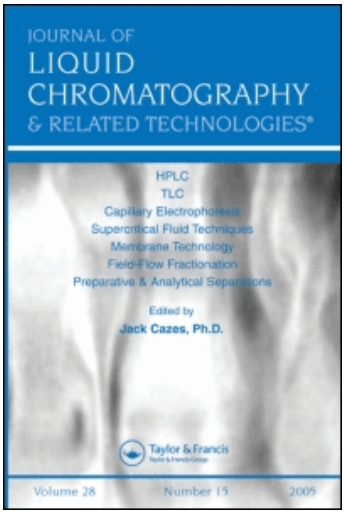

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Sadek, Paul C. , Carr, Peter W. and Bowers, Larry W.(1985) 'The Significance of Metallophilic and Silanophilic Interactions is Reversed Phase Hplc', Journal of Liquid Chromatography & Related Technologies, 8: 13, 2369 — 2386
To link to this Article: DOI: 10.1080/01483918508076576
URL: http://dx.doi.org/10.1080/01483918508076576

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(13), 2369-2386 (1985)

THE SIGNIFICANCE OF METALLOPHILIC AND SILANOPHILIC INTERACTIONS IN REVERSED PHASE HPLC

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ABSTRACT

The poor peak shape encountered for amines in reversed phase high performance liquid chromatography (RPLC) is widely recognized. In order to improve chromatographic efficiency, the mechanism of the broadening must be understood. In addition to the silanol groups on the support surface, metal sites have been implicated as possible adsorption sites for amines.

We have investigated the relative role of metallophilic and silanophilic interactions in the non-ideal behavior of amines and proteins. Stainless steel frits appear to have a deleterious effect on column efficiency via both mechanical and chemical interactions. The use of stainless steel meshes or screens is recommended. The residual metal sites on the support material have been found to play an insignificant role in solute retention or peak broadening. Minimization of silanophilic interactions is therefore the key to increasing efficiency in the separation of basic amines.

 0148-3919/85/0813-2369$3.50/0

INTRODUCTION

Many studies have shown that RPLC of basic amines and of peptides and proteins exhibits less than ideal behavior (1-7). Frequently this non-ideality is observed as extreme tailing of small solutes or as mass recovery of less than 80% for proteins, especially when the amount of protein injected is less than 5 µg (8-16). Hysteresis effects are also commonly observed (17,18), in which repetitive injection of the protein results in a monotonic increase in peak area and peak height which eventually yields reproducible, but incomplete, protein recovery. Such results indicate the presence of a substantial number of irreversible adsorption sites in the chromatographic system. It is important to identify the nature of these sites if they are to be eliminated.

Many researchers attribute the above non-idealities solely to hydrogen bond interaction between the amine moiety and the residual silanol groups on the support surface (3,19-23). If silanophilic interactions are responsible, a number of permanent and dynamic modification schemes have been proposed. Treatment of the base silica before stationary phase bonding by heating and acid washing, and end capping after bonding is frequently used, but results in derivatization of only about 50% of the silanol groups. Perhaps more distressing is the fact that these treatments tend to degrade with column use, faster than loss of column efficiency for common test probes. Amine compounds, buffered at low pH in the mobile phase, can be used as dynamic masking agents (26). Multidentate amines have been shown to be stronger blocking agents than monoamines (27,28).

More recently, metallic impurities in the base silica have been proposed as significant contributors to chromatographic non-idealities (29-35). Verzele and co-workers analyzed two representative RP-HPLC packing materials for metal content and found twelve metal species at the 1 ppm level or greater and an additional 23 elements at the 1 ppb level or above (33). These investigators used β-diketones to probe the trace metal content of the

silica surface. They found that acid washing the base silica resulted in lower heavy metal content and better peak shapes for the β-diketones. Unfortunately, since β-diketones are also weak hydrogen bond acceptors, it is impossible to deconvolve the role of silanophlic and metallophilic interactions with these compounds. In addition, the columns used contained stainless steel frits.

Trumbore (33) and Cramer and coworkers (34) used metallophilic compounds to verify the existence of this type of interction. In the former case, up to 20% of an 8 µg injection of ferritin was lost in open stainless steel tubing. Cramer et al. (34) were able to demonstrate both retention and peak shape changes for the iron complexing agent deferoxamine as solute concentration was varied. The source of the iron in the system was not clearly demonstrated. Shih and Carr (35) showed that the column frits produced a serious interference in the separation of metal dithiocarbamates. We have demonstrated previously that a significant loss of protein can occur in stainless steel frits (36).

The present study was undertaken to evaluate the relative importance of metallophilic and silanophilic interactions in peak tailing and hysteresis effects.

EXPERIMENTAL

The chromatographic system has been previously described (28). Two 5 cm x 4.6 mm column blanks were packed with Chromegabond C10, 10 µ (ES Industries; Marleton, NJ) using the upward slurry packing technique. Methanol served as both the slurry and the packing solvent. One column was fitted with 0.5 µ stainless steel frits (Alltech; Deerfield, IL) and the other with 2 µ stainless steel meshes (Shandon; Sewickley, PA). In order to create effective sealing with the meshes, the column endfittings were altered. The inlet end had both a 2 µ mesh and a Knauer distributor plate (Chromapon; Whittier, CA), the plate being placed between the mesh and the column tubing. The outlet fitting had only the mesh.

The following test solutes were used: benzyl cyanide, anisole, ethylbenzene, chlorobenzene, 2-picoline, pyridine, 2-aminopyridine, phenol, 1-naphthylacetic acid, 4-aminobenzophenone, allantoin, 1-naphthalenemethylamine, benzyl mercaptan, trifluoroacetic acid, 4-dimethylaminopyridine, 1-(2-pyridyl)-piperazine (Aldrich; Milwaukee, WI); nitrobenzene, toluene, 2-nitrodiphenylamine, 2,2'-bipyridine (Eastman Kodak; Rochester, NY); uracil, benzenethiol (MCB Chemical; Norwood, OH); benzene (Baker; Phillipsburg, NJ); butyrophenone (Pierce; Rockford, IL); naphthalene (Fisher; Fair Lawn, NJ); caffeine (Merck; Rahway, NJ); 2,6-diaminopyridine (Reilly; St. Paul, MN); ovalbumin (Sigma; St. Louis, MO); methanol (Mallinckrodt; Paris, KY); glacial acetic acid (Spectrum Chemicals; Gardena, CA); ammonium pyrrolidine dithiocarbamate (Hach; Ames, IA).

Methanol and water were passed through 0.45 μ Zetapor filters (AMF Cuno Division; Meriden, NJ). All solutes were made up in the appropriate solvent. All directly compared results were run with the same sample on the same day. Triplicate injections were done for each sample.

Benzenethiol and benzyl mercaptan were injected onto the column and allowed to interact for various periods of time. The delay time was accomplished through the use of a Rheodyne 7010 bypass valve (Rheodyne; Cotati, CA). After time delay periods had elapsed, the flow was again directed through the column and the solute allowed to elute. The solvent was 60/40 methanol/water with 25 mM acetic acid buffer.

Spectroscopy was done on a GCA-McPherson UV/vis Model EU-701-50, Model EU-721-11 Model sample chamber and a Model EU-701-30 photomultiplier module (GCA; Acton, MA). 2,2'-bipyridine (bipy) and 4-dimethylaminopyridine (DMAP) standards of 1×10^{-5} to 1×10^{-4} M in 10 μM increments were prepared in 45/55 methanol/water. Bipy and DMAP samples were measured at 279 nm and 257 nm, respectively. Stainless steel 0.5 μ frits (six sets of two) and 2 μ meshes (two sets of two) were cleaned in a 50/50 HNO_3/H_2O solution for 15 minutes with ultrasonication. All

sets were thoroughly rinsed and then equilibrated with 3 ml of 6 x 10^{-5} M bipy in 5 ml Falcon 2063 tubes (VWR Scientific; San Francisco, CA). A vacuum was pulled to insure complete solute penetration into the frits. The samples were allowed to equilibrate for 30 minutes with intermittent agitation. Two blanks (no frits or meshes) were treated in the same fashion. The supernatant was removed and the absorbance measured. The same frits were cleaned as above and used for testing DMAP adsorption.

Ovalbumin was used as received (Sigma; St. Louis, MO) and labelled with ^{125}I using iodogen (37). A 200 ng aliquot in water/0.1% trifluoroacetic acid (TFA) was equilibrated with frits and meshes. A vacuum was pulled to insure that liquid penetrated the pores. After 30 min the supernatant was poured off and two one ml rinses with agitation were carried out. All solutions were then counted.

RESULTS AND DISCUSSION

Metallophilic Interactions in Frits

A series of static and dynamic experiments were performed in order to ascertain and verify the extent to which metal sites, present in stainless steel frits and packing material, could affect a chromatographic separation. The static experiments consisted of monitoring the adsorption of two basic amines, known to be good complexing agents, and one protein on stainless steel frits and stainless steel meshes. The dynamic experiments compare two columns packed with the same batch and lot of support material. One column contained the traditional stainless steel frits while the other column was modified to accommodate the low surface area stainless steel meshes.

Static Experiments. Tables 1 and 2 show the results of a one hour equilibration of known amounts of amine and protein with frits and meshes. In the case of bipy, a very strong metal complexing agent, the amount adsorbed is about 14 times greater on the frits than on the meshes. DMAP shows lower overall

TABLE 1. Static Loss of Amines to Frits and Meshes[a]

	bipy		DMAP	
	Ave	nmoles ads	Ave	nmoles ads
New Frits	0.753	12.4	0.374	4.7
New Meshes	0.8045	0.7	0.386	0.8
Used Frits	0.775	7.4	0.371	5.7
Blanks	0.8075		0.3885	
All Frits[b]		9.9 ± 0.4		5.2 ± 0.6
All Meshes		0.7 ± 0.4		0.8 ± 0.6

[a]45/55 methanol water as solvent

[b]average for all six sets of frits

TABLE 2. Static Ovalbumin Adsorption to Frits and Meshes[a]

	total cpm[b]	cpm in orig. tube	cpm in rinse	cpm ads	% sample ads	pmoles ads
Frits	78752	2273	68231	8248[c]	10.5	0.46
Frits	80816	4302	68517	7997[c]	9.9	0.44
Meshes	78744	2020	75907	847	1.1	0.05
Meshes	76202	1691	73585	681	0.9	0.05

[a]20 µl of 1 mg/ml ovalbumin + 2 ml H_2O/0.1% TFA

[b]Before addition of frits and meshes

[c]Total counts observed were 2902 and 3160 for 1 and 2, respectively. Thick stainless steel frits severely attentuate signal. 72 hours soak in 50/50 HNO_3/H_2O at 60°C recoveries >80% of the counts.

adsorption, but the level is still 6.5 times higher on the frits than on the meshes.

The frits definitely adsorbed more bipy than DMAP, but statistically equivalent amounts of each were adsorbed onto the meshes. Undoubtedly, 316 stainless steel shows metal site heterogeneity, containing mainly the transition metals Cr, Ni, Mo, and Mn. Therefore, the large difference in amount of bipy and DMAP adsorbed on the frits can be interpreted as being due to the presence of a large number of heterogeneous sites of widely different binding strengths. Bipy, a bidentate ligand, can successfully bind to fairly weak sites whereas the monodentate DMAP cannot. No definite conclusion can be made with respect to the metal sites in meshes since the uncertainty in the amount adsorbed is large enough to preclude differentiation between Bipy and DMAP.

The same pattern is found for ovalbumin (see Table 2). Significantly more ovalbumin is adsorbed to the frits than to the meshes. The number of nanomoles adsorbed for either the protein or the small amines indicates much less than monolayer coverage occurs, implying that single or clustered adsorption sites exist at widely separated positions on the surface.

Dynamic Experiments. Eighteen test solutes were used to characterize the fritted and meshed columns (see Table 3). Nine solutes, either nonpolar or aprotic, were chosen to assess the reproducibility of the packing procedure. Through the comparison of the number of plates generated over the range of k' values (0 to 6.5), it is apparent that the meshed column is substantially more efficient than the fritted column at k' values less than 2. The A/B parameter was significantly larger on the fritted column than on the meshed column. The number of plates generated (N) by solutes whose k' is less than two is substantially larger for the meshed column. These differences are not due to extra-column effects since the added volume generated by the frit is a very small percentage of the total column volume. It would be unlikely for the mesh and frit to cause such a significant difference in the packing structure at the outlet of the column, but a signifi-

TABLE 3

Chromatographic Characteristics of Solutes*

		A/B[a]		$w_{1/2}$[b]		N[c]	
	k'	Frit	Mesh	Frit	Mesh	Frit	Mesh
Uracil	0.0	1.70	1.05	3.93	3.04	110	177
Allantoin	0.01	1.27	0.64	4.03	3.40	520	11850
Caffeine	0.15	1.33	0.64	3.97	3.37	11530	15871
Benzyl Cyanide	0.76	1.33	0.86	5.03	4.73	16740	18460
Nitrobenzene	1.46	1.53	0.95	6.48	6.10	19740	21430
Anisole	1.92	1.47	0.93	6.73	6.35	25790	28170
Benzene	2.00	1.43	0.88	6.57	6.47	28480	28810
Butyrophenone	3.07	1.49	0.86	8.97	8.90	28160	27940
Toluene	3.36	1.54	0.92	8.75	8.97	33880	33870
Chlorobenzene	3.80	1.84	1.12	9.77	9.45	32960	33990
Naphthalene	6.18	1.25	0.82	13.96	13.80	36180	35210
Ethylbenzene	6.46	1.09	0.82	14.00	13.94	38600	37170
2,6-Diaminopyridine[d]	0.45	4.85	4.75	23.07	14.87	650	1240
Pyridine	0.52	2.53	1.40	6.37	5.57	6960	8930
2-Aminopyridine	0.56	5.18	5.48	16.63	10.83	1270	2520
2-Picoline	0.88	5.20	3.28	11.37	8.03	3730	7070
4-Aminobenzophenone	1.01	1.31	0.79	5.57	5.50	17080	17950
2,6-Diaminopyridine[e]	1.56	4.27	4.60	33.63	33.33	820	730
1-Naphthylene-methylamine	6.75	5.85	5.73	99.83	79.80	210	310
2-Nitrodiphenylamine	8.98	1.43	1.02	9.63	0.47	36100	32630

*Mobile phase was 60/40 methanol/water; flow rate 1 ml/min. The k' values are the average for both columns.
[a]Asymmetry factor
[b]Peak width at half height (mm at 5 cm/min)
[c]Plates/meter
[d]2.5 mM
[e]0.5 mM

cant difference could occur at the head of the column. The frit may be much less effective at the mechanics of spreading the solute plug than the mesh/distributor plate combination.

In general, the basic amine solutes also show improved chromatographic efficiency (i.e., N increases) at low k' values when the

mesh is used. Since removal of the frit is the only significant change in the system, the decreased chromatographic efficiency for both nonpolar and amine solutes must be due to the presence of the frit. Unfortunately, the amines which exhibit the largest differences in N all have low k' values so it is difficult to reach a conclusion as to whether the improvement in their N values on the mesh columns is beyond what should be expected due to mechanical improvements alone.

Silanophilic and Metallophilic Interactions in Columns

The effect of solute concentration on the retention and peak shape of the eluite was investigated using columns equipped with frits. Figures 1 and 2 are plots of k' as a function of solute concentration for DAP, phenol, bipy, and 1-(2-pyridyl)-piperazine (PYPIP) with and without 15 μM ammonium pyrrolidine dithiocarbamate (APDC) present in the mobile phase.

The k' of phenol is totally independent of both concentration and of the presence of APDC in the mobile phase. Karger et al. (38), found alkylated phenols to be unaffected by the presence of residual silanol groups. Phenols are not strong hydrogen bond acceptors but they are, however, strong donors. Silanol groups must therefore act as very weak hydrogen bond acceptors.

Both bipy and DMAP show very steep increases in k' as the concentration is lowered below 1 mM. PYPIP shows the same behavior but at a concentration level three-fold higher than bipy and DAP. As stated earlier, silanol groups most likely have a wide range of pK_a values, and presumably hydrogen bond donating capability. Only a small fraction of the total number of residual silanol groups needs to be strong hydrogen binding sites to produce the effects discussed above. For all of the low concentrations of the amines, the addition of APDC dramatically decreased the solute k' values. At the higher concentrations of the amine solutes, APDC has virtually no effect on k'.

Treating Figures 1 and 2 as "titration curves," it is possible to estimate the number of active sites present in the system. The

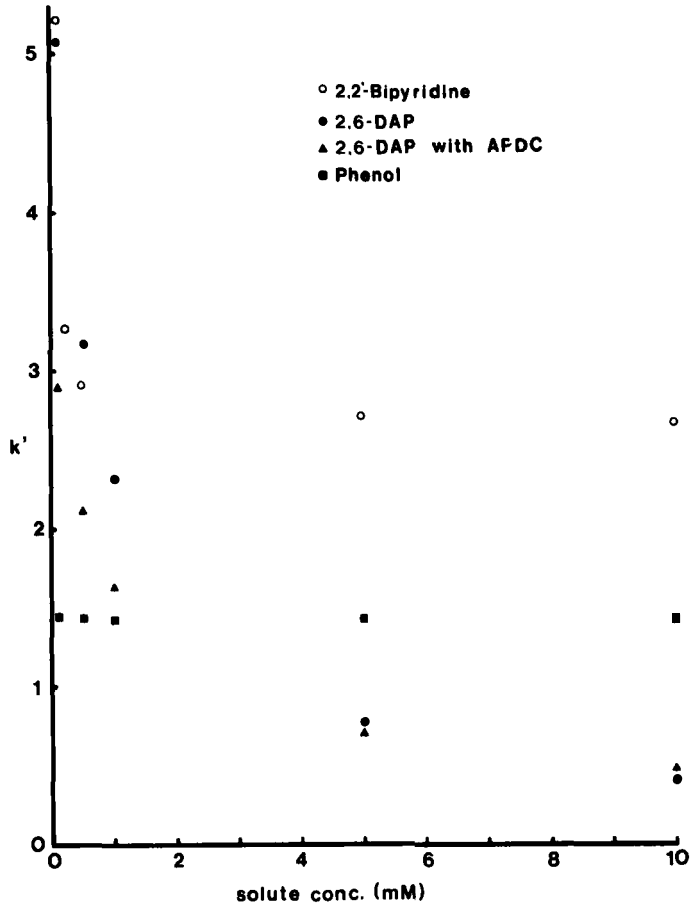

FIGURE 1. Plot of k' versus solute concentration (20 µl of the concentration listed) for various basic solutes and phenol. Solvent: 60/40 methanol/water, flow 1 ml/min. ADPC is present at the 15 µM level where indicated.

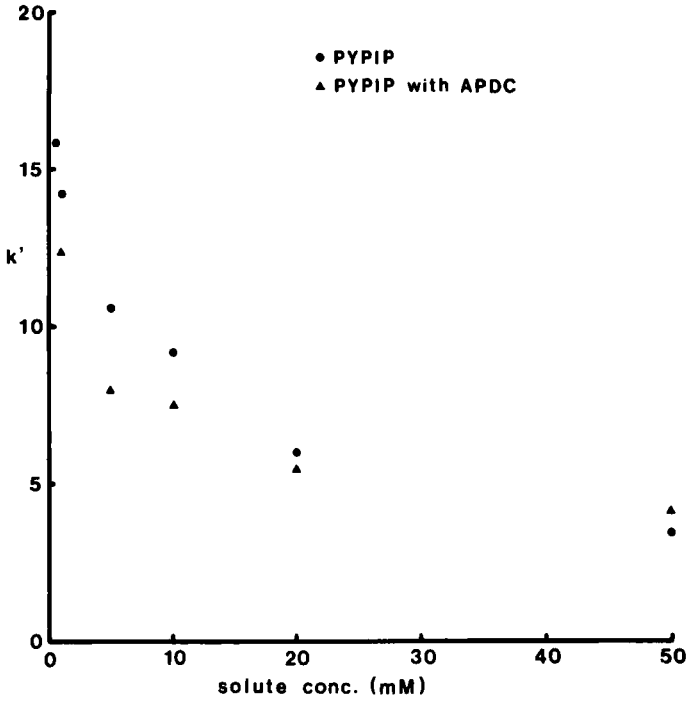

FIGURE 2. Plot of k' versus concentration for PYPIP with and without 15 μM ADPC. Other conditions the same as in Figure 1.

values were obtained by drawing lines through the first two and last two points in the plot and using the intersection as the "endpoint." The results were: bipy -- 12 nmoles; DMAP -- 18 nmoles; PYPIP -- 60 nmoles. These numbers represent the upper limit for the total number of sites. Although these numbers are clearly the same order of magnitude as the static experimental results for metal frits, it is unlikely that the majority of amine molecules which come into contact with the metal sites will have the proper orientation to undergo a strong irreversible interaction. This may not be the case with proteins since they are greatly influenced by surface sheer (39) and may easily undergo multiple interactions at the frit surface. It is unclear from the

present studies whether the frits or the packing material were responsible for the tailing and adsorption.

Metallophilic Interactions

In order to determine the significance of the metal sites in the base silica in solute retention, two strong metal complexing compounds, benzenethiol and benzyl mercaptan, were used. Aryl thiols, like phenol, are weak hydrogen bond acceptors, thus any peak distortion should be solely the result of metallophilic interactions. Injection of benzenethiol resulted in a peak shape similar to that of the nonpolar solutes in Table 2. Benzyl mercaptan behaved in a similar manner.

To exclude the possibility that the peak shape distortion was masked by slow interaction kinetics, a series of injections were made after which the flow was diverted around the column to allow 5, 15, 30 or 60 minute contact times. After the delay, mobile phase flow was again directed through the column. There was no change in the k' of 2.1 regardless of the length of the contact interval. The k' was also unaltered down to a concentration fourfold smaller than that used for phenol or the amine compounds in the above experiments. The peak height decreased monotonically with increasing contact time, but peak area remained constant, as shown in Figure 3. Similar results were observed for benzyl mercaptan, an even stronger metal complexing agent. We attribute this broadening to longitudinal diffusion. Packing material slurried with benzenethiol for 48 hours also failed to show the characteristic blackening observed for thiol/metal complexes.

If irreversible adsorption at metallic sites were to occur, a hysteresis effect with an increase in peak area with repeated injections should result. Within the reproducibility of the measurements, this was not observed for triplicate injections regardless of the delay time. The fact that no trend was observed on increasing contact times eliminates the possibility of a relatively slow adsorption process masking the irreversible desorption. For a slow desorption process, peak tailing would be

expected for a peak with a k' of 2.1. It is clear from Figure 3 that no significant tailing occurred. In the limit of extremely slow desorption, the peak area should decrease with increasing contact time. Since this was not observed, any irreversible adsorption sites present are not detectable by chromatographic experiments. Thus, for a strong metal complexing agent, there does not appear to be a metallophilic interaction with the packing material which results in non-ideal behavior.

CONCLUSIONS

There are a number of components in an HPLC column which affect chromatographic behavior. The effect of the bed terminating device, frits or screens, is evidenced in two ways. First, the efficiency for non-polar or apolar compounds improves, probably due to improved deposition of the sample onto the head of the column. This effect was most pronounced for early eluting peaks and in the symmetry of almost all peaks. Second, there does appear to be metallophilic interaction between amine compounds and frits which can broaden and tail the peaks. Again, the effect is most apparent in weakly retained peaks where interaction with other column components is minimized. We feel that screens will in all cases be superior to frits for amine or protein separations (36).

The interactions of amines with the chromatograhic support is complex. The curvature in the k' versus amine concentration plot again illustrates that more than on mechanism is operating. The behavior of amines can be compared to that of alcohols, phenols, and protonated thiols. The latter groups are readily chromatographed by silica-based RP-HPLC. The fact that all of these groups are poor hydrogen bond acceptors but good donors implies that silanol groups are strong hydrogen bond donors and poor acceptors, as would be expected from their Bronsted acidity. Thus, depending on the pK_a of the silanol groups and the mobile phase pH and ionic strength, both hydrogen bonding and ionic interactions can occur with amine functional groups.

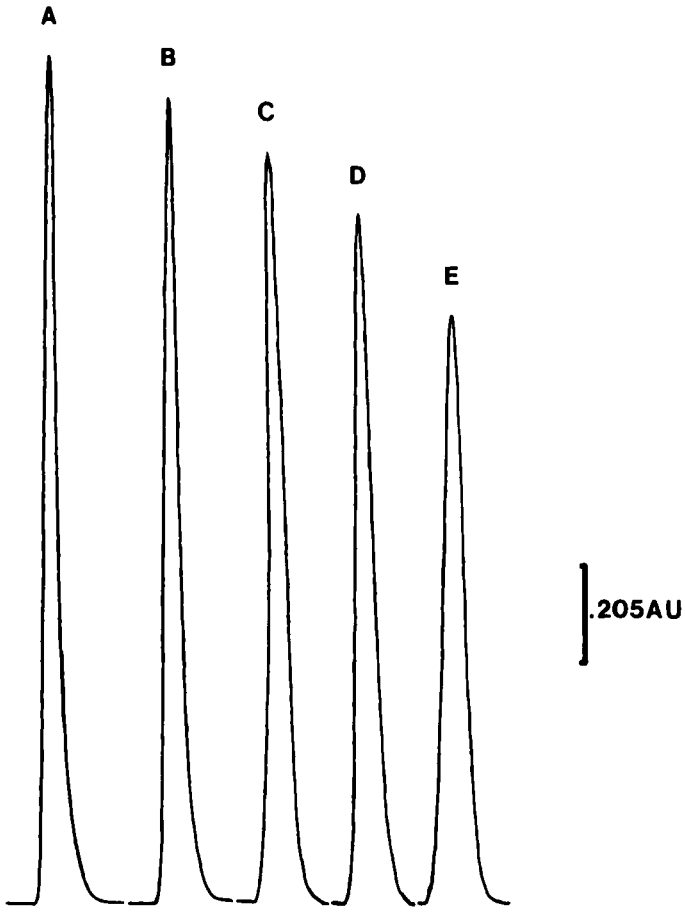

FIGURE 3. Peak profiles for benzenethiol eluted with 60/40 methanol/water, 25 mM acetic acid. Flow rate was 1 ml/min. The delay times are: A = 0 (directly eluted); B = 5 min; C = 15 min; D = 30 min; E = 60 min.

Finally, we have shown metallophilic interactions on the packing material to be of little significance for the majority of compounds. This may be the result of the presence of passive species on the surface, physical inaccessibility of the metal sites, or a surface concentration too low to be of any consequence in the separation process.

From the above results, the retention mechanism of amines and other hydrogen bond accepting species in RPLC is a convolution of only hydrophobic and silanophilic interactions. The relative contribution of these processes to the peak shape and retention is determined by the silanophilicity of the solute, the concentration of the solute, and the concentration of residual silanol groups on the surface.

ACKNOWLEDGEMENTS

We wish to thank the 3M Company for the use of their laboratory for the radioactive work. This work was supported by a National Science Foundation grant (CHE-8205187).

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