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ROLE OF BUFFER CATIONS IN THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AROMATIC AMINES*

I. METHANOL-RICH ELUENTS

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SUMMARY

The role of buffer cations in the retention of aromatic amines in reversed-phase high-performance liquid chromatography was investigated. A methanol-rich eluent was used with a LiChrosorb RP-18 column. The eluent contained varying amounts of H_3PO_4, NaH_2PO_4, $NaClO_4$, $KClO_4$ and tetramethylammonium (TMA^+) chloride. Log k' of aromatic amines at constant pH decreases linearly with increasing log (cation concentration). The retention-decreasing effect of inorganic buffer cations increases in the order $H^+ < Na^+ < K^+$, irrespective of the type of organic amine solute. The effect of TMA^+ depends on the structure of the amine solute. The pH of the eluent at constant alkali metal ion concentration also influences the k' of amines. The maximum of the log k' *versus* pH curves could be interpreted by considering the change in the ionization of the surface silanols and the pK-dependent protonation of the amine solutes.

INTRODUCTION

The separation of biogenic amines is an important and frequent task in high-performance liquid chromatography (HPLC). Generally, such separations are effected by reversed-phase (RP)-HPLC with or without ion-pairing reagents. Most aromatic amines, however, tail badly on alkylsilica packings. Numerous masking agents have been used to improve the separations, the most popular being primary, secondary, tertiary and quaternary amines[1-7].

High concentrations of phosphoric acid or dihydrogen phosphate are also used to improve the peak shapes of amines. In examining the separation of catecholamines, Hansson *et al.*[2] noted that the addition of a high concentration of sodium sulphate to the aqueous eluent greatly influenced the k' values and that the nature of the change varied with the different alkylsilica stationary phases. They concluded that

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the general decrease in k' was in agreement with the predictions of the hydrophobic retention model of RP-LC[8]. They ascribed the slight pH-induced increase in the k' of dopamine to a decrease in the degree of protonation of dopamine. However, the addition of methanesulphonic acid at the same pH increased the k', which contradicts the previous explanation (cf., Figs. 4 and 5 in ref. 2).

Hancock et al.[3] studied the retention of various oligopeptides with methanol–water (1:1) as the eluent at pH 4, containing various amines and also Li^+, Na^+, K^+ and Cs^+ as buffer cations. The retention-modifying effect of the cations was attributed to the formation of hydrophilic ion pairs. The effect of buffer cation concentration on the k' values was not studied.

Asmus and Freed[4] used several inorganic and organic medium-to-strong acids as ion-pair reagents to control the separation of catecholamines in pure aqueous eluents. At an acid concentration of 0.1 mole/l and pH 3 the k' values of catecholamines decreased in the order $Cl_3CCOOH > HNO_3 > H_2SO_4 > H_3PO_4 > CH_3COOH$. This order was attributed to the different ion-pairing ability of the anions. The pH of the acidic mobile phase was adjusted to 3 by addition of sodium hydroxide and so, as the acid dissociation constants were different, the eluents contained widely varying amounts of sodium.

Eksborg and Ekquist[5] examined the effect of the tetrabutylammonium (TBA$^+$) cation on the retention of tetracyclines and interpreted the results by the two-site adsorption model[6,7]. When sodium salts were added to the eluent the k' of tetracyclines decreased significantly with increasing salt concentration, and the effect was almost masked when the eluent also contained a comparable amount of TBA$^+$.

The publications of Nahum and Horváth[9] and Bij et al.[10] prompted us to investigate in more detail the role of surface silanols and various additives in RP systems. We noted that when potassium iodide was used for the determination of the dead volume[11] in freshly activated columns[9], the retention times of amines became irreproducible for a fairly long period of time. This led to the investigation of the role of buffer cations in RP systems.

Bidlingmeyer et al.[12] described the separation of organic amines on "naked" silica using aqueous RP eluents. They found that both the ionic strength and the pH of the eluent had a profound influence on the retention of cationic amines, which they attributed to ion exchange with the silanol groups of naked silica.

EXPERIMENTAL

Experiments were carried out with a custom-made liquid chromatograph consisting of a Model OE-312 pump (Labor MIM, Budapest, Hungary), a Rheodyne 7010 six-port injection valve with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a 250 × 4.6 mm I.D. stainless-steel column packed with 10-μm LiChrosorb RP-18 (Merck, Darmstadt, G.F.R.), a Model OE-308 variable-wavelength UV detector (Labor MIM) and a Model A-25 dual-channel recorder (Varian, Walnut Creek, CA, U.S.A.).

The column temperature was maintained at 30.0 ± 0.5°C by a Type U 10 circulating water-bath (MLW, Medingen, G.D.R.) and a water-jacket. The eluents were prepared as described in ref. 13. The column was "activated" prior to use as

described in ref. 9. The amounts of the aromatic amines injected were kept as small as possible by working at the highest sensitivity setting of the detector.

The pH of the eluent was measured with an OP 208 precision digital pH meter (Radelkisz, Budapest, Hungary) and a combined glass electrode calibrated with pH 4.0 and 7.0 aqueous buffers.

The chemicals were obtained from Reanal (Budapest, Hungary) and were used without further purification.

The solutes studied and their pK values are listed in Table I.

TABLE I

AROMATIC AMINES TESTED AND THEIR pK VALUES[14]

Amine	pK	Amine	pK
3,4-Dichloroaniline	2.9–3.0	N,N-Dibenzylamine	8.5
4-Chloroaniline	4.0	Benzylamine	9.3–9.6
Aniline	4.6	N-Methylbenzylamine	9.6
N,N-Dimethylaniline	5.1	p-Nitro-α-aminoacetophenone	>8
N,N-Diethylaniline	6.6		

RESULTS

As silanophilic interactions are said to be the strongest in water-lean eluents[9,10], the experiments discussed here were carried out with a 94% (v/v) methanol-containing aqueous–organic eluent.

At first, the concentration of orthophosphoric acid (H_3PO_4) was varied in the range 0–50 mmole/l to see whether the retention behaviour of aromatic amines followed the pattern noted by Asmus and Freed[4] for catecholamines. The log k' values of both low and high pK aromatic amines are plotted against the eluent concentration of H_3PO_4 in Fig. 1. The apparent pH values, which were measured in the eluent, and which varied between 3.0 and 4.2, are also shown in Fig. 1. With high pK amines, i.e., protonated over the entire range tested here, log k' decreases with increasing H_3PO_4 concentration. For low pK amines the retention at first increases with increasing H_3PO_4 concentration (decreasing pH), then it starts to decrease. These observations may be interpreted by considering the change in the degree of protonation of the silanol groups and the amines. The pK of the silanol groups of naked silica in aqueous solutions is about 6.5 (ref. 15). With increasing pH the silanol groups of the alkylsilica packing behave as the functional groups of a weak cation exchanger: the ion-exchange capacity of RP-18 silica increases with increasing pH[16].

For high pK amines, i.e., fully protonated amines (Nos. 4–6), an increasing acid concentration causes a monotonous decrease in the retention, because the number of available ion-exchange sites decreases with increasing H_3PO_4 concentration. For the low pK amines (Nos. 1–3), an increasing acid concentration (decreasing pH) increases the degree of protonation, so the retention increases. However, above 25 mmole/l H_3PO_4 the dissociation of surface silanols decreases, i.e., the number of available ion-exchange sites decreases, resulting in a net decrease in log k'.

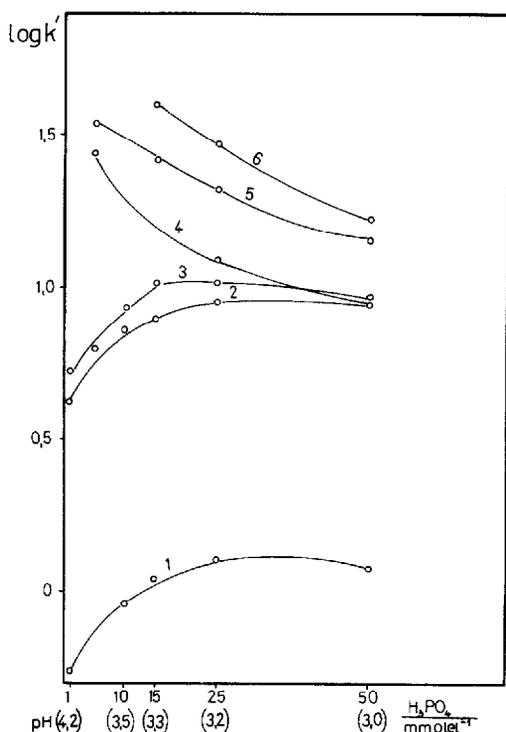The unexpected retention order of benzylamine and dibenzylamine, the latter

Fig. 1. Retention of aromatic amines as a function of the H_3PO_4 concentration of the eluent. Eluent: 94% (v/v) methanol, 1–50 mmole/l H_3PO_4; pH 4.2–3.0. 1, 3,4-Dichloroaniline; 2, 4-chloroaniline; 3, aniline; 4, dibenzylamine; 5, benzylamine; 6, p-nitro-α-aminoacetophenone.

being more retained, also suggests that hydrophobic effects are of secondary importance. (The observed retention order also excludes the likelihood of hydrophilic ion-pair formation with $H_2PO_4^-$ as a retention-governing mechanism.)

To shed light on the role of phosphate ion, the overall phosphate concentration was maintained constant at 50 mmole/l, and the concentration of sodium dihydrogen orthophosphate (NaH_2PO_4) was varied between 5 and 20 mmole/l. The results are shown in Fig. 2. The eluent pH also varies, between 3.9 and 4.5, with NaH_2PO_4 concentration, as shown in Fig. 2. Now the retention of each amine is greatly reduced. To show that this change is not connected with the hydrophobic eluting power of the eluent, the k' of n-decylbenzene, which is constant over the entire range, is also included in Fig. 2.

It can be seen that the retention-modifying factor is not the overall phosphate concentration, but rather the concentration of sodium ion. However, in these experiments the pH of the eluent also changed with sodium concentration. To distinguish the role of Na^+ and pH, another series of measurements was carried out. A constant perchloric acid ($HClO_4$) concentration (10^{-4} mole/l) was maintained, and $NaClO_4$ was added to the system. In the $NaClO_4$ concentration range 0–7.5 mmole/l the pH of the eluent was constant at 3.6 ± 0.1. The log k' values are shown in Fig. 3. Again, the constant log k' of n-decylbenzene indicates that we are not dealing with a mobile phase ionic-strength effect. The log k' of each amine decreases very steeply,
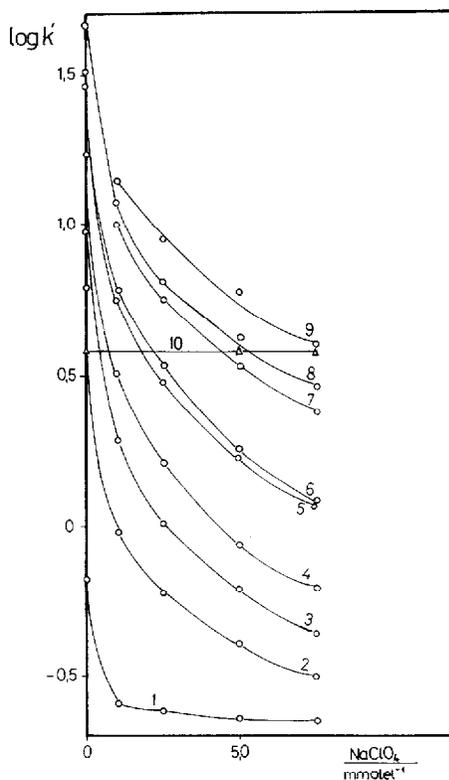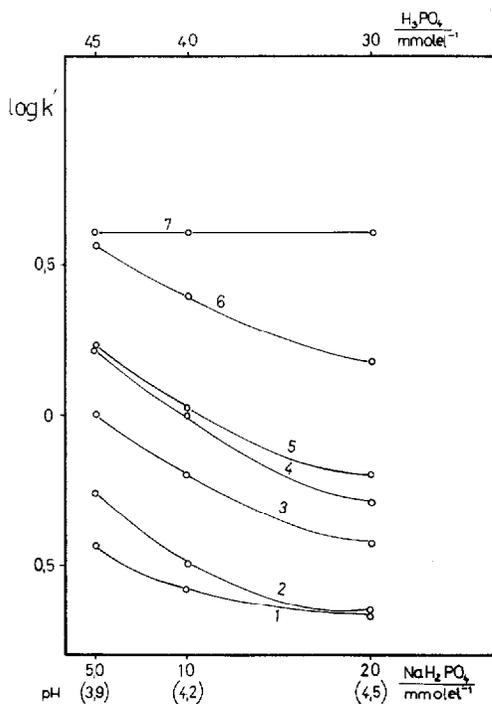
Fig. 2. Retention of aromatic amines as a function of the NaH_2PO_4 concentration of the eluent. Eluent: 94% (v/v) methanol, 5–20 mmole/l NaH_2PO_4; total phosphate concentration 50 mmole/l; pH 3.9–4.5. 1, 4-Chloroaniline; 2, aniline; 3, dibenzylamine; 4, p-nitro-α-aminoacetophenone; 5, benzylamine; 6, N-benzylmethylamine; 7, n-decylbenzene.

Fig. 3. Retention of aromatic amines as a function of the $NaClO_4$ concentration of the eluent. Eluent: 94% (v/v) methanol, 0.1 mmole/l $HClO_4$ (pH 3.6 ± 0.1), 0–7.5 mmole/l $NaClO_4$. 1, 3,4-Dichloroaniline; 2, 4-chloroaniline; 3, aniline; 4, dibenzylamine; 5, benzylamine; 6, p-nitro-α-aminoacetophenone; 7, N,N-diethylaniline; 8, N-Benzylmethylamine; 9, N,N-dimethylaniline; 10, n-decylbenzene.

even at very low Na^+ concentrations (higher $NaClO_4$ concentrations could not be examined because the solubility of $NaClO_4$ in 94% methanol is low).

Fig. 4 shows the log k' values replotted as a function of the logarithm of sodium ion concentration. The circles represent the $NaClO_4$ data and the triangles the NaH_2PO_4 values. It can be seen that irrespective of the type of anion and the slight difference in the pH values, the points scatter around the same straight lines. Also, the slopes of the log k' versus log C_{Na} curves are practically identical, showing that the same mechanism is operative in each instance. This behaviour is typical of ion exchange.

Having concluded that the concentration of the buffer cation profoundly influences the retention of aromatic amines we investigated the effect of the chemical nature of the cation. The log k' values obtained with $KClO_4$ are plotted in Fig. 5. Although the anion is identical, there is a further decrease in the retention when the
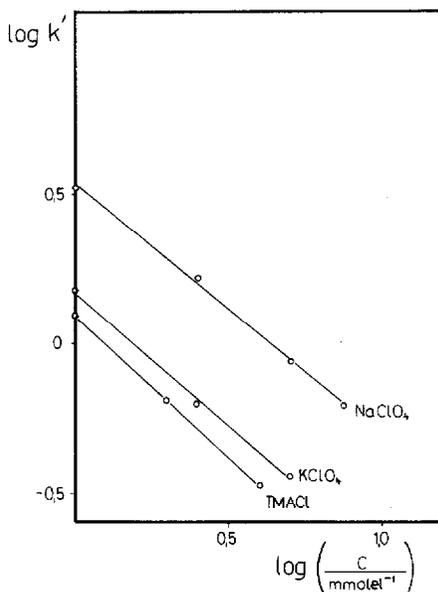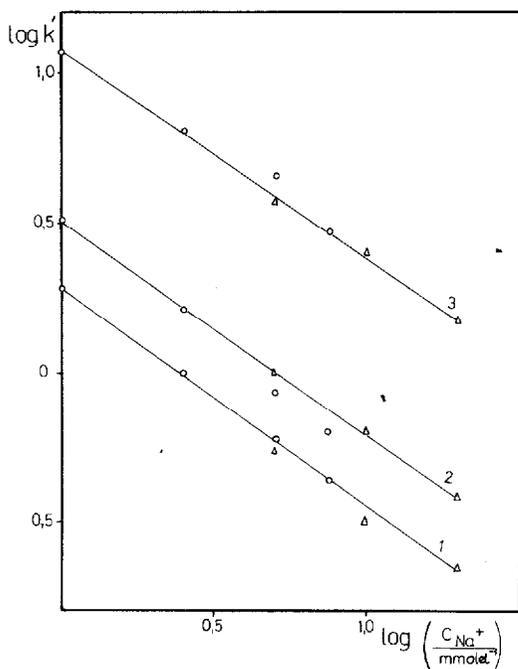
Fig. 4. Retention of aromatic amines as a function of the Na^+ concentration of the eluent. ○, Data from Fig. 3 (anion: ClO_4^-); △, data from Fig. 2 (anion: $H_2PO_4^-$).

Fig. 5. Retention of dibenzylamine as a function of the cation concentration of the eluent. Eluent: 94% (v/v) methanol, 0.1 mmole/l $HClO_4$, 0–10 mmole/l $NaClO_4$, $KClO_4$, tetramethylammonium chloride; pH 3.6 ± 0.1.

cation is K^+. The slope is almost the same as with Na^+. Thus, it can be concluded that both the type and the concentration of the buffer cation influence the retention of aromatic amines.

Next, tetramethylammonium chloride was added to the eluent. The log k' values obtained with the various cations are summarized in Table II.

Compared with K^+, the TMA^+ cation further decreases the log k' of secondary and tertiary amines (Fig. 5), while the log k' values of primary amines are between those obtained with Na^+ and K^+ as eluent buffer cations (Fig. 6). Probably the reversal is related to the steric difference between primary, and secondary and tertiary amines (the pK value of the amine does not influence this phenomenon).

In eluents that contain only H_3PO_4 the cation, *i.e.*, the competitor of amine solutes, is H^+. It would be instructive to establish the relative retention-modifying strength of H^+, Na^+ and K^+. The following estimation might serve as a first approximation: The first dissociation constant of H_3PO_4 in 90% (v/v) methanol is of the order of 10^{-6} (ref. 17). Thus, the concentration of H^+ in 10 mmole/l H_3PO_4 is not less than 10^{-4} mole/l. When the log k' of dibenzylamine in 10 mmole/l H_3PO_4 is plotted in Fig. 5, it is far above the extrapolated Na^+ line. Therefore, the order of competition strength of the cations on RP-18 alkylsilica is $H^+ < Na^+ < K^+$. This

TABLE II

LOG k' VALUES OF AROMATIC AMINES AS A FUNCTION OF THE CATION CONCENTRATION OF THE ELUENT

Eluent: 94% (v/v) methanol, 0.1 mmol/l $HClO_4$; pH 3.6 ± 0.1.

| Solute	Log k'									
	$NaClO_4$ (mmole/l)				$KClO_4$ (mmole/l)			$TMACl$ (mmole/l)		
	1	2.5	5	7.5	1	2.5	5	1	2	4
4-Chloro-aniline	−0.03	−0.23	−0.39	−0.50	−0.32	−0.57	−0.67	−0.18	−0.32	−0.43
Aniline	0.28	0.01	−0.21	−0.36	−0.06	−0.43	−0.66	0.02	−0.21	−0.39
Benzylamine	0.75	0.47	0.23	0.07	0.25	−0.15	−0.40	0.40	0.11	−0.07
N,N-Diethyl-aniline	1.00	0.75	0.53	0.38	0.55	0.21	0.0	0.26	−0.01	−0.27
N,N-Dime-thylaniline	1.14	0.95	0.77	0.60	0.66	0.37	0.20	0.30	0.12	0.05
N,N-Diben-zylamine	0.51	0.22	−0.07	−0.20	0.19	−0.20	−0.44	0.09	−0.19	−0.48
N-Benzyl-methylamine	1.07	0.81	0.66	0.47	0.59	0.27	0.05	0.55	0.30	0.08
p-Nitro-α-aminoace-tophenone	0.77	0.53	0.26	0.09	0.21	−0.20	−0.48	0.46	0.22	0.04
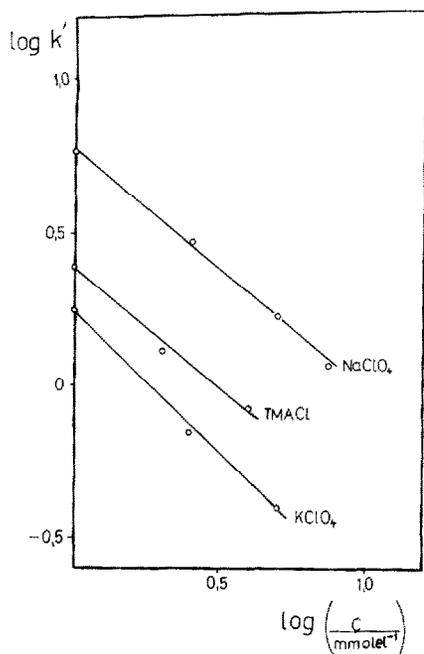
Fig. 6. Retention of benzylamine as a function of the cation concentration of the eluent. Conditions as in Fig. 5.

is the usual order of binding observed with naked silicas at pH $\leq$ 7 in neat aqueous solutions[18].

To test the effect of pH, another series of eluents was prepared with a constant $KClO_4$ concentration (1 mmole/l). The measured pH was varied between 2.3 and 6.2 by adding $HClO_4$. Again, n-decylbenzene was included to detect solvophobic changes. The log k' values of the low pK amines are shown in Fig. 7. The curves show a maximum, which can be interpreted by considering the increase in the amount of the available ion-exchange sites (rising portion of the curve) and by the decrease in amine protonation at higher pH (the points are connected by a broken line to indicate that the number of data is insufficient for the determination of the exact location of the maxima).

The log k' values of the high pK amines are shown in Fig. 8. Their degree of protonation does not change in the range tested, so the increase and subsequent levelling off of log k' at higher pH is caused by the increase in the number of available ion-exchange sites.

At most of the pH values examined here, the retention of a protonated amine is believed to be caused mainly by ion exchange. However, hydrophobic interactions could also contribute to the retention, and the extent of this contribution depends on the degree of ionization of the solute, and its hydrophobicity. This means that the retention data can be fitted to the two-adsorption site models[5-7,9,10]. This data set is especially suitable for testing with the method proposed in ref. 10, because the
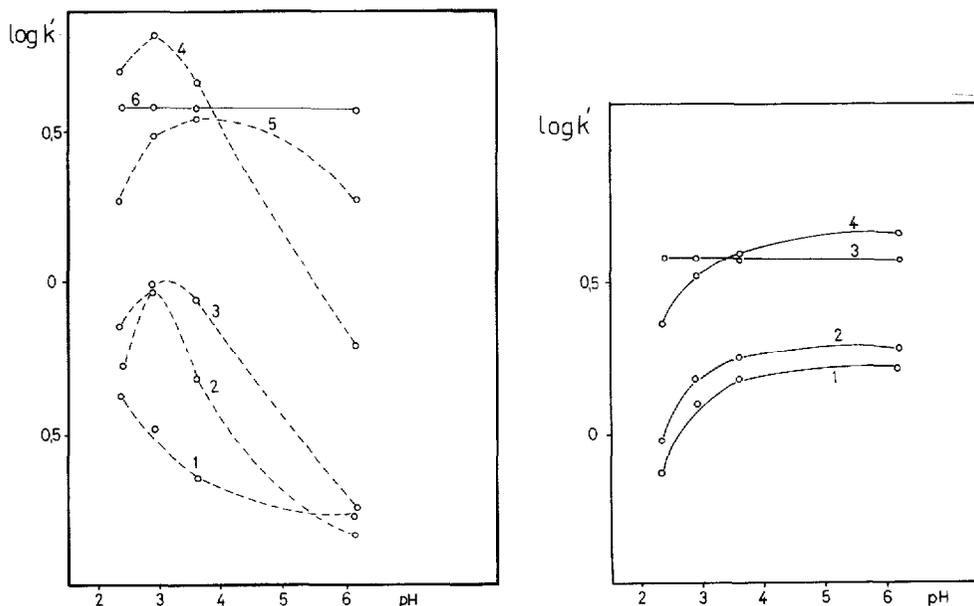

Fig. 7. Retention of low pK aromatic amines as a function of the pH of the eluent. Conditions: 94% (v/v) methanol, 1 mmole/l $KClO_4$; pH 2.3–6.2, adjusted by addition of $HClO_4$. 1, 3,4-Dichloroaniline; 2, 4-chloroaniline; 3, aniline; 4, N,N-dimethylaniline; 5, N,N-diethylaniline; 6, n-decylbenzene.

Fig. 8. Retention of high pK aromatic amines as a function of the pH of the eluent. Conditions as in Fig. 7. 1, Dibenzylamine; 2, benzylamine; 3, n-decylbenzene; 4, N-benzylmethylamine.

binding of Na^+ and K^+ is not assisted by hydrophobic adsorption on the bonded alkyl chains, in clear contrast to most organic masking agents[10,19]. According to ref. 10, the two-site adsorption can be described by the following linearized equation:

$$\frac{A}{k'_0 - k'} = \frac{1}{k'_2 K_A} + \frac{A}{k'_2}$$

where k'_0 is the measured k' value in the absence of a competing masking agent, k' is the measured k' value in the presence of a competing masking agent, k'_2 is the so-called silanophilic contribution to the actual k' and K_A is the binding constant of the competing masking agent.

When the chromatographic conditions are identical, except for the chemical identity of the competing agent, then the "silanophilic" retention contribution, k'_2, is characteristic of the solute only, and is independent of the masking agent (this implies that the binding of the masking agent does not alter the binding energy of the solute, only the number of the available binding sites is decreased; this condition is not necessarily fulfilled in each instance).

Data from Table II were tested with the above equation. The linear fit was always excellent, the correlation coefficients being better than 0.999. The data obtained are presented in Table III. The k' values measured in the absence of competing agents (k'_0) are also included. It can be seen that the non-hydrophobic retention contribution always accounts for almost the entire retention: the hydrophobic contribution is generally less than 2%. The measured k'_0 of N,N-dibenzylamine is slightly lower than expected. This indicates the limitation of the approach proposed in ref. 10, i.e., the fit is very sensitive for the k'_0 values and the true k'_0 values are often difficult to obtain experimentally.

The solute-independent binding values of Na^+ and K^+ are listed in the last two columns of Table III. Except for a few obvious outliers, the Na^+ data scatter around 4.5 and the K^+ data around 13.5. There is no apparent solute-dependent trend in the scatter of either K_{Na}^+ or K^+. K^+ is 3 times as high as K_{Na}^+, in agreement with the detected, stronger effect of the potassium cation.

Hence the above analysis also supports the interpretation that under the chromatographic conditions tested here the aromatic amines are retained by an almost "pure" ion-exchange mechanism, and it explains the excellent linearity of the log k' versus log C_{cation} curves obtained, which is characteristic of ion-exchange reactions.

TABLE II

NON-HYDROPHOBIC RETENTION CONTRIBUTION OF AROMATIC AMINES

Conditions as in Table II.

Solute	k'_0	$k_2^{Na^+}$	$k_2^{K^+}$	K_{Na}^+ (mmole^{-1})	K^+ (mmole^{-1})
Aniline	9.37	9.22	9.34	4.17	10.92
4-Chloroaniline	6.22	6.02	6.10	6.22	16.4
N,N-Dimethylaniline	28.5	27.32	27.78	1.06	6.23
Benzylamine	28.6	28.25	28.57	4.12	17.50
N-Methylbenzylamine	46.3	45.04	46.29	3.00	10.80
N,N-Dibenzylamine	16.9	16.95	17.00	4.91	12.25
p-Nitro-α-amino-acetophenone	32.6	32.36	32.57	4.41	28.80

CONCLUSION

Investigation of the effects of the concentration and type of buffer cation on the reversed-phase retention of both low and high pK aromatic amines in water-lean eluents has shown that the addition of H_3PO_4 to the eluent leads to pK-dependent maxima in the log k' versus $C_{H_3PO_4}$ curves. The behaviour is interpreted as ion exchange on a weak-acid cation exchanger.

The type of anion has no noticeable effect on the retention of amine solutes. At constant pH the log k' versus log C_{cation} relationships are linear. The log k' versus pH curves of the low pK amines pass through a maximum, whereas those of the high pK amines increase, then level off at high pH. The retention-decreasing effect of the cations increases in the order $H^+ < Na^+ < K^+$, in agreement with the binding order observed on naked silica. The two-adsorption site models can be fitted well to the experimental data, and the hydrophobic retention contributions are negligible. The binding constants of the competing agents increase in the order $Na^+ < K^+$.

These observations strongly suggest that under the experimental conditions used here protonated aromatic amines are retained on C_{18} alkylsilica mainly by ion exchange.

As alkylsilica responded very sensitively to small changes in the cation concentration of the eluent, and especially to the type of buffer cation, we believe that much more attention should be paid to the so-far neglected inorganic buffer cations of reversed-phase eluents.

Further work is under way with more aqueous eluents and the results will be published in a forthcoming paper.

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