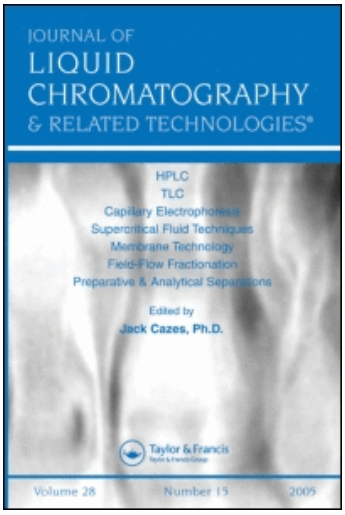

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REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING CARBOXYLIC ACIDS IN THE MOBILE PHASE

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ABSTRACT

This report describes the use of different carboxylic acids as mobile phase modifiers. The effect on retention of acid chain length, pH, and eluent composition for a series of phenylalkanols, phenol, and the amines aniline, N-methylaniline, and benzylamine is discussed. The retention of both neutral and positively charged compounds is influenced by the dissociation equilibrium of the carboxylic acid in the mobile phase. By using 1-pentanol to coat excess exposed silanol groups on the reversed phase column used, the inflection in the retention of both neutral and charged solutes as pH is changed occurs at the pK_a of the acid in the mobile phase. In addition, by using an acid and amine with the same or similar pK_a values, selective ion-pairing of this pair over others with dissimilar pK_a values can be promoted. Application of this technique to the selective retention of amino acids and peptides was unsuccessful.

INTRODUCTION

Ion-pairing partition high-performance liquid chromatography, as introduced by Eksborg and Schill (2,3) has been applied to

numerous separations in recent years (4-6). The theory of the retention mechanism of this process involves various equilibria including ionization of both sample and counter-ion species, partitioning of both species and their ion-pair between stationary and mobile phases, in-situ ion exchange equilibria, adsorption processes, micelle formation, and complexation between the species involved (7). Control of the separation process can be accomplished through careful selection of eluent pH, counter ion concentration, and solvent composition to establish an optimum equilibrium position for separation.

The counter ions used for retention of basic samples have typically been the alkyl sulfonic acids (8-12) or sulfates (13, 14), di- or tricarboxylic acids (15,16), picric acid (17), or perchloric acid (18,19). The pH of the mobile phase has been chosen for protonation of the analyte base and full ionization of the counter ion. By choosing counter ions with low pK_a values, such as the sulfonic acids or alkyl sulfonates, complete ionization could be assured over a wide pH range. Therefore, ion-pairing of a number of basic samples with different pK_a values could be attained in complex mixtures (20-22).

In the present study, we have investigated the use of carboxylic acids as ion-pair reagents under conditions of partial ionization. While the ionized species was expected to act as an ion pairing reagent, it was thought that the neutral species would act as an organic modifier of the mobile phase, similar to methanol or

acetonitrile when used as organic modifiers in reversed phase high-performance liquid chromatography.

EXPERIMENTAL

Materials: Solvents and reagents were gold label spectrophotometric grade and reagent grade respectively, from Aldrich Chemical Company (Milwaukee, WI), and were used without further purification. Columns were 30 x 0.42 mm (i.d.) packed with 10$_\mu$ Sherisorb ODS (Phase Separations, Queensferry, Clwyd., UK) as described below. The chromatographic system consisted of a Waters ALC 202 liquid chromatograph (Waters Associates, Milford, MA, USA), equipped with a model 660 solvent programmer, two model 6000 pumps, a 254 nm differential UV detector, R401 differential refractive index detector, and a U6K 2 mL sample loop injector. Retention volumes were measured by collecting and measuring the eluent at the exit port from the time of injection to the top of each peak using 10 mL graduated cylinders. The void volume was measured using a saturated NaCl/H_2O solution. In general, the void volume was independent of the mobile phase composition, and the standard value of 2.92 mL was used in all calculations. Mobile phases were prepared for a pressurized solvent reservoir (24) in 500 mL aliquots. For all aqueous mobile phases, the organic salt or acid was weighed and diluted to about 450 mL, the pH adjusted to the desired value using 1.0 M HCl or concentrated NH_4OH, and then the solution was diluted to exactly 500 mL. The

pH did not change after this second dilution. When using 1-pentanol stationary phases, the mobile phase was saturated with stationary phase before this second dilution. The 70%/30% (v/v%) H_2O/MeOH solutions were prepared by diluting the organic salt or acid with 150 mL methanol and 350 mL H_2O, and then adjusting the pH. This "apparent pH" was measured using a Beckman Zeromatic pH meter (Fullerton, CA). Mobile phases were degassed for 10 minutes using an ultrasonic bath (L & R Manufacturing, Kearney, NJ) before pumping.

Stationary Phase Coating

Coating with the stationary phase 1-pentanol or 1-octanol was performed using a modified in-situ method of Kirkland and Dilks (25). The column was equilibrated with absolute ethanol, followed by 100 mL of a 10% (vol %) solution of the stationary phase in acetone at a flow rate of 1.0 mL/min. Finally, the mobile phase (aqueous solution) saturated with the stationary phase was pumped through the column at a flow rate of 2.0 mL/min, until a stable baseline was obtained. The passage of about 100 mL of mobile phase was usually required. Control of the temperature was essential, and each mobile phase was kept at the same temperature as the column.

The amount of stationary phase on the column was determined by gas chromatography after elution of the stationary phase with absolute ethanol. A Hewlett Packard HP3380A integrating gas chromatograph (Hewlett-Packard, Palo Alto, CA) was used for analysis.

The loading was 0.095 gram/gram of support for 1-pentanol and 0.077 gram/gram of support for 1-octanol.

Column Packing

30 x 0.42 mm (i.d.) columns were slurry packed as follows. 4.0 grams of dried 10μ Sherisorb ODS was weighed into a 75 mL graduated test tube. 15 mL of dry spectrophotometric grade methanol was added to the support and the mixture shaken for 30 min. on a rotary shaker. This mixture was allowed to settle on an angle and the top layer of methanol was removed by pipette as much as possible. 25 mL of redistilled cyclohexanol was then added to the wetted support, and this mixture placed on a rotary shaker for 30 min. The column interior was cleaned with conc. HNO_3, distilled H_2O, and acetone, and dried in a stream of $N_{2(g)}$. The cyclohexanol-support mixture was degassed in an ultrasonic bath for 8 min. The mixture was then added to a solvent reservoir connected between a model 6000 pump and the column with "no dead volume" fittings between the column and reservoir. The reservoir was completely filled with fresh cyclohexanol, leaving no air in the reservoir. The pump was primed with n-heptane, and this solution pumped through the column and reservoir as quickly as possible maintaining maximum pressure at or near 6000 psi. The column was heated from the bottom as the support was packing with a heat gun (Master Appliance Corporation, Racine, WI) being careful only to heat already packed portions of the column. The packing was completed when pump pressure dropped to normal values

(1000 psi). This packing procedure showed excellent results, with column plate counts in excess of 18200/m, obtained using naphthalene as solute and 80%/20% (v/v%) MeOH/H_2O as eluent.

RESULTS AND DISCUSSION

The chromatographic behavior of phenol and phenylalkanols (i-iii) was investigated as a function of pH in a reversed phase system with an aqueous 0.4 M ammonium acetate solution as mobile phase. The pronounced change in retention as pH is changed is shown in Figure 1.

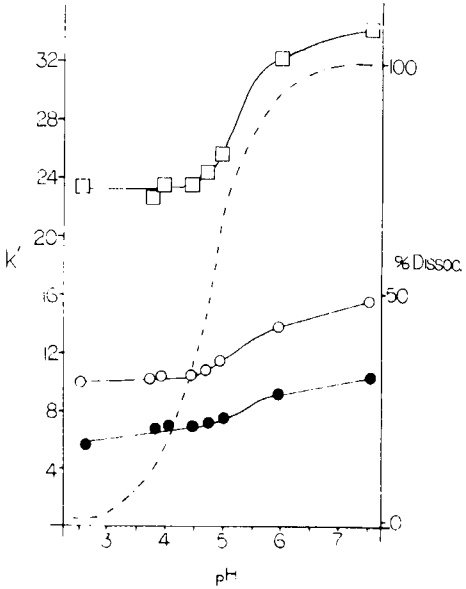


FIGURE 1

Effect of pH on the capacity factor for phenol (●), benzyl alcohol (O), and phenethyl alcohol (□) using a 0.40 M ammonium acetate mobile phase. A calculated dissociation curve for acetic acid is also shown (—·—).

The capacity factor shows a sigmoidal increase with pH and an inflection point at about pH = 5.2. A calculated dissociation curve for acetic acid is also shown, with its inflection point at 4.75. An explanation for this change in retention at or near the pK_a of the organic acid modifier of the mobile phase is found in the degree of dissociation of the acid at various pH's. At low pH, a neutral species exists in solution acting as an organic modifier of the mobile phase and lowering its polarity. At high pH, the carboxylate salt is the predominant species acting to increase the ionic strength of the mobile phase and increase retention of these neutral solutes through a "salting out" mechanism or by increasing the surface tension of the mobile phase (23).

To mimic the behavior of the equilibrium system in Figure 1, mobile phases were prepared containing calculated concentrations of ethanol and ammonium chloride equal to the equilibrium concentrations of acetic acid and acetate ion, respectively, at various pH's. Results for the retention of phenol (i) and phenylalkanols (ii, iii) using these solutions are shown in Figure 2. The shape of the curves and the magnitude of the effect exhibited by ethanol and ammonium chloride are similar to that of the ammonium acetate system.

The same comparison was made for the retention of (i-iii) using a 1-butanol and ammonium chloride system to mimic the retention of valeric acid at various pH values. The curves were also similar in shape and the magnitude of their effect.

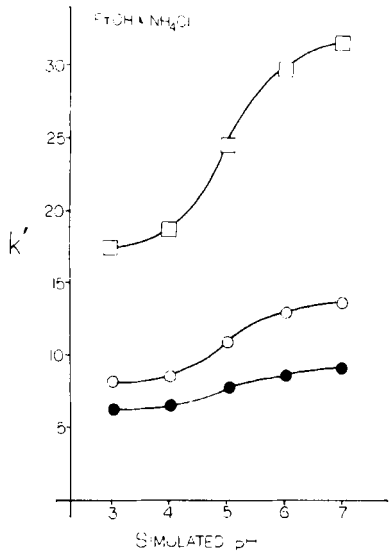


FIGURE 2

Capacity factor for phenol (●), benzyl alcohol (○), and phenethyl alcohol (□) using solutions containing ethanol and ammonium chloride in the same concentrations as acetic acid and acetate ion of Figure 1.

As additional evidence that retention is controlled by dissociation of the acid, the retention of the alcohols (i) and (ii) was examined as a function of pH using a mobile phase containing malonic acid. These results are shown in Figure 3. The curves show two inflection points at pH's of 3.3 and 5.8. These inflection points correspond to the two pK_a's for malonic acid of 2.83 and 5.69 respectively. As before, calculated dissociation curves for the two ionized species are also shown for comparison.

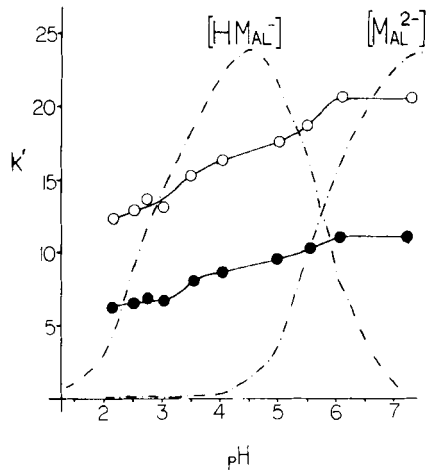
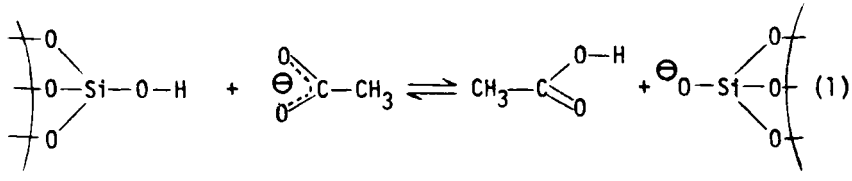


FIGURE 3

Effect of pH on the capacity factor for phenol (●) and benzyl alcohol (O) using an aqueous 0.4 M malonic acid mobile phase. The dissociation curves for the mono and dianion species of malonic acid are also shown (—·—).

The noticable difference between the point of inflection in these graphs, using both malonic and acetic acid, and the pK_a values for these acids can be explained by the acidity of residual exposed silanol functions on the column which supress the ionization of the acid through their own ionization, as is shown in Equation 1 below.



Coating the column with a 1-pentanol or 1-octanol stationary phase, in addition to the C-18 bonded phase, succeeded in blocking the effect of these acidic silanol moieties and the inflection in the curves for retention of both neutral and positively charged solutes in the presence of carboxylic acids, more closely paralleled the dissociation of the acid used. An example is shown in Figure 4 for the effect of pH on the retention of amines. By using 1-pentanol in the stationary phase, the inflection for the retention of these amines occurs at the pK_a of valeric acid (pK_a = 4.82), which was used in the mobile phase.

The use of 1-pentanol in the mobile phase also serves to solubilize valeric or octanoic acids used as well as maintain a saturated coating on the stationary phase. The effect of a sat-

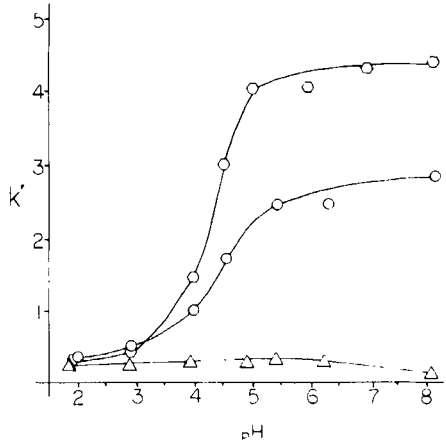


FIGURE 4

Effect of pH on the capacity factor for phenylalanine(△), benzyl amine (○), and aniline (⬡) using an aqueous 0.4 M sodium valerate mobile phase and a 1-pentanol coated stationary phase.

urated pentanol mobile phase alone causes a marked reduction in the retention of all solutes by approximately half their value in a pure water mobile phase, and its effect on the ion-pairing and in-situ ion exchange processes is unknown. However, it does not seem to affect the ionization equilibria of the species in solution as is evidenced by the foregoing data for the pK_a's of the various acids. An attempted use of a nonaqueous solvent system resulted in a suppression of ionization of the acid. When a 70%/30% (v/v%) H_2O/MeOH solvent system was used in conjunction with ammonium valerate as the mobile phase for the separation of phenol and phenylalkanols (i-iii), the inflection in the curve of pH versus k' occurred at about 6.5, and an even greater shift in the dissociation equilibrium was found for acetic acid. Use of a non-aqueous solvent in conjunction with carboxylic acids as mobile phase modifiers is therefore not useful for ion-pairing because too high a pH is needed to produce replete ionization of the acid. Present reversed phase columns preclude the use of pH's above 8.

Finally, it was of interest to explore the case where pK_a's of solute bases were similar to the pK_a of the acid in the mobile phase. From the preceding results for alcohols and amines, one would expect that a maximum in retention can occur at a pH equal to the pK_a of both species. At this pH, a maximum amount of both ionized species of the ion-pair exist in solution. Such a maximum did occur for the retention of amines (vi) and (vii), as is shown in Figure 5. Both aniline (vi) and N-methylaniline (vii)

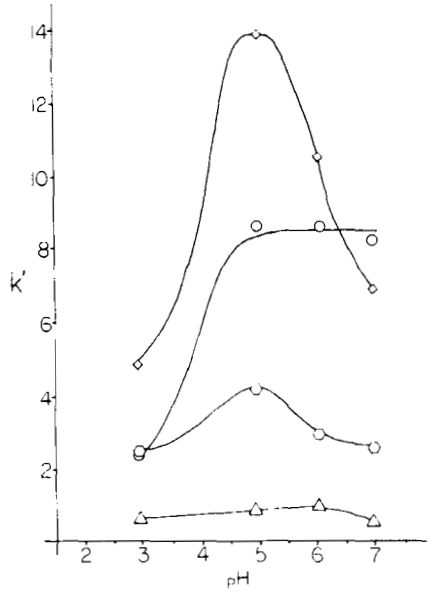


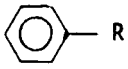
FIGURE 5

Selective ion-pairing using a 10 mM sodium octanoate mobile phase and a 1-pentanol coated stationary phase: (△) phenylalanine; (⬡) aniline; (○) benzylamine; (◇) N-methylaniline.

have similar pK_a's to the pK_a of octanoic acid used in the mobile phase. The pK_a of benzylamine (v), on the other hand, is much higher (see Table 1). Over the entire pH range shown, benzylamine exists as its protonated ammonium species, and therefore it exhibits similar behavior to that discussed earlier, being controlled by the dissociation of the acid. For aniline and N-methylaniline new factors enter into the equilibria. The curves in Figure 5 for these compounds can be thought of as containing three different sections; below, at, and above pH 4.8, which is

TABLE 1

Analyte Species And Their Ionization Constants

cmpd.	name	R	K_a	pK_a
				
i	phenol	OH	1.28×10^{-10}	9.89
ii	benzyl alcohol	CH_2OH		
iii	phenethyl alcohol	CH_2CH_2OH		
iv	phenylalanine	$CH_2CH(NH_2)COOH$	2.63×10^{-3}	2.58
			5.75×10^{-10}	9.24
v	benzylamine	CH_2NH_2	4.67×10^{-10}	9.33
vi	aniline	NH_2	2.34×10^{-5}	4.63
vii	N-methylaniline	$NHCH_3$	1.41×10^{-5}	4.85

the common pK_a of both the bases and the octanoic acid in the mobile phase. At pH's below 4.8, the amines are protonated, and neutral octanoic acid exists in the mobile phase. At pH's above 4.8, the octanoic acid exists as its octanoate ion and the amines are neutral species. At a pH equal to about 4.8, the maximum amount of both ionized species exists in the mobile phase. Maximum ion-pairing of these species at this pH, or maximum in-situ ion-exchange is responsible for the maximum in retention observed. By choosing appropriate acid-base pairs of this type, with common pK_a values, one should be able to selectively promote

ion-pairing of this pair over others in a mixture by adjusting the pH of the eluent.

Attempted use of decanoic acid as a paired-ion reagent failed due to the insolubility of this acid in saturated 1-pentanol or 1-octanol solutions.

Using these same carboxylic acids as ion-pair reagents for the retention of amino acids or peptides was unsuccessful. Examples of these experiments are shown in Figures 4 and 5 for the retention of phenylalanine (iv). In general, these amphoteric compounds were unaffected by the type of acid used or pH, and their retention volumes were short in all cases. This behavior is consistent with the foregoing explanations given for the retention behavior of neutral and charged solutes. These amphoteric species exist as charged solutes at all pH's, and cannot exhibit the "salting out" effect of neutral solutes. Ion-paired species, if formed, contain a second charge and are therefore repelled by the hydrophobic column. Low retention is therefore shown at all pH values.

Current research is underway to develop ion-pairing reagents for amino acids and peptides. It is expected that this technique will prove useful in the separation of compounds on the basis of their pK_a values.

Reversed phase columns with stability at high pH values will be needed for development of the use of alkylamines as organic modifiers of the mobile phase in a partial ionization mode for the separation of ionized acids and other anionic species.

ACKNOWLEDGMENT

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