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LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

VIII. RETENTION BEHAVIOUR OF AMINO COMPOUNDS IN CATION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The use of crown ethers in the mobile phase with cation-exchange chromatography has been investigated. The capacity factors of primary amino compounds, which form host–guest complexes with 18-membered crown ethers, were significantly increased when 18-crown-6 or dicyclohexyl-18-crown-6 was added to the mobile phase. The degree of retention enhancement varied with the molecular structure of the guest compound, and also with the concentration of the crown ether and protons, the composition of the organic modifier and the nature of added electrolytes in the mobile phase. The retention mechanism in this particular system is discussed and the practical applicability to the separation of amino compounds is demonstrated.

INTRODUCTION

Recently, in high-performance ion-exchange liquid chromatography, ion exchangers in which the ion-exchange group is chemically bonded to totally porous silica microspheres have been widely used instead of cross-linked polystyrene ion-exchange resins because of the large number of theoretical plates, adequate ion-exchange capacity, high pressure resistance and rapid equilibration of the ion-exchange reaction. However, these ion exchangers allow limited selection of pH compared with the latter. It is well known in ion-exchange chromatography that the proton concentration, the nature and ionic strength of added electrolytes and the concentration of the organic solvent in the mobile phase affect the retention of sample solutes. For instance, ionic organic compounds can be separated by controlling the pH, ionic strength and/or concentration of the organic solvent of the mobile phase in high-performance ion-exchange chromatography, though there may also be a slight hydrophobic effect[1,2].

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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[3] or immobilized on the stationary phase[4,5]. Since then 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 of anions with a common cation. Shono and co-workers separated alkali and alkaline-earth metal ions with poly(12-crown-4)-modified silica which has a great affinity for Na^+[6], and separated nitrophenol isomers with poly(vinylbenzo-18-crown-6)-modified silica and a potassium chloride-containing mobile phase, where the electrostatic interaction between the K^+ complexed with the benzo-18-crown-6 moiety and the negative charges or dipoles of the nitrophenol isomers is predominant[7]. Furthermore, they separated alkali metal ions by using dodecyl-18-crown-6, which is highly lipophilic, dynamically coated on the stationary phase by hydrophobic interaction[8]. Igawa *et al.*[9] prepared a polyamide-type crown ether resin coated on silica and separated some anions. Detailed investigations of the retention behaviour of various amino compounds in reversed-phase liquid chromatography with mobile phases containing crown ethers were made by Nakagawa *et al.*[10–15].

In high-performance cation-exchange chromatography with crown ether-containing mobile phases, it is expected that the retention behaviour of amino compounds depends on the changes in hydrophobic interaction with the resin matrix as well as on ionic adsorption onto the ion-exchange site, because not only the hydrophobicity but also the ionic properties of protonated primary amino compounds can be affected by complex formation with crown ethers. The present study deals with the retention behaviour of amino compounds (aromatic amines, amides) in high-performance cation-exchange chromatography with mobile phases containing crown ethers and various alkali metal chlorides or ammonium chloride. The analytical applicability of the proposed method is demonstrated.

EXPERIMENTAL

Reagents and materials

The amino compounds and inorganic salts of reagent grade were obtained from commercial sources. They were used as supplied. 18-crown-6 (18-C-6) and dicyclohexyl-18-crown-6 (DC-18-C-6) were from Nippon Soda (Tokyo, Japan). DC-18-C-6 was used without separation of the A,B-isomers. Glass-distilled deionized water and glass-distilled methanol for high-performance liquid chromatography (HPLC) (Katayama Chemicals) were used to prepare the mobile phases. Reagent grade hydrochloric acid was used to adjust the pH of the mobile phase.

Chromatography

A liquid chromatograph (LC-3A; Shimadzu, Kyoto, Japan) equipped with a refractive index detector (SE-51; Showa Denko, Tokyo, Japan) or a variable-wavelength UV detector (SPD-2A; Shimadzu) was used for the measurements of the capacity factors of crown ethers and amino compounds. The stationary phase was a cation exchanger (Nucleosil 10SA, propyl phenyl sulphonate type; Macherey Nagel, Düren, F.R.G.) packed in a stainless-steel tube (50 mm × 4.6 mm I.D.). The operating conditions are given in Table I.

TABLE I

HPLC CONDITIONS

Column temperature: 40°C. Flow-rate: 1.0 ml/min.

Experiment (Detection)	*Mobile phase*	*Data given in*
k' of crown ether (RI)	Methanol–water (60:40); [LiCl], [NaCl], [KCl], [NH_4Cl] or [RbCl] = 20 m*M* or no salts	Table II
k' *vs.* crown ether concentration (UV 254 nm)	Methanol–water (60:40); [18-C-6] or [DC-18-C-6] = 0–15 or 0–20 m*M*; [LiCl], [NaCl], [KCl], [NH_4Cl] or [RbCl] = 20 m*M*	Figs. 1–3
k' *vs.* pH (UV 254 nm)	Methanol–water (60:40); [LiCl] = 20 m*M*; [18-C-6] = 5 m*M* or [DC-18-C-6] = 3 m*M* or no crown ethers	Figs. 4, 5
k' *vs.* % methanol (UV 254 nm)	Methanol–water (40:60 to 90:10); [LiCl] = 20 m*M*; [18-C-6] = 5 m*M* or [DC-18-C-6] = 3 m*M* or no crown ethers	Fig. 6

The samples were dissolved in methanol, and the minimum amount required for detection was applied to the chromatograph. The capacity factors, k', were calculated from $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the sample and of a non-adsorbed substance (water or methanol), respectively, averaged over repeated measurements at the top of the elution curve. Both water and methanol gave almost identical t_0 values.

RESULTS AND DISCUSSION

Capacity factors of crown ethers

The capacity factors of 18-C-6 and DC-18-C-6 were measured by the use of mobile phases containing 20 m*M* LiCl, NaCl, NH_4Cl, RbCl or no salts. The results are shown in Table II. It was found that the two crown ethers were scarcely retained on the cation-exchange stationary phase when the mobile phase contained lithium chloride or no salts. However, the addition of NaCl, RbCl, KCl or NH_4Cl to the mobile phase resulted in increased capacity factors in this order, which reflects the stability of the complex formed between the cations and the 18-membered crown ethers. Thus, it follows that the magnitude of the increase in the capacity factors for the crown ethers depends on the stability of the complex, and that the hydrophobic

TABLE II

CAPACITY FACTORS, k', OF 18-CROWN-6 AND DICYCLOHEXYL-18-CROWN-6

Stationary phase: Nucleosil 10SA (50 mm × 4.6 mm I.D.). Mobile phase: methanol–water (60:40) (pH 3.0 with hydrochloric acid). Flow-rate: 1.0 ml/min. Column temperature: 40°C. Detection: RI.

	No salts added	*20 mM LiCl*	*20 mM NaCl*	*20 mM KCl*	*20 mM NH_4Cl*	*20 mM RbCl*
18-crown-6	0.58	0.36	2.49	2.82	3.02	2.73
Dicyclohexyl-18-crown-6	0.68	0.44	1.98 2.82	2.78	3.13	2.43

interactions between the crown ethers and the resin matrix of the stationary phase are very weak under these HPLC conditions. It is not clear from Table II why DC-18-C-6, which is a mixture of A,B-isomers, gave two peaks only when the mobile phase contained 20 m*M* sodium chloride.

Effect of crown ether concentration

The capacity factors of various amino compounds are shown in Fig. 1 as a function of the concentration of 18-C-6 or DC-18-C-6 in the mobile phase (pH 3.0) containing 20 m*M* lithium chloride. The capacity factors of primary amines increased with increasing concentration of the crown ethers, and gradually approached maxima. There was almost no difference in the effects of 18-C-6 and DC-18-C-6, in contrast to reversed-phase liquid chromatography where the capacity factors of primary amines were increased much more by addition of DC-18-C-6 than 18-C-6[10]. Thus, it follows that the ionic interaction between the protonated amino group of the guest molecule and the cation-exchange site of the resin is enhanced by complex formation with the crown ether, and that the contribution of the ionic effect predominates over that of the hydrophobic interaction with the resin matrix. On the other hand, the

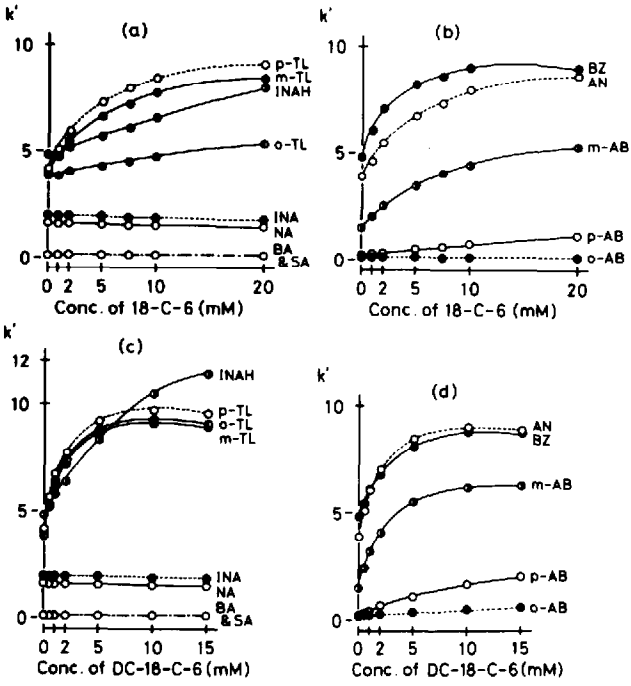

Fig. 1. Effect of the concentrations of 18-crown-6 and dicyclohexyl-18-crown-6 on the capacity factors of amino compounds. Stationary phase: Nucleosil 10SA (50 mm × 4.6 mm I.D.). Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* lithium chloride and < 20 m*M* crown ether. Flow-rate: 1.0 ml/min. Column temperature: 40°C. Detection: UV at 254 nm. Abbreviations: AN = aniline hydrochloride; BZ = benzylamine hydrochloride; SA = salicylamide; BA = benzamide; TL = toluidine; AB = aminobenzoic acid; INAH = isonicotinic acid hydrazide; INA = isonicotinamide; NA = nicotinamide.

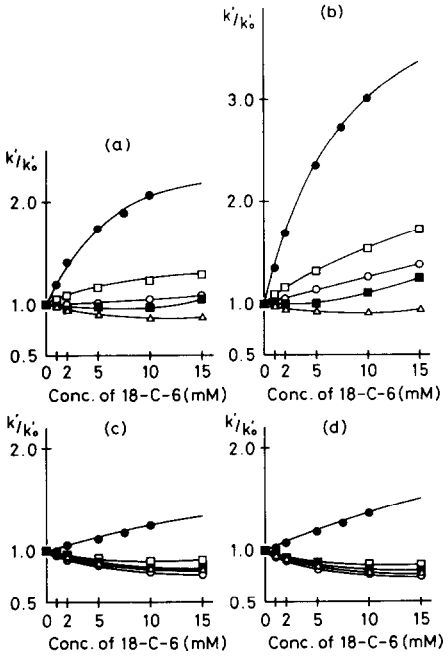

Fig. 2. Effect of added salts on the degree of retention enhancement *vs.* 18-crown-6 concentration for (a) aniline, (b) *m*-aminobenzoic acid, (c) *o*-toluidine and (d) isonicotinic acid hydrazide. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* LiCl (●), NaCl (□), NH_4Cl (○), KCl (△) or RbCl (■) and 0–15 m*M* 18-C-6. For other conditions, see Fig. 1.

capacity factors of amides such as isonicotinamide, nicotinamide, salicylamide and benzamide were almost unchanged, because they were not protonated under these mobile phase conditions.

With toluidine and aminobenzoic acid isomers (Fig. 1a and b) it is noticeable that the capacity factors of *ortho*-isomers in the 18-C-6-containing mobile phase are much lower than those of other isomers. This demonstrates the steric effects on the complexation with the crown ether. However, when DC-18-C-6 was used, the capacity factor of *o*-toluidine became as large as those of other isomers (Fig. 1c), whereas that of *o*-aminobenzoic acid remained much lower (Fig. 1d). This suggests that hydrophobic interaction may occur between the methyl group of toluidine and the cyclohexyl groups of DC-18-C-6, and that the carboxyl group of aminobenzoic acid tends to interfere with complex formation.

The effects of added salts on the degree of retention enhancement, k'/k'_0, caused by 18-C-6 are shown in Fig. 2, where k'_0 is the capacity factor observed with the mobile phase not containing 18-C-6. Fig. 2a and b indicates that the capacity factors of aniline and *m*-aminobenzoic acid underwent greater increases than did those of *o*-toluidine and isonicotinic acid hydrazide (INAH) upon the addition of salts, the magnitude of the increase depending on the kind of salt, in the order $Li^+ > Na^+ > NH_4^+ \geqslant Rb^+ > K^+$. For o-toluidine and INAH the corresponding sequence was $Li^+ > Na^+ \geqslant Rb^+ \geqslant K^+ \geqslant NH_4^+$. It is also found that the capacity factors for these solutes decreased upon addition of 18-C-6 in the presence of cations, except

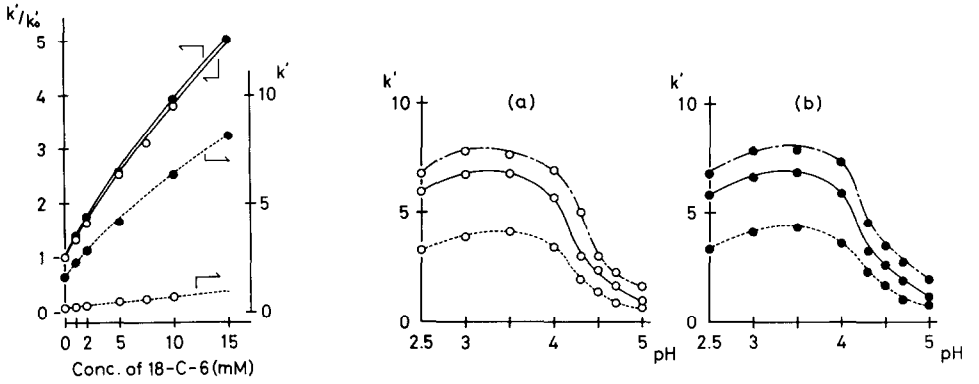

Fig. 3. Effect of added Li^+ on the capacity factor and the degree of retention enhancement *vs.* 18-crown-6 concentration for *p*-aminobenzoic acid. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing 0 m*M* (●) or 20 m*M* (○) lithium chloride and 0–15 m*M* 18-C-6. For other conditions, see Fig. 1.

Fig. 4. Effect of pH on the capacity factors of (a) aniline and (b) *m*-toluidine. Mobile phase: methanol–water (60:40, v/v) containing 20 m*M* lithium chloride and 5 m*M* 18-C-6 (——), 3 m*M* DC-18-C-6 (– · –) or in the absence of crown ether (------). For other conditions, see Fig. 1.

Li^+. Therefore, it is considered that, except for Li^+, the cations compete with the solutes in complex formation with the crown ether. It is interesting that when the mobile phase contained KCl or RbCl the capacity factors slightly decreased with an initial increase in 18-C-6 concentration, followed by a gradual increase at higher concentrations.

When the mobile phase contained no salts, the capacity factors of the solutes, except salicylamide and benzamide, were much larger than those obtained with the mobile phase containing 20 m*M* lithium chloride. The capacity factor and the degree of retention enhancement for *p*-aminobenzoic acid are shown in Fig. 3 as a function of the concentration of 18-C-6 in the mobile phase containing 20 m*M* lithium chloride

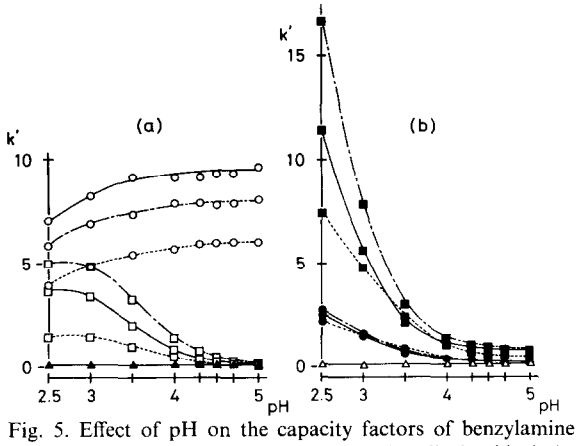

Fig. 5. Effect of pH on the capacity factors of benzylamine (○), isonicotinic acid hydrazide (■), *m*-aminobenzoic acid (□), nicotinamide (●), salicylamide (▲) and benzamide (△). For other conditions, see Fig. 4.

or no salts. There is almost no difference in the degree of retention enhancement between the cases of 20 m*M* lithium chloride and no salts, indicating that Li^+ does not compete with the solutes in complex formation with the 18-membered crown ether.

Effect of pH

The dependence of the capacity factor on pH between 2.5 and 5.0 was investigated by using mobile phases containing 5 m*M* 18-C-6, 3 m*M* DC-18-C-6 or no crown ethers in the presence of 20 m*M* lithium chloride. The results are shown in Figs. 4 and 5. In all cases, the observed profiles of *k'* *vs.* pH were almost unaffected by the presence of a crown ether in the mobile phase.

Under the chromatographic conditions employed in this experiment, protonation of the amino group of the guest molecule affects not only the ionic adsorption onto the cation-exchange site but also the complex formation with the 18-membered crown ether. Therefore, the capacity factors are generally expected to increase with increasing proton concentration. The capacity factors for aniline, *m*-toluidine (Fig. 4) and *m*-aminobenzoic acid (Fig. 5a) increased markedly with an initial decrease in pH of the mobile phase, followed by a gradual approach to maxima between pH 3 and 4. This behaviour is opposite to that observed in reversed-phase liquid chromatography[10], where the capacity factors decreased with increasing proton concentration. The slight decrease in the capacity factors for aniline and *m*-toluidine at low pH may be due to the competition of protonated bases with hydronium ions for the cation-exchange site[16,17]. The *k'* *vs.* pH profile of benzylamine in Fig. 5a exhibited a decrease in the capacity factor at pH < 3.5, because its pK_a value is larger than the pH examined. On the contrary, INAH and nicotinamide (Fig. 5b) showed a

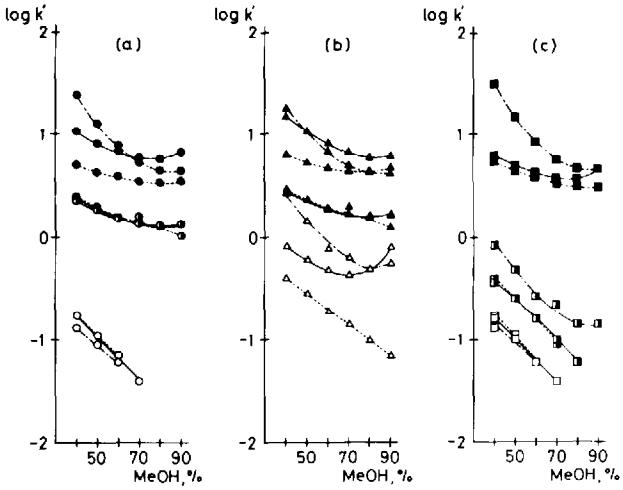

Fig. 6. Effect of methanol concentration on the capacity factors of amino compounds. Mobile phase: methanol–water (40:60 to 90:10, v/v) (pH 3.0 with hydrochloric acid) containing 20 m*M* lithium chloride and 5 m*M* 18-C-6 (——), 3 m*M* DC-18-C-6 (– · –) or in the absence of crown ether (------). For other conditions, see Fig. 1. Key: (●) aniline hydrochloride; (▲) benzylamine hydrochloride; (■) *o*-toluidine; (◑) nicotinamide; (▲) isonicotinamide; (◨) *o*-aminobenzoic acid; (○) salicylamide; (△) *p*-aminobenzoic acid; (□) benzamide.

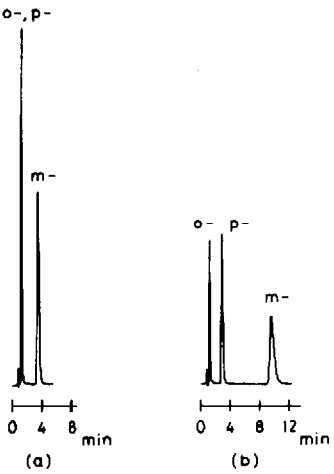

Fig. 7. Separation profile of aminobenzoic acid isomers. Mobile phase: methanol–water (60:40, v/v) (pH 3.0 with hydrochloric acid) containing (a) 20 m*M* LiCl and (b) 20 m*M* LiCl and 10 m*M* 18-C-6. For other conditions, see Fig. 1.

monotonous increase in k' with decreasing pH, because their pK_a values are lower than the pH. The capacity factors of salicylamide and benzamide (Fig. 5a and b) remained low and constant over the whole pH region examined, suggesting that there are no appreciable interactions with the 18-membered crown ethers. These k' *vs.* pH profiles indicate that when the capacity factor of an amino compound depends on the crown ether concentration it is also equally affected by the proton concentration.

Effect of solvent

The solvent effect on the capacity factor was investigated using mobile phases having various ratios of methanol–water (40:60 to 90:10, v/v) containing 5 m*M* 18-C-6, 3 m*M* DC-18-C-6 or no crown ethers at pH 3.0 in the presence of 20 m*M* lithium chloride. The results are shown in Fig. 6. In general, the log k' values decreased with increasing concentration of methanol, when the mobile phase contained no crown ethers. On the other hand, when a crown ether was added to the mobile phase, the decrease in log k' was followed by a slight increase at $>80\%$ methanol.

In ion-exchange liquid chromatography it is generally expected that the capacity factor of an organic cation is affected not only by the ionic adsorption on the stationary phase but also by the hydrophobic adsorption onto the resin matrix[18,19]. When the dielectric constant of the mobile phase decreases with increasing methanol concentration, it is expected that the ionic adsorption becomes much stronger and the hydrophobic adsorption weaker. Accordingly, the capacity factor varies depending on the balance of these opposing effects. The decrease in log k' values indicates that the elution ability of the mobile phase was enhanced with increasing methanol concentration and exceeded the ionic interaction with the ion-exchange site. At high methanol concentrations, the ionic interaction becomes stronger, so that the log k' values tend to increase. The magnitude of the decrease or increase in log k' is amplified by using mobile phases containing crown ethers, where both the ionic and hydrophobic properties of the guest compounds become stronger upon complex for-

mation. Salicylamide and benzamide, which are not retained on the cation-exchange stationary phase and do not form complexes with crown ethers, exhibited extremely small log k' values because of a slight hydrophobic interaction. Therefore, they showed linear decreases in log k' as in the reversed-phase mode, and the log k' values were almost unchanged despite the presence of crown ethers. Similar results were obtained with nicotinamide and isonicotinamide.

The elution profiles of aminobenzoic acid isomers obtained by using mobile phases with and without 18-C-6 are compared in Fig. 7. It is seen that *ortho-* and *para*-isomers are completely separated within 10 min by addition of 18-C-6.

CONCLUSIONS

In high-performance cation-exchange chromatography with mobile phases containing crown ethers the capacity factor of a guest compound bearing a primary amino group was increased by complex formation with the 18-membered crown ethers, because the hydrophobic adsorption onto the resin matrix as well as the ionic adsorption onto the ion-exchange site become stronger. When various alkali metal cations or ammonium were added to the mobile phase, except for Li^+ they competed with the guest compound in complex formation with the crown ether, resulting in a suppression of the increase in the capacity factor.

Complex formation with the crown ether increased with an initial decrease in the pH of the mobile phase, so that the capacity factor of the guest compound increased. However, a slight decrease in the capacity factor was observed at lower pH owing to the lowering of the ion-exchange ability of the stationary phase.

When methanol is added to the mobile phase, the ionic adsorption becomes stronger and the hydrophobic adsorption becomes weaker. Therefore, the capacity factor varied depending on the balance of these effects.

Thus, the capacity factor of a primary amine depends on the concentrations of the crown ether, proton and methanol in the mobile phase. On the contrary, that of an amide is affected only by the methanol concentration. Generally, the complexation of crown ethers with cations is weaker in water, which is a highly polar solvent, compared with methanol. Lithium does not compete with amino compounds in complex formation with 18-membered crown ethers. Therefore, it is considered that high-performance cation-exchange chromatography with water–methanol mobile phases containing 18-membered crown ethers and lithium chloride is useful for separating amino compounds.

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