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

II*. METHANOL-LEAN ELUENTS

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SUMMARY

The retention of low, medium and high p*K* aromatic amines was investigated by using a LiChrosorb RP-18 column and acidic aqueous methanol eluents. The eluents also contained (at constant pH) varying concentrations of sodium, potassium and tetramethylammonium (TMA) salts. Salt addition decreased the retention of protonated amines and, to a smaller extent, that of the polar, non-ionic solutes. At low concentrations the retention-decreasing effect of the cations increased in the order $H^+ < Na^+ < K^+ < TMA^+$. At constant metal ion concentration and increasing pH, log k' passed through a maximum, and the shape of the curve depended on the relative magnitude of the p*K* of the amine solute and the silanol group. The addition of 20 mmole/l of potassium bromide effectively suppressed the minimum of the log k' *vs.* C_{methanol} curves. Ion exchange between the protonated amines and the dissociated silanol group is assumed to cause the observed phenomena.

INTRODUCTION

In Part I[1], the role of the buffer cation in the retention of aromatic amines in reversed-phase high-performance liquid chromatography (RP-HPLC) was discussed. A LiChrosorb RP-18 column and 94% (v/v) methanol–water eluents with varying concentrations of H_3PO_4, $HClO_4$, NaH_2PO_4, $NaClO_4$, $KClO_4$ and tetramethylammonium chloride (TMA) were applied. Using a series of aromatic amines with p*K* of −4.5 to 12, we found that in these methanol-rich eluents the logarithm of the capacity factor (k') at constant pH decreased linearily with the logarithm of the cation concentration, and the retention-decreasing effect of the cation became stronger in the order H^+, Na^+, K^+, irrespective of the structure of the amine solute. At constant metal ion concentration the log k' *vs.* pH curves passed through a maximum. Ion exchange with the dissociated silanol groups of the reversed-phase packing, pH-dependent ionization of the surface silanols and p*K*-dependent protonation of the amine

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* For Part I, see ref. 1.

solutes explained these phenomena. In order to gain further insight into the role of the eluent cations, experiments have been carried out with additional aqueous eluents over the methanol concentration range 0–100% (v/v) and these results are reported here.

Since the completition of Part I, further papers addressing certain aspects of the role of residual silanol groups have been published, and these are briefly reviewed here.

Jansson *et al.*[2] used aqueous phosphate buffer eluents (pH 2.2) and naked LiChrosorb Si-60 silica columns. They concluded that the addition in low concentrations of either N,N-dimethyloctylamine or N,N,N-trimethyloctylamine modifiers significantly reduced the retention of several amine solutes. From the adsorption isotherms of the amine modifiers and from the $1/k'$ *vs.* amine modifier concentration curves of the solutes, they concluded that part of the modifier was very strongly bound to the dissociated silanol groups of the silica surface and that there was a very steep decrease in the k' of the aromatic amine solutes. They also noted that sodium, added in low concentrations, likewise reduced the retention of the amine solutes.

Gill *et al.*[3] examined the retention and peak shapes of 2-phenylethylamine drugs on ODS-Hypersil packing. They used aqueous methanolic eluents which contained orthophosphoric acid, sodium hydroxide and aliphatic primary, secondary and tertiary amine additives. For comparison purposes the amine derivatives were replaced with sodium and potassium ions. In acidic ($pH < 3$) eluents, where the 2-phenylethylamine solutes were protonated, the addition of each modifier, including sodium and potassium, decreased the retention and improved the peak shape. The effect of the modifier greatly depended on its structure (chain length, primary *vs.* secondary to tertiary amine), and was stronger than that of the inorganic cation. However, in addition to the organic amine modifier, each eluent also contained sodium (from pH adjustment), so that composite effects were observed.

Flanagan *et al.*[4] used naked Spherisorb S5W silica and acidic methanol eluents (1.85 mmole/l perchloric acid) for the analysis of basic drugs. They concluded that maximum retention was observed at intermediate pH values. At low pH, retention was low because few of the silanol groups were ionized, and at high pH basic drugs were not protonated. They noted that the addition of sodium hydroxide to the eluent altered the retention volumes of basic drugs, but no detailed data were given. The retention changes were attributed to pH changes only.

Recently, Van Lancker *et al.*[5] investigated the separation of anthracyclines on Spherisorb 5S-ODS by using acidic (pH 2.5) acetonitrile–water eluents of constant ionic strength ($I = 0.03$, adjusted with sodium sulphate). They noted that Spherisorb ODS was more suitable than irregular ODS silicas for this type of separation. This can be rationalized by Engelhardt and Müller's observation[6] that Spherisorb, slurried in deionized water, gave a basic solution, whereas most irregular silicas gave neutral or slightly acidic supernatants. Van Lancker *et al.*[5] noted that the log k' *vs.* acetonitrile concentration curves of anthracyclines with NH_2 groups passed through a minimum in pH 2.5 eluents. They rationalized this observation by a dual hydrophobic–silanophilic retention mechanism. They maintain that silanophilic interactions operate mostly in acetonitrile-rich eluents whereas at higher water concentrations silanols are masked and hydrophobic interactions become dominant. However, the latter hypothesis is contradicted by Fig. 4 in ref. 5, which shows a large decrease in the retention of NH_2-bearing anthracyclines with respect to the retention of a non-protonated derivative on addition of 1.5 mmole/l of nonylamine to the eluent. The largest decrease is observed on the water-rich side, indicating that there is a strong silanophilic contribution, which is largely suppressed by 1.5 mmole/l of nonylamine. In acetonitrile-rich eluents the amount of nonylamine adsorbed by Spherisorb ODS from the 1.5 mmole/l eluents is insufficient for the effective masking of the silanols, hence higher retentions are observed.

In conclusion, we can state that there is ample evidence in the literature indicating the effectiveness of the dual retention mechanism over the entire concentration range of hydroorganic eluents, warranting a detailed study of the role of the buffer cation in the retention of aromatic amines.

EXPERIMENTAL

The experimental set-up described in Part I[1] was used. Eluents were prepared as described in ref. 7.

In order to characterize the stationary phase more fully, the carbon and hydrogen content of the LiChrosorb RP-18 silica (Charge No. 1163534, Batch No. VV 2249) were determined (courtesy of Dr. Th. Welsch, University of Leipzig, G.D.R.). The carbon and hydrogen contents were 16.3 and 3.2% (w/w), respectively. The concentration of the accessible residual silanol groups was also determined by the methyllithium method as described by Welsch and Frank[8], and was found to be 567 μmole of OH groups per gram of RP-18. When suspended in aqueous methanol[8], the material became wetted at a methanol concentration of 55.7% (v/v).

The pH of the eluent reported here is the apparent pH, measured by a combined glass electrode, calibrated with aqueous buffers of pH 4.0 and 7.0. If needed, the pH values can be corrected to thermodynamically meaningful pH values as described by Leitold and Vigh[9].

The solute studied and their p*K* values are listed in Table I.

As the retention volumes of aromatic amines changed with the amount of solute injected, measurements were carried out at the highest sensitivity setting of the UV detector. Generally, 0.2–1 nmole were injected.

RESULTS AND DISCUSSION

As in Part I[1], the effects of the eluent pH on the retention of low and high p*K* aromatic amines was investigated first. The base eluent contained 35% (v/v) methanol and 20 mmole/l of potassium perchlorate, to which varying amounts of perchloric acid were added to obtain apparent pH values in the range 2–6. Some of the results are shown in Fig. 1.

The constant log k' values of benzene (Bz) indicate that the hydrophobic eluting power of the eluent and the hydrophobic retentivity of the RP-18 silica remain constant while the pH is increased by decreasing the concentration of added perchloric acid (10–0.01 mmole/l). The retention of non-ionic, but polar, benzyl alcohol (BzOH) also remains constant, indicating that polar contributions to its retention remain unchanged while the eluent pH increases.

2,4-Dinitroaniline (4) is not protonated in the range tested ($pK = -4.3$); con-

TABLE I

AROMATIC AMINE SOLUTES SYMBOL AND pK VALUES

No. symbol	*Solute*	*pK*
1	Aniline	4.6
2	3-Nitroaniline	2.5
3	4-Chloroaniline	4.0
4	2,4-Dinitroaniline	-4.3
5	2,6-Dimethylaniline	3.55; 3.95
6	2,6-Diethylaniline	n.a.*
7	3,4-Dichloroaniline	3.0
8	3,5-Dichloroaniline	2.4
9	N,N-Dimethylaniline	5.1
10	N,N-Diethylaniline	6.6
11	Benzylamine	9.3–9.6
12	N,N-Dibenzylamine	8.5
13	2,6-Diaminotoluene	n.a.
14	*o*-Phenylenediamine	pK_1 4.47; pK_2 0.67
15	*p*-Phenylenediamine	pK_1 6.2; pK_2 2.7
16	*p*-Nitro-α-aminoacetophenone	>8
Bz	Benzene	
BzOH	Benzyl alcohol	
PhEtOH	α-Phenylethanol	
PNAF	*p*-Nitroacetophenone	
C_2NO_2	Nitroethane	
C_3NO_2	Nitropropane	

* n.a. = not available.

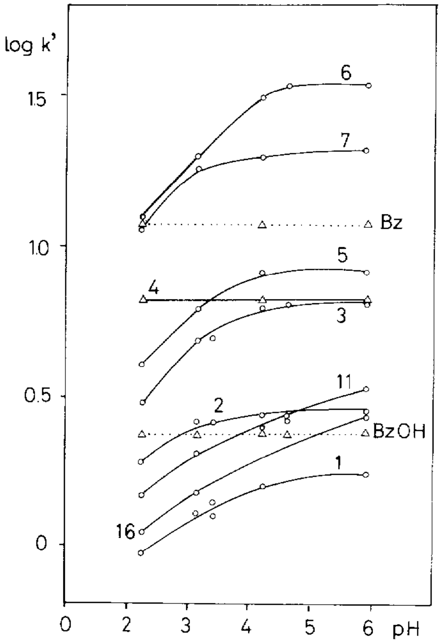


Fig. 1. Retention of aromatic amines as a function of eluent pH. Eluent: 35% (v/v) methanol, 20 mmole/l $KClO_4$. Symbols as in Table I.

sequently, it behaves like benzyl alcohol, a polar, non-ionic solute, and its retention is constant. 3-Nitroaniline (2), 3,4-dichloroaniline (7), 4-chloroaniline (3), 2,6-dimethylaniline (5), 2,6-diethylaniline (6) and aniline (1), with aqueous p*K* values ranging from 2.5 to 4.6, show a broad *k'* plateau (or maximum) with increasing pH, while the high p*K* (above 8) amines *p*-nitro-α-aminoacetophenone (16) and benzylamine (11) show increasing retention over the pH range tested.

Compared with the pH dependence in methanol-rich eluents (Figs. 7 and 8 in Part I[1]) we can conclude that in 35% (v/v) methanol eluents the *k'* maxima of the low p*K* amines and the plateaux of the high p*K* amines were shifted towards higher apparent pH values.

Next, the potassium perchlorate concentration of the 35% (v/v) methanol eluent was changed. An apparent pH of 3.4 ± 0.1 was maintained by the addition of perchloric acid (0.5 mmole/l in the final eluent). The log *k'* values are shown in Fig. 2. The retention of benzene (Bz) is again constant, indicating that the change in ionic strength on addition of 0–20 mmole/l of potassium perchlorate did not alter hydrophobic retention in the system. The polar, non-ionic solutes *p*-nitroacetophenone (PNAF) and benzyl alcohol (BzOH) show slightly decreased retention, indicating that changes in the ionic environment on the surface of the reversed-phase silica also influence the retention of non-ionic, polar solutes. Low p*K* amines, such as 3-nitroaniline (3) with an aqueous p*K* of 2.5, respond only slightly to the K^+ concentration. Medium to high p*K* amines, such as aniline (1) with an aqueous p*K* of 4.6 and benzylamine (11) with an aqueous p*K* of 9.5, experience a dramatic de-

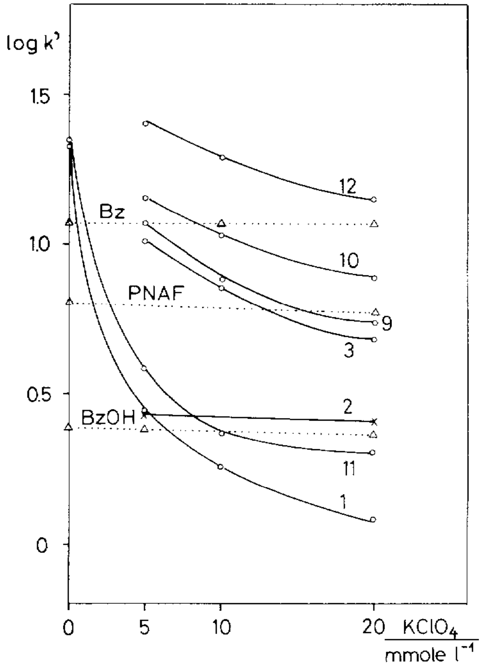


Fig. 2. Retention of aromatic amines as a function of $KClO_4$ concentration of the eluent. Eluent: 35% (v/v) methanol, pH 3.4 ± 0.1 (0.5 mmole/l H_3PO_4).

TABLE II

RETENTION OF AROMATIC AMINES AS A FUNCTION OF THE SALT CONCENTRATION IN 35% (v/v) METHANOL, 0.5 mmole/l $HClO_4$ ELUENTS (pH 3.4 ± 0.1)

Solute	*Log k'*								
	C_{NaClO_4} *(mmole/l)*			C_{KClO_4} *(mmole/l)*			C_{TMACl} *(mmole/l)*		
	5	*10*	*20*	*5*	*10*	*20*	*5*	*10*	*20*
1	0.65	0.52	0.42	0.44	0.27	0.09	0.28	0.21	0.10
16	0.94	0.77	0.57	0.59	0.34	0.13	0.50	0.34	0.14
3	1.31	1.15	0.99	1.01	0.85	0.69	0.89	–	0.78
2	0.49	–	–	0.43	–	0.41	–	–	0.41
5	–	–	–	1.07	0.90	0.76	0.88	0.80	0.70
9	1.3	–	1.09	1.06	0.87	0.74	0.75	0.64	0.50
10	–	–	–	1.15	1.03	0.89	0.84	0.69	0.52
12	–	–	–	1.40	1.29	1.15	1.22	1.10	0.93

crease in their retention. This can be rationalized as in Part I[1]. The ion-exchange-mediated retention of protonated amines is sensitive to the cation concentration of the mobile phase. In general, protonated amines with log *k'* larger than 0.75 (*e.g.*, 3, 9, 10 and 12) could not be reasonably eluted with salt-free eluents.

In 35% (v/v) methanol, unlike in 94% (v/v) methanol at pH 3.6[1], the elution order of dibenzylamine (12) and benzylamine (11) and that of N,N-diethylaniline

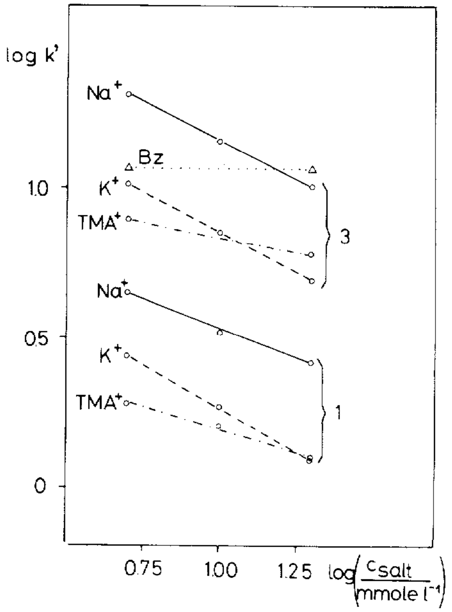


Fig. 3. Retention of aromatic amines as a function of salt concentration. Eluent: 35% (v/v) methanol, pH 3.4 ± 0.1 (0.5 mmole/l $HClO_4$).

TABLE III

RETENTION OF AROMATIC AMINES IN 35% (v/v) METHANOL, 10 mmole/l $HClO_4$ ELUENTS (pH 2.2)

Salt concentration: 5 mmole/l.

Solute	*Log k'*	
	$KClO_4$	*KBr*
1	0.12	0.13
11	0.30	0.29
9	0.75	0.75
7	1.15	1.15
6	1.20	1.18

(10) and N,N-dimethylaniline (9) corresponds to that of the expected hydrophobic order.

These experiments were repeated with sodium perchlorate and tetramethylammonium chloride in the 35% (v/v) methanol eluents. The apparent pH was again kept at 3.4 (0.5 mmole/l of perchloric acid). The results are summarized in Table II.

In Fig. 3 the log *k'* of two NH_2-bearing amines, 4-chloroaniline (3) and aniline (1), are plotted against the salt concentration of the eluent. Compared with the 0.5 mmole/l perchloric acid–35% (v/v) methanol eluent, both Na^+ and K^+ decrease the observed retention. Similar to the 94% (v/v) eluent, K^+ decreases the retention more than Na^+. At low concentrations, TMA^+ decreases the retention even more than Na^+ and K^+.

In order to learn more about the role of the anion, experiments were carried

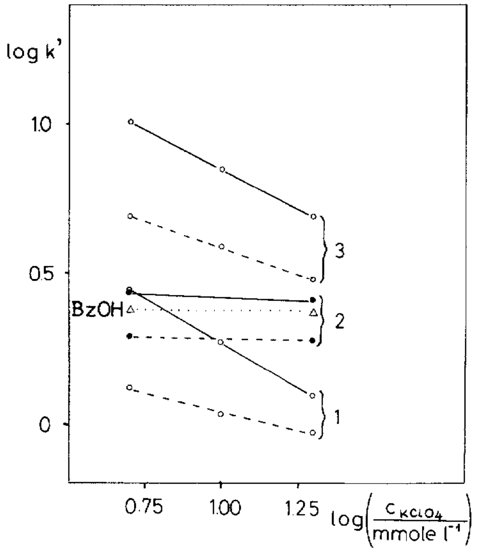


Fig. 4. Retention of aromatic amines as a function of $KClO_4$ concentration in 35% (v/v) methanol eluents at pH 3.4 and 2.2 (0.5 and 10 mmole/l $HClO_4$, respectively). ——, pH 3.4, – – –, pH 2.2.

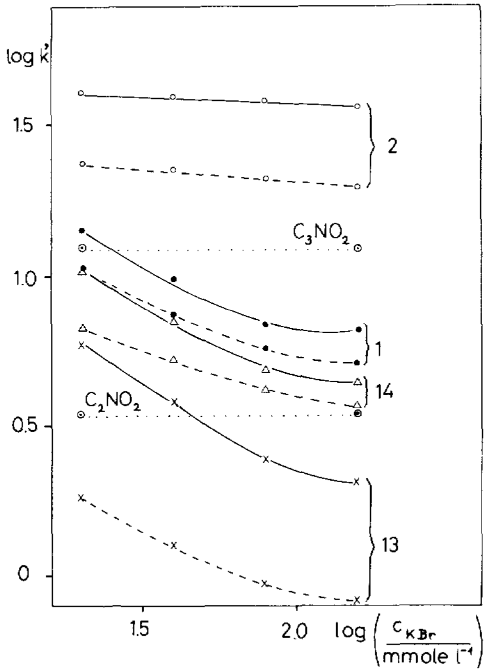


Fig. 5. Retention of aromatic amines as a function of KBr concentration in neat aqueous eluents of pH 3.4 and 2.5 (0.5 and 5 mmole/l $HClO_4$, respectively). ——, pH 3.4; – – –, pH 2.5.

out with 10 mmole/l perchloric acid (pH 2.2)–5 mmole/l salt–35% (v/v) methanol eluents. In one series the salt was potassium perchlorate and in the other potassium bromide. The log k' values obtained are listed in Table III. It can be seen that identical retentions are obtained with both anions.

The retentions in eluents of pH 3.4 (0.5 mmole/l perchloric acid) and 2.2 (10 mmole/l perchloric acid) are compared in Fig. 4. It can be seen that, while the retention of the neutral solute, benzyl alcohol, is constant, amines (even the low pK 3-nitroaniline) are eluted earlier in eluents of pH 2.2. Retention still depends on the

TABLE IV

RETENTION OF AROMATIC AMINES IN NEAT AQUEOUS ELUENTS, 0.5 mmole/l $HClO_4$ (pH 3.4)

Solute	*Log k'*							
	c_{KBr} *(mmole/l)*				c_{TMACl} *(mmole/l)*			
	20	*40*	*80*	*160*	*20*	*40*	*80*	*160*
15	0.0	−0.20	−0.38	−0.41	−0.76	−0.87	–	–
13	0.77	0.58	0.39	0.32	0.35	−0.02	−0.09	−0.10
14	1.03	0.85	0.69	0.65	0.72	0.39	0.29	0.27
1	1.15	0.99	0.84	0.82	0.62	0.54	0.42	0.43
2	1.61	1.59	1.58	1.56	1.56	1.55	1.52	1.53

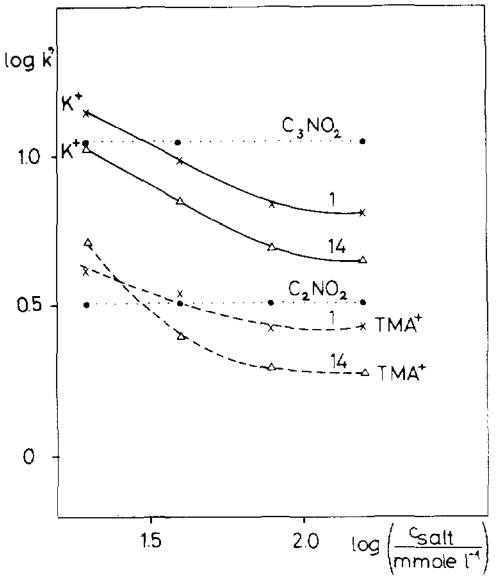


Fig. 6. Retention of aromatic amines as a function of salt concentration in neat aqueous eluents at pH 3.4 (0.5 mmole/l $HClO_4$).

K^+ concentration, although to a smaller extent. This observation can be rationalized by the smaller ion-exchange capacity of RP-18 at lower pH.

Similar experiments were carried out with neat aqueous eluents of pH 3.4 (0.5 mmole/l perchloric acid) and pH 2.5 (5 mmole/l perchloric acid). Some of the results are shown in Fig. 5. Nitroethane (C_2NO_2) and nitropropane (C_3NO_2) were used as non-ionic, polar reference compounds. Their retention was constant, irrespective of the eluent pH and the salt concentration. The retention of low p*K* 3-nitroaniline (2) decreased only slightly with increasing K^+ concentration, while that of the medium to high p*K* amines aniline (1), 2,6-diaminotoluene (13) and *o*-phenylenediamine (14), decreased more. Above 100 mmole/l of potassium bromide this decrease became less steep. The retention also decreased at lower pH, in agreement with Fig. 4.

Similar results were obtained when K^+ was replaced with TMA^+, except that retention was lower with TMA^+ than with K^+ (Table IV, Fig. 6).

These observations are in agreement with the postulated retention mechanism, *i.e.*, ion exchange of the protonated amine with the dissociated silanols of the RP-18 packing. In earlier studies some of these solutes displayed the minimum-point behaviour characteristic of the dual retention mechanism[10]. It therefore seemed worthwhile to examine the methanol dependence of the retention of aromatic amines in more detail.

Eluents with varying methanol concentrations were prepared, each containing 0.5 mmole/l of perchloric acid and 20 mmole/l of potassium bromide. The log k' *vs.* C_{methanol} curves are shown in Fig. 7 (the apparent pH values of the eluents are also included). It can be seen that the characteristic minimum behaviour of amines is absent. Slightly non-linear but monotonous curves were obtained. The addition of 20 mmole/l of potassium bromide at pH 3.3 cannot fully repress the ionic contribution to retention and restore the hydrophobic retention order (Figs. 8 and 9).

In Fig. 8 the log *k'* of N,N-dimethylaniline (9) and N,N-diethylaniline (10) is plotted against methanol concentration. Notwithstanding the presence of 20 mmole/l of K^+, in methanol-rich eluents N,N-dimethylaniline is retained more strongly than N,N-diethylaniline, whereas below 40% (v/v) methanol concentration the combined effect of K^+ and water restores the hydrophobic retention order. However, there is still a significant polar contribution to retention, because, on addition of 160 mmole/l of potassium bromide to the neat aqueous pH 3.4 eluent, the retention of 2,6-diaminotoluene (13), *p*-phenylenediamine (15) and *p*-nitro-α-aminoacetophenone (16) is decreased by about 0.4 log *k'* units. The log *k'* values in the absence of salt in 94% (v/v) methanol are also shown in Fig. 9.

Some of the experiments were repeated with columns that were packed with another batch of octadecylsilica [carbon content 17.3%, hydrogen content 3.24%, hydroxyl group concentration by the methyllithium method[8] 660 μmole/g, methanol concentration of wetting point 55.4% (v/v)]. Non-polar solutes (alkylbenzenes, iodoalkanes, etc.) showed almost identical retention volumes, but the absolute *k'* values of amines differed occasionally by as much as 40%. Nevertheless, the tendencies observed were similar with both batches of octadecylsilica.

On completion of each series of experiments the columns were washed with 10 mmole/l perchloric acid in the respective eluent, then equilibrated with the salt-free

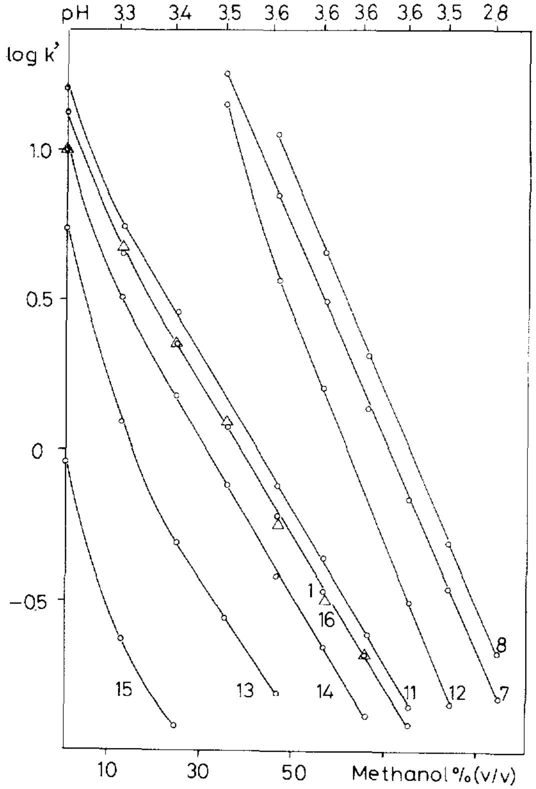
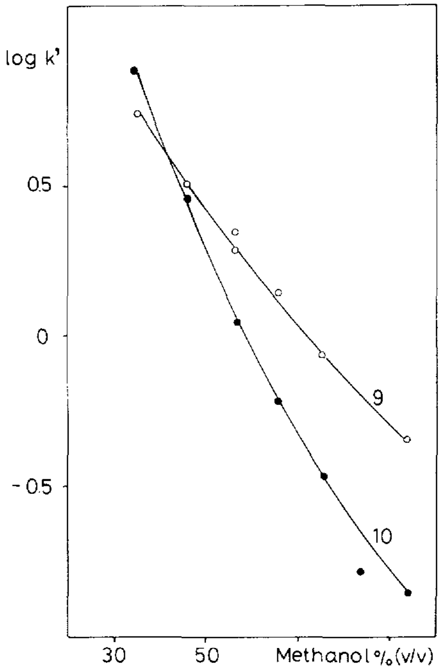


Fig. 7. Retention of aromatic amines as a function of methanol concentration of the eluent (0.5 mmole/l $HClO_4$, 20 mmole/l KBr).



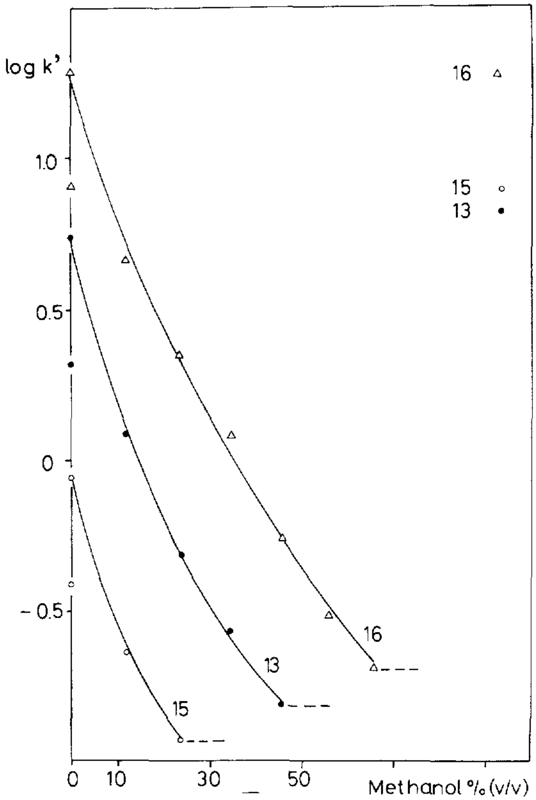


Fig. 8. Retention of N,N-dimethylaniline and N,N-diethylaniline as a function of methanol concentration of the eluent (0.5 mmole/l $HClO_4$, 20 mmole/l KBr).

Fig. 9. Retention of aromatic amines as a function of methanol concentration of the eluent (0.5 mmole/l $HClO_4$, 20 mmole/l KBr). Single points in the case of 94% (v/v) methanol were obtained without any salt. Single points in the case of 0% (v/v) methanol were obtained with 160 mmole/l KBr.

base eluents. Selected amine solutes were reinjected and the constancy of their k' value was checked. If necessary, washing was continued until the original k' values were re-established within ±10%. Regeneration was always complete, except after the use of 160 mmole/l TMA^+ in neat aqueous eluent. More elaborate washing schemes also failed with TMA^+. This aspect is under further investigation and will be dealt with a forthcoming paper.

CONCLUSION

The retention to RP-18 silica of low, medium and high pK amines was investigated over the 0–94% (v/v) methanol concentration range. In acidic eluents (0.5–10 mmole/l perchloric acid), in the absence of salts or competing organic cations, the retention of amine solutes as a function of the methanol concentration of the eluent passed through a minimum. This type of retention behaviour was explained by the dual hydrophobic–silanophilic retention mechanism proposed in the literature. At each methanol concentration, the log k' values decreased approximately linearly with the logarithm of the salt concentration of the acidic eluent (apparent pH held constant at 3.5). At a constant, low salt concentration, the retention-decreasing power of the cations increased in the order $Na^+ < K^+ < TMA^+$. The type of anion in the salt had no apparent effect on the log k' of the amine solutes.

At constant metal ion concentrations, the log k' *vs.* pH curves passed through a maximum. The shape of the curve depended on the pK of the amine and the methanol concentration of the eluent.

The addition of even moderate concentrations of salts to the eluent eliminated the minimum of the log k' *vs.* C_{methanol} curves, but the expected hydrophobic retention order could not always be observed.

These phenomena can be explained by assuming that the retention of amines by reversed-phase silicas (and also by naked silica) is governed primarily by ionic interactions between the protonated amine and the dissociated silanol group of silica. Various shapes of the log k' *vs.* pH curves in eluents of varying methanol concentration (at constant metal ion concentration) can be rationalized by the relative changes in the pK of the amine solute and the silanol group. If $pK_{\mathrm{silanol}} - pK_{\mathrm{amine}} \gg 0$, then the log k' is almost independent of the eluent pH (Fig. 1, solute 4). If $pK_{\mathrm{silanol}} - pK_{\mathrm{amine}} \approx 0$, then log k' passes through a maximum with increasing pH, and the length of the plateau depends on the $pK_{\mathrm{silanol}} - pK_{\mathrm{amine}}$ value (Figs. 7 and 8 in ref. 1 and Fig. 1 here, solutes 1, 2, 3, 5 and 6). If $pK_{\mathrm{silanol}} - pK_{\mathrm{amine}} \ll 0$, then log k' increases with increasing pH over the experimentally available range.

Contrary to views in the literature[11], the "silanophilic", *i.e.*, ion-exchange, contribution to the retention of amines by reversed-phase silicas is large over the entire methanol concentration range, even with neat acidic eluents (Fig. 9). This ionic contribution can be largely suppressed by higher concentrations of inorganic and organic cations.

Although to a smaller extent, the retention on reversed-phase of non-ionic, polar solutes also depends on the type and concentration of the cation in the eluent. Therefore, greater attention should be paid to the retention-modifying effects of salts used in reversed-phase liquid chromatographic systems.

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