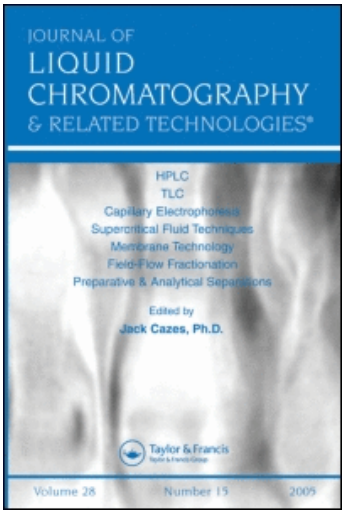

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ABSTRACT

The chromatographic performance of metal chelating analytes on a C18 functionalized styrene-divinylbenzene stationary phase is studied. The influence of metal ions impurities on their peak shapes and the metal sorption properties of such a stationary phase are investigated. Quantitative evidence is obtained to support the proposition that sorption of metal ions onto the stationary phase results in a chromatographic performance loss of the investigated metal chelating analytes. Since their retention is a function of strong complexing interactions and van der Waals forces, the mixed retention mechanism is best described as one of "metal interaction." Also when the base material is itself strongly hydrophobic and displays no ionic character whatsoever, pure reversed phase interactions may not be always achievable.

INTRODUCTION

Rationalisation of the puzzling retention behaviour of metal chelating substances is a particular challenge and a long time burden[1-2] to the separation scientist. The development of High Performance Liquid Chromatographic (HPLC) analytical methods for complex forming analytes has often met with difficulty due to poor chromatographic efficiency.[3-9] When metal ions have been deliberately incorporated into a stationary phase to achieve high selectivity[10,11] the separations were characterised by poor efficiency and frequently by severe band asymmetry.

A number of strategies have been attempted to improve poor peak shape of complexing compounds. The negative influence of metal ions, which may be present as eluent impurities, or be solubilized from the wetted stainless steel parts in the HPLC apparatus,[8] on analyte chromatographic performance, has been minimised (i) by adding a structurally related or unrelated complexing agent in the mobile phase[3-9,12] to prevent complexation of the analytes with metal cations or (ii) by pre-equilibration of the column with a proper complexing compound,[13-14] to presaturate the analyte binding sites.

Pyridinedicarboxylic (PDAs) and pyridinecarboxylic (PMAs) acids can be considered highly eligible model compounds in the chromatographic study of such kinds of analytes because the chelating isomers yield much poorer performance than the non complexing isomers.[15-16]

Experiments designed to deconvolve the metallophilic and silanophilic interactions, via the use of a C_{18} functionalized styrene-divinylbenzene stationary phase, are described here. To our knowledge, the influence of metal ion impurities on the chromatographic performance of metal chelating analytes on a strongly hydrophobic and non-ionogenic stationary phase, has never been elucidated. The metal ion sorption properties of such a stationary phase are presented.

EXPERIMENTAL

Apparatus

A 1090 series II Hewlett Packard (Palo Alto, CA, USA) high pressure liquid chromatograph with a diode array detector and variable volume 25-μL syringe based auto-injector (Rheodyne sample injection valve Model 7010) was used. The analyses were run at ambient temperature under isocratic conditions. The detector was operated at 270 nm.

Chromatographic Conditions

A 15 cm x 4.6 mm I.D (316 stainless steel) column packed with 9 μm Polyspher (styrene-divinylbenzene copolymers) C_{18}, (120 Å pore diameter), purchased from Merck was used. The eluent flow-rate was 0.800 mL/min. The HPLC experimental arrangement was then modified: injections were made in the chromatographic system with the substitution of a 107 cm x 0.25 mm I. D. 316 stainless steel capillary obtained from Hewlett Packard (Palo Alto, CA, USA) in place of the chromatographic column. When this capillary was used the eluent flow rate was 0.062 mL/min (this value was calculated on the basis of the equation between flow rates ratio and total void volumes ratio, in the two systems in order to mantain the same eluent velocity).

The eluent systems consisted of a) 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15, v/v; b) 81.6 mM phosphate buffer pH 7.2, containing 200 ppb copper (II) - methanol, 85:15 v/v; c) 81.6 mM phosphate buffer pH 7.2, containing 100 ppb each of chromium (III), iron (III), copper (II), manganese (II), nickel (II), lead (II), zinc (II) - methanol, 85:15 v/v.

All solutions were filtered through a 0.45 μm pore size regenerated cellulose filter (Schleicher&Schuell, Dassel, Germany). All pH values reported were those of the aqueous solutions prior to the addition of methanol. The hold-up time (t_O) was determined by injecting 25 μL of water and measuring the time from injection to the first deviation from the baseline.

Prior to use, the reversed phase column or the open tubular capillary was equilibrated for one hour with the uncontaminated solvent system to be used. Equilibration was established by obtaining similar results in duplicate runs at a 15 min interval. When the metal-contaminated mobile phase was used, repeated runs were performed until the column could be considered equilibrated.

The peak asymmetry factor (AF) was quantitatively expressed as the ratio of the peak half widths at 10% of peak height.

Chemicals

All isomers of pyridinedicarboxylic acid and pyridinecarboxylic acid (2,4-PDA was in the monohydrate form), benzoic acid, acetone, were purchased from Aldrich (Milwaukee, WI, USA); potassium dihydrogen phosphate and disodium monohydrogen phosphate and each metal standard solution (1 g/L) were purchased from Merck (Darmstadt, Germany). All chemicals were of the best available quality and used without further purification. Water was produced by a Milli-Q 185 system (Millipore, Bedford, MA, USA). All analytes were dissolved in the mobile phase and filtered through a 0.2 μm pore size nylon filter (Lida, Kenosha, WI, USA).

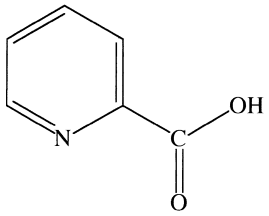

Figure 1. Part structure of heterocyclic acid series of metal chelating analytes.

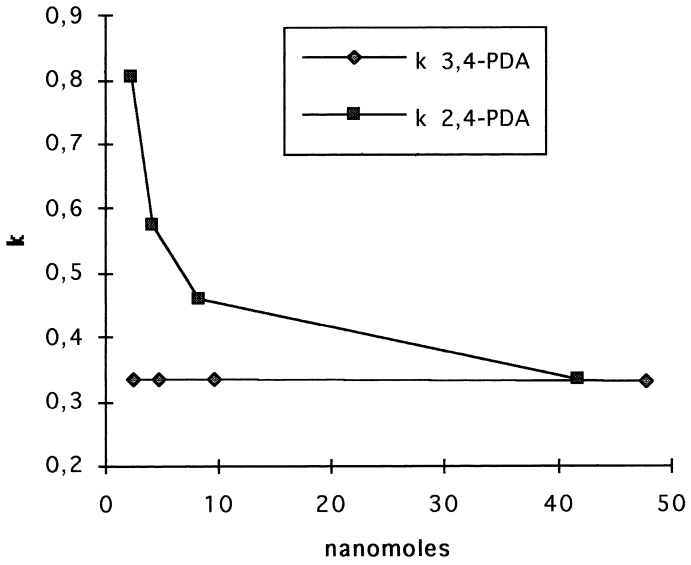

Figure 2. Influence of sample size on retention of 2,4-PDA and 3,4-PDA.Condition: column, Polyspher RP-18 15 cm x 4.6 mm I. D.; flow rate 0.800 mL/min; injections of 1.3-2.5-5.0-25.0 µL of 2,4-PDA (0.31 mg/mL) and 3,4-PDA (0.32 mg / mL) in 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15, v/v.

RESULTS AND DISCUSSION

As already found for a conventional C18 stationary phase bonded to silica,[12,17] the appearance of severe band asymmetry was compound dependent: it was specific for complexing analytes featuring carboxylic group adjacent to heterocyclic nitrogens, (Figure 1), confirming that the analyte chelating properties and not simple silanophilic interactions were responsible for the poor peak shape. For the sake of conciseness, in the present study, we will focus on

Table 1

Influence of Sample Size on N and AF of 2,4- and 3,4-PDA*

	2,4-PDA (nmoles)		3,4-PDA (nmoles)	
	41.86	4.186	47.87	4.787
N[a]	156	30	670	842
AF[a]	4.8	11.1	1.8	1.8

* Conditions: Column, Polyspher RP-18 15 cm x 4.6 mm I.D.; flow rate 0.800mL/min; injections of 25 μL or 2.5 μL of 2,4-PDA (0.31 mg/mL), 3,4-PDA (0.32 mg/mL). Eluent: 81.6 mM phosphate buffer bH 7.2 - methanol 85:15, v/v. [a] Mean values for duplicate injections.

the chromatographic behavior of 2,4- and 3,4-PDA, which can be respectively considered representatives of chelating and non chelating isomers.[15-16] The effect of sample size on the retention and peak shape of the eluite was investigated to discern between kinetic and simple non-linear tailing.

As it can be seen from Figure 2 and Table 1 metal chelating isomers showed increasing retention and asymmetry with decreasing sample size. While the former can also be explained by a non linear adsorption isotherm the latter is in contrast with this simple hypothesis because non-linear tailing should decrease as the amount of the injected sample is lowered and this is the opposite of what we observe.

Moreover the chromatographic peaks of complex forming analytes were characterised by a progressive loss of column efficiency with decreasing sample size, as shown in Figure 3. Conversely, non metal chelating isomers and a related compound such as benzoic acid did not show these anomalies. This underlines that the chromatographic behaviour is largely impaired by the ligand activity and simple non linear tailing can not play an outstanding role in the genesis of complexing analytes peak tailing.

These experimental findings can be explained by taking into account the kinetic-stochastic mechanism proposed by Giddings and Eyring[18] and later re investigated by Fornsted et al.,[19-20] according to which adsorption occurs on two types of sites, one of which, characterised by high adsorption energy and hence a slow mass transfer kinetic, can be relatively scarce.

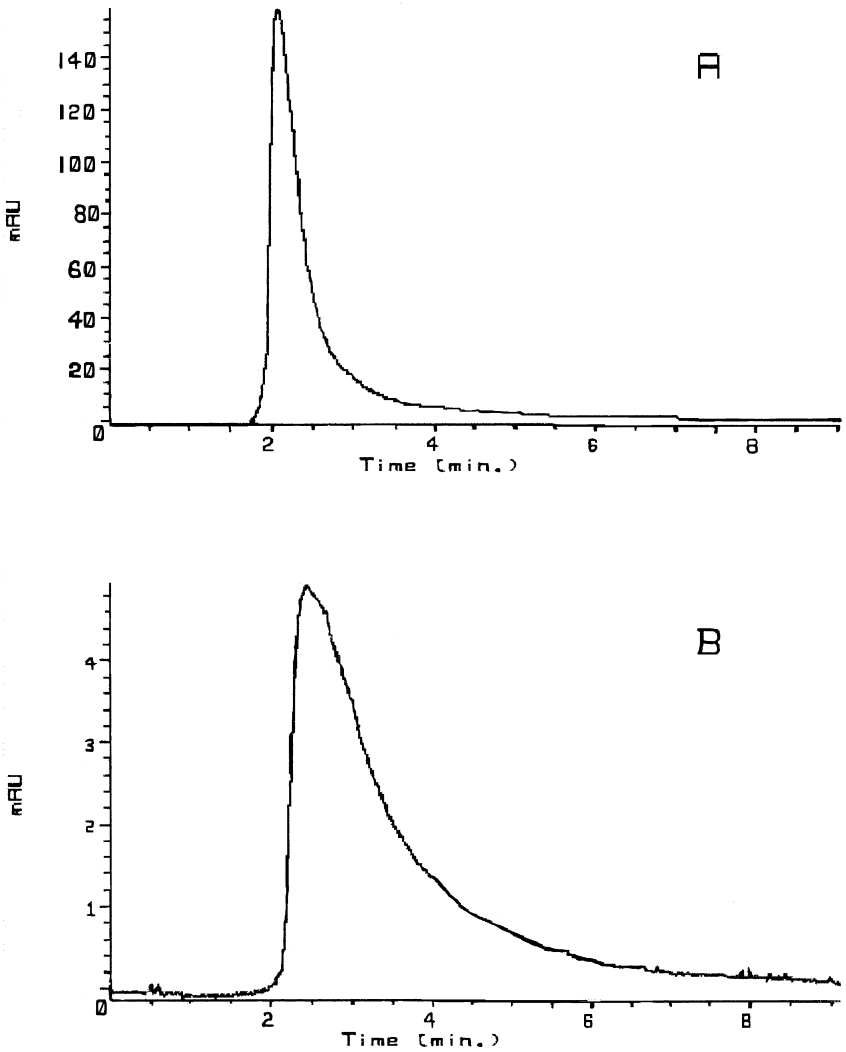

Figure 3. Experimentally obtained peaks for 2,4-PDA at high and low injected amount. Condition: column, Polyspher RP-18 15 cm x 4.6 mm I. D.; flow rate 0.8 mL/min; injections of 25.0 (A) and 2.5 μL (B) of 2,4-PDA (0.31 mg/mL) in 81.6 mM phosphate buffer pH 7.2, methanol, 85:15, v/v.

Table 2

Influence of Flow Rate on Asymmetry*

AF[a]	Flow Rate	
	0.3 (mL/min)	0.8 (mL/min)
2,4-PDA	8.8	11.1
3,4-PDA	1.8	1.8

* Conditions: Column, Polyspher rP-18 15 cm x 4.6 mm I.D.; injections of 2.5 μL of 2,4-PDA 0.31 μg/ μL) and 3,4-PDA (0.32 μg/μL) in 81.6 mM phosphate buffer pH 7.2 - methanol 85:15, v/v. [a] Mean values for duplicate injections.

The effect of this "tail-producing site" which involves selective or specific interactions, including complexation,[19] is to increase analyte retention with decreasing sample size; since the adsorption energy on active sites is high, these sites are saturated also at low sample sizes, even if sample concentration is small enough to cause the non selective sites to operate under linear conditions. As the amount of the injected sample is lowered, a larger percentage of the injected analyte molecules would be strongly adsorbed and held for a long time on these kind of sites which increasingly dominate the retention, hence an increase of the capacity factor and peak asymmetry, as well as a column efficiency loss are observed. On the converse, at relatively high ligand loading mainly solvophobic interactions determine the magnitude of retention and peak shape, since the active sites are quite scarce.[19]

This rationale is confirmed by the influence of eluent velocity on the peak asymmetry. As it may be seen in Table 2, the tailing of the chelating isomer was especially prominent at high flow rate while the asymmetry of the non chelating one was independent on the flow rate. Since kinetic tailing is expected to increase at high flow rate and, on the converse, simple non linear tailing will show little difference,[21] for 2,4-PDA the kinetic origin of tailing was confirmed.

The question raised by these experimental findings is how to account for more than one kind of adsorbing sites on the stationary phase which is itself non-ionic and non-ionogenic. Non ionic adsorption interactions, in ion exchange chromatography of inorganic ions on SDVB based resins, have been observed and attributed to π-π interactions between ions and the aromatic resin backbone.[22] The Lewis base activity of the π electron density on the aromatic ring of the SDVB copolymer has been reported to make it possible for it to undergo weak hydrogen bonding.[23]

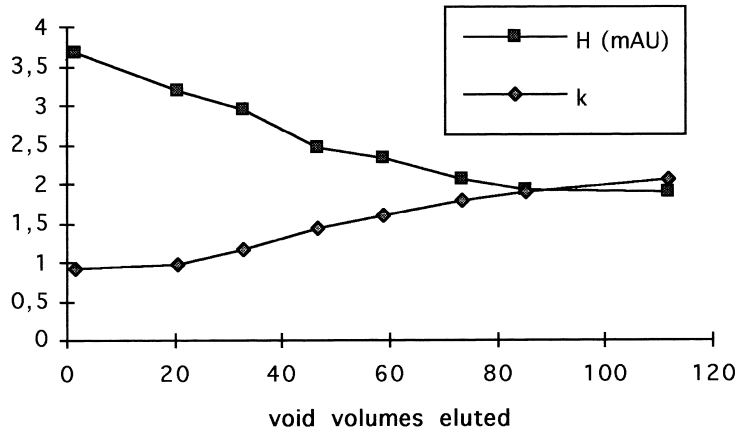

Figure 4. Courses of the capacity ratio (k) and peak heigth (H, milli Absorbance Units) of 2,4-PDA as a function of the eluted number column volumes of copper containing eluent. Condition: column, Polyspher RP-18 15 cm x 4.6 mm I. D.; flow rate 0.8 mL/ min; injections of 2.5 μL of 2,4-PDA (0.31 mg/mL) in 81.6 mM phosphate buffer, pH 7.2, containing copper ions 200 ppb - methanol, 85:15, v/v.

Moreover, such copolymers have been predicted and found to contain polar impurity surface sites[23] and it has been noticed that, for high ionic strength, the ionic concentration inside the resin may eventally be higher than that in the eluent.[24] Hence, even if only C-C and C-H bonds are present in stationary phase matrix, aromatic backbone may adsorb metal ions thereby dynamically generating active sites for complexing compounds.

On the basis of the mixed adsorption mechanism on the stationary phase, we investigated the effect of switching from the mobile phase containing no added metal ions ("blank" eluent which the column was first equilibrated for 1 h with) to the same eluent containing copper (II) ions at 200ppb level.

As it is shown in Figure 4 for 2,4-PDA, a progressive increase of retention can be observed; it may also be noted that the peak height continuously decreases. The poor efficiency arises from slow rate of desorption of ligands strongly bound to metal sites.

Since equilibrium was obtained after *ca.* 120 void volumes were eluted, quantitative evidence was obtained to support the hypothesis that a progressive sorption of copper ions onto the stationary phase resulted in the chromatographic performance loss.

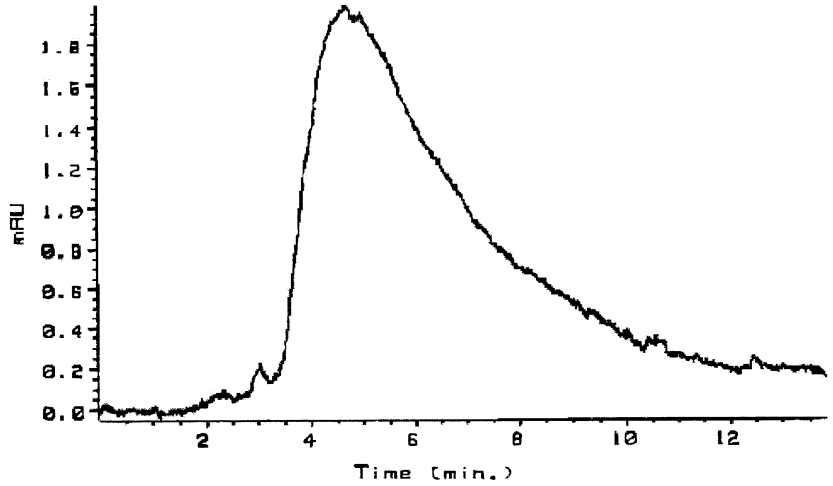

Figure 5. Influence of copper ions in the eluent on the chromatographic peak of 2,4-PDA. Condition: column, Polyspher RP-18 15 cm x 4.6 mm I. D.; flow rate 0.8 mL/ min; injections of 2.5 μL of 2,4-PDA (0.31 mg/mL) in 81.6 mM phosphate buffer, pH 7.2, containing copper ions 200 ppb- methanol, 85:15, v/v.

At the selected pH, bidentate PDAs give complexes[25] of the kind $[Cu(PDA)^{3}]^{4-}$, when the mole ratio between copper and the selected ligand is less than 0.05, as in our case. A complexation reaction in the mobile phase would not explain the observed increase in retention, since the reaction product would be in a higher charge status, hence a decrease in retention should be observed.

As Figure 5 underlines, the peak shape 2,4-PDA eluted with the copper containing mobile phase is less L-shaped and asymmetric (AF= 6.0) than that eluted by the same eluent containing no added metal (AF=11.1) which is shown in Figure 3, panel B. Even if surprising, this is not unexpected since when the contribution of the slow sites becomes small, as in the latter eluent, the upper part of the band becomes narrow and significant tailing appear only close to baseline;[19-20] on the contrary, the increased number of selective sites which are progressively generated when the column is eluted with the copper containing eluent, decreases the concentration overload at these sites and this results in a lower AF.

However, at the same time, the addition of copper ions to the eluent results in a strong efficiency loss which impairs the performance of the chromatographic peak of 2,4-PDA: this can be explained by the slow mass transfer kinetics of the dynamically generated ligand selective sites.

Table 3

Influence of Copper Ions in the Eluent on Retention Moduli*

Analyte	Retention Modulus[a]
2,4-PDA	3.80
3,4-PDA	1.05
Benzoic Acid	1.03
Acetone	1.01
Benzilammine	0.99

*Conditions: column, Polyspher RP-18 15 cm x 4.6 mm I.D.; flow rate 0.800 mL/min; injectons of 2.5μL of 2,4-PDA (0.31 μg/μL), 2.5 μL of 3,4-PDA (0.32 μg/μL), 25 μL benzoic acid (0.31 μg/μL), 25 μL of acetone (0.79 μg/μL) and 25 μL of benzylamine (0.98μg/μL) in 81.6 mM phosphate buffer pH 7.2 - methanol 85:15, v/v. See text for explanation of retention modulus. [a] Mean values for duplicate injections.

Table 3 details the consequences of copper addition to the eluent on the chromatographic behavior of non-metal chelating analytes. The enhancement in retention upon copper presence in the eluent is conveniently expressed by the modulus, η, that is defined by

$$\eta = k/k_0$$

where k and k_0 are the retention factors of the tested analyte with and without copper ions in the eluent both measured under otherwise identical conditions. It is evident that non metal chelating analytes are not influenced by the copper presence in the eluent.

However, it was intended to do more for a pinpointing of the exact source of complexing compound selective adsorption. The use of the syringe based auto sampler provided by the manufacturer, prevents the contact between the sample and the injection valve body and hence adsorption on potentially active sites, such as the rotor, would not be possible.[26] To investigate the relative importance of the stationary phase and the column walls, we decided to inject analytes in the chromatographic system with the substitution of a capillary open capillary tube (107 cm x 0.25 mm I. D., 316 stainless steel) for the analytical column. We decided to maintain in the capillary open tube the same eluent velocity which was present in the analytical column in order to reproduce the same, if any, influence of the wetted stainless steel column walls and HPLC tubing on the chromatographic performance of metal chelating isomers.

Table 4

Influence of Copper Ions in the Eluent on the Peak Shape of 2,4-PDA Eluted from a Capillary Open Tube*

	Peak Height[a] (mAU)	AF[a]	Half Height Bandwidth[a] (min)
"Blank" Eluent	256 ± 2	1.4 ± 0.2	0.61 ± 0.07
Copper Containing Eluent	254 ± 5	1.5 ± 0.2	0.62 ± 0.04

* Conditions: 107 cm x 0.25 mm I.D. stainless steel capillary in place of the chromatographic column; flow rate 0.062 mL/min; injections of 2.5 μL of 2,4-PDA (0.31 μg/μL) in 81.6 mM phosphate buffer pH 7.2 methanol, 85:15, v/v ("blank" eluent) and in 81.6 mM phosphate buffer pH 7.2 containing 200 ppb copper (II) - methanol, 85:15, v/v (copper containing eluent).
[a] Mean values and standard deviations for triplicate injections.

Table 5

Influence of Copper Ions in the Eluent on the Peak Shape of 3,4-PDA Eluted from a Capillary Open Tube*

	Peak Height[a] (mAU)	AF[a]	Half Height Bandwidth[a] (min)
"Blank" Eluent	134 ± 3	1.7 ± 0.2	0.56 ± 0.06
Copper Containing Eluent	135 ± 4	1.7 ± 0.2	0.57 ± 0.05

* Conditions: 107 cm x 0.25 mm I.D. stainless steel capillary in place of the chromatographic column; flow rate 0.062 mL/min; injections of 2.5 μL of 3,4-PDA (0.32 μg/μL) in 81.6 mM phosphate buffer pH 7.2 methanol, 85:15, v/v ("blank" eluent) and in 81.6 mM phosphate buffer pH 7.2 containing 200 ppb copper (II) - methanol, 85:15, v/v (copper containing eluent).
[a] Mean values and standard deviations for triplicate injections.

As may be seen from the comparison of numerical data for 2,4-PDA and 3,4-PDA in Table 4 and 5 the chromatographic peak shape is not strongly compound dependent and the performance of the complexing isomer is not worse than that of non chelating ones. It is clear that system design sources of

tailing[27] are almost uninfluential. Moreover, a comparison of the asymmetry factors obtained for the same conditions with the analytical column (Table 1), underlines that, while the skewness of the chromatographic peak of 3,4-PDA did not appreciably change, the performance of the metal chelating 2,4-PDA was widely improved when the capillary open tube was used, thereby indicating that the outstanding tailing source was mainly in the stationary phase and not in the wetted stainless steel parts of the chromatographic equipment.

The capillary open tube was then equilibrated with the copper containing eluent. As it is shown in Table 5 and 6 respectively for 2,4-PDA and 3,4-PDA, peak heights, widths, and asymmetry, with or without copper ions in the eluent, are not statistically different at the 95% confidence level. No trends in peak height or width (i.e. continual decrease or increase) was observed, on the contrary of that shown in Figure 4. We can interpret these data as indicating that the stainless steel wetted surfaces can not play a significant role in adsorption of copper ions and hence in ligand selective sites generation.

In order to definitively demonstrate the sorption of metal ions onto the C18 functionalized SDVB stationary phase we injected 25μL of chromium (III) 1000ppm first in the column conditioned with the mobile phase containing no added metal ion and then equilibrated with the same mobile phase deliberately contaminated with all the most common transition metal ions at 100 ppb level each. We choose chromium (III) as test ion because of its good absorbing properties in the visible range: the detector was operated at 430nm to deconvolve its absorption from those of the nitrate counter ions. As it is shown in Figure 6 an improvement in peak shape and an increase in peak area can be observed with the metal containing eluent. This was interpreted to further support the sorption properties of the stationary phase: when the column was conditioned with the metal containing eluent, the sample was eluted with a better peak shape since metal ions in the eluent compete with chromium ions for the binding with the stationary phase.

CONCLUSIONS

For metal chelating compounds kinetic tailing and other chromatographic anomalies can be observed when metal impurities are present in the chromatographic system and a strongly hydrophobic and non ionic stationary phase is used.

Two hypotheses can be taken into account to explain their origin: 1) the complex form prior to sorption, in the mobile phase; 2) metal ions are first sorbed onto the stationary phase thus generating highly selective, active sites for complexing compounds. It can be suggested that both occur and the extent to which one is more significant than the other depends on the metal and complexing analyte nature and concentration, on how rapid the rate of equilibration in the mobile phase is compared to the time scale of the

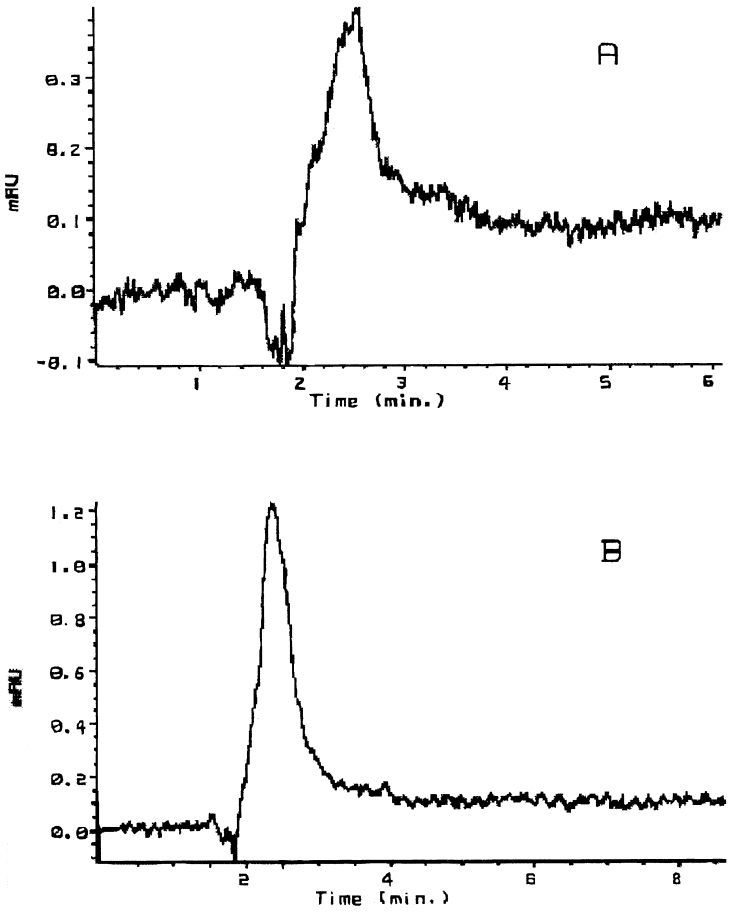

Figure 6. Influence of metal ions in the eluent on the chromatographic peak of Cr (III). Condition: A: column, Polyspher RP-18 15 cm x 4.6 mm I. D.; flow rate 0.8 mL/ min; injections of 25 µL of Cr (III) (1000 ppm) in 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15, v/v. B: as A except the buffer contains the following metals at a concentration of 100ppb each: chromium (III), iron (III), copper (II), manganese (II), nickel (II), lead (II), zinc (II).

chromatographic separation. We gave evidence that, for the range of compounds we tested, the second hypothesis is the correct one and, since retention is a function of strong complexing interactions and van-der-Waals forces the mixed retention mechanism is best described as one of "metal interaction."

It is worthwhile to underline that the experimental results herein described beg the question of the real possibility to achieve pure reversed phase interactions, without any mixed mechanism, also when the base material is itself strongly hydrophobic and display no ionic character whatsoever.

ACKNOWLEDGMENTS

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