Journal of Chromatography, 211 (1981) 1–13
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,716

LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

I. CAPACITY FACTORS OF AMINO COMPOUNDS ON A HYDROPHOBIC STATIONARY PHASE

TERUMICHI NAKAGAWA*, HIROSHI MIZUNUMA, AKIMASA SHIBUKAWA and TOYOZO UNO

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606 (Japan)

(Received February 9th, 1981)

SUMMARY

The retention behaviour of amino compounds (aromatic amines, amides, amino acids) in reversed-phase liquid chromatography with mobile phases containing crown ethers (18-crown-6 or dicyclohexyl-18-crown-6) have been investigated. The chromatographic process is viewed in terms of simultaneous equilibria involving protonation of the solute molecule, complexation of the cationic solute with the crown ether and associations of the complex and free species with the hydrophobic stationary phase. From these equilibria, an expression for the capacity factor as a function of the proton and crown ether concentrations in the mobile phase is derived. The equation predicts that the capacity factor should initially increase with increasing crown ether concentration at constant pH followed by a regular decrease at higher concentrations, and that the dependence of the capacity factor on the proton concentration should be rectangular hyperbolic at constant concentration of crown ether. The experimental results obtained at various crown ether concentrations at constant pH and at various pH values at constant concentration of crown ether are in accord with the predicted profiles. The analytical applicability of this method has been demonstrated in the reversed-phase separation of amino compounds.

INTRODUCTION

Marked progress in the reversed-phase liquid chromatography of ionizable solutes has been achieved by the introduction of ion-pair systems, where the solute retention on a hydrophobic stationary phase is significantly enhanced by ion-pair formation with a hydrophobic counter ion added in the mobile phase. A variety of practical applications has been described[1], and the retention mechanisms in this particular mode have been investigated on the basis of solvophobic theory in terms of the capacity factor[2–4].

0021-9673/81/0000–0000/$02.50

In 1967 crown ethers were first introduced by Pedersen[5]. Since then much attention has been paid to their analytical applications, that is to the specific cation-anchoring ability of crown ethers in solvent extraction, ion selective electrodes and chromatography. The progress in this field was recently reviewed by Kolthoff[6]. The use of crown ethers in liquid chromatography was first demonstrated by Cram and co-workers, who achieved optical resolution of amino acids and their ester salts through chiral recognition by a crown ether which was contained in the mobile phase[7] or immobilized on the stationary phase[8,9]. The retention mechanism of alkali metals in ion-exchange chromatography was studied by using a crown ether-containing solution as mobile phase[10]. A detailed examination of the association of metal cations with crown ethers was made by Horváth *et al.*[11], where the association constants were measured by liquid chromatography of the crown ethers using an aqueous cation-containing solution as mobile phase and a non-polar bonded stationary phase. Recent developments have included the use of immobilized crown ethers as stationary phases.

The aim of the present work was to investigate the retention behaviour of amino compounds in reversed-phase liquid chromatography with mobile phases containing crown ethers, and to demonstrate the analytical applicability of the proposed method.

THEORETICAL

In reversed-phase liquid chromatography it is expected that the retention of a cationic solute will be significantly affected by the addition of a crown ether to the mobile phase, and that the change will be dependent on the degree of complex formation between them. If the solute is a cationic organic substance, such as the amino compounds used in this work, the complexation with the crown ether is affected by the pH of the mobile phase, since the non-ionized form makes a much smaller contribution to the complex formation. Therefore, as depicted in Fig. 1, the chromatographic process comprises simultaneous equilibria involving protonation of the amino compound, complex formation between the cation and the crown ether and association of the complex and free species with the hydrophobic stationary phase. Since the solute concentration in liquid chromatography is usually very low, it is postulated that the complex formation has a 1:1 stoichiometry[12]. From the equilibria involved (see Fig. 1) and the definition of capacity factor, *i.e.*

$$k = \varphi \cdot \frac{[LS]_s + [LSH^+]_s + [LCSH^+]_s}{[S]_m + [SH^+]_m + [CSH^+]_m} \tag{1}$$

it follows that

$$k = \varphi\,[L]_s \cdot \frac{K_a K_{LS} + A\,[H^+] + B\,[C]_m\,[H^+]}{K_a + [H^+] + K_{CSH}\,[C]_m\,[H^+]} \tag{2}$$

where φ is the phase ratio, $[L]_s$ is the concentration of free stationary phase, $[C]_m$ is the concentration of free crown ether in the mobile phase, K_a is the dissociation constant of the protonated solute and the other K values are formation constants specified in

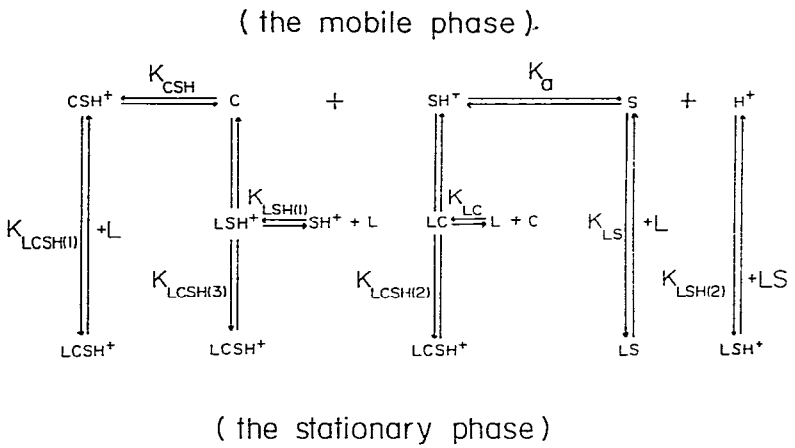


Fig. 1. Schematic illustration of the equilibria involved in a reversed-phase chromatographic process with a crown ether-containing mobile phase. S = Non-ionized solute; SH^+ = protonated solute; C = crown ether; CSH^+ = complex of crown ether + protonated solute; L = hydrophobic stationary phase; LS = non-ionized solute bound to stationary phase; LSH^+ = protonated solute bound to stationary phase; $LCSH^+$ = complex bound to stationary phase; K_a = dissociation constant of protonated solute; other K values = formation constants of specified equilibria.

Fig. 1; A represents $K_{LSH(1)}$ or $K_{LSH(2)}K_aK_{LS}$, and B denotes $K_{LCSH(1)}K_{CSH}$, $K_{LCSH(2)}K_{LC}$, $K_{LCSH(3)}K_{LSH(1)}$ or $K_{LCSH(3)}K_{LSH(2)}K_aK_{LS}$. The amount of stationary phase bound to solute is expected to represent only a small part of the total, $[L_T]$:

$$[L_T] = [L]_s + [LC]_s \tag{3}$$

Substituting for $[L]_s$ in eqn. 2 and introducing K_{LC}, the capacity factor is finally expressed by:

$$k = \varphi [L_T] \cdot \frac{K_aK_{LS} + A [H^+] + B [C]_m [H^+]}{(1 + K_{LC} [C]_m)(K_a + [H^+] + K_{CSH} [C]_m [H^+])} \tag{4}$$

Eqn. 4 yields several expressions for the capacity factor depending on the choice of the constants A and B. These constants reflect the formation processes of the crown ether complexes and of their binding with the hydrophobic stationary phase. Although each of the processes may be involved in the actual chromatographic system, it seems unlikely that the cationic solute is bound first to stationary phase followed by complex formation with the crown ether.

In the present investigation, we observed the effect of varying the crown ether concentration on the capacity factor of the amino compounds at relatively high proton concentration. Since the amino group is almost completely protonated under these conditions, the simplified model in Fig. 1 at constant pH leads to the expression

$$k = \frac{k_0 + \varphi [L_T] D [C]_m}{(1 + K_{CSH} [C]_m)(1 + K_{LC} [C]_m)} \tag{5}$$

where k_0 is capacity factor for $[C]_m = 0$ and D represents $K_{LCSH(1)}K_{CSH}$, $K_{LCSH(2)}K_{LC}$ or $K_{LCSH(3)}K_{LSH}$. Eqn. 5 predicts that the value of the capacity factor at constant pH first increases with increasing crown ether concentration, reaches a maximum and then decreases. In cases where the concentration of crown ether may be regarded as constant, eqn. 4 reduces to

$$k = \frac{k_0 + \varphi [L_T] F [H^+]}{E (K_a + G [H^+])} \tag{6}$$

where $E = 1 + K_{LC} [C]_m$, $F = A + B [C]_m$ and $G = 1 + K_{CSH} [C]_m$. Eqn. 6 suggests that the dependence of the capacity factor on the proton concentration is rectangular hyperbolic at constant concentration of crown ether.

EXPERIMENTAL

Liquid chromatography

A Twincle liquid chromatograph (Jasco, Tokyo, Japan) equipped with a Uvidec-100 III variable-wavelength UV detector (Jasco) was used for the measurements of capacity factor. The stationary phase was Nucleosil 10 C_{18} (Mackerey, Nagel & Co., Düren, G.F.R.) packed in a stainless-steel tube (15 cm × 4 mm I.D.). The mobile phases were prepared by dissolving known amounts of crown ether in a mixture of water and methanol, the pH of which was varied between 2.1 and 4.8 by addition of HCl. The mixing ratio (v/v) of water–methanol was 1:1 for mobile phases containing 18-crown-6 and 1:2 for those containing dicyclohexyl-18-crown-6. The flow-rate was maintained at 0.7 ml/min, and all the operations were carried out at ambient temperature.

Materials

Amino acids, their ester hydrochlorides, aniline hydrochloride, benzamide and salicylamide of reagent grade were obtained from Wako (Osaka, Japan), and *o*-, *m*- and *p*-toluidines, *o*-, *m*- and *p*-aminobenzoic acids and benzylamine hydrochloride were purchased from Nakarai (Kyoto, Japan). 18-crown-6 of reagent grade and dicyclohexyl-18-crown-6 were obtained from Nippon Soda Co. (Tokyo, Japan). Dicyclohexyl-18-crown-6 was used without separation of the A,B-isomers.

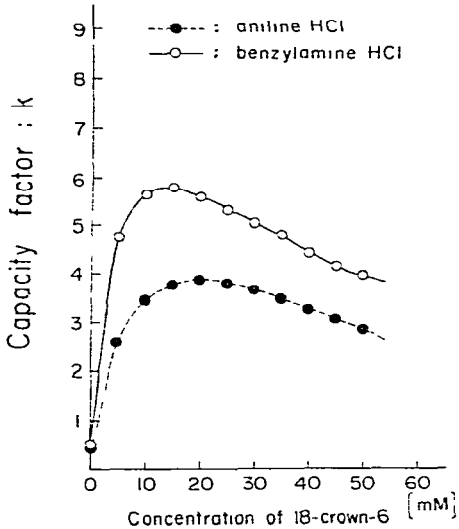
Measurement of capacity factor

The solute was dissolved in a small portion of mobile phase without crown ether, and the minimum amount required for UV detection was injected so as to maintain linearity of the chromatographic system. The retention time, t_R, was repeatedly measured at a peak of the elution curve, and the average value was used to calculate the capacity factor, $k = (t_R - t_0)/t_0$, where t_0 is the retention time of potassium dichromate.

RESULTS

Effect of crown ether concentration

The dependence of the capacity factor on the concentration of crown ether was



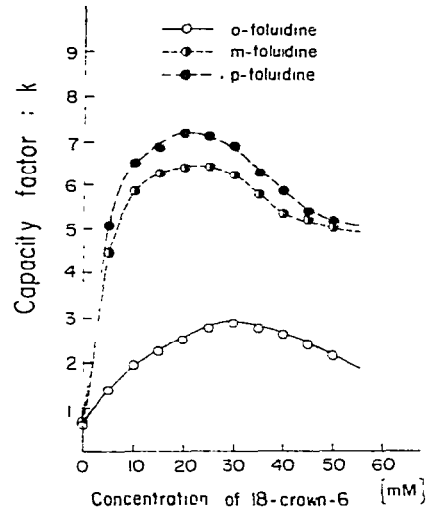
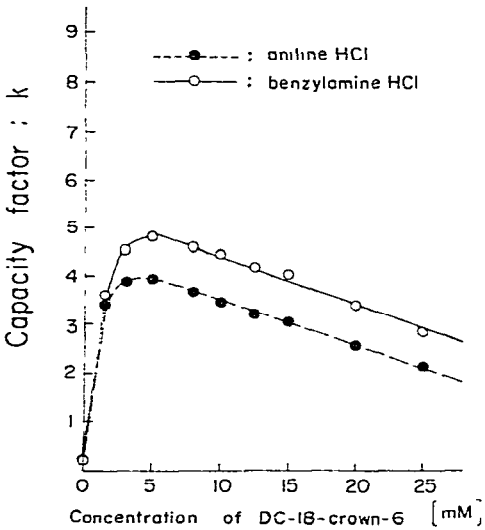


Fig. 2. Effect of 18-crown-6 concentration on the capacity factors of aniline and benzylamine at pH 3.0.

Fig. 3. Effect of 18-crown-6 concentration on the capacity factors of toluidine isomers at pH 3.0.

investigated by using mobile phases containing 0–50 m*M* crown ethers at pH 3.0. The observed profiles are shown in Figs. 2–13. Figs. 2 and 3 give the results for aniline, benzylamine and isomers of toluidine in mobile phases containing 18-crown-6 and Figs. 4 and 5 show those obtained in mobile phases containing dicyclohexyl-18-



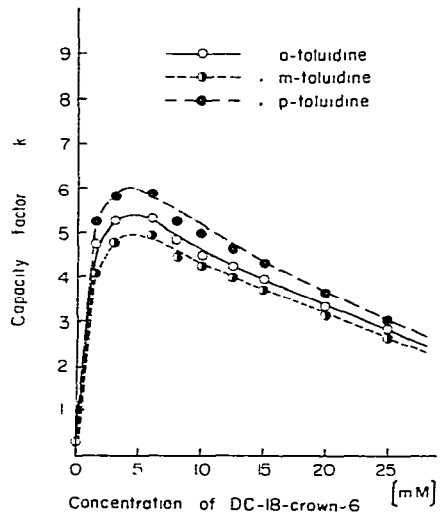


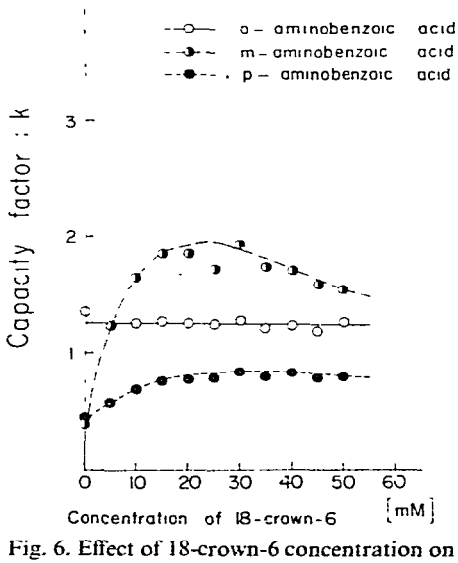
Fig. 4. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of aniline and benzylamine at pH 3.0.

Fig. 5. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of toluidine isomers at pH 3.0.

crown-6. It is clear that the retention behaviour of these solutes is in agreement with that predicted by eqn. 5. While there are almost no differences between aniline and benzylamine with respect to the concentration of crown ether at the maximum k value, the capacity factor of benzylamine is always larger than that of aniline over the range of crown ether concentration examined, suggesting that benzylamine has the higher hydrophobicity, possibly due to the presence of a methylene group. Although from Figs. 2 and 5 it appears that there are almost no significant differences in the magnitude of the capacity factor for any solute between mobile phases containing 18-crown-6 or dicyclohexyl-18-crown-6, the capacity factors actually obtained in mobile phases containing dicyclohexyl-18-crown-6 were much larger than those in 18-crown-6 when the methanol contents were the same. The methanol content in the former mobile phase was, therefore, adjusted to be twice that in the latter in order to give appropriate retention times. This difference in the effect of the crown ethers is obviously due to the strong hydrophobicity of dicyclohexyl-18-crown-6.

With toluidine isomers, it is noticeable that the capacity factor of *o*-toluidine in the 18-crown-6-containing mobile phase (Fig. 3) is much lower than those of the other isomers. This strange behaviour suggests the existence of steric effects on the complexation with the crown ether and on the affinity for the stationary phase. These effects may be used to advantage for the specific separation of *o*-toluidine.

The results for aminobenzoic acids are shown in Figs. 6 and 7, where the capacity factors of the *o*- and *p*-isomers exhibit different behaviours to that predicted by eqn. 5. The gentle slope for *p*-aminobenzoic acid (Fig. 6) suggests that the complexation with 18-crown-6 is weak and/or a low affinity for the stationary phase; the *o*-isomer seems quite indifferent to 18-crown-6. A comparison of the results for the *o*-isomers of toluidine and aminobenzoic acid shows that substitution at the *o*-position



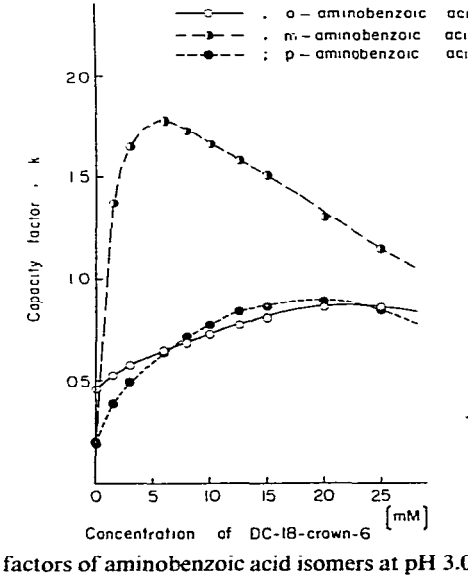


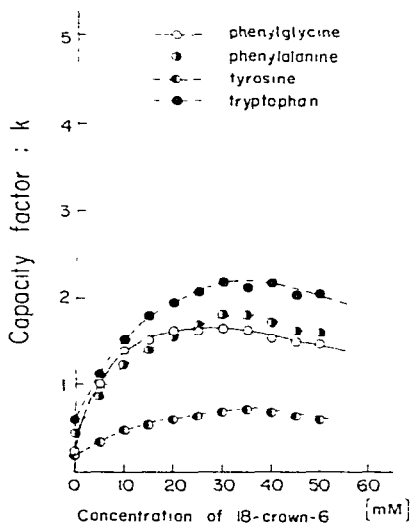
Fig. 6. Effect of 18-crown-6 concentration on the capacity factors of aminobenzoic acid isomers at pH 3.0.

Fig. 7. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of aminobenzoic acid isomers at pH 3.0.

of aniline interferes with the complex formation between the ionized amino group and the crown ether, the degree of interference being dependent on the nature of the substituent. The carboxyl group of aminobenzoic acid, which must be partially ionized in this mobile phase, is likely to interfere with complex formation, although hydrogen bonding between the free carboxyl group and the crown ether may make a small contribution to the complex formation. It is not clear why *m*-aminobenzoic acid exhibits a higher capacity factor than the *p*-isomer, but the present results suggest a promising method for the liquid chromatographic separation of aminobenzoic acid isomers.

Figs. 8 and 9 illustrate the dependence of the capacity factors of amino acids on the concentrations of crown ethers, and Figs. 10 and 11 show the corresponding results for hydrochlorides of amino acid methyl esters. The concentration of dicyclohexyl-18-crown-6 at the maximum k values of the amino acids (Fig. 9) is generally lower than that of 18-crown-6, but there are no marked differences between amino acids. This is possibly because these amino acids have the same configuration of the amino and carboxyl groups, *i.e.*, they are α-amino acids. Therefore the difference between the hydrophobicities of the aromatic groups seems responsible for the differences in magnitude of the capacity factor. This is confirmed by the results given in Figs. 10 and 11. Comparisons between Figs. 8 and 10 and between Figs. 9 and 11 indicate that the expected increase in hydrophobicity of the amino acids upon esterification of the carboxyl group results in pronounced enhancement of the capacity factor.

As seen in Figs. 12 and 13, the retention behaviour of amides is different from that of amines. The observed changes in the capacity factors of benzamide and salicylamide indicate that these solutes have almost no interaction with both 18-crown-6 and dicyclohexyl-18-crown-6. This can be related to the difference in basicity of the amino group between amines and amides.



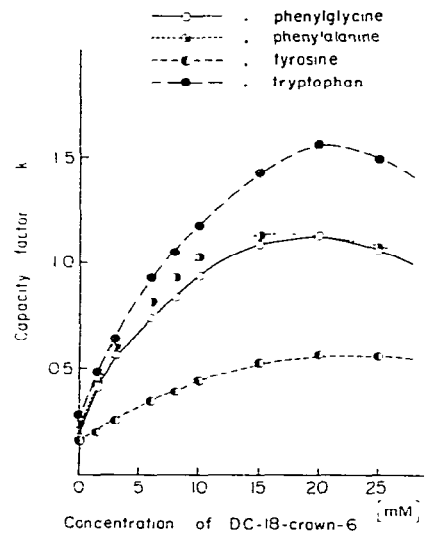
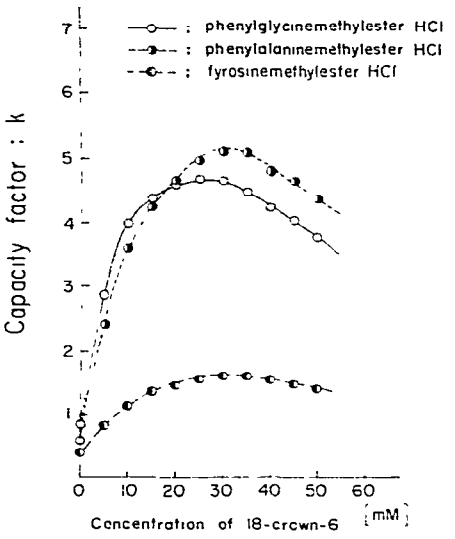


Fig. 8. Effect of 18-crown-6 concentration on the capacity factors of amino acids at pH 3.0.
Fig. 9. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of amino acids at pH 3.0.



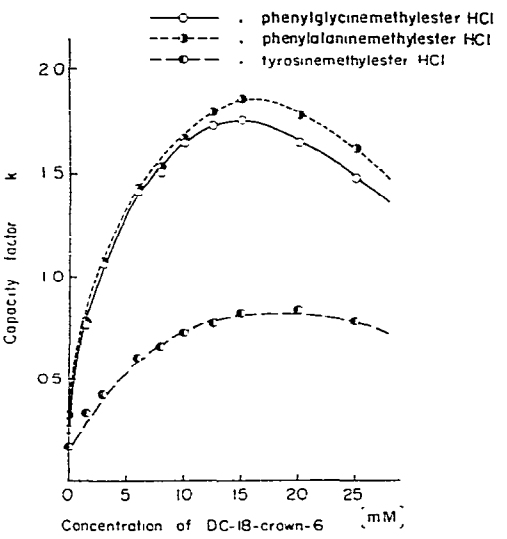
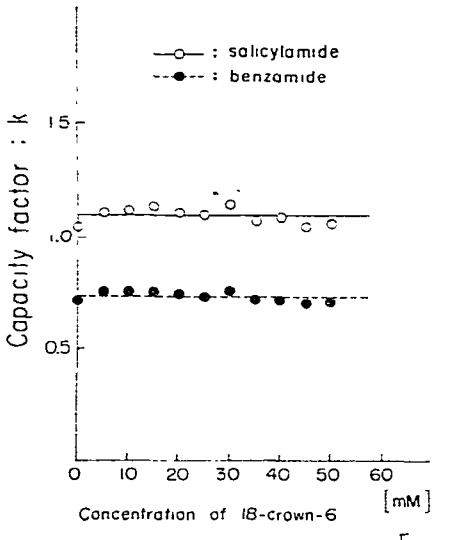


Fig. 10. Effect of 18-crown-6 concentration on the capacity factors of amino acid methyl esters at pH 3.0.

Fig. 11. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of amino acid methyl esters at pH 3.0.

Effect of pH

The dependence of the capacity factor on pH between 2.2 and 4.1 was investigated by using mobile phases containing 5 m*M* 18-crown-6. The results are shown in Figs. 14–19. All the observed profiles except those for *m*- and *p*-aminoben-



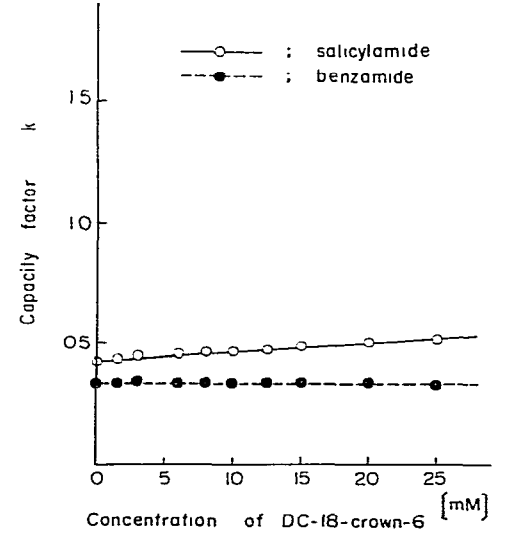


Fig. 12. Effect of 18-crown-6 concentration on the capacity factors of benzamide and salicylamide at pH 3.0.

Fig. 13. Effect of dicyclohexyl-18-crown-6 concentration on the capacity factors of benzamide and salicylamide at pH 3.0.

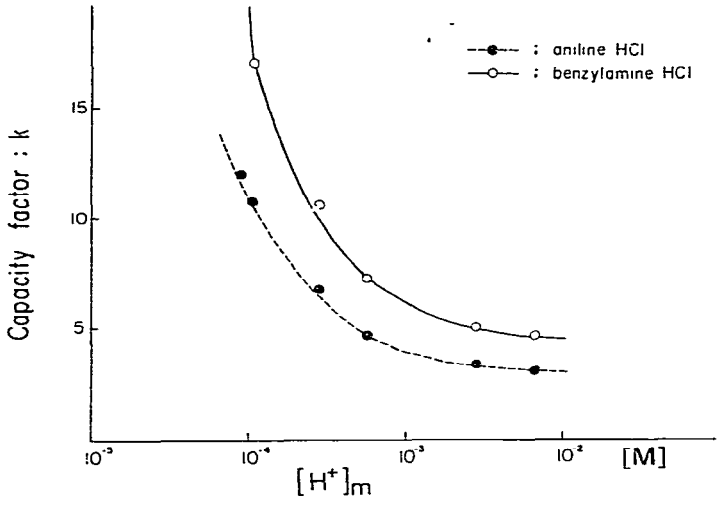


Fig. 14. Effect of hydrogen ion concentration on the capacity factors of aniline and benzylamine at 5 m*M* 18-crown-6.

zoic acids, salicylamide and benzamide exhibit rectangular hyperbolic curves as predicted by eqn. 6, that is, the capacity factor decreases inversely with increasing proton concentration. The magnitude of the decrease is dependent on the nature of the solute species and is almost the same for analogous compounds. The capacity factors of aniline, benzylamine (Fig. 14), toluidines (Fig. 15) and amino acid methyl esters (Fig. 17) show large decreases between pH 3 and pH 4, and converge at lower pH. From a comparison of the results for amino acids (Fig. 16) and their methyl esters, it appears that the free α carboxylic acid group exerts an effect which reduces the pH dependence of the capacity factor of the amino acids over the pH range examined. Similar results are found for the *o*- and *p*-isomers of aminobenzoic acid

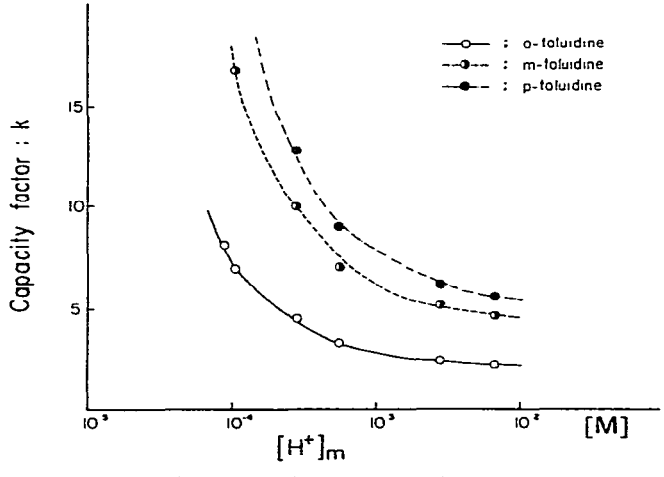


Fig. 15. Effect of hydrogen ion concentration on the capacity factors of toluidine isomers at 5 m*M* 18-crown-6.

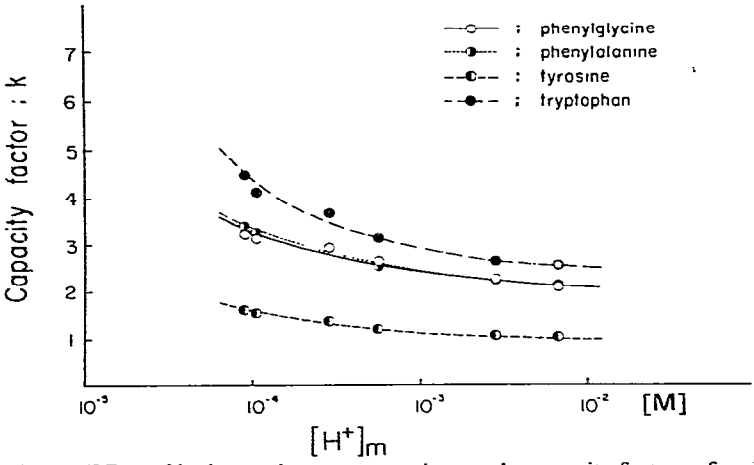


Fig. 16. Effect of hydrogen ion concentration on the capacity factors of amino acids at 5 m*M* 18-crown-6.

(Fig. 18), which show no dependence on pH, whereas the *m*-isomer exhibits an appreciable dependence in accord with eqn. 6. This behaviour of *m*-aminobenzoic acid is similar to that observed in Fig. 6. The capacity factors of benzamide and salicylamide (Fig. 19) remained constant with pH, suggesting that there are no appreciable interactions between these amides and 18-crown-6 dependent on proton concentration.

These pH profiles indicate that amino compounds whose capacity factors are dependent on the crown ether concentration are also dependent on proton concentration.

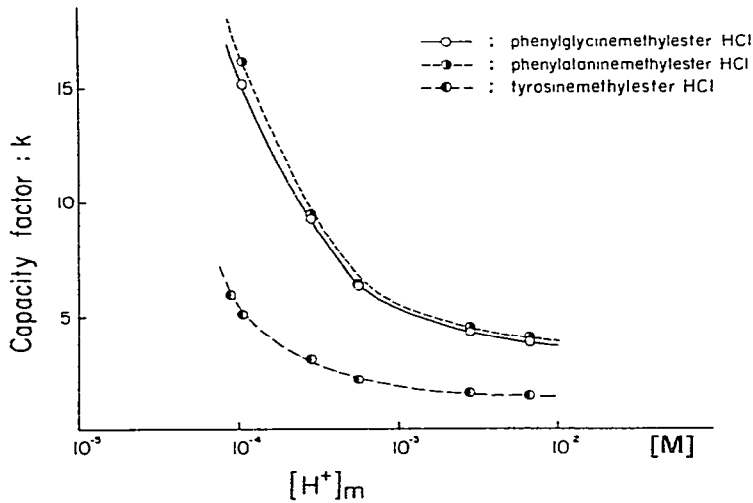


Fig. 17. Effect of hydrogen ion concentration on the capacity factors of amino acid methyl esters at 5 m*M* 18-crown-6.

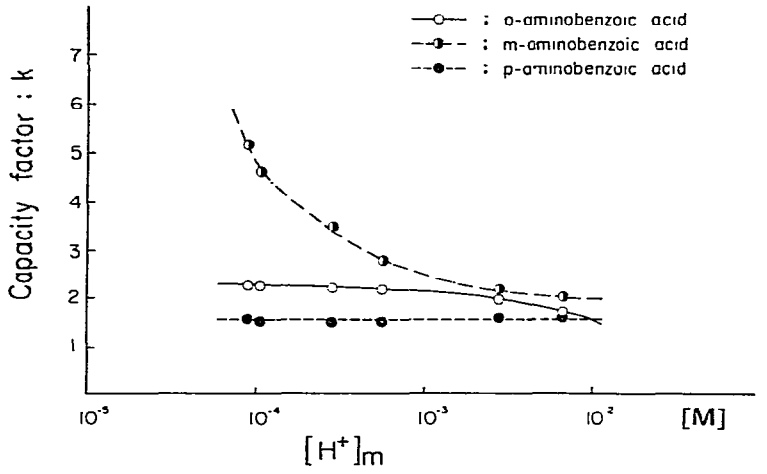


Fig. 18. Effect of hydrogen ion concentration on the capacity factors of aminobenzoic acid isomers at 5 m*M* 18-crown-6.

Effect of salt

A preliminary examination of the salt effect on the capacity factor was conducted by using mobile phases containing 0–30 m*M* KCl and 20 m*M* 18-crown-6 at pH 3.2. The results obtained with toluidine isomers are shown in Fig. 20, where it is seen that the capacity factor rapidly decreases at very low concentrations of KCl but gradually approaches a plateau at higher concentrations. The curves are slightly convex in the range 5–25 m*M* KCl. Although these results may be related to the strong affinity of K^+ for 18-crown-6, a detailed consideration of this point will be reserved for a subsequent paper, which will also include the effects of organic modifiers in crown ether-containing mobile phases.

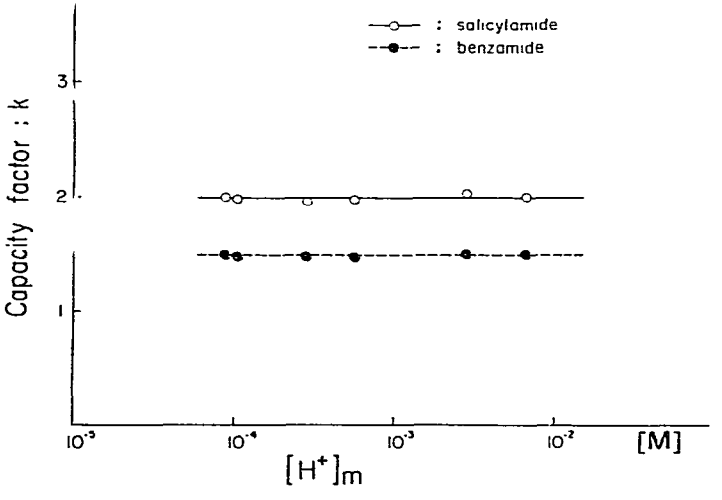


Fig. 19. Effect of hydrogen ion concentration on the capacity factors of benzamide and salicylamide at 5 m*M* 18-crown-6.

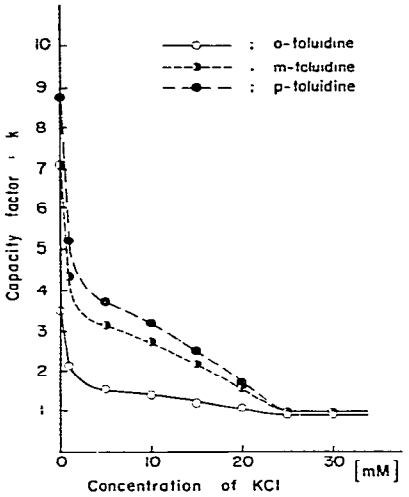


Fig. 20. Effect of KCl concentration on the capacity factors of toluidine isomers in reversed-phase liquid chromatography with a mobile phase containing 20 m*M* 18-crown-6 at pH 3.2.

DISCUSSION

Attempts to incorporate selective interactions into chromatographic systems have resulted in great advantages in separation chemistry. Ion-pair chromatography is a technique in which the interaction of an ionized solute with a counter ion in the mobile phase is utilized to manipulate the selectivity of solute retention on a hydrophobic stationary phase. Consideration of the mechanisms in combination with a variety of experimental observations has enabled the elucidation of the retention behaviour of ionizable solutes as a function of the concentrations of counter ion, proton and organic modifiers, etc. in the mobile phase.

On the other hand the use of crown ethers in liquid chromatography has been limited chiefly to the separation of inorganic cations on crown ether-bonded stationary phases, and no previous publication has referred to the utility of crown ether-containing mobile phases for the analysis of cationizable organic substances. The present results indicate that the use of such phases in reversed-phase liquid chromatography has the advantage of specific separation of organic amino compounds. Because of the similarity in modelling of the chromatographic process, the present expressions for the capacity factor are the same as those previously given for ion-pair chromatography. However, the rôle of the crown ether is different from that of the counter ion in ion-pair chromatography in that the former is a hydrophobic non-electrolyte which can participate in stereospecific interactions with organic cations. These properties promise a wider applicability of the present method, the selectivity of which should be enhanced by use of various derivatives of crown ethers and cryptands.

REFERENCES*

1 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
2 Cs. Horvath, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
3 Cs. Horvath, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
4 M. T. W. Hearn, B. Grego and W. S. Hancock, *J. Chromatogr.*, 185 (1979) 429.
5 C. J. Pedersen, *J. Amer. Chem. Soc.*, 89 (1967) 7017.
6 I. M. Kolthoff, *Anal. Chem.*, 51 (1979) 1R.
7 L. R. Sousa, D. H. Hoffman, L. Kaplan and D. J. Cram, *J. Amer. Chem. Soc.*, 96 (1974) 7100.
8 G. Dotsevi, Y. Sogoh and D. J. Cram, *J. Amer. Chem. Soc.*, 97 (1975) 1259.
9 G. Dotsevi, Y. Sogoh and D. J. Cram, *J. Amer. Chem. Soc.*, 98 (1976) 3038.
10 W. H. Delphin and E. P. Horwitz, *Anal. Chem.*, 50 (1978) 843.
11 Cs. Horváth, W. Melander and A. Nahum, *J. Chromatogr.*, 186 (1979) 371.
12 R. M. Izatt, N. E. Izatt, B. E. Rossiter and J. J. Christensen, *Science*, 199 (1978) 994.

* *Editor's Note:* See also W. J. T. Brugman and J. C. Kraak, *J. Chromatogr.*, 205 (1981) 170.