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Journal of Chromatography A, 847 (1999) 233–244

JOURNAL OF  
CHROMATOGRAPHY A

## Sulphonated azoligand for metal ion determination in ion interaction chromatography

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### Abstract

An azosulphonated ligand, namely cyclo-tris-7-(1-azo-8-hydroxynaphthalene-3,6-disulphonic acid), Calcion, which is generally used as a metallochromic indicator for metal ion titrations, has been studied in order to verify its suitability as a new ligand for metal ion separation in a liquid chromatography technique based on the ion interaction mechanism. Before chromatographic method development, studies on the complexation capabilities of Calcion with  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  metal ions were performed, determining the stability constants of the complexes between such metal ions and the ligand. The procedure is based on ion-exchange chromatography and involves the retention of the negatively charged complexes on an anion-exchange resin and the determination of free and complexed metal by inductively coupled plasma atomic emission spectrometry. Chromatographic separation of  $\text{Ni}^{2+}$ – $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ – $\text{Al}^{3+}$  was reached, using a silica-based octyl stationary phase, through pre-complexation with Calcion, and UV–visible detection of the resulting anionic complexes. The effect of the main mobile phase parameters (concentration of the ion interaction reagent tetrabutylammonium chloride, organic modifier methanol and pH) on  $k'$  of analytes was studied and optimization of the metal ion separation was achieved. In order to obtain the simultaneous determination of metal ions, the concentration of  $\text{CH}_3\text{OH}$  and pH gradient elution were also considered. The method developed has been applied to tap water analysis. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Water analysis; Metal cations; Calcion

### 1. Introduction

Metal complexes with sulphonated azo dyes are extensively used in analytical chemistry for ion-exchange preconcentration [1–3], chromatographic separations [4,5], voltammetric [6,7], spectrophotometric [8,9] and titrimetric [10,11] procedures.

Such ligands are characterized by the presence of an azo-group connecting two differently substituted aromatic rings, with at least one sulphonato group,

which allows the species to dissolve in water and gives rise to a negative charge on the molecule.

Complexation occurs through the azo moiety and the other chelating groups (e.g.,  $-\text{OH}$ ,  $-\text{OCH}_3$ ) *ortho* to it. The stability of the complexes depends on the nature of the metal ions and on the ligand structure. Knowledge of the constants aids in selecting the proper metal–ligand pair when devising a chromatographic preconcentration or separation procedure for a mixture of metal ions [12].

Liquid chromatography, which is widely used as a trace organic method, has proved to be suitable for the separation and simultaneous determination of metal ions that have similar chemical properties. Among the analytical approaches used to determine

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metal ions in liquid chromatography, ion interaction chromatography (IIC) provides efficient and sensitive separations, even in complex matrices [13,14].

In the IIC mechanism, a suitable pairing ion is added to the mobile phase, usually a strong base cation (tetraalkylammonium ion), which is considered to give a dynamic equilibrium between the eluent and the stationary phase [15]. Retention is due to electrostatic and hydrophobic interactions between the stationary phase and the species originated by the pairing ion and an analyte of opposite charge [16,17].

Metal ions can be separated as simple, hydrated ions or as anionic complexes using suitable ligands. The ligands used in the IIC of metal ions, using direct UV–Vis detection, should preferably form easily detectable species and, for this reason, the ones most commonly used are dithiocarbamates [18], quinolines [19] and 4-(2-pyridylazo)resorcinol [20].

The aim of this work was to test the chromatographic performance of Calcion, cyclo-tris-7-(1-azo-8-hydroxynaphthalene-3,6-disulphonic acid), which is generally used as a metallochromic indicator for metal ion titrations [21]. Calcion, also indicated as Calcichrome, was first introduced by Close and West [22] as a reagent that was specific mainly for calcium determination. The structure proposed in their study and later confirmed by Allan et al. [23] by means of optical absorption, proton NMR experiments, was a macrocyclic type. Recent literature suggests that Calcion is not a tetrameric structured compound [24] and that the most common Calcion applications still deal with calcium-selective determinations [25].

Since, in the current literature, there was no evidence for the use of Calcion as a ligand in liquid chromatography, and due to the incomplete literature data available, we have determined the stability constants of its complexes with  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  in order to verify its suitability for chromatographic separation and determination. The constants were determined by a procedure developed in our laboratories [26,27] that uses an ion-exchange mechanism involving the retention of the negatively charged complexes on an anion-exchange resin and the determination of free and complexed metal by inductively coupled plasma (ICP) spectrometry.

The preliminary study evaluating stability constants allowed us to choose the best experimental

conditions to develop the separation of  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  as anionic complexes by an ion interaction mechanism. Separation was performed on a silica-based  $\text{C}_8$  stationary phase. The effect of the main mobile phase parameters (e.g., concentration of ion interaction reagent–tetrabutylammonium chloride, organic modifier–methanol and pH) on capacity factors of analytes was studied and the optimization of metal ion separation was achieved. In order to obtain the simultaneous determination of the species considered, the use of different  $\text{CH}_3\text{OH}$  concentrations and pH gradients have also been evaluated. The method developed has been applied to the determination of metal ions in a tap water sample.

## 2. Experimental

### 2.1. Instrumentation

For the determination of stability constants, polypropylene columns (Bio-Rad Econo-columns) were slurry-packed with 0.5 g of macroporous Bio-Rad AG MP-1 100–200 mesh anion-exchange resin. The resin was cleaned with 10 ml of methanol (in order to remove organic or hydrophobic molecules), high purity water (HPW), 10 ml of 2 M  $\text{HNO}_3$  and preconditioned with HPW buffered at the desired pH. The treatment with nitric acid converted the resin from a chloride to a nitrate form, in order to avoid competitive complexation phenomena due to chloride ions. The flow-rate through the columns was 1.2 ml/min.

Metal ions were then determined by ICP atomic emission spectrometry (AES) with a Plasma Liberty 100 Varian spectrometer at the following wavelengths: 396.15 nm for Al, 324.75 nm for Cu, 259.94 nm for Fe and 231.60 nm for Ni. The operating conditions were: radio frequency power, 1.00 kW; nebulizer pressure, 150 kPa; slit width, 0.080 nm; coolant flow-rate, 15.0 l/min; observation height above the load coil, 3.00 mm.

For the chromatographic determination of the complexes, the chromatographic apparatus was a Varian (Walnut Creek, CA, USA) Model 9012 equipped with a Varian Model 9050 variable wavelength spectrophotometric detector and a Vista CDS 401 system (Varian) for data recording. The injection

system included a Rheodyne valve 7125 Model with a 100- $\mu$ l loop. The column used was a 10- $\mu$ m LiChrospher 100 RP-8 (250 $\times$ 4 mm I.D.) coupled with a 5- $\mu$ m LiChrospher 100 RP-8 (4 $\times$ 4 mm I.D.) guard column, both from Merck (Darmstadt, Germany).

The eluent flow-rate was kept at 1.0 ml/min and experiments were performed at room temperature. After preliminary experiments, wavelength detection was set at 510 nm. Retention times were the means from triplicate injections and the dead volume, evaluated by injection of water, was 2.4 ml. The experiments performed throughout this work showed that the sensitivity of detection was higher for  $\text{Al}^{3+}$  than for  $\text{Fe}^{3+}$ .

## 2.2. Reagents and solutions

Analytical grade reagents were used.  $\text{CH}_3\text{COOH}$ ,  $\text{HNO}_3$  and  $\text{HCOOH}$ , were obtained from Carlo Erba.  $\text{NaH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaOH}$  and  $\text{Na}_2\text{B}_4\text{O}_7$  were from Merck. Cyclo-tris-7-(1-azo-8-hydroxynaphthalene-3,6-disulphonic acid), Calcion or Calcichrome (Fig. 1), was purchased by Aldrich (Milwaukee, WI, USA).

Metal ion solutions were obtained by dilution of concentrated stock solutions (Titrisol Merck). During evaluation of stability constants, 10 mM solutions of the ligand were prepared in HPW. The following

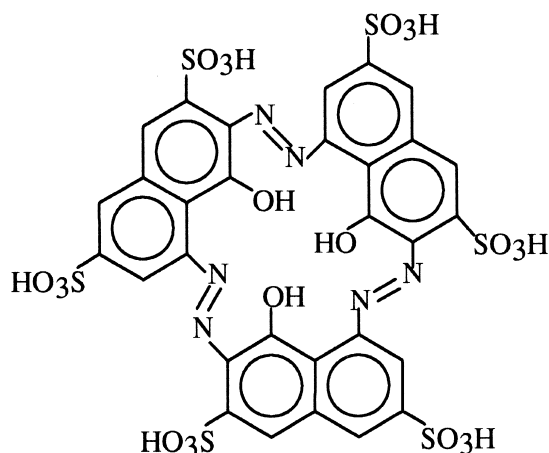


Fig. 1. Molecular structure of the ligand Calcion (Calcichrome).

$1.0 \cdot 10^{-2}$  M buffers were employed:  $\text{HCOOH}/\text{HCOONa}$ , pH 3;  $\text{CH}_3\text{COOH}/\text{CH}_3\text{COONa}$ , pH 4–6;  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ , pH 7–8;  $\text{Na}_2\text{B}_4\text{O}_7$ , pH 9. Buffers with pH values of 1 and 2 were obtained by the addition of  $\text{HNO}_3$  and  $\text{CCl}_3\text{COOH}$ , respectively, while pH values higher than 9 were obtained by the addition of  $\text{NaOH}$ .

All aqueous solutions were prepared using high purity water obtained using a Milli-Q system (Millipore, Bedford, MA, USA). Eluents were filtered through 0.22  $\mu$ m filters. Tetrabutylammonium (TBA) chloride was from Fluka (Buchs, Switzerland), while methanol was from Carlo Erba (Milan, Italy). Acetic acid and disodium hydrogenphosphate, which were used for the preparation of buffers, were from Merck. Standard solutions of analytes of the correct concentration were prepared in the presence of an excess of Calcion, buffered at the same pH as that of the mobile phase.

## 2.3. Procedure for determination of stability constants

The test solutions described below were obtained by mixing the appropriate volumes of buffer, ligand and concentrated solutions of metal ion and diluting as required. All experiments were performed in triplicate.

Test solutions (100 ml) containing equimolar ratios of ligand and metal ion ( $1.0 \cdot 10^{-5}$  M for Cu and Fe;  $2.0 \cdot 10^{-5}$  M for Ni and Al) and  $5.0 \cdot 10^{-2}$  M buffer were driven through the columns packed with the anion-exchange resin. The resin was rinsed with HPW buffered at the same pH in order to remove traces of the test solution, and the retained metal was eluted with 10 ml of 2 M  $\text{HNO}_3$ , thus obtaining an enrichment factor of ten. The acid was left in contact with the resin for 4 h in order to ensure complete release of the metal.

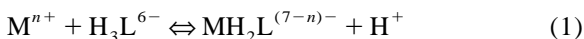
The acidic eluates obtained from three different columns and the test solutions driven through the resin were analysed by ICP–AES. The total (retained plus unretained) metal recovery was quantitative, within experimental error. Separate calibrations with matrix-matched standards were performed for the two kinds of samples. Blanks were run at all pH values investigated.

### 3. Results and discussion

#### 3.1. Thermodynamic, apparent and conditional stability constants

The theoretical background is described, with activities represented by round brackets ( $\circ$ ) and molar concentrations by square brackets [ $\square$ ]; stability constants will be referred to as ‘thermodynamic’ when expressed as a function of activities and ‘apparent’ when expressed as a function of concentrations.

Calcichrome can be represented as  $\text{H}_3\text{L}^{6-}$ , with the three protons belonging to the hydroxo-groups involved in metal complexation. During coordination of a metal ion,  $\text{M}^{n+}$ , one proton from a naphthyl group is released [28], according to the following equilibrium



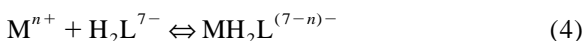
the constant of which is represented by

$$K' = \frac{(\text{MH}_2\text{L}^{(7-n)-})(\text{H}^+)}{(\text{M}^{n+})(\text{H}_3\text{L}^{6-})} \quad (2)$$

Considering the dissociation constant  $K_{a1}$  for the ligand, Eq. (2) can be rewritten as:

$$\frac{K'}{K_{a1}} = K^\circ = \frac{(\text{MH}_2\text{L}^{(7-n)-})}{(\text{M}^{n+})(\text{H}_2\text{L}^{7-})} \quad (3)$$

$K^\circ$  is the thermodynamic stability constant of the complexation reaction:



Introducing activity coefficients, Eq. (3) can be rewritten in the following log form:

$$\log K^\circ = \log \beta_{\text{MH}_2\text{L}^{(7-n)-}} + \log \frac{f_{\text{MH}_2\text{L}^{(7-n)-}}}{f_{\text{M}^{n+}} f_{\text{H}_2\text{L}^{7-}}} \quad (5)$$

where  $\beta_{\text{MH}_2\text{L}^{(7-n)-}}$  is the apparent formation constant of the complex  $\text{MH}_2\text{L}^{(7-n)-}$ .

The conditional stability constant is given by

$$\beta_c = \frac{[\text{MH}_2\text{L}^{(7-n)-}]}{[\text{M}^{n+}]' [\text{H}_2\text{L}^{7-}]'} = \beta_{\text{MH}_2\text{L}^{(7-n)-}} \alpha_{\text{M}(\text{OH})} \alpha_{\text{L}} \quad (6)$$

where  $[\text{M}^{n+}]'$  and  $[\text{H}_2\text{L}^{7-}]'$  represent the concentrations of ligand and metal not involved in

complex formation;  $\alpha_{\text{MOH}} = [\text{M}^{n+}] / [\text{M}^{n+}]' = 1 / (1 + \sum_i \beta_i [\text{OH}^-]_i)$ ;  $\alpha_{\text{L}} = [\text{L}] / [\text{L}]' = K_{a1} / ([\text{H}^+] + K_{a1})$ , since Calcichrome can be considered as a monoprotic acid (it releases one proton during complexation).

The uncomplexed metal exists as free hydrated ion or bound to  $\text{OH}^-$  groups. Due to the low concentrations involved, the presence of polynuclear hydroxo complexes can be neglected. The uncomplexed ligand is present in differently protonated species depending on the pH of the medium.

Finally,  $K^\circ$  can be rewritten in the form

$$\begin{aligned} \log K^\circ = & \log \beta_{\text{cMH}_2\text{L}^{(7-n)-}} \\ & + \log \left( 1 + \sum_i \beta_i [\text{OH}^-]^i \right) \\ & - \log \frac{k_{a1}}{[\text{H}^+] + k_{a1}} + \log \frac{f_{\text{MH}_2\text{L}^{(7-n)-}}}{f_{\text{M}^{n+}} f_{\text{H}_2\text{L}^{7-}}} \quad (7) \end{aligned}$$

Many terms in Eq. (7) are known or can be calculated:  $\beta_i$  and  $k_{a1}$  are available from the literature, activity coefficients can be obtained from the ionic strength ( $I$ ) of the solution according to the Debye–Huckel equation (for  $I$  between 0.001 and 0.1  $M$ ) [29], and  $[\text{H}^+]$  and  $[\text{OH}^-]$  from the pH of the solution. The values of  $\beta_i$  and  $k_{a1}$  for the metals and ligands of interest are collected in Table 1. The concentrations of the free and complexed metal and of the free ligand have been determined experimentally, according to the following procedure.

When an equimolar solution of negatively charged ligand and metal ion is driven through a column packed with an anion-exchange resin, the ligand (free and complexed) is retained on the resin. The ICP–AES analysis of the column output gives the concentration of metal ion that is not complexed by the ligand, whereas the complexed fraction is ob-

Table 1  
Calcichrome dissociation constant and Al, Cu, Fe and Ni hydroxo complexes stability constants

Species	Constant
Calcichrome	$k_{a1} = 10^{-7.2}$
Cu	$\beta_1 = 10^{6.3}$ ; $\beta_2 = 10^{12.8}$ ; $\beta_3 = 10^{14.5}$ ; $\beta_4 = 10^{16.4}$
Ni	$\beta_1 = 10^{4.1}$ ; $\beta_2 = 10^{8.0}$ ; $\beta_3 = 10^{11.0}$
Al	$\beta_1 = 10^{9.01}$ ; $\beta_2 = 10^{18.7}$ ; $\beta_3 = 10^{27.0}$ ; $\beta_4 = 10^{33.0}$
Fe	$\beta_1 = 10^{11.81}$ ; $\beta_2 = 10^{22.3}$ ; $\beta_3 = 10^{32}$ ; $\beta_4 = 10^{34.4}$

tained from the analysis of the acidic eluate. The concentration of the free ligand is simply obtained by subtraction. In this way, all of the terms are known for the calculation of  $\beta_c$ ,  $\beta$  and  $K^\circ$ .

The calculation is more complicated in the presence of metal anionic or neutral hydroxo species, which are retained on the resin together with  $\text{MH}_2\text{L}^{(7-n)-}$  by ion exchange and adsorption mechanisms, respectively. Of the four ions investigated, Fe and Al form  $\text{Fe}(\text{OH})_3$ ,  $\text{Fe}(\text{OH})_4^-$ ,  $\text{Al}(\text{OH})_3$  and  $\text{Al}(\text{OH})_4^-$ , while Cu and Ni give rise to  $\text{Cu}(\text{OH})_2$  and  $\text{Ni}(\text{OH})_2$  [30]. An equation, developed in order to take account of the presence of such hydroxo complexes [31], was applied, making computation of the apparent and thermodynamic stability constants possible (Table 2).

Analysis of the acidic eluates showed that the fraction of metal retained by the resin, which corresponds to the complexes with the ligand of interest plus its neutral and anionic hydroxo species, increases with pH. Correspondingly, the concentration of the unretained metal, i.e. free metal ion and cationic hydroxo complexes, decreases with decreasing acidity. The stability constants were evaluated from data obtained at pH values where both free and complexed metal exist at sufficiently high concentrations in order to minimise errors in their analytical determination. The results obtained for  $\beta$  and  $K^\circ$  at the different pH values were averaged. The experimentally obtained fraction of retained metal vs. pH was compared with the one theoretically computed from the metal complexation, metal hydrolysis and ligand dissociation constants. Experimental and theoretical results for iron are similar for Calcichrome complexes over the whole pH range investigated. Discrepancies were found at low pH with Al, probably due to the presence of unexpected cationic hydroxo species, which cause a decrease of the complexed percentages.

Table 2

Values of  $\log \beta$  and  $\log K^\circ$  for metal ions complexes with Calcichrome

Metal	$\log \beta$	$\log K^\circ$
Cu	$7.15 \pm 0.73$	$9.11 \pm 0.13$
Ni	$4.61 \pm 0.53$	$7.91 \pm 0.57$
Al	$7.74 \pm 0.65$	$11.8 \pm 1.0$
Fe	$10.2 \pm 0.2$	$12.6 \pm 1.1$

In order to plan the subsequent chromatographic research, the fractions of each metal ion complexed by the ligand and by the  $\text{OH}^-$  ions as a function of pH were calculated (Figs. 2 and 3). As expected, for each metal ion, the fraction of hydroxo species increases with pH, whereas the percentage of azo

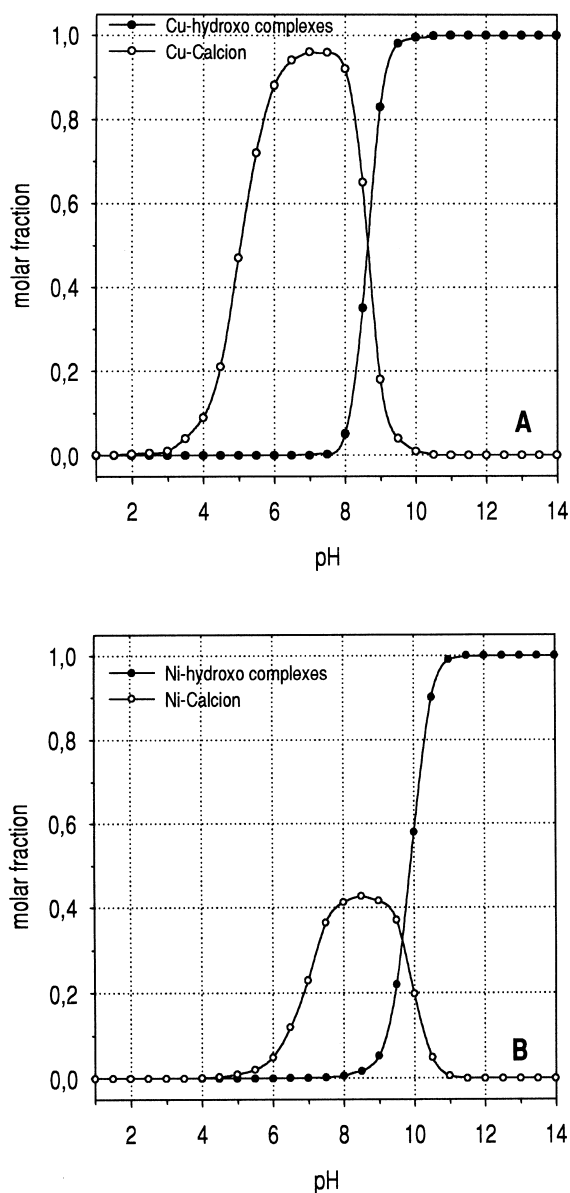


Fig. 2. Distribution of molar fractions of (A) Cu and (B) Ni with hydroxo complexes and Calcion according to the stability constants calculated.

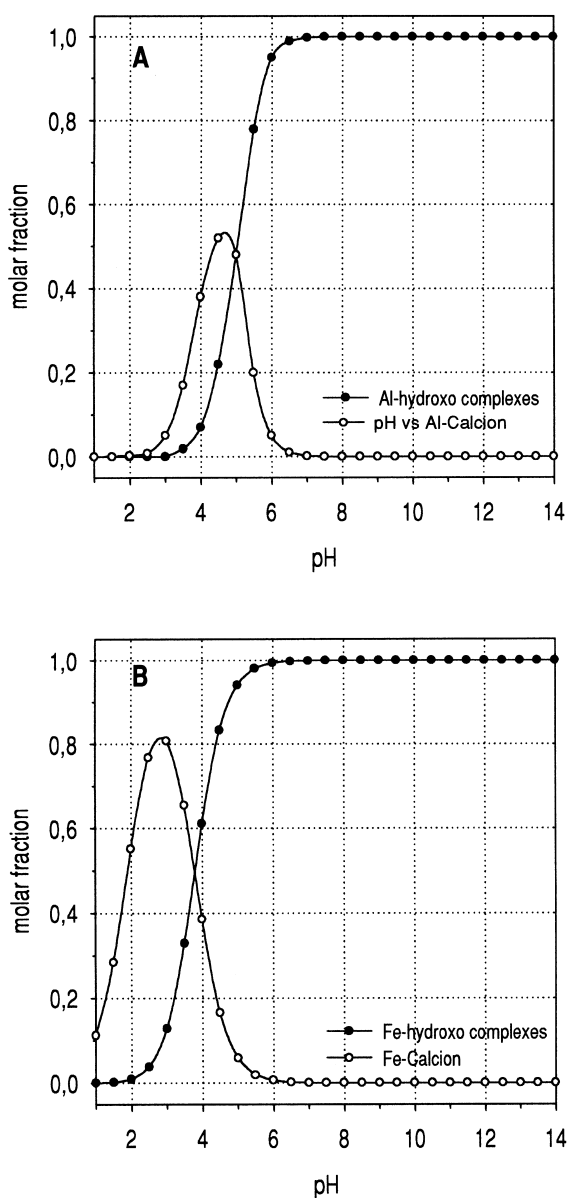


Fig. 3. Distribution of molar fractions of (A) Al and (B) Fe with hydroxo complexes and Calcion according to the stability constants calculated.

complexes increases up to a maximum and then goes down, due to the competition of metal hydrolysis. The trivalent metals (Fig. 3A and 3B), whose  $\beta$  values are higher than those for divalent ones, are significantly complexed at lower pH values, but the fraction of azo complexes rapidly decreases with

decreasing acidity because hydrolysis constants also have high values.

The stability constants obtained were compared and confirmed with those obtained by two different spectrophotometric methods [31] within a 9% R.S.D.

### 3.2. Chromatographic separation

In order to characterise the suitability of Calcion as a ligand for the chromatographic separation of metal ions, and taking into account that no experimental data were available to set a research plan, some preliminary experiments were performed. A mobile phase of water–methanol (40:60, v/v) containing 40 mM acetic acid, 10 mM TBA, 25 mM NaCl and  $5.0 \cdot 10^{-4}$  mM Calcion, pH 4.5, was first used. Standard solution of 10 ppm  $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  precomplexed with  $5.0 \cdot 10^{-4}$  M Calcion were injected individually. According to previous results, at pH 4.5,  $\text{Ni}^{2+}$  is weakly precomplexed by Calcion (Fig. 2B) and, therefore, it was not injected under these experimental conditions.

Under these mobile phase conditions, Cu coeluted with the excess of ligand ( $k' = 0.3$ ), while peaks of Al ( $k' = 0.2$ ) and Fe ( $k' = 0.2$ ) were not completely resolved from the ligand peak. The absence of any other peaks was verified by recording the chromatogram of each analyte for 1 h after the last peak eluted.

After this preliminary investigation, further experiments were performed in order to improve the separation, by studying the effects of different mobile phase parameters.

### 4. Effect of TBA

The effect of the ion interaction reagent was studied in the range 15–25 mM TBA, keeping the concentrations of the other mobile phase components listed in the previous paragraph constant. Even if the higher TBA concentrations led to longer retention times for all of the species considered, the increase in retention and the yield in selectivity were not so enhanced. In fact, at every mobile phase composition evaluated, the separation between trivalent metal ions and Calcion did not improve and Cu still coeluted with the excess of ligand. The expected

increase in retention was decreased due to the strong elution power of the mobile phase induced by the high methanol content (60%), which attenuated any other effect of other mobile phase parameters that control retention in ion interaction chromatography. Further experiments were performed using 25 mM TBA; this concentration was enough to ensure retention, reproducible retention times and response with the injected sample mass [32].

## 5. Effect of CH<sub>3</sub>OH

According to previous experiments, the content of organic modifier was an important parameter governing the retention of metals complexed by Calcion. The effect of methanol content on capacity factors of analytes was studied covering the range 40–60%. Taking into account previous results on the pH values required to ensure a high degree of complex formation for each metal ion with Calcion (Figs. 2 and 3), two different sets of measurements were planned and performed.

The effect of methanol on the retention of trivalent metal ions was evaluated at pH 4.5, while the behaviour of divalent metal ions was investigated at pH 7.5, where recovery studies showed that even complexation of Ni occurs, as previously shown. Trivalent metal ions were not studied at pH 7.5 due to large competition in hydroxo complex formation for Al<sup>3+</sup> and Fe<sup>3+</sup>, as can be seen from Figs. 3A and 3B).

### 5.1. Trivalent metal ions

As shown in Fig. 4, retention of Al–Calcion complex is lower than that of Fe–Calcion species and their chromatographic behaviour is quite different for CH<sub>3</sub>OH concentrations lower than 50%. As the methanol content decreases, the Calcion peak rapidly shifts towards higher retention times, reaching elution times longer than 1 h at 40% CH<sub>3</sub>OH. Moreover, it was noted that, at low percentages of CH<sub>3</sub>OH, Calcion gives rise to three peaks, probably due to impurities in the commercial reagent (its declared purity is about 60%), whose retention behaviour is diversified at decreasing elution power.

The increase in elution times caused by the

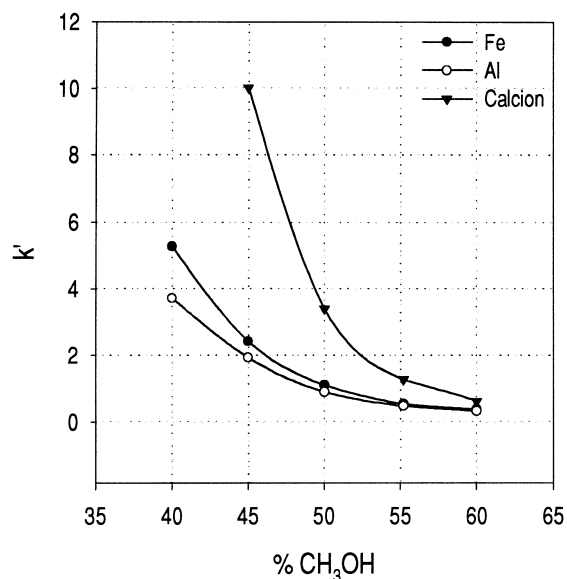


Fig. 4. Effect of CH<sub>3</sub>OH content on the  $k'$  values of Fe<sup>3+</sup> and Al<sup>3+</sup>. Mobile phase: 40 mM acetic acid, 25 mM TBA, 25 mM NaCl and  $5.0 \cdot 10^{-7}$  M Calcion, pH 4.5. Content of methanol is as shown. Stationary phase: 10  $\mu$ m LiChrospher 100 RP-8 (250  $\times$  4 mm I.D.). Detection was at  $\lambda = 510$  nm.

reduction in the percentage of organic modifier is due to preferential interactions between lipophilic analytes and the stationary phase, as is usual in an ion interaction mechanism.

The best separation between Al<sup>3+</sup> and Fe<sup>3+</sup> complexes was obtained for 45% CH<sub>3</sub>OH ( $k'_{Al} = 2.2$ ,  $k'_{Fe} = 2.9$ ); under these conditions, peaks of the excess of ligand were eluted in the range 20–37 min. At lower organic modifier content, even if a better separation between metal ions was obtained, as previously mentioned, ligand retention times were very long, precluding chromatographic analysis. At 50% CH<sub>3</sub>OH, the analysis time was about 15 min, but the separation of the metal ions was not complete.

### 5.2. Separation of Fe<sup>3+</sup> and Al<sup>3+</sup> by a CH<sub>3</sub>OH concentration gradient

The results obtained suggested the use of a gradient run in order to obtain lower analysis times without losing selectivity. During the gradient runs, the methanol content was increased in order to

increase the elution strength of the mobile phase, which would quicken the elution of compounds that were more retained by the column. Gradient profiles were chosen in order to evaluate both the effect of duration of gradient stages and the effect of methanol content in the different steps on analyte separation.

Five linear gradient profiles were studied; after each gradient separation, a mobile phase whose composition matched that of the initial conditions of the gradient was passed through the column for 30 min in order to equilibrate it.

Table 3 reports the gradient profiles and the corresponding selectivities between Fe and Al complexes, calculated as  $k'_{\text{Fe}}/k'_{\text{Al}}$  and total analysis time.

Gradients 1 and 2 show the effect of the variation of eluent composition on a longer period of time. As indicated in Table 3, selectivity and time analysis slightly increase when the mobile phase is changed on a longer period (10 min for gradient 2). Keeping the time for eluent variation constant, it was decided to evaluate the effect of higher methanol content in the initial step of the gradient (gradient 3). Comparing results obtained using gradients 2 and 3, we can state that selectivity and analysis times are complexively unaffected by higher initial concentrations of methanol.

Results obtained thus far show that selectivity can be improved by enhancing the interaction between analytes and the stationary phase. In order to avoid longer analysis times, with gradient 4, eluent composition was changed in the first 10 min of the chromatographic run, but planned to reach higher methanol content (60% CH<sub>3</sub>OH). Both selectivity and analysis times improved, confirming that these conditions leave enough time for the column to differentiate between the behaviour of analytes.

Analysis times were significantly reduced, without losing selectivity, which actually increased when the interactions between analytes and the stationary phase were raised in the first step of the separation, by working at constant changing CH<sub>3</sub>OH concentration and then the eluent composition is changed in a very short time (1 min, gradient 5).

As shown in Table 3, optimization of the gradient enabled us to reach selectivities that were comparable to those of isocratic runs, but provided shorter elution times with significant advantages for routine applications.

An example of the chromatogram obtained for the separation of metal ions by gradient 5 is shown in Fig. 5.

### 5.3. Divalent metal ions

After optimization of the separation of trivalent metal, the study was extended to divalent metal ions Cu and Ni. In order to ensure Ni complexation, a pH value of 7.5 was chosen. Since a Ni–Calcion complex had not been studied at this stage, preliminary experiments were performed in order to characterise its chromatographic behaviour. The mobile phase used was a water–methanol (50:50, v/v) solution containing 40 mM disodium hydrogenphosphate, 25 mM TBA, 25 mM NaCl and  $5.0 \cdot 10^{-7}$  M Calcion, pH 7.5. Cu and Ni peaks were identified by spiking increasing concentrations of each metal ion. Under the experimental conditions used, the ligand exhibited two peaks ( $k'_1=0.9$  and  $k'_2=1.6$ ). The Cu–Calcion complex coeluted with the first peak of the ligand, while Ni–Calcion species were only partially resolved from the same ligand peak. Therefore,

Table 3

Compositions of the different linear methanol concentration gradients studied. Methanol concentration is expressed as a percentage. Selectivity and total analysis times are also shown for each gradient

Time (min)	Gradient 1	Gradient 2	Gradient 3	Gradient 4	Gradient 5
0	30	30	40	40	45
5	30	30	40		
10	50			60	45
11					60
15		50	50		
Selectivity (Fe–Al)	1.0	1.1	1.1	1.2	1.3
Analysis time (min)	25	26	27	22	16



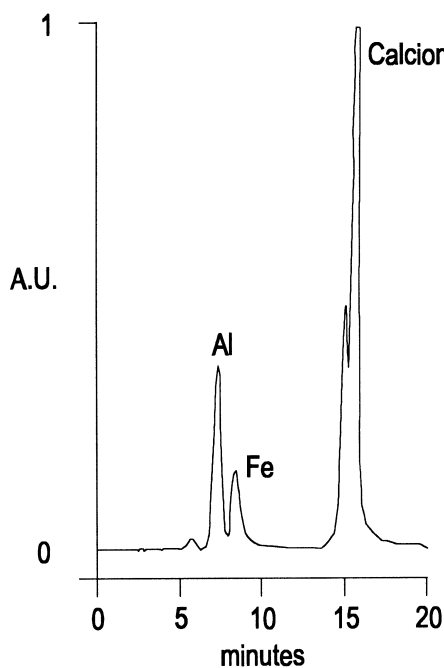


Fig. 5. Separation of 5 ppm Al and 10 ppm Fe, complexed with Caclon, using an optimal methanol gradient (gradient 5). Other conditions are the same as those given in Fig. 4.

separation between the two metal ions is not possible using these starting conditions.

Since methanol was found to be one of the parameters with the greatest influence on the retention of trivalent metal ions, a study of the effect of methanol concentration (39–60, v/v) on Cu and Ni  $k'$  values was performed. Other mobile phase conditions were kept constant (as mentioned above). The results obtained are shown in Fig. 6. As previously verified, with decreasing methanol content, the retention of ligand increases more rapidly than for the complexes. In particular, at 39% CH<sub>3</sub>OH, Caclon elutes after about 50 min. At every mobile phase composition studied, Ni–Caclon elutes before Cu–Caclon. Experiments showed that separation among metal ions and the excess of ligand can be achieved with up to 50% CH<sub>3</sub>OH, even if the best results are obtained for 47% CH<sub>3</sub>OH, as shown in Fig. 7. Under these experimental conditions, analysis times were relatively short, in fact, the last peak eluted at about 12 min, while the selectivity was  $\alpha = (k'_{\text{Cu}}/k'_{\text{Ni}}) = 1.4$ .

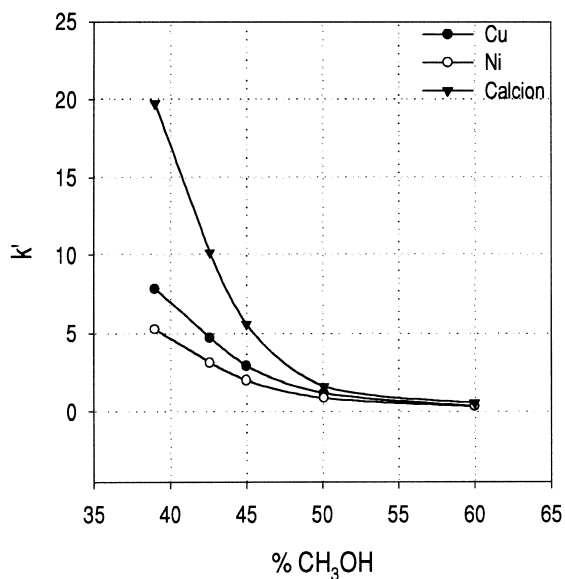


Fig. 6. Effect of CH<sub>3</sub>OH content on the  $k'$  values of Ni<sup>2+</sup> and Cu<sup>2+</sup>. Mobile phase: 40 mM disodium hydrogenphosphate, 25 mM TBA, 25 mM NaCl and 5.0·10<sup>-7</sup> M Caclon, pH 7.5. Methanol concentrations are as shown. Other conditions are the same as those given in Fig. 4.

For divalent metal ions, the effect of a gradient of organic modifier concentration was evaluated. For the gradient compositions investigated, there were no improvements in separation and in selectivity and, therefore, further experiments were performed at 47% CH<sub>3</sub>OH, where trivalent metal ions can be successfully separated.

## 6. Effect of pH

It is well known that pH affects the conditional stability constants of each complex, affecting in turn the possibility of their chromatographic determination. In order to obtain a simultaneous separation of the four metal ions considered, the effect of pH (4.5–7.5) on the separation of metal ion–Caclon complexes was studied. The mobile phase used for this set of measurements was 47–53% (v/v) methanol in water containing 25 mM TBA, 25 mM NaCl, 5.0·10<sup>-7</sup> M Caclon and 40 mM buffer solution. Depending on the pH value being investigated, different buffer solutions were used. An acetic buffer

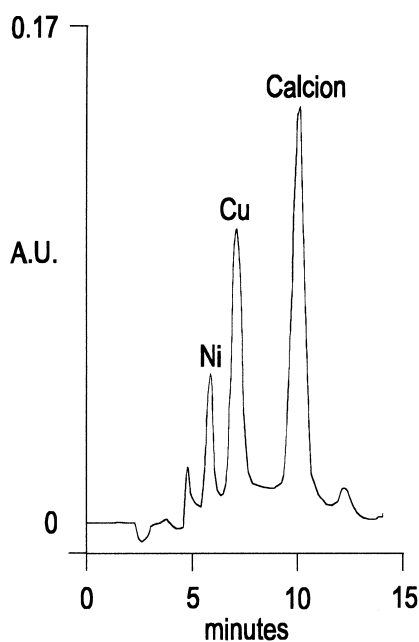


Fig. 7. Optimised separation of  $\text{Ni}^{2+}$  (2 ppm) and  $\text{Cu}^{2+}$  (2 ppm) precomplexed with  $1.6 \cdot 10^{-4}$  M Calcion. Mobile phase: 53–47% (v/v) methanol in water containing 40 mM disodium hydrogenphosphate, 25 mM TBA, 25 mM NaCl and  $5.0 \cdot 10^{-7}$  M Calcion, pH 7.5. Other conditions are the same as those given in Fig. 4.

was employed in the pH range 4.5–6.0, while a hydrogenphosphate buffer was used to perform experiments between pH 6.5 and 7.5. The results obtained are shown in Fig. 8.

Experimental data show that Al–Calcion complex elutes only at pH values of 4.5 and 5.5. At pH values higher than 6.0, it cannot be eluted or detected. As previously shown (in Fig. 3A), the formation of hydroxo complexes with Al is effective at pH 5, reducing the formation of Al–Calcion complex, whose molar fraction reaches a maximum for pH included between 4 and 5.5. The same considerations can be adduced for Fe–Calcion complex. In fact, the fraction of 1:1 (metal:ligand) complex has a maximum for pH 2–4 (Fig. 3B). Beyond this value, the formation of hydroxo complexes occurs. Chromatographic experiments performed at pH 7.0 and 7.5 revealed the presence of a peak when Fe was injected. The chromatographic behaviour is now completely different from that obtained at pH values lower than 5.5. In fact, at higher pH values, the

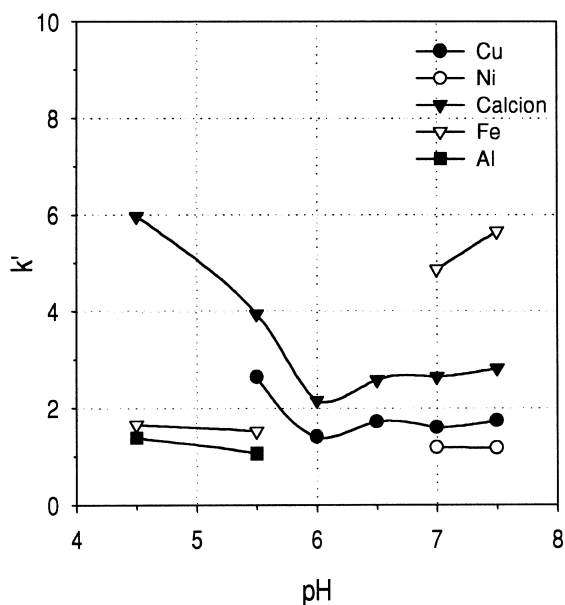


Fig. 8. Effect of pH on  $k'$ . Mobile phase: 53–47% (v/v) methanol in water containing 25 mM TBA, 25 mM NaCl,  $5.0 \cdot 10^{-7}$  M Calcion and 40 mM buffer solution. Buffers used: acetic (pH 4.5, 5.0 and 6.0) and hydrogenphosphate (pH 6.5 and 7.5). Other conditions are the same as those given in Fig. 4.

complex is more retained than the ligand, suggesting the presence of a more hydrophobic or highly charged species at different metal:ligand stoichiometries.

The chromatographic data obtained for Ni showed that its complex with Calcion can only be determined at pH values higher than 7.0, in agreement with the species distributions obtained (Fig. 2B), which indicate that Ni–Calcion has its maximum formation between pH 7.0 and 10. According to these studies, the formation of hydroxo complexes starts at  $\text{pH} \geq 9.0$ . An agreement between the chromatographic data and the calculated molar fraction for the Cu–Calcion complex has also been obtained. In fact, our previous studies indicated that the complex is formed in the range of pH values between 5.0 and 8.0 (Fig. 2A).

Nevertheless, the overall effect of an increase in the pH of the mobile phase on retention is a decrease in  $k'$  up to pH 6.0. Higher pH values cause an increase in retention. This behaviour can be explained by considering that, at such values, deprotonation of an OH group ( $\text{p}K_{\text{H}_3\text{I}} = 7.1$  [21]) present in the molecular structure of the ligand occurs, leading

to stronger electrostatic interactions with the pairing ion.

From the data obtained, it was found that, in the pH range studied, it is not possible to obtain the simultaneous separation of the four metal ions considered, even if, at pH 5.5, at least Fe-, Al- and Cu–Calcion complexes can be determined in the same chromatographic run. At pH values higher than 7.0, the simultaneous determination of Ni-, Cu- and Fe–Calcion complexes is feasible.

### 6.1. Separation by gradient pH

Taking into account the results obtained at different pH values, the use of a gradient pH for the

separation of all metal ions considered was evaluated. Considering that previous results pointed out that a pH value of 7.5 was suitable for divalent metal ions, while more acidic conditions were required for the elution of trivalent metal ions, the following gradient was imposed:  $t=0$ , pH 7.5;  $t=5$  min, pH 7.5;  $t=6$  min, pH=4.0. The mobile phase was a methanol–water mixture containing 40 mM buffer (pH 4.0, acetic buffer; pH 7.5, hydrogenphosphate buffer), 25 mM TBA and 25 mM NaCl. Considering that stability constants are higher for trivalent than for divalent metal ions and that complexation is expected to occur more extensively for trivalent metal ions with increasing acidity, the gradient was imposed from less acidic to more acidic pH values.

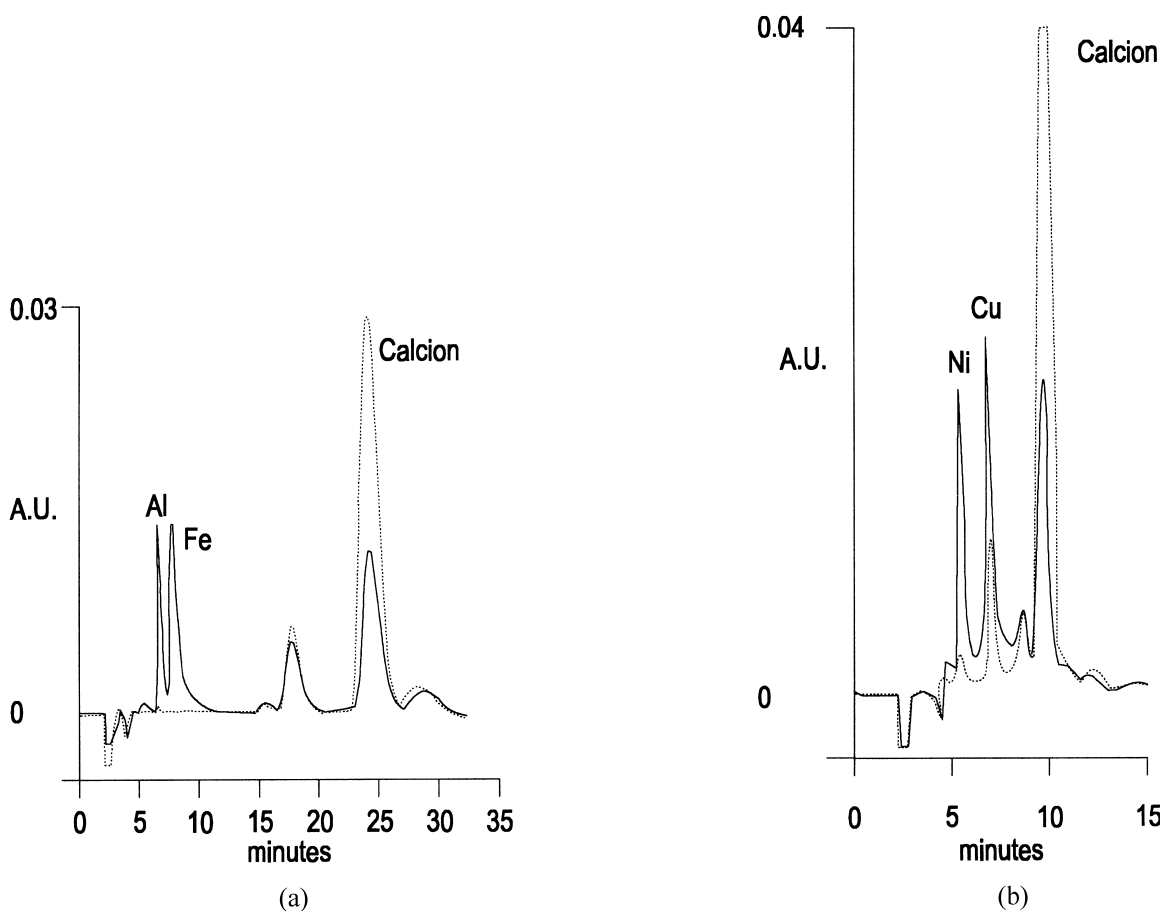


Fig. 9. Determination of  $\text{Al}^{3+}$  and  $\text{Fe}^{3+}$  at pH 4.5 (A) and of  $\text{Ni}^{2+}$  and  $\text{Cu}^{2+}$  at pH 7.5 (B) in a tap water sample. Mobile phase: 53–47% (v/v) methanol in water containing 40 mM buffer, 25 mM TBA, 25 mM NaCl and  $5.0 \cdot 10^{-7}$  M Calcion. Tap water as such and spiked with 1 ppm of each metal ion. Other conditions as the same as those given in Fig. 4.

With such a gradient, the elution of divalent metal ions is expected while trivalent metal ions remain as hydroxo complexes. The rapid change in pH towards acidic values ensures that trivalent metal ions form complexes with Calcion and then can be eluted.

The gradient imposed confirmed that the elution order was: Ni-, Cu- and Fe–Calcion complexes, while complexation and elution of Al–Calcion did not take place, since the kinetics of complex formation are slower than the elution time. Our results are in agreement with those of Ishii and Einaga [33] who showed that, for Al–Calcion at pH 6.0, about 20 min are required to reach a steady state.

### 6.2. Real sample analysis

After the development and optimization of the chromatographic separation of Ni<sup>2+</sup>-, Cu<sup>2+</sup>-, Al<sup>3+</sup>- and Fe<sup>3+</sup>–Calcion complexes, the method was used to verify its suitability in the determination of these metal ions in a tap water sample.

Tap water was filtered through 0.22 μm filters and added to 7.0·10<sup>-5</sup> M Calcion and 1 ppm of each metal ion. The sample was then injected and eluted at pH values of 4.5 and 7.5 for the determination of trivalent (Fig. 9A) and divalent (Fig. 9B) metal ions, respectively; a blank was eluted under the same experimental conditions. Relative standard deviations [R.S.D. (%)] for the determination of metal ions were 11.7, 9.3, 2.6 and 4.9% (*n*=4) for Fe, Al, Cu and Ni, respectively.

As shown in the chromatograms, the analysis does not present difficulties for the determination of metal ions, showing the feasibility of the method developed throughout this work.

### Acknowledgements

Financial support from the National Research Council (CNR, Rome) and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) is gratefully acknowledged.

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