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Short Communication

High-performance liquid chromatographic separation of metal ions using aspartic acid β-hydroxamate as a model siderophore

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ABSTRACT

Aspartic acid β-hydroxamate (ASPHA) is used for the precolumn formation and separation of the metal chelates of Al(III), Co(II), Cu(II), Fe(III) and Mo(IV) by ion-pairing reversed-phase liquid chromatography on a polymeric column. Better selectivity and detectibility at 313 nm were achieved using ASPHA than with acetohydroxamate, N-methylpropionohydroxamate or α-glutamic γ-monohydroxamate. The ASPHA chelates are separated in about 7 min using a PLRP-S 5 μm (15 cm × 4.6 mm I.D.) column with water–acetonitrile (98:2) as the mobile phase containing $5 \cdot 10^{-3}$ *M* ASPHA, $5 \cdot 10^{-3}$ *M* tetrabutylammonium perchlorate and $1 \cdot 10^{-3}$ *M* acetate buffer (pH 7) at 1 ml/min. Retention times and selectivity can be varied by changing pH, buffer ion and ion-pairing agent concentrations.

INTRODUCTION

The use of naturally occurring metal sequestering agents for the liquid chromatographic analysis of trace metal ions is an approach worthy of greater investigation. In the microbial world metals such as Fe(III) are transported or "partitioned from" the aqueous environment through hydrophobic membranes by the use of special chemical agents called siderophores [1]. The unique selectivity of the agents toward metal ions should be an aid in the separation process. From the chemical structures of isolated siderophores it is clear that a major role in coordination is played by hydroxamate, catecholate and amino acid functional groups [2]. In this work, a low-molecular-mass model of a siderophore with similar metal coordination properties is shown to be useful for the chromatographic separation of transition metals.

Separations and determinations of metal ions by high-performance liquid chromatography have been carried out using many novel approaches based on ion-exchange, ligand-exchange, normal-phase, reversed-phase and ion-pairing mechanisms [3–12]. Of particular relevance here is the work of Palmieri and Fritz [10] who studied the separation of Zr(IV), Hf(IV), Fe(III), Nb(V), Al(III) and Sb (III) on a polymeric column using a synthetic water-soluble complexing agent N-methylfurohydroxamic acid. This work extended further the areas of application of hydroxamic acids in the analysis of

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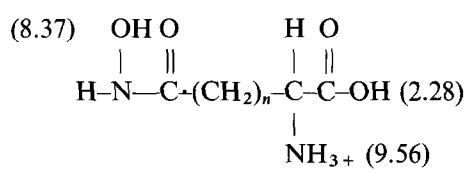
metal ions which include classical [13,14], atomic absorption spectrometry [15], flow injection analysis [16] and voltammetry [17]. As the method of analysis moves from classical to instrumental, the reagents have changed from being in the main water insoluble, *e.g.* N-benzoylphenylhydroxamic acid to water soluble. The purpose of this work is to highlight the use of a water-soluble amino acid hydroxamate, aspartic acid β-hydroxamate (shown below with pK_a values) (ASPHA, $n = 1$) for the precolumn formation and separation of the metal chelates of Al(III), Co(II), Cu(II), Fe(III) and Mo(IV).



The reagent is a model of the naturally occurring siderophore Pseudobactin which contains aspartic acid and hydroxamate functional groups [2].

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Pye-Unicam LC 3-XP pump equipped with a Rheodyne model 7125 syringe loading sample injector with a 20-μl loop. A guard column, hand-packed with Amberlite XAD-2 preceeded the analytical column, containing PLRP-S 5 μm polystyrene–divinylbenzene (15 cm × 4.6 mm I.D.), from Polymer Labs. A Pye-Unicam LC 3-UV detector was employed. The detector output was recorded on both a Philips PM 8251 recorder and a Hewlett-Packard model 3390A integrator.

Chemicals

DL-Aspartic acid β-hydroxamate monohydrate (ASPHA) was obtained from Sigma (Poole, UK). Tetrabutylammonium iodide (TBAI) was purchased from Koch-Light Labs. (Colnbrook, UK) and tetrabutylammonium perchlorate (TBAP) from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile obtained from Rathburn (Walkerburn, UK) was used.

Metal ion stock solutions (0.1 *M* in 0.02 *M* HCl) were prepared from AnalaR-grade metal ion salts. Al(III), Co(II), Cu(II) and Fe(III) stock solutions were prepared from their nitrates while the Mo(VI) solution was prepared from sodium molybdate. Deionised water from an Elgastat spectrum water purification unit, with a resistivity of 18 MΩ/cm or better, was used throughout the experiment.

Procedure

Eluents were filtered through a 0.45-μm filter and degassed under vacuum on a Millipore filtration unit before use. The chromatographic behaviours of metal ions were investigated by injecting their precomplexed solutions. Aqueous eluent (2% CH_3CN) containing $5 \cdot 10^{-3}$ *M* ASPHA and $5 \cdot 10^{-3}$ *M* pairing ions (either TBAI or TBAP) with pH values of 6, 7, 8 and 9 were initially employed. The eluent flow-rate was 1 ml/min, with detection at 313 nm.

The influence of buffer concentration and the % organic modifier on the chromatographic behaviour of the ASPHA chelates of Al(III), Co(II), Cu (II), Fe(III) and Mo(IV) were examined. These studies were carried out with an eluent containing $5 \cdot 10^{-3}$ *M* ASPHA and $5 \cdot 10^{-3}$ *M* pairing ion at pH 7.0. The dependence of retention for those metal ASPHA chelates on the pairing-ion concentration was also studied using an aqueous eluent (2% CH_3CN) containing $1 \cdot 10^{-3}$ *M* acetate buffer pH 7.0 with and without $5 \cdot 10^{-3}$ *M* ASPHA.

Quantitative analysis of ASPHA chelates of Al (III), Co(II), Cu(II), Fe(III) and Mo(VI) was studied using both separate and mixed solutions of the metal chelates. The various concentrations of metal ions, both individual and as mixtures of Al(III), Co (II), Cu(II) and Mo(VI), and of Al(III), Fe(III), Cu (II) and Mo(VI) were prepared by taking appropriate aliquots of the stock solution, diluting to volume with an aqueous solution of ASPHA to yield mol ratios of ASPHA:total metal ≥5:1. The pH of the metal chelate solution was adjusted to the desired pH (eluent pH) by microlitre additions of 5.0 *M* NaOH. The quantitative analyses were carried out using the eluent of 2% CH_3CN in $1 \cdot 10^{-3}$ *M* acetate buffer pH 7.0 containing $5 \cdot 10^{-3}$ *M* TBAP and either with or without $5 \cdot 10^{-3}$ *M* ASPHA at the detection wavelength of 313 nm. Repetitive injections ($n \geq 5$) for each solution were performed for the statistical calculation of standard deviation and relative standard deviation (R.S.D.). Calibration plots were obtained using linear regression analysis.

The limit of detection (LOD) was defined as the metal ion concentration which gave a peak height three times the background noise.

RESULTS AND DISCUSSION

Initial studies on the chromatography of metal ions on octadecylsilica using in-situ complexation with acetohydroxamic acid (AHA = $CH_3CONHOH$) indicated that while baseline separation and UV–visible detection of Fe(III) and V(V) was achievable using a water–methanol mobile phase, other metal ions such as Cu(II) and Co(II) yielded poor retention and detectibility in the 254–500 nm range. In addition, bands obtained for Fe(III) and V(V) were broad on octadecylsilica, an effect observed by other workers [10]. Improvements in retention using N-methylpropionohydroxamic acid (NMPHA = $CH_3CH_2CON(OH)CH_3$) as an alternative mobile phase additive, were obtained. Apart from the increase in hydrophobicity, the higher chelate stability of the N-substituted complexes together with a changeover to the use of a polymeric column (PLRP, 5 μm polystyrene–divinylbenzene) lead to sharper peaks and better detectibility for the injected mixture of Fe(III) and V(V). A typical mobile phase consisted of water–acetonitrile (98:2) with $5 \cdot 10^{-3}$ *M* NMPHA and 0.02 *M* acetate buffer (pH 5.6). In order to increase the range of metal ions amenable to this in-situ complexation approach using hydroxamic acids, attention turned to amino acid hydroxamates and in particular to aspartic acid β-hydroxamate, which has the potential to form more intensely absorbing chelates with certain metal ions. Immediate improvements to the retention (in conjunction with ion pairing) and detectibility of Cu(II) were observed.

Separation conditions for metal–ASPHA chelates

To obtain the optimal conditions for the separation of the metal–ASPHA chelates, the eluent parameters including pH, pairing ion and organic modifier were studied. As metal complexation by amino hydroxamic acids in aqueous solution occurs through the amino nitrogen and the oxygen of the deprotonated NHO^- group [18], the α-carboxy of the ASPHA should be completely deprotonated in the pH range studied in these experiments (pH 6.0–9.0), yielding net negatively charge complexes. With TBAP containing mobile phases, all of the metal–ASPHA chelates studied showed a decrease in the retention time with increasing pH with the exception of Fe(III) chelate, as shown in Fig. 1. This latter behaviour suggests that the Fe(III)-ASPHA increases in hydrophobicity as the pH is increased either through further coordination by the ASPHA ligand, *e.g.* tridentate or through further ion pairing. For the metals studied, which span a range of oxidation states, a variety of simultaneously existing metal complexes species are expected to occur over this pH range. As such it is a surprise that a trend is observed at all and further species distribution analysis is underway using potentiometry to throw further light on the nature of complexation of the metals [19]. A mobile phase pH of 7.0 was chosen for further studies.

The ASPHA ligand present in the mobile phase should display enough buffer capacity in the pH range studied (pH 6.0–9.0) for the control of complexation and ion-pairing mechanisms. However, the addition of acetate buffer produced useful peak

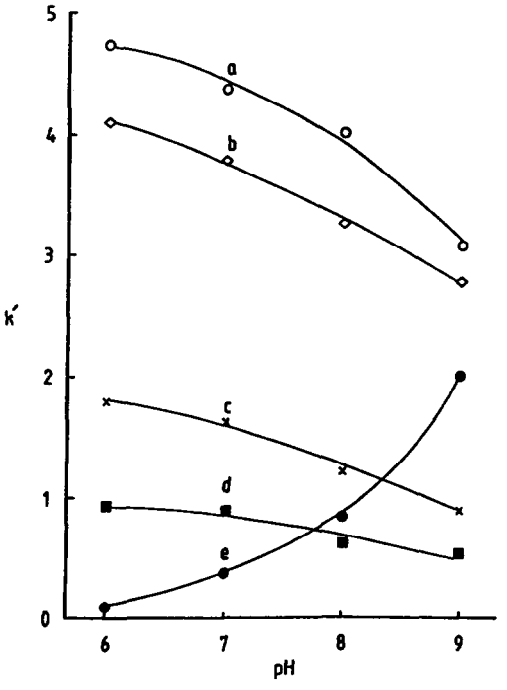


Fig. 1. Dependence of capacity factors (k') on pH for metal–ASPHA chelates using TBAP-based mobile phases: (a) Mo(IV), (b) Cu(II), (c) Al(III), (d) Co(II) and (e) Fe(III).

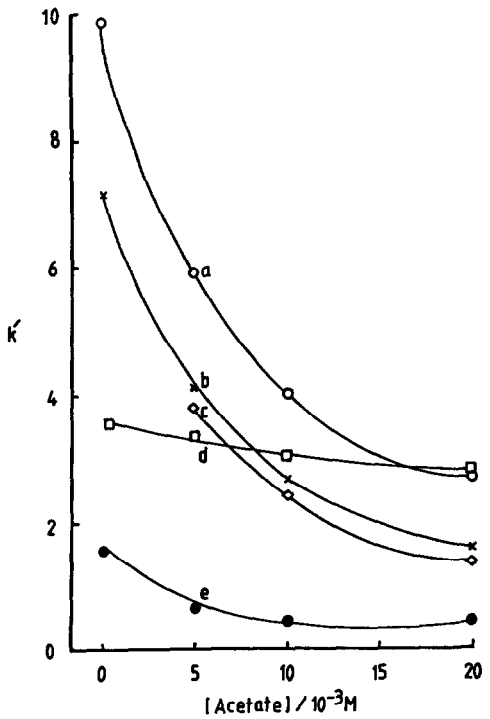


Fig. 2. Dependence of capacity factor (k') on acetate buffer concentration using TBAI-based mobile phase (pH 7.0): (a) Mo(VI), (b) Cu(II), (c) Ni(II), (d) Al(III) and (e) Co(II).

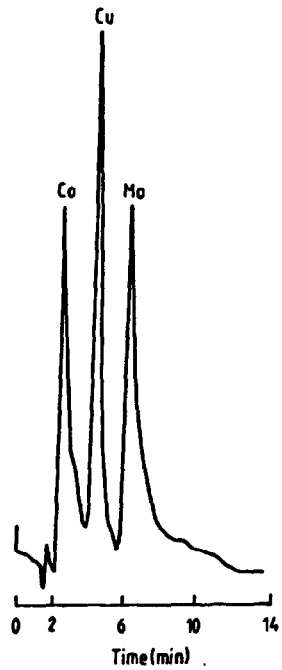


Fig. 3. Chromatographic separation of Co(II), Cu(II) and Mo (VI) as ASPHA chelates: eluent 2% CH_3CN, $5 \cdot 10^{-3}$ *M* ASPHA, $5 \cdot 10^{-3}$ *M* TBAI, $2 \cdot 10^{-2}$ *M* acetate buffer pH 7.0; flow-rate 1 ml/min, 20 μl $2.0 \cdot 10^{-4}$ *M* Co(II), Cu(II) and $6.0 \cdot 10^{-4}$ *M* Mo(VI).

shape and sensitivity improvements but with a concomitant reduction in retention. Fig. 2 shows the dependence of capacity factor on the acetate concentration and a typical separation of Co(II), Cu(II) and Mo(VI) is given in Fig. 3. This effect, of decreasing k' with increasing buffer concentration, is consistent with a reduction in the hydrophobicities of the metal–ASPHA chelates. However, the situation is likely to be more complex considering the number of equilibria involved. Effects of buffer concentration are also mentioned by other researchers [20–22].

Ion-paired metal–ASPHA chelates

Tetrabutylammonium salt, $(C_4H_9)_4N^+$ or $(but)_4N^+$, was used as the pairing ion and the effects of its concentration as well as the type of co-anion on the chromatographic behaviour of the metal–ASPHA chelates were investigated. Fig. 4 shows the influence of pairing-ion concentration on the retention of the metal–ASPHA chelates. In the absence of pairing ion little or no retention is observed for these water-soluble complexes. A sharp rise in retention of the metal–ASPHA chelates is observed for the pairing-ion concentration range of $0.01 \cdot 10^{-4}$–$1.0 \cdot 10^{-4}$ *M*. A maximum point is reached at a concentration of *ca.* $1 \cdot 10^{-4}$ *M* TBAP after which the retention begins to drop as the concentration of the pairing-ion increases.

The parabolic behaviours or bell-shaped curves for the dependence of retention on the pairing-ion concentration were also observed by previous workers. Several mechanisms have been proposed to explain this behaviour including the formation of micelles or less-retained compounds [22–24]. However, a competition effect of the co-ion or counter-ion of the pairing-ion has also been suggested [21].

In contrast, increases in retention on ion-pair formation were much less when α-glutamic γ-monohydroxamate (GLUHA, $n = 2$) was used instead of ASPHA, in particular for Cu(II). In fact with the exception of Mo(VI), the capacity factors for the

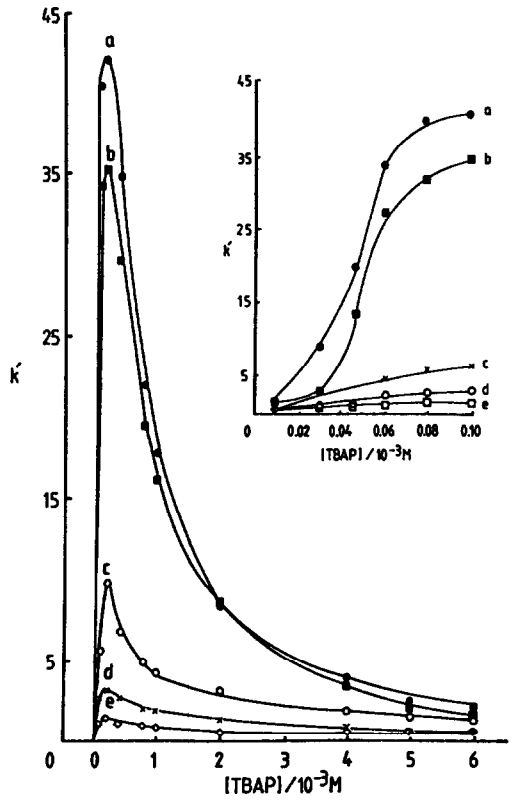


Fig. 4. Dependence of capacity factor (k') on pairing-ion concentration (TBAP): (a) Mo(VI), (b) Cu(II), (c) Al(III), (d) Co(II) and (e) Fe(III).

series of metals studied all remained below 2.5 on a similar variation of TBAP concentration. The additional methylene group in GLUHA has a major effect on chromatographic retention of the resulting chelates.

It has been suggested that the choice of co-ion or counterion (type, concentration) of the pairing-ions would strongly influence retention [20,21,23]. In the present work, iodide and perchlorate co-anions of the tetrabutyl ammonium salts, *i.e.* TBAI and TBAP were investigated. Perchlorate co-anion provides better chromatography *i.e.* conveniently shorter retention times, less peak tailing and an enhancement of sensitivity. The superiority of perchlorate (ClO_4^-) over iodide (I^-) can be attributed to its qualities as a good inorganic pairing ion [10,25].

The need for precomplexation of metal ions before injection in HPLC has been recognised [7,8]. Typically a 5-fold excess of ligand is recommended. The advantages of the presence of ligand in the mobile phase have already been well established [1,8], and include the enhancement of the stability of complexes and the promotion of re-formation of the complexes if dissociation takes place. However, in the case of kinetically inert complexes, the addition of ligand into mobile phase is unnecessary [10]. In this work, a mobile phase containing 5 m*M* ASPHA was compared with a mobile phase with no added ASPHA and the chromatographic behaviours of the metal–ASPHA chelates examined. Without ASPHA in the mobile phase, Mo(VI) was not detectable, whereas Fe(III) was detected with very poor reproducibility. The chromatographic behaviour of the other chelates, *i.e.* Al(III), Co(II) and Cu(II) was not affected by the removal of ligand from the mobile phase. These results are in keeping with dissociation of the Mo(VI)– and Fe(III)–ASPHA chelates and the suggestion that the other metal ions, Al(III), Co(II) and Cu(II), form kinetically inert chelates with ASPHA [6,7].

Determination of metal ions

The separation and determination of Al(III), Co (II), Cu(II), Fe(III) and Mo(IV) as their ASPHA chelates were carried out on a PLRP-S column using the chosen mobile phase of 2% CH_3CN, $1 \cdot 10^{-3}$ *M* acetate buffer pH 7.0 containing $5 \cdot 10^{-3}$ *M* TBAP and $5 \cdot 10^{-3}$ *M* ASPHA. Under these conditions, good separations of Al(III), Co(II), Cu(II) and Mo(VI), and of Al(III), Fe(III), Cu(II) and Mo (VI) were achieved. In both mixtures, quite good separation between the Al(III), Co(II) or Fe(III) and Cu(II) was obtained. However, incomplete resolution of Cu(II) and Mo(VI) chelates with a resolution of *ca.* 1.20 was obtained. Fig. 5 shows typical chromatograms obtained from this system for which Co(II) and Fe(III) were the shortest retained chelates, followed by Al(III) and Cu(II) and Mo (VI), the longest retained chelate.

Using the chromatographic conditions above, the quantitative aspects for these metal–ASPHA chelates were examined. Both separate solutions and mixtures of metal chelates were examined. Peak height and peak area measurements were employed for the calibration plots. Calibration plots of peak height *versus* concentration gave correlation coeffi-

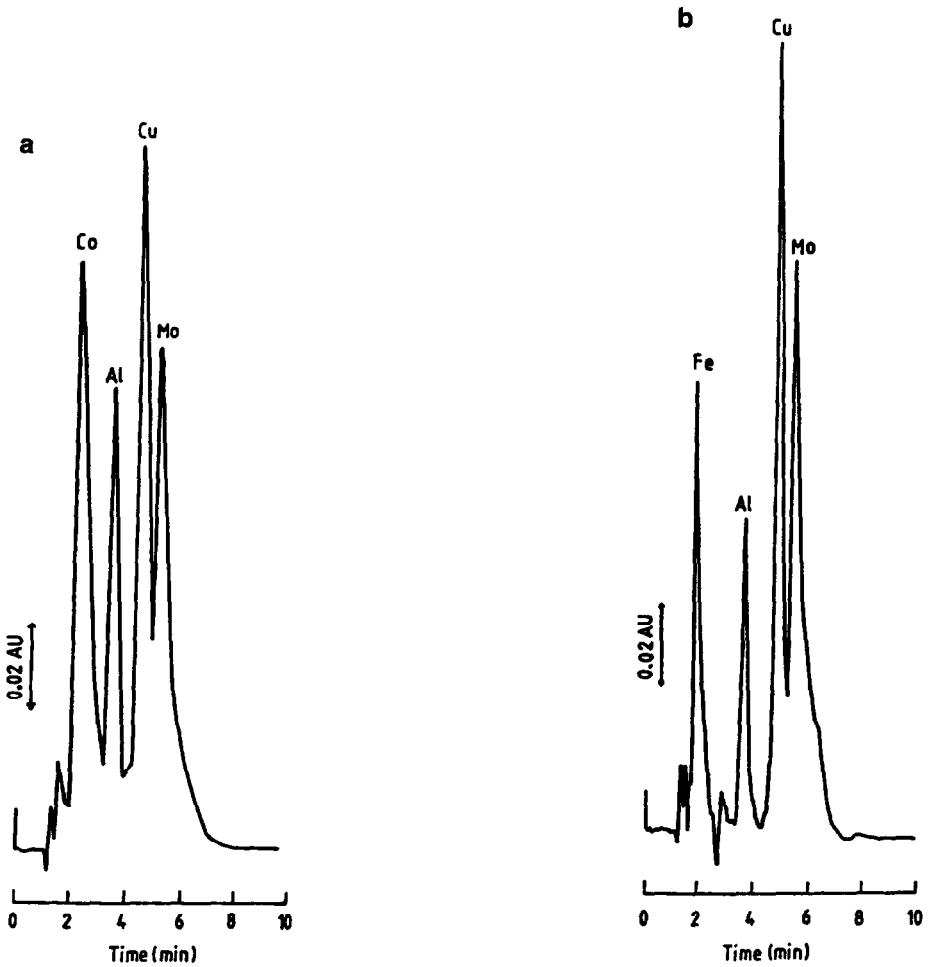


Fig. 5. Chromatographic separations of (a) Co(II), Al(III), Cu(II) and Mo(VI) and (b) Fe(III), Al(III), Cu(II) and Mo(IV) as ASPHA chelates: eluent 2% CH_3CN, $5 \cdot 10^{-3}$ *M* ASPHA, $5 \cdot 10^{-3}$ *M* TBAP, $1 \cdot 10^{-3}$ *M* acetate buffer pH 7.0; flow-rate 1 ml/min, 20 μl $1.6 \cdot 10^{-4}$ *M* Co(II), $4.6 \cdot 10^{-5}$ *M* Fe(III), $1.5 \cdot 10^{-3}$ *M* Al(III), $8.0 \cdot 10^{-5}$ *M* Cu(II) and $1.6 \cdot 10^{-4}$ *M* Mo(VI).

cients in 0.9980–0.9998 range and good reproducibility and linear ranges were obtained. A signal-to-noise ratio of 3.0 was used to calculate the following limit of detection values: Al(III), $7.2 \cdot 10^{-5}$ *M*; Co (II), $1.6 \cdot 10^{-5}$ *M*; Cu(II), $6.2 \cdot 10^{-6}$ *M*; Fe(III), $5.8 \cdot 10^{-6}$ *M* and Mo(IV), $1.3 \cdot 10^{-5}$ *M*.

In addition, there is some evidence of a partial redox reaction taking place in the mixed solutions of metal ASPHA chelates. The re-injection of sample solutions, after standing for 24 h leads to noticeable changes. The Cu(II) peak seemed to disappear whereas the Mo(VI) peak slightly increased and the Co(II) and Fe(III) peaks began to split. However, these effects could be ignored if freshly prepared mixture solutions were used.

Trace analysis of Cu(II) in water samples

The method was applied to the analysis of Cu(II) in samples of laboratory tap water, following preconcentration using solid-phase extraction as previously reported [26]. The Cu(II) ions, preconcentrated from a 250-ml sample at pH 5, were eluted with acid into 10 ml. Following precomplexation with ASPHA, 20-μl samples were injected into the HPLC system. Levels of metal ion in the original water sample, determined by this method, com-

pared favourably with the values obtained by flame atomic absorption spectroscopy. The following data pairs are good illustrative examples of the results ($\times 10^{-6}$ *M*) obtained for three different water samples: (liquid chromatography–atomic absorption spectrometry) 3.75, 3.60; 4.66, 4.75; 4.33, 4.24.

REFERENCES

1 J. B. Neilands, *Ann. Rev. Biochem.*, 50 (1981) 715.
2 M. Teintze, M. B. Hossain, C. L. Barnes, J. Leong and D. van der Helm, *Biochemistry*, 20 (1981) 6446.
3 J. N. King and J. S. Fritz, *Anal. Chem.*, 59 (1987) 703.
4 H. Ge and G. G. Wallace, *Anal. Chem.*, 60 (1988) 830.
5 D. F. Hilton and P. R. Haddad, *J. Chromatogr.*, 361 (1986) 141.
6 Y. Shijo and T. Shimizu, *Analyst*, 113 (1988) 1201.
7 J. W. O'Loughlin and R. S. Hanson, *Anal. Chem.*, 52 (1982) 2263.
8 X. X. Zhang, M. S. Wang and J. K. Cheng, *Anal. Chem.*, 60 (1980) 1670.
9 S. Inoue, N. Hashimoto, S. Hoshi and M. Matsubara, *Talanta*, 32 (1985) 1093.
10 M. D. Palmieri and J. S. Fritz, *Anal. Chem.*, 59 (1987) 2226.
11 H. Hoshino, K. Nakano and T. Yotsuyanagi, *Analyst*, 115 (1990) 133.
12 M. V. Main and J. S. Fritz, *Anal. Chem.*, 61 (1989) 1272.
13 Y. K. Agrawal and S. A. Patel, *Rev. Anal. Chem.*, 4 (1980) 237.
14 Y. K. Agrawal and R. D. Roshania, *Bull. Soc. Chim. Belg.*, 89 (1980) 159.
15 P. Allain, Y. Mauras, G. Beaudeau and P. Hingouet, *Analyst*, 111 (1986) 531.
16 A. Senior and J. D. Glennon, *Anal. Chim. Acta*, 196 (1987) 333.
17 P. K. Bhansaii and B. I. Nemade, *J. Electrochem. Soc. (India)*, 32 (1983) 135.
18 D. A. Brown, M. V. Chidambaram and J. D. Glennon, *Inorg. Chem.*, 19 (1980) 3260.
19 M. P. Ryan and J. D. Glennon, *Anal. Proc.*, 29 (1992) 16.
20 C. P. Terweig-Groen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 161 (1978) 69.
21 Z. Iskandarani and D. J. Pietrzyk, *Anal. Chem.*, 54 (1982) 1065.
22 J. H. Knox and R. A. Hartwick, *J. Chromatogr.*, 204 (1981) 3.
23 B. A. Bidlingmeyer, S. A. Deming, W. P. Price, Jr., B. Sachok and M. Petrusek, *J. Chromatogr.*, 186 (1979) 419.
24 J. H. Knox and G. Lalrd, *J. Chromatogr.*, 122 (1976) 17.
25 I. M. Johansson, K. G. Wahlund and G. Schill, *J. Chromatogr.*, 149 (1978) 281.
26 J. D. Glennon and S. Srijaranai, *Analyst*, 115 (1990) 627.