. The calculations also show that at pH 3.5 all dissolved SHAM is present in the free form and not in form of Cu-complexes. The calculated pH dependence agrees well with observed IMAC retention of SHAM at pH 7 and the incomplete elution at pH 3.5 (the measured recovery rate was 85%).

To our knowledge there are no published data that confirm the formation of a ternary IDA–Cu–γ-Glu-Cys complex. However, a copper-glutathione complex has been isolated and characterized [38,39].

4.2. Soil solution separation

The IMAC analysis of soil solution resulted in three separate peaks, reflecting a corresponding diversity of functional groups. Based on our results with the model ligands we concluded that the fraction which was not retained on IMAC contained all those compounds that do not form a ternary complex with IDA–Cu. Apart from components that do not form complexes with Cu at all, these are mostly ligands with carboxylic functional groups. The elution times of the retained fraction of soil solution corresponded well with the elution times of the model ligands.

The first retained peak had an elution time comparable to proteins and peptides. The second retained peak had an elution time indicative of compounds containing N-heterocyclic aromatic structure. The latter are functional groups also present in humic and fulvic acids [40]. Compared to the model ligand chromatograms, soil solution samples exhibited broader peaks, indicating a mixed composition of closely related ligands of very similar copper affinities.

Our results demonstrate that IMAC can be used as a qualitatively and quantitatively effective tool for the fractionation of copper complexing ligands from soil solution capable of forming ternary complexes with a structure “ligand 1–Cu–ligand 2”.

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