Journal of Chromatography, 280 (1983) 31–42
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,123

LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

IV*. ROLE OF pH, ALKALI METAL ION AND PAIRING ANION IN RETENTION OF GUEST SUBSTANCES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received June 27th, 1983)

SUMMARY

The effects of pH and added electrolytes (alkali metal chlorides, ion-pair agents) on the retention of various organic guest compounds in reversed-phase high-performance liquid chromatography with crown ether-containing mobile phases have been investigated. The capacity factor (k) of a guest compound bearing a primary amino group was markedly increased by the presence of a crown ether (18-crown-6) in the mobile phase the pH of which was varied between 2.4 and 4.9 by addition of hydrochloric, perchloric, or trichloroacetic acid. The pH *vs.* k profiles indicated that the retention of a guest ammonium cation associated with the crown ether was decreased with increasing proton concentration owing to ion exclusion. However, in the mobile phase acidified with trichloroacetic acid (TCA), a slight increase in the k value was observed in the low-pH region (<3.5) owing to ion-pairing between the crown ether-associated ammonium cation and the TCA anion. The addition of a hydrophobic anion (*e.g.* heptanesulphonate) to the crown ether-containing mobile phase caused the retention of the primary amino compound to be more enhanced than in normal reversed-phase ion-pair chromatography, and the degree of change in the retention was characteristic of the class of amino group and the chemical structure around the amino group in the guest molecule. The addition of an alkali metal ion to the crown ether-containing mobile phase caused the guest ammonium cation to compete with the alkali metal ion in binding to the crown ether, resulting in a change in the retention which depended on the kind of alkali metal ion and the structure of the guest molecule; addition of K^+ or Rb^+ gave rise to a significant decrease in the k values of primary amino compounds, while Na^+ produced almost no apparent change, and Li^+ brought about an enhanced retention. The guest compounds bearing either a sterically hindered primary amino group or a secondary amino group received very weak effects from any of the alkali metal ions tested.

* Part III, see ref. 6.

0021-9673/83/$03.00

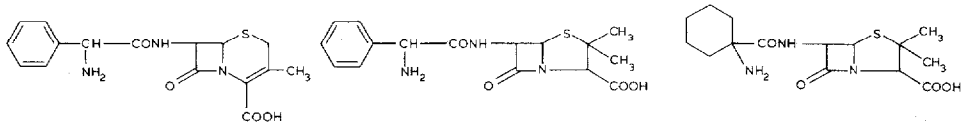
INTRODUCTION

Host–guest interactions incorporated into chromatography provide high selectivity for the separation of a particular substance from a complex mixture. Use of a crown ether as a host has also been evaluated in the liquid chromatography of various guests. The specific cation-anchoring ability of crown ethers used as stationary ligands or as components of mobile phases has been utilized for the separation of various inorganic and organic cations as well as anions with a common cation. Recent developments have been described in review articles[1–3]. In previous papers, we reported investigations on the retention behaviours of amino compounds (amines, amino acids, amides, etc.) in reversed-phase liquid chromatography with crown ether-containing mobile phases[4], and demonstrated the applicability of the proposed method to the analyses of β-lactam antibiotics[5] and catecholamines[6]. The present investigation deals with the role of pH and added electrolytes in the retention of guest compounds in this particular system, and emphasizes the synergistic effect of host–guest interactions and ion-pairing in structure-recognizing separations of amino compounds.

EXPERIMENTAL

Reagents and materials

Alkali metal chlorides, hydrochloric acid, and sodium 1-heptanesulphonate of reagent grade were obtained from Nakarai Chemicals (Kyoto, Japan) and were used as supplied; 18-crown-6 was the product of Nippon Soda Co. (Tokyo, Japan). The guest materials [aniline, benzylamine, N-methylaniline, N-methylbenzylamine, *o*-, *m*- and *p*-toluidine, benzoic acid and some antibiotics such as ampicillin (ABPC), cephalexin (CEX) and ciclacillin (ACPC)] were obtained from commercial sources and were used without further purification. Glass-distilled water and methanol were used to prepare the mobile phases for high-performance liquid chromatography (HPLC).



Measurements of capacity factors

A liquid chromatograph (Trirotar III, Jasco, Tokyo, Japan) equipped with a UV detector (Uvidec 100-III, Jasco) was used for the measurements of the capacity factors. The operating conditions are given in Table I. The guest materials were dissolved in a small volume of mobile phase, and the minimum amount required for UV detection was used in order to maintain linearity of the chromatographic system. The capacity factors were calculated from the equation, $k = (t_R - t_0)/t_0$, where t_R is the retention time of a guest compound averaged over repeated measurements at the top of the elution curve and t_0 is that of a non-absorbed substance.

TABLE I

HPLC CONDITIONS

Detection: UV, 210 or 220 nm. Flow-rate: 1.0 ml/min. Column temperature: 40°C.

Experiment	*Mobile phase*	*Stationary phase*
Effect of alkali metal ion	Water–methanol (55:45); pH 2.5 by hydrochloric acid; [18-crown-6]=0 or 10 m*M*; [LiCl], [NaCl], [KCl] or[RbCl]=0–40 m*M*	Develosil ODS 10 (20 cm × 4.6 mm I.D.)
Effect of pH	Water–methanol (55:45); pH=2.4–5.6 by hydrochloric acid, perchloric acid, or TCA; [18-crown-6]=0 or 20 m*M*.	Develosil ODS 10 (20 cm × 4.6 mm I.D.)
Effect of ion pairing	Water–methanol (55:45); pH=2.5 by hydrochloric acid; [18-crown-6]=0 or 20 m*M*; [$C_7H_{15}SO_3Na$]=0–20 m*M*	Develosil ODS 10 (15 cm × 4.6 mm I.D.)

RESULTS AND DISCUSSION

Effect of alkali metal ion

The effect of alkali metal ion on the retention of guest compounds was investigated by using mobile phases containing 0–40 m*M* lithium, sodium, potassium or rubidium chlorides in the absence and presence of 10 m*M* 18-crown-6, the pH of which was adjusted to 2.5 by addition of hydrochloric acid. Fig. 1 shows the changes in the *k* values of benzylamine, N-methylbenzylamine and benzoic acid as a function of Rb^+

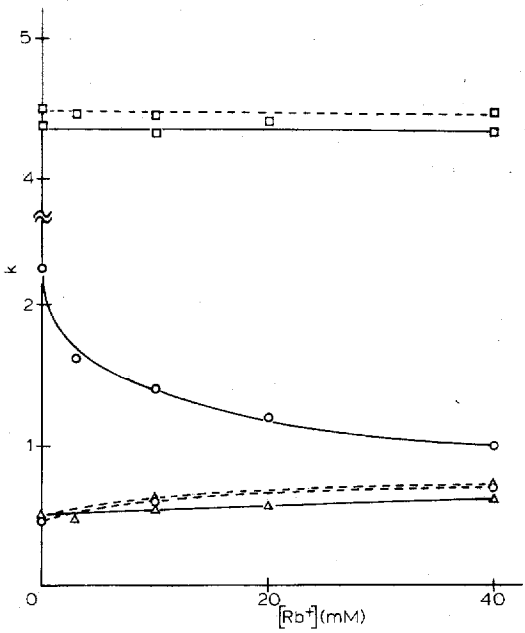


Fig. 1. Effect of Rb^+ concentration on the capacity factors of benzylamine (○), N-methylbenzylamine (△) and benzoic acid (□) observed with the mobile phases (pH 2.5 with hydrochloric acid) containing 10 m*M* 18-crown-6 (——) and in the absence of crown ether (– – –).

concentration. When the mobile phase did not contain 18-crown-6 (as indicated by broken curves), the k values of both amines slightly increased with increasing Rb^+ concentration, whereas no apparent change was observed in the k value of benzoic acid. This conforms to the fact that the ionized species suffer stronger salting-out effects than the neutral ones in reversed-phase chromatography. On the other hand, when 10 m*M* 18-crown-6 was added to the mobile phase (as indicated by solid curves), the k value of benzylamine alone markedly decreased with increasing concentration of Rb^+, while the value itself became larger for the whole range of Rb^+ concentration than that obtained by using the mobile phase in the absence of crown ether. However, the k *vs.* $[Rb^+]$ curves for N-methylbenzylamine and benzoic acid indicate that the k values were slightly decreased by the presence of 18-crown-6. This suggests that these compounds have little or no interaction with 18-crown-6, and that the salting-out effect may be weakened by association of the Rb^+ with the 18-crown-6.

The changes in the k values of various guests evaluated in terms of k_{40}/k_0 are given in Table II, where k_0 and k_{40} denote the respective capacity factors measured in the absence and presence of 40 m*M* of each ion. It is found that when the mobile phase did not contain 18-crown-6, the degree of enhancement of the k value due to salting-out effects appeared almost indifferent to the kind of added ions and the class of amino group of the guest compounds. However, the presence of 18-crown-6 in the mobile phase exerted quite a different effect on the k value; for instance, as can be seen in Fig. 2 which demonstrates the change in the k value of ABPC as a function of the concentration of four alkali metal ions, addition of K^+ and Rb^+ to the mobile phase containing 10 m*M* 18-crown-6 gave rise to a significant decrease in the k value, while Na^+ produced almost no apparent change and Li^+, on the contrary, brought about enhanced retention. The sequence of this ion effect agrees with that of the stabilities of the complexes between the alkali metal ion and 18-crown-6 (*i.e.* $K^+ > Rb^+ > Na^+ > Li^+$)[7], suggesting competition between the alkali metal ion and the guest ammonium in binding to 18-crown-6. It is interesting to find in Fig. 2 that the shape of the k *vs.* $[K^+]$ curve for ABPC obtained by using the mobile phase without containing 18-crown-6 (as indicated by a broken curve) is gradually increased with increasing concentration of K^+, and, in the region above 30 m*M*, it converges to the same level as

TABLE II

CHANGES IN CAPACITY FACTORS (k_{40}/k_0) BY ADDITION OF ALKALI METAL IONS TO THE MOBILE PHASES IN THE ABSENCE AND PRESENCE OF 18-CROWN-6

The values of k_0 and k_{40} are the capacity factors measured with the mobile phases containing 0 and 40 m*M* of each ion, respectively.

Guest compound	*0 mM 18-crown-6*				*10 mM 18-crown-6*			
	Li^+	*Na^+*	*K^+*	*Rb^+*	*Li^+*	*Na^+*	*K^+*	*Rb^+*
CEX	1.42	1.41	1.39	1.38	1.37	0.90	0.39	0.51
ABPC	1.47	1.47	1.44	1.43	1.34	0.89	0.39	0.51
ACPC	1.41	1.41	1.40	1.36	1.40	1.14	1.00	1.00
Benzylamine	1.54	1.44	1.51	1.49	1.21	0.81	0.32	0.44
p-Toluidine	1.53	1.54	1.50	1.50	1.16	0.68	0.31	0.40
N-Methylbenzylamine	1.45	1.43	1.41	1.39	1.38	1.18	1.18	1.18

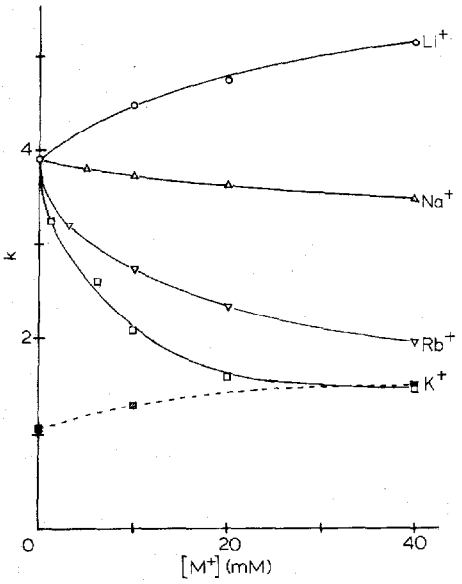


Fig. 2. Effect of alkali metal ion concentration on the capacity factor of ABPC observed with mobile phases (pH 2.5 with hydrochloric acid) containing 0–40 m*M* Li^+ (○), Na^+ (△), K^+ (□, ■) and Rb^+ (▽) in the absence (– – –) and presence (——) of 10 m*M* 18-crown-6.

that observed with the 18-crown-6-containing mobile phase. The use of other alkali metal ions in the absence of 18-crown-6 gave almost the same k values as K^+.

These results indicate that the salt added to the crown ether-containing mobile phase exerted different effects on the retention of guest compounds on the hydrophobic stationary ligand; one effect is to decrease the k value by competing with the guest ammonium ion in binding to the crown ether, and the other is to increase it by salting-out. As the latter effect is enhanced by increasing the concentration of the alkali metal ion but is almost independent of their nature, the shape of the k *vs.* $[M^+]$ curves in Fig. 2 depends largely on the former effect; the rapid initial decrease in the k value in the low-concentration region of K^+ and Rb^+ could be due to strong competition with the ammonium in binding to the 18-crown-6, which is followed by a plateau where the two effects are balanced. This state will probably be followed by gradual increase in the k value in the higher concentration region where the curve may conform with the extrapolated part of the broken curve. Sodium ion has such a weak binding to 18-crown-6 as to give no apparent change in the k value, and Li^+, which is known not to associate with 18-crown-6, exerts solely the salting-out effect, the shape of the k *vs.* $[Li^+]$ curve in Fig. 2 therefore appearing similar to the broken curve.

The change in the k values for several guest compounds in the presence of 10 m*M* 18-crown-6 are also shown in Table II in terms of k_{40}/k_0. It is found that primary amino compounds except ACPC suffered from effects similar to ABPC as mentioned above, but the degree of the effects varied depending on the relative stability of the complex. As shown in Fig. 3, ACPC, which is known to have a weak interaction with 18-crown-6 on account of sterically hindered primary amino group[5], exhibited a slight decrease in the k value on addition of K^+ and Rb^+; in addition, the k value itself became

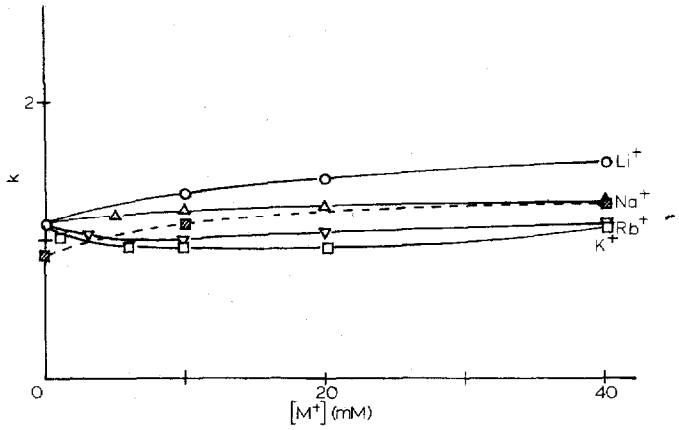


Fig. 3. Effect of alkali metal ion concentration on the capacity factor of ACPC observed with mobile phases (pH 2.5 with hydrochloric acid) containing 0–40 m*M* Li^+ (○), Na^+ (△), K^+ (□) and Rb^+ (▽) in the absence (- - -) and presence (——) of 10 m*M* 18-crown-6.

smaller by addition of K^+ than those indicated by the broken curve. This suggests that the salting-out effect is apparently weakened by association of K^+ with 18-crown-6. The guest compounds bearing either secondary amino groups or no amino group suffered from little or no salting-out effect.

The above discussion on the salt effects is limited to the role of alkali metal ions, because these salts involve, as the common anion, Cl^- which is so hard a base as to scarcely exert any ion-pair effect on the retention of the guest ammonium. The role of soft anions is discussed below from the view-point of ion-pair effects.

Effect of pH

It is known that in reversed-phase liquid chromatography, the retention of amino compounds on a hydrophobic stationary phase decreases with increasing proton concentration because of the stronger hydration of ammonium than neutral species[8]. This is basically true for the present case, which involves a crown ether as a neutral host substance, although the retention itself of a guest compound having a primary amino group is significantly enhanced by association with a crown ether dissolved in the mobile phase and absorbed onto the stationary phase. Naturally, the retention of the non-ionized amino group is known to hardly be affected at all by crown ethers[4].

Fig. 4 shows the relationship between the *k* value of benzylamine and the pH of the mobile phase in the absence and presence of 20 m*M* 18-crown-6, when the pH was varied between 2.4 and 5.6 by addition of hydrochloric acid, perchloric acid or trichloroacetic acid (TCA). It is found that the retention was enhanced markedly by the presence of 18-crown-6 over the whole pH range, and that a drastic decrease in the *k* value due to ion exclusion was found with increasing proton concentration, up to pH 4. The kind of acid used was also responsible for a change in the *k* value at a given pH; the *k* value obtained by using the mobile phase acidified with TCA was always higher than those found using hydrochloric or perchloric acids. These *k vs.* pH profiles were very similar for all the primary amino compounds, but differed from those of the guest compounds which make little or no interaction with crown ether. For instance,

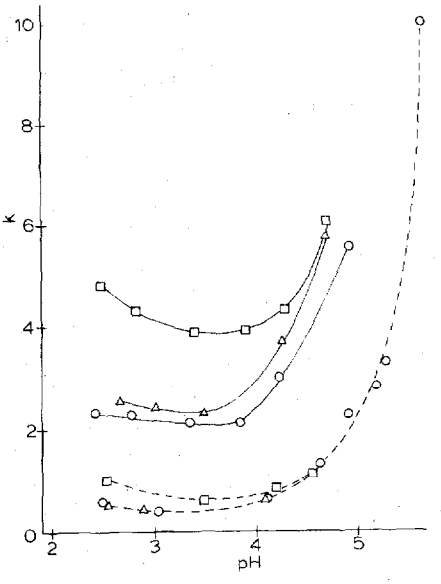


Fig. 4. Effect of pH on the capacity factor of benzylamine observed with mobile phases acidified with hydrochloric acid (○), perchloric acid (△), or TCA (□) in the absence (– – –) and presence (——) of 20 m*M* 18-crown-6.

the *k vs.* pH curves for N-methylbenzylamine shown in Fig. 5 indicate that there was almost no change in the *k* value when 18-crown-6 was added to the mobile phase. However, despite the presence of 18-crown-6, ion exclusion took place with increasing proton concentration up to pH 4, and the *k* value observed using the mobile phase acidified with TCA was higher than those found using hydrochloric and perchloric acids. An inverse relationship was obtained with respect to benzoic acid; as shown in

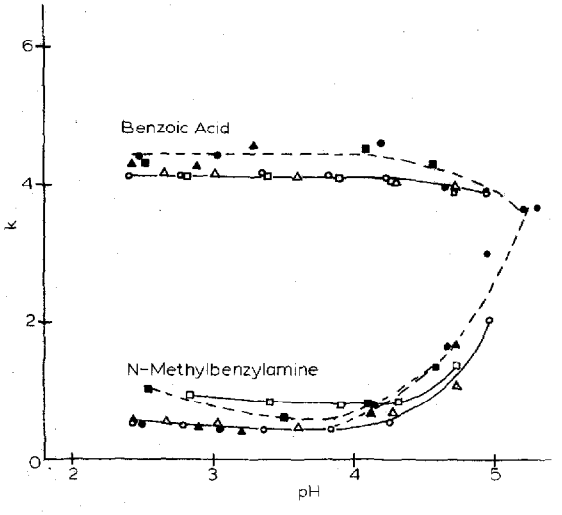


Fig. 5. Effect of pH on the capacity factors of N-methylbenzylamine and benzoic acid observed with mobile phases acidified with hydrochloric acid (○, ●), perchloric acid (△, ▲) and TCA (□, ■) in the absence (– –) and presence (——) of 20 m*M* 18-crown-6. The open symbols specify the data points belonging to the solid curves.

Fig. 5, the k value of benzoic acid was lowered by the presence of 18-crown-6 and was almost independent of pH and nature of the acid. This is possibly because benzoic acid competes with 18-crown-6 in binding to the hydrophobic ligand. A slight decrease in the k value at pH > 4 is obviously a result of the ion exclusion effect.

Effect of ion pairing

In the previous papers[4–6], we reported that in reversed-phase liquid chromatography using crown ether-containing mobile phases, the retention of primary amino compounds on a hydrophobic stationary phase is enhanced by increasing the concentration and hydrophobicity of the crown ether, and that a more hydrophobic crown ether causes the capacity factor to reach a higher maximum value at a lower crown ether concentration. A similar effect has been demonstrated for a hydrophobic counter ion in normal reversed-phase ion-pair chromatography[9]. In the present system which involves both a crown ether and a counter ion in the aqueous mobile phase, it is expected that the number of ion pairs may be increased, because the association of the ammonium cation with the crown ether brings about dehydration of the counter anion. Therefore, the increase in the number of ion-pairs, as well as in the hydrophobicity of guest ammonium ion itself, may exert a synergistic effect on the specific retention of the amino compounds.

As can be seen from Fig. 5, the k value for benzylamine at a given pH varies with the acid used, *i.e.* the k *vs.* pH curves obtained by using the mobile phases acidified with hydrochloric and perchloric acids appear almost parallel, both showing a reciprocal decrease in slope with increasing proton concentration. The curves obtained with TCA appear higher than those with using hydrochloric and perchloric acids, and exhibit a significant increase at lower pH regions(<3.5). Such differences in the k *vs.* pH profile for different acids become more explicit when 18-crown-6 is involved in the mobile phase.

These results suggest that the enhancement of the ion-pair effect caused by crown ethers may be much pronounced by increasing the hydrophobicity of the counter ion, and that this effect may be specific to primary amino compounds. Therefore, we used heptanesulphonate ($C_7H_{15}SO_3^-$) as a hydrophobic counter ion and measured the k values of several guest compounds as a function of the sulphonate concentration in the mobile phase at pH 2.5.

Fig. 6 shows the k *vs.* [$C_7H_{15}SO_3^-$] curves for ABPC, CEX and ACPC (β-lactam antibiotics, each bearing a primary amino group). When the mobile phase did not contain 18-crown-6, the k values for ABPC and CEX, as expected, increased with increasing sulphonate concentration, approaching maxima at 20 m*M*. Such increases in the k values due to ion-pairing were further enhanced by addition of 20 m*M* 18-crown-6 to the mobile phase, which brought about higher maximum values at lower sulphonate concentrations (*ca.* 10 m*M*). On the other hand, there appeared to be no significant change in the k value for ACPC. This is a result of the weak association of the amino group with 18-crown-6 owing to steric hindrance[5].

A similar steric effect was also observed with the *o*-, *m*- and *p*-isomers of toluidine (Figs. 7 and 8). In a normal ion-pair system, the ion-pair effect on the retention of these isomers is almost of the same order of magnitude, so that the separation of their peaks is not very much improved by increasing the sulphonate concentration. However, when 20 m*M* 18-crown-6 was added to the mobile phase, the k values for

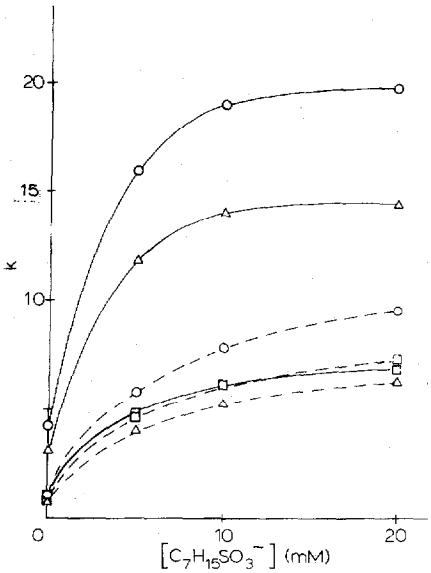


Fig. 6. Effect of heptanesulphonate concentration on the capacity factors of ABPC (○), CEX (△) and ACPC (□) observed with mobile phases (pH 2.5 with hydrochloric acid) containing 20 m*M* 18-crown-6 (——) and in the absence of crown ether (– – –).

the *p*- and *m*-isomers increased markedly to afford complete separation, whereas that for the *o*-isomer was less enhanced. This is possibly because the methyl group of *o*-toluidine hinders the association of the amino group with the 18-crown-6[4].

Fig. 8 demonstrates the above profiles for the separation of the toluidine isomers. In a normal reversed-phase mode using a mobile phase of water–methanol (55:45), the isomer peaks were not resolved. The addition of 10 m*M* heptanesulphonate to the mobile phase (Fig. 8a) exerted ion-pair effects of almost the same intensity for the

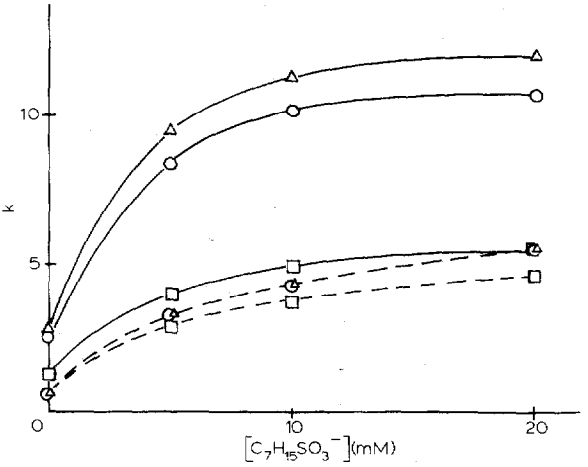


Fig. 7. Effect of heptanesulphonate concentration on the capacity factors of *o*- (□), *m*- (○) and *p*- (△) isomers of toluidine observed with mobile phases (pH 2.5 with hydrochloric acid) containing 20 m*M* 18-crown-6 (——) and in the absence of crown ether (– – –).

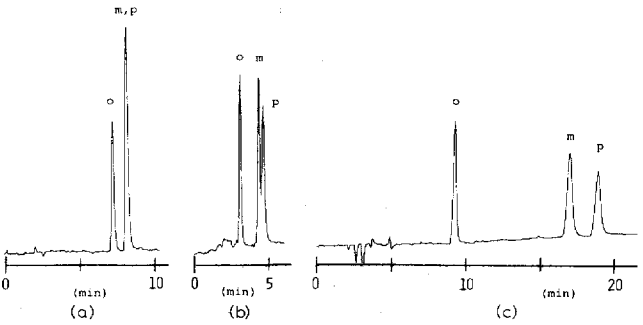


Fig. 8. Separation profiles of the *o*-, *m*- and *p*-isomers of toluidine. Mobile phases containing (a) 10 m*M* heptasulphonate, (b) 20 m*M* 18-crown-6 and (c) 10 m*M* heptasulphonate and 20 m*M* 18-crown-6. HPLC conditions: mobile phase, water–methanol (55:45); pH 2.5, hydrochloric acid; flow-rate, 0.7 ml/min; stationary phase, Chemcosorb ODS-H, 5 μm (15 cm × 4.6 mm I.D.); detection, UV 210 nm; column temperature, 40°C.

m- and *p*-isomers but a weaker effect for the *o*-isomer. The addition of 18-crown-6 instead of the sulphonate (Fig. 8b) revealed the difference in the complexing ability between the isomers, causing the peaks to be separated with relatively short retention times. With the mobile phase containing both sulphonate and crown ether (Fig. 8c), the isomers underwent a synergistic effect which resulted in their complete separation from each other.

The particular role of crown ethers in the ion-pair effect is also shown in Fig. 9, where k *vs.* $[C_7H_{15}SO_3^-]$ profiles obtained both in the absence and presence of 18-crown-6 are compared for primary and secondary amines. The interesting point that arises from Fig. 9 is that the k value for N-methylaniline decreases upon addition of 18-crown-6, while that of aniline, as expected, markedly increases. This may be explained as follows. A secondary ammonium group interacts so weakly with 18-crown-6 that the 18-crown-6 prefers rather to associate with Na^+ (this having entered the mobile phase along with the heptanesulphonate) or to be absorbed onto the stationary phase. This latter effect reduces the surface area of the stationary phase on which the N-methylaniline can be retained. Also, the salting-out effect is weakened by association

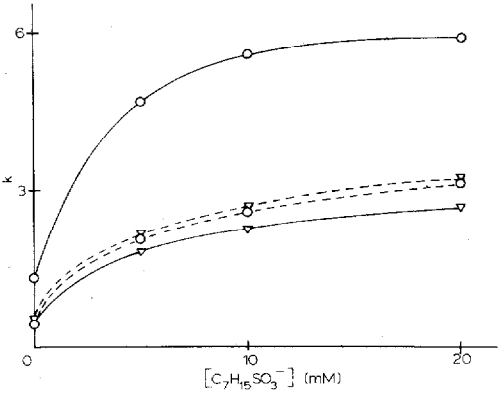


Fig. 9. Effect of heptanesulphonate concentration on the capacity factors of aniline (○) and N-methylaniline (▽) observed with mobile phases (pH 2.5 with hydrochloric acid) containing 20 m*M* 18-crown-6 (——) and in the absence of crown ether (– – –).

of the Na^+ with the 18-crown-6. The resulting crown ether-associated Na^+ forms an ion-pair with the heptanesulphonate, so that the ion-pairing between the N-methylaniline and the sulphonate apparently decreases. Therefore, if the potassium salt of heptanesulphonate is used as the pairing-agent instead of the sodium salt, the *k* value for N-methylaniline will be much lowered. Thus, the *k* value for N-methylaniline becomes lower by the presence of 18-crown-6 than that found in normal reversed-phase ion-pair chromatography.

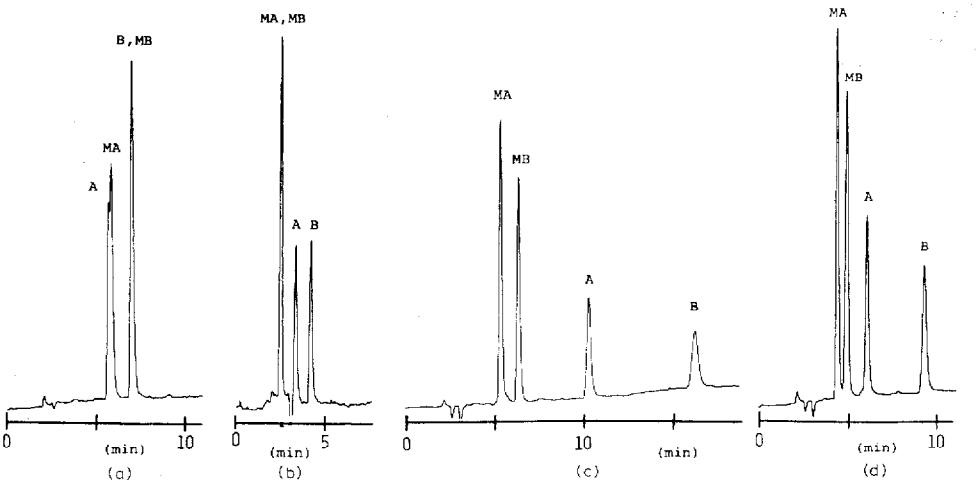


Fig. 10. Separation profiles of aniline (A), benzylamine (B), N-methylaniline (MA) and N-methylbenzylamine (MB). Mobile phases containing (a) 10 m*M* heptanesulphonate, (b) 20 m*M* 18-crown-6, (c) 10 m*M* heptane sulphonate and 20 m*M* 18-crown-6 and (d) 3 m*M* heptanesulphonate and 5 m*M* 18-crown-6. HPLC conditions as in Fig. 8.

Such a situation is clearly shown in Fig. 10, which compares elution profiles for aniline, benzylamine, N-methylaniline and N-methylbenzylamine observed with mobile phases containing heptanesulphonate, 18-crown-6 or both. When the mobile phase contained neither sulphonate nor crown ether, the amine peaks were not separated. Addition of 10 m*M* heptanesulphonate alone to the mobile phase (Fig. 10a) revealed a difference in the hydrophobicities of aniline and benzylamine (also between their N-methyl derivatives), but was incapable of distinguishing between primary and secondary amines. Addition of 20 m*M* 18-crown-6 alone to the mobile phase (Fig. 10b) revealed the complexing ability of the primary amines, the difference in their hydrophobicities being retained, whereas the secondary amines were indifferent of these effects. Addition of both sulphonate and 18-crown-6 to the mobile phase (Fig. 10c) exerted a synergistic effect on the retention of the amines, resulting in complete separation with longer retention times than would be expected from the sum of the individual effects. The retention and separation of these amines can be manipulated by changing the concentrations of the sulphonate and 18-crown-6. For instance, Fig. 10d shows that use of 3 m*M* heptanesulphonate and 5 m*M* 18-crown-6 leads to complete separation within 10 min.

It is concluded from these results that the addition of a crown ether to the mobile phase used in ion-pair chromatography gives rise to a specific ion-pair effect which recognizes the chemical structure around the amino group in a guest molecule.

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