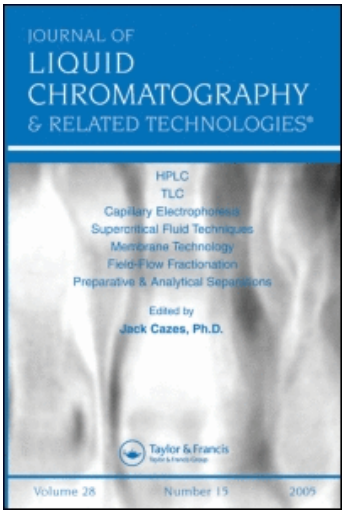

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 22 February 1999

To cite this Article Cecchi, T. , Pucciarelli, F. , Passamonti, P. and Ferraro, S.(1999) 'INFLUENCE OF METAL IMPURITIES SORPTION ONTO A SILICA BASED C_{18} STATIONARY PHASE ON THE HPLC OF METAL CHELATING ANALYTES', Journal of Liquid Chromatography & Related Technologies, 22: 3, 429 — 440
To link to this Article: DOI: 10.1081/JLC-100101670
URL: http://dx.doi.org/10.1081/JLC-100101670

J. LIQ. CHROM. & REL. TECHNOL., 22(3), 429–440 (1999)

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ABSTRACT

The chromatographic performance of metal chelating analytes on a C_{18} silica based stationary phase was studied. The influence of metal ions impurities on their peak shapes and the metal ions exchange properties of such a stationary phase were investigated. Evidence was obtained to support the proposition that sorption of metal ions onto the stationary phase base 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".

INTRODUCTION

The use of metal-modified silica[1-7] or mobile phase[8-9] has often attracted interest as a possible way to achieve highly selective separations The drawback of this approach may be poor chromatographic performance if the rate of desorption of ligands strongly bound to metals is slow compared to the

chromatographic process time scale. Rapid adsorption-desorption kinetics, which are a prerequisite for high chromatographic efficiency, can be achieved only for weaker binding metals.[8] On the other hand, even in absence of metal assisted chromatography, rationalization of the puzzling retention behaviour of metal chelating substances has been a particular challenge and a long time burden[10-11] to the separation scientist since the development of HPLC analytical methods for them has often met with difficulty due to poor chromatographic efficiency and severe band asymmetry.[12-18]

A number of strategies have been attempted to improve their peak shape. 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,[17] on analyte chromatographic performance, has been minimized by using a related or unrelated complexing agent in the mobile phase[12-19] to prevent complexation of the analytes with metal cations or by pre-equilibration of the column with a proper complexing compound,[20-21] to pre-saturate the analyte binding.

Pyridinedicarboxylic (PDAs) and pyridinecarboxylic (PMAs) acids can be considered highly eligible test substances in the chromatographic study of such kind of analytes because the performance of chelating isomers is much poorer than that of isomers whose complexing properties are low or non-existent.[22-23]

Since good chromatographic performance is a prerequisite for high sensitivity, experiments designed to shed light on the chromatographic behaviour of the selected model compounds are described.

It is the aim of this contribution to present and asses the metal ion impurities exchange properties of the silica based reversed stationary phase and to gain insight into the resulting mixed retention mechanism for metal chelating analytes. Some hypotheses on the genesis of bad peak shape, tailing, and other chromatographic anomalies are proposed.

EXPERIMENTAL

Apparatus

A Varian high pressure liquid chromatograph model 5000 equipped with a Rheodyne sample valve injector with 50 μL loop (Model 7125) was used. A Hewlett Packard 8452A diode array spectrophotometer equipped with a 30 μL flow cell (10 mm optical path) and with external computer control (HP 89531A MS-DOS - UV/VIS operating software) was used as detector.

The analyses were run at room temperature under isocratic elution conditions. The analyses were run at ambient temperature under isocratic elution condition. The detector was operated at 270 nm.

Chromatographic Conditions

All experiments were carried out with a commercial stainless steel column (25 cm x 4.6 mm I.D.), packed with 5 μm Res Elut 5 C_{18}, for reverse phase chromatography, purchased from Varian. The eluent flow-rate was 0.9 mL/min. The eluent systems consisted of a) 153.2 mM phosphate buffer pH 7.3, b) 153.2 mM phosphate buffer pH 7.3, containing chromium (III), iron (III), copper (II), manganese (II), nickel (II), lead (II), zinc (II) at a concentration of 100 ppb each.

All solutions were filtered through a 0.2 μm pore size cellulose nitrate filter (Whatman).

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 was equilibrated with the uncontaminated solvent system to be used for one hour. 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 the isomers of pyridinedicarboxylic acid (3,4-, 2,5-, 2,6-, 3,5-, 2,3- and 2,4-PDAs - 2,4-PDA was in the monohydrate form) and pyridinecarboxylic acid (2-, 3-, 4-PMAs), were purchased from Aldrich (Milwaukee, WI, USA); potassium dihydrogen phosphate and disodium monohydrogen phosphate, sodium chloride and each metal standard solutions 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). Test substances were dissolved in the mobile phase and were filtered through a 0.2 μm pore size nylon filter (Lida, Kenosha, WI, USA).

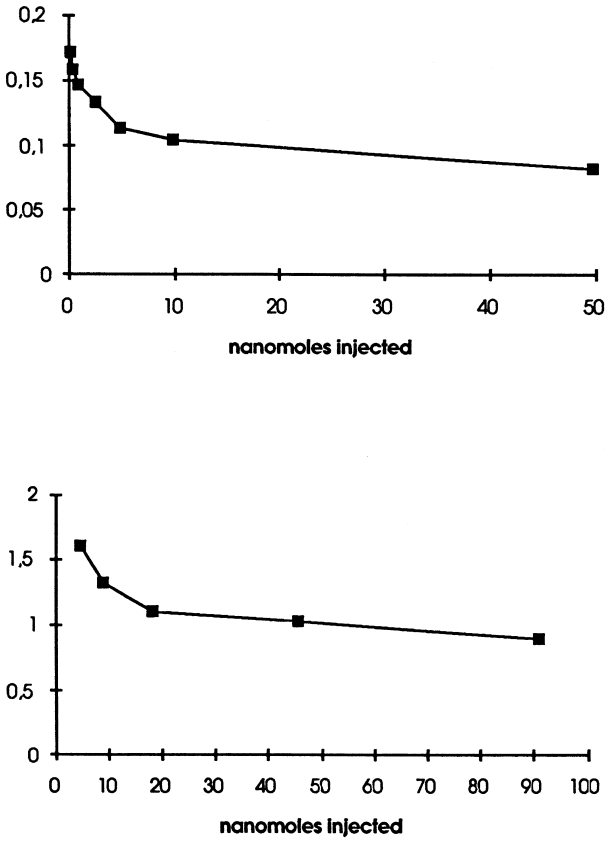

Figure 1. Influence of sample size on retention of 2,4-PDA (0.368 μg / μL, top panel) and 2-PMA (0.448 μg / μL, bottom panel). Conditions: column, Res Elut 5 C_{18}, 25 cm x 4.6 mm I.D. 5 μm (Varian); flow rate 0.9 mL/ min; injections volume, 25.0 μL; mobile phase, 153.2 mM phosphate buffer pH 7.3.

RESULTS AND DISCUSSION

As already demonstrated,[19] severe band asymmetry of the chromatographic peaks of the tested compounds, was related to complex forming properties and not simple silanophilic interactions.

The effect of solute concentration on the retention and on the peak shape of the eluent was investigated to discern between kinetic and simple non-linear tailing. As it can be seen from Figure 1 for 2,4-PDA and 2-PMA, metal chelating isomers showed a progressive increase of retention with decreasing

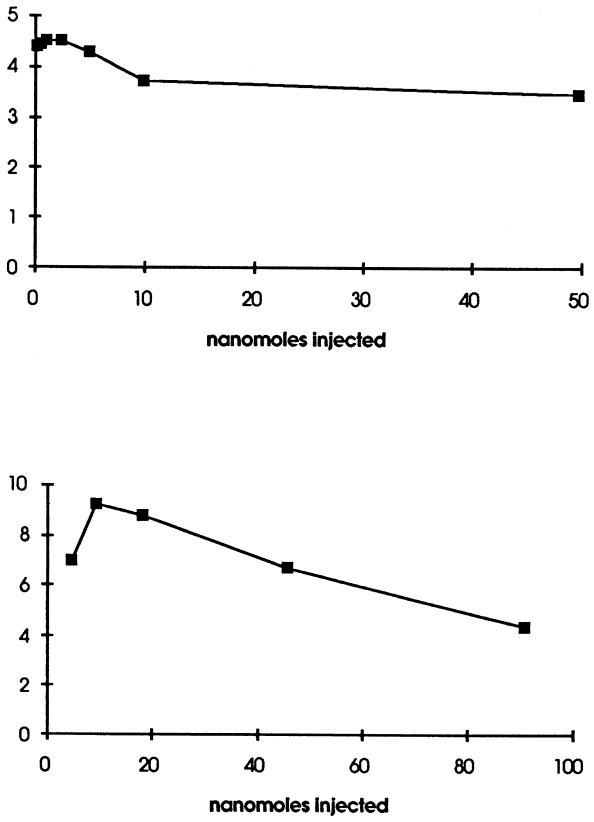

Figure 2. Influence of sample size on asymmetry factor of 2,4-PDA (0.368 μg / μL, top panel) and 2-PMA (0.448 μg / μL, bottom panel). Condition: column, Res Elut 5 C_{18}, 25 cm x 4.6 mm I.D. 5 μm (Varian); flow rate 0.9 mL/ min; injections volume, 25.0 μL; mobile phase, 153.2 mM phosphate buffer pH 7.3.

sample size. While this phenomenon can also be explained by a non linear adsorption isotherm, the course of asymmetry factors outlined in Figure 2 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 not what we observed. The asymmetry factor initially increases and then decreases as the analyte concentration decreases.

Moreover the chromatographic peaks of complex forming analytes were characterised by a progressive loss of column efficiency with decreasing sample size as shown in Table 1. Conversely, non metal chelating isomers did not show

Table 1

Influence of Sample Size on Plate Number*

	Original Sample	1:5 Dilution	1:10 Dilution
N of 2,4-PDA (0.368 μg/μL)	529	469	391
N of 2-PMA (0.448 μg/μL)	582	119	44

* Conditions: Columns: 25 cm x 4.6 mm I.D. 5μm Res Elut 5 C_{18}, Varian (C_{18}-silica). Injection volume: 25 μL; eluent flow-rate: 0.9 mL/min. Eluent: 153.2 mM phosphate buffer pH 7.3.

these anomalies. This underlines that the chromatographic behaviour is largely impaired by the ligand activity and simple nonlinear tailing of the reversed phase sites 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[24] and later re investigated by Fornsted et al.[25-26] It is assumed that the adsorbent surface is covered with a large proportion of non selective sites characterised by low-energy interactions and a relatively scarce number of active sites. These "tail producing sites" involve specific interactions, including complexation.[25] Since the adsorption energy is high these selective sites usually provide slow mass transfer kinetics. Once adsorbed, molecules would be held very tightly until desorption finally occurs into the rear part of the zone: the resulting concentration increase for the back boundary would appear as a tailing phenomenon.

We think that the rationale to account for more than one kind of adsorbing site for complexing test substances on the reversed stationary phase is given by the ion-exchange[27] properties of unreacted silanols which are in the ionized form, its pKa value being 4.5.[28] The affinity of dissociated silanol for metal ions should be higher if compared to that one for hydrogen ions, since the former are in a higher charge status.

Hence, cationic metal impurities can undergo an ion exchange process, thereby, providing the dynamic genesis of active sites for chelating compounds. The already reported positive influence of the eluent pH increase on the

chromatographic performance of metal chelating test substances[16,19] confirms our hypothesis, since a pH increase leads to the formation of hydroxo-complexes thereby reducing free metal activity.

The effect of the "tail-producing site" is easily predicted to increase analyte retention with decreasing sample size and this effect, as evidenced in Figure 1, may not be so little as previously predicted:[26] 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 kinds 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.

Another point of interest which comes out from the comparison of Figure 1 and 2 is the continuos increase of retention concomitant with the final asymmetry factor decrease. This can be explained, again, by a mixed retention mechanism according to which retention switches, as the sample size is reduced, from being dominated by hydrophobic forces to strong, ligand selective interactions. Since the relatively scarce ligand selective sites are characterized by high adsorption energy, they are practically saturated even at low sample sizes, and may operate under nonlinear conditions even if the reversed phase sites operate under linear conditions.[25] The further decrease of sample size lowers the concentration overload of the active sites and this results in a further increase of retention together with a final improvement in peak shape.

The kinetic origin of tailing is confirmed by the influence of eluent velocity on the peak asymmetry. Kinetic tailing is expected to be especially prominent at high flow rate; on the converse, non linear tailing will show little difference.[29] It was found that the mean value of the asymmetry factor, for duplicate injections of 25 μL of 2,4-PDA (0.368 μg / μL), changed from 3.47 to 3.29 when the flow rate was reduced from 0.9 mL/min to 0.5 mL/min and the same mobile phase (153.2 mM phosphate buffer pH 7.3) was used. It does follow that the tailing has a kinetic and not a simple non-linear origin.

The course of retention factors for 2,4-PDA and 2-PMA in Figure 1 may be treated as a titration curve[30-31] of the ligand selective sites present in the system. By drawing lines through the first two and the last two points in the plot, the end point is given by the intersection. Hence, an estimate of the upper limit for the total number of sites prone to metallophilic interactions with chelating compounds, when the column was equilibrated with the described mobile phase, was *ca* 2 nanomoles (nmoles) for 2,4-PDA and *ca* 12 nmoles for

2-PMA. This result is not unexpected. The lack of the negative inductive influence of the second carboxylic group results in a better chelating attitude of 2-PMA respect to 2,4-PDA, hence, the former can "titrate" a higher number of active sites. We, therefore want to underline that, since the tail producing sites are compound selective, their rough quantitation is test compound dependent. Furthermore, it is noteworthy that the higher number of active site "titrated" by 2-PMA, can easily be related to higher asymmetry of the chromatographic peak of the former, when its sample size was similar to that one of the latter (see Figure 2).

Adsorption interactions between the heterocyclic nitrogen electron pair and the iron free d orbitals[32] on the stainless steel parts of the HPLC apparatus can not be ruled out, but the complexing compound specificity (which is not the one expected for heterocyclic nitrogen electron pair and the iron free d orbitals interactions)[32] of the chromatographic anomalies supports the fact that they arise from ligand-metal ions interactions. The reported need for a complexing additive in the mobile phase,[12-19] with respect to peak shape of chelating analytes confirms that the presence of cationic metal species is the cause of the poor chromatographic performance of this kind of analytes.

On the basis of this mixed adsorption mechanism on the stationary phase, we now want to discuss 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 chromium (III), iron (III), copper (II), manganese (II), nickel (II), lead (II), zinc (II).

When metal ions were added at a 10 ppb level, no significant changes of the chromatographic parameters of complexing test substances could be observed, thereby indicating that the buffer metal content ranged in this order of magnitude, as expected according to the declared buffer metal impurities.

In order to stress their influence, each metal ion was then added at a concentration of 100 ppb. As it is shown in Figure 3 for 2,4-PDA, a progressive increase of retention can be observed. Since equilibrium, as evidenced by the achievement of constant retention, was obtained after *ca.* 80 void volumes were eluted, quantitative evidence was obtained to support the hypothesis that a progressive ion exchange process of metal ions on residual silanolates resulted in the chromatographic performance loss. The plate number changed from 268 for the non metal modified mobile phase to 64 when the column was fully equilibrated with the metal containing eluent. This strong column efficiency loss, which arises from the slow rate of desorption of ligands strongly bound to metal sites, progressively impairs the performance of the chromatographic peak of 2,4-PDA since the more the column is run with the metal ions containing mobile phase, the more ligand selective sites are generated and dominate retention.

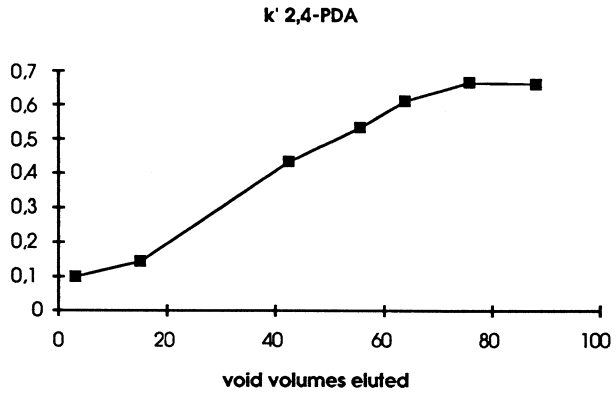

Figure 3. Courses of the capacity ratio (k) of 2,4-PDA (0.278 μg / μL) as a function of the eluted void volumes of metal ions containing eluent. Condition: column, Res Elut 5 C_{18}, 25 cm x 4.6 mm I.D. 5 μm (Varian); flow rate 0.9 mL/ min; injections volume, 25.0 μL; mobile phase, 153.2 mM phosphate buffer pH 7.3 containing chromium (III), iron (III), copper (II), manganese (II), nickel (II), lead (II), zinc (II) at a concentration of 100 ppb each.

Non metal chelating analytes did not show these chromatographic anomalies. When NaCl was added to the uncontaminated eluent to give the same ionic strength of the metal contaminated mobile phase, the peak shape of metal chelating isomers did not change, thereby indicating that ionic strength increase was not responsible for the observed chromatographic anomalies.

The column, after being conditioned with the metal containing eluent, remains altered in its performance also after the substitution of the non metal containing mobile phase for the metal containing one. To restore the original performance, metals could be displaced from the column by running it with HCl pH 2.7 for half an hour at a flow rate of 0.9 mL/min. This can be interpreted as evidence of a very strong binding of metals on residual silanols. When the free energy favor the solution state, as in the case of metal ions at low pH, the hydrogen ions would successfully compete with metal ions for the residual silanolate groups and metals would be displaced.

CONCLUSION

Two hypotheses can be taken into account to explain the origin of the negative influence of metal ion impurities in the eluent on the HPLC of chelating compounds: 1) the complex form prior to sorption, in the mobile phase; 2) metal ions undergo an ion-exchange process on residual ionized

silanols, thereby 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 ions nature and concentration, on how rapid the rate of equilibration in the mobile phase is compared to the time scale of the chromatographic separation, and on the possibility that species bound to the stationary phase are involved in the complex formation. We gave evidence that, for the range of compounds we tested, the second hypothesis is the correct one when metal ions are present as impurities in the chromatographic system. The results suggest that, since retention of the chelating analytes is a function of strong complexing interactions and van-der-Waals forces, the mixed retention mechanism is best described as one of "metal interaction."

ACKNOWLEDGMENTS

We thank MURST and CNR for financial support.

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Received January 23, 1998
Accepted June 5, 1998
Manuscript 4727

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