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## Reversed-phase liquid chromatography of polar benzene derivatives on poly(vinylbenzo-18-crown-6)-immobilized silica as a stationary phase

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### ABSTRACT

A packing in which poly(vinylbenzo-18-crown-6) is immobilized covalently to silica was tested for its usefulness as a stationary phase in the reversed-phase liquid chromatography of polar disubstituted benzene derivatives. Both electrostatic and hydrophobic interactions contribute considerably to the chromatographic separation. When potassium cations, which are complexed by the crown ether moiety on the stationary phase, are added to the mobile phase during chromatography using the poly(crown ether)-immobilized silica, positive charges are generated on the surface of the stationary phase and some electrostatic interaction occurs between polar disubstituted benzene derivatives and the stationary phase. Hence the retention behaviour of polar organic compound can be controlled easily by adding metal cations to the mobile phase. This type of reversed-phase chromatography using poly(crown ether)-immobilized silica is useful for separating positional isomers of various disubstituted benzene derivatives.

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### INTRODUCTION

Various crown ether-immobilized silicas have been synthesized [1–5], and were designed as stationary phases for the high-performance liquid chromatographic (HPLC) separation of cations, particularly alkali and alkaline earth metal cations. Poly(crown ether)-immobilized silicas can be synthesized easily by copolymerization between vinyl-modified silica and crown ether vinyl monomers [6]. The ion chromatographic behaviour of poly(crown ether)-immobilized silica reflects the cation-complexing abilities of the corresponding poly(crown ether)s. The poly(crown ether)-immobilized silicas turned out to be promising stationary phases for ion chromatography [7–9]. Poly(crown ether)-immobilized silicas generally contain both hydrophobic and hydrophilic moieties. There is, therefore, a possibility of applying poly(crown ether)-immobilized silica to stationary phases in reversed-phase liquid

chromatography [10]. In conventional reversed-phase liquid chromatography using octadecylsilylated silica as the stationary phase, the interaction between the solute and the stationary phase is based mainly on hydrophobic interactions. On the other hand, in reversed-phase liquid chromatography on poly(crown ether)-immobilized silica, both electrostatic and hydrophobic interactions are expected to play an important role in the separation. In this paper, we report on the chromatographic retention behaviour of poly(benzo-18-crown-6)-immobilized silica in the reversed-phase liquid chromatography of disubstituted benzene derivatives, which are difficult to separate by conventional reversed-phase liquid chromatography. The effect of the addition of metal salts and the effect of the pH of the mobile phase in this reversed-phase liquid chromatography is also described. The chromatographic behaviour was compared with that for poly(benzoglyme-6)- and polystyrene-immobilized silica.

## EXPERIMENTAL

### *Synthesis of poly(vinylbenzo-18-crown-6)-immobilized silica*

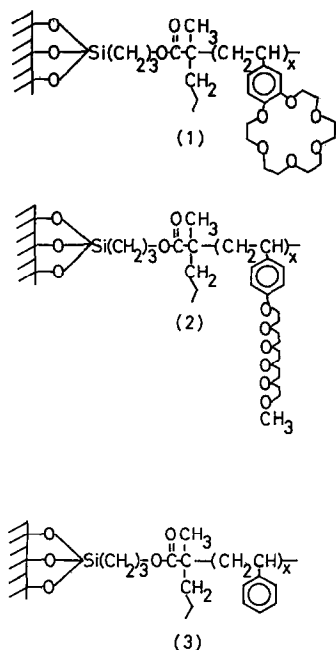
Silica gel for HPLC (10  $\mu\text{m}$ , spherical, Wako LC-10) was activated in concentrated hydrochloric acid. To this activated silica, a toluene solution of 3-(methacryloyloxy)propyltrimethoxysilane was added and the mixture was refluxed. The resulted vinyl-modified silica was washed successively with toluene, chloroform and methanol and then dried overnight under vacuum at 80°C. A glass tube containing a mixture of vinyl-modified silica and vinylbenzo-18-crown-6 [11–13] and  $\alpha,\alpha'$ -azobisisobutyronitrile dissolved in toluene was degassed by the freeze-thaw method and sealed. The polymerization was carried out by shaking the sealed tube in an incubator at 80°C for 21 h. After the polymerization, the modified silica was washed successively with toluene, chloroform and methanol and then dried overnight under vacuum at 80°C. The crown ether content of the poly(vinylbenzo-18-crown-6)-immobilized silica, which was calculated from the weight increase and carbon elemental analysis, was 0.57 mmol/g.

### *Synthesis of poly(vinylbenzoglyme-6)- and polystyrene-immobilized silica*

Vinylbenzoglyme-6 or styrene, instead of vinylbenzo-18-crown-6, was employed for copolymerization with the vinyl-modified silica. The polymerization was carried out in a similar manner to that for the vinylbenzo-18-crown-6 system. The glyme content of poly(vinylbenzoglyme-6)-immobilized silica was 0.37 mmol/g and the styrene content of polystyrene-immobilized silica was 1.19 mmol/g.

### *Chromatography*

The HPLC system consisted of a pumping system (Waters Assoc. Model 6000A), a sample injector (Waters Assoc. Model U6K) and a UV detector (Oyo-Bunko Uvilog-7). The stainless-steel columns (150 mm  $\times$  4 mm I.D.) were packed with the modified silicas 1, 2 and 3 in Scheme 1 using the balanced-density slurry technique. The mobile phases were generally methanol–water mixtures and the aqueous components were adjusted to pH 5 [acetic acid–lithium acetate (0.2 M)] or pH 8 [0.1 M tris(hydroxymethyl)aminomethane–HCl]. Chromatography was performed at a flow-rate of 1.0 ml min<sup>-1</sup>. The organic compounds used as the solutes were phenol, benzene and the *ortho*, *meta* and *para* isomers of nitrophenol, aminobenzoic acid, nitrobenzoic



Scheme 1.

acid, iodoaniline, toluidine and nitroaniline. The sample concentration and volume injected were 0.2 mM and 5  $\mu$ l, respectively, in each instance.

## RESULTS AND DISCUSSION

### *Interaction between poly(vinylbenzo-18-crown-6)-immobilized silica and solutes*

Liquid chromatography of the *ortho*, *meta* and *para* isomers of disubstituted benzene derivatives was carried out by using poly(vinylbenzo-18-crown-6)-immobilized silica as the stationary phase and methanol–water mixtures as the mobile phase. The dependence of the retention of the nitrophenol isomers on the mobile phase composition is shown in Fig. 1. The retention of nitrophenol was generally diminished by increasing the methanol fraction of the mobile phase at pH 5 or pH 8. This phenomenon was also observed in the chromatography of other disubstituted benzene derivatives such as iodoaniline and toluidine. It is suggested from this result that the chromatographic retention of the solutes on the stationary phase is derived basically from hydrophobic interactions. There might be some other interaction such as hydrogen bonding between the ring oxygen of the crown ether moiety on the stationary phase and the polar group of the solutes. However, the contribution of the hydrogen bonding to the retention is possibly small under the mobile phase conditions involving highly polar solvents such as methanol–water mixtures.

On the column of poly(vinylbenzo-18-crown-6)-immobilized silica, the three isomers of nitrophenol were eluted in the order *ortho* < *meta* < *para* at pH 5. The same

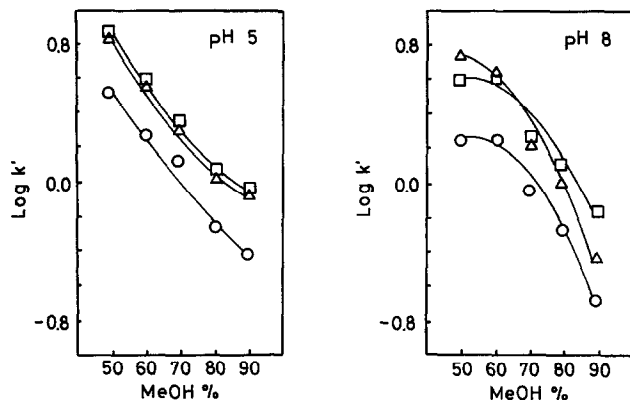


Fig. 1. Dependence of retention of nitrophenol isomers ( $\circ$  = *ortho*;  $\triangle$  = *meta*;  $\square$  = *para*) on the methanol (MeOH) content in the mobile phases on poly(vinylbenzo-18-crown-6)-immobilized silica. For experimental conditions, see text.

elution order holds for polystyrene-immobilized silica, which does not contain any crown ether moiety. A reversal of the retention order of the *para* and *meta* isomers was observed at an eluent pH of 8 with methanol concentrations less than 60%. Moreover, the shapes of the retention curves at pH 5 and 8 are different, especially for the *ortho* and *para* isomers.

We speculate the cause as follows. From a rough calculation, the proportion of the anionic form of *m*-nitrophenol is 28% in water at pH 8, compared with 86 and 92% for *o*- and *p*-nitrophenol, respectively. It is reasonable that the hydrophobic interaction with the stationary phase is weaker for the anionic than for the neutral forms. Also, the electrostatic repulsive interaction with the crown units is stronger for the anionic forms. The dissociation of the solutes is considered to reduce as the methanol concentration increases. Consequently, the *ortho* and *para* isomers are less retained than the *meta* isomer at pH 8, especially in the lower methanol concentration range, because of their greater dissociation. At pH 5, on the other hand, the three isomers are hardly dissociated (<1%). This supposedly gives rise to the above-mentioned reversal of the elution order and difference in the shapes of the retention curves. It is therefore suggested that the hydrogen bonding between the crown ether moiety and the hydroxy group of nitrophenol is not very important.

#### *Effect of addition of metal salts to the mobile phase*

We previously carried out ion chromatography of alkali metal cations by using poly(vinylbenzo-18-crown-6)-immobilized silica as the stationary phase [8]. It became apparent that  $K^+$  is retained strongly on the crown ether stationary phase, because the benzo-18-crown-6 moiety on the stationary phase forms a stable complex with  $K^+$ . We attempted to improve the separation of the *ortho*, *meta* and *para* isomers of disubstituted benzene derivatives by reversed-phase liquid chromatography by taking advantage of the cation-complexing ability of the stationary phase. The effect of the addition of crown ether-complexing cations to the mobile phase was therefore

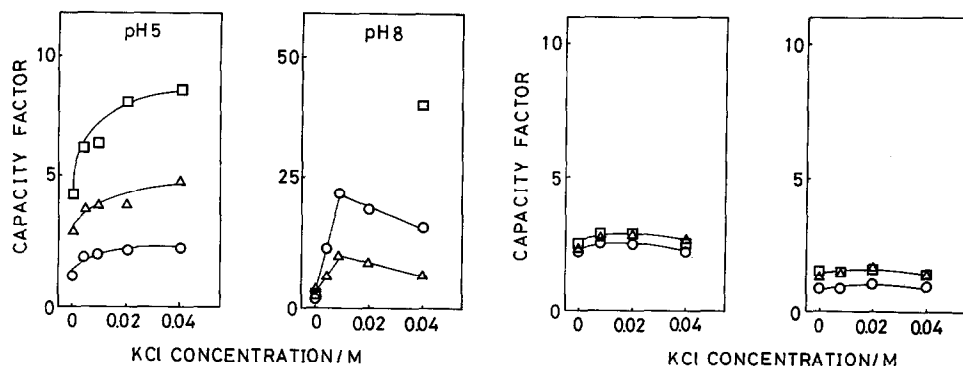


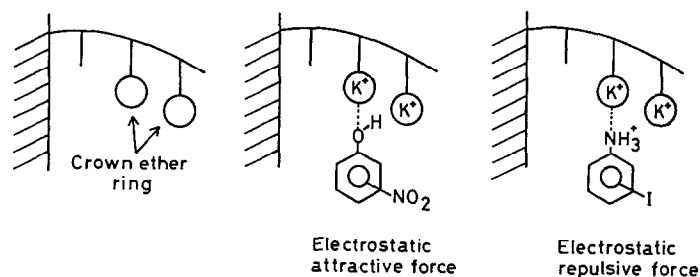
Fig. 2. Dependence of retention of nitrophenol isomers ( $\circ$  = *ortho*;  $\Delta$  = *meta*;  $\square$  = *para*) on KCl concentration in methanol-water (60:40, v/v) on poly(vinylbenzo-18-crown-6)-immobilized silica.

Fig. 3. Dependence of retention of nitrophenol isomers ( $\circ$  = *ortho*;  $\Delta$  = *meta;  $\square$  = *para*) on KCl concentration in methanol-water (pH 5) (60:40, v/v) on poly(vinylbenzoglyme-6)-immobilized silica (right) and polystyrene-immobilized silica (left).*

examined in the chromatography of anionic solutes such as nitrophenol and aminobenzoic acid isomers on the poly(crown ether)-immobilized silica.

The dependence of the retention times of nitrophenol in the potassium chloride concentration in the methanol-water (60:40) mobile phase is depicted in Fig. 2. On adding a small amount of potassium chloride to the aqueous component of the mobile phase at pH 5 or 8, the retention of nitrophenol isomers was increased substantially, especially at pH 8. However, when polystyrene- or poly(vinylbenzoglyme-6)-immobilized silica was used instead of the poly(crown ether)-immobilized silica, such a marked change in the retention behaviour was not observed (Fig. 3).

The retention enhancement may be explained as illustrated in Scheme 2.  $K^+$  in the mobile phase is complexed strongly by the benzo-18-crown-6 moiety on the stationary phase in poly(vinylbenzo-18-crown-6)-immobilized silica, which is thus positively charged. Therefore, an electrostatic attractive force between the positive charge and the polar group (OH) of nitrophenol is generated, which in turn enhances the retention of each isomer. In spite of the addition of potassium chloride to the mobile phase, the retention behaviour was not changed with either poly(vinylbenzo-



Scheme 2.

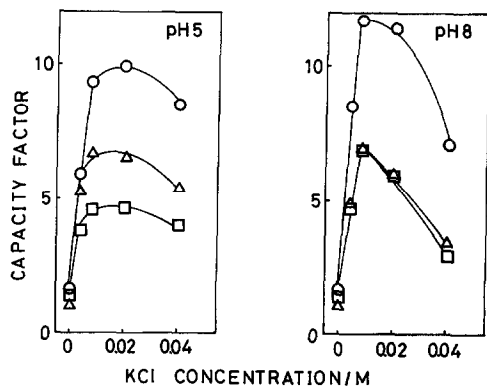


Fig. 4. Dependence of retention of aminobenzoic acid isomers on KCl concentration. Details as in Fig. 2.

glyme-6)- or polystyrene-immobilized silica. As neither the benzoglyme-6 nor the styrene moiety on the stationary phase can form stable complexes with  $K^+$ , the stationary phases do not acquire positive charges on addition of potassium chloride to the mobile phase. Therefore, electrostatic interactions do not seem to occur between the solutes and the poly(vinylbenzoglyme-6)- and polystyrene-immobilized silica stationary phases. As described above, most of the *ortho* and *para* isomers are dissociated at pH 8. It is reasonable that the anionic forms are retained much more strongly than the neutral forms by the positively charged stationary phase. The *ortho* isomer is, therefore, retained more strongly than the less dissociated *meta* isomer.

Similar phenomena were observed for aminobenzoic and nitrobenzoic acid isomers. Fig. 4 shows the considerable retention enhancement for the former acid on addition of potassium chloride. In contrast with nitrophenol, the increased retentions at pH 5 and 8 are similar in this instance. This is ascribed to the much smaller  $pK_a$  values of the aminobenzoic acid isomers than those of the nitrophenol isomers, *i.e.*, each acid isomer is dissociated more than 50% even at pH 5 and almost quantitatively at pH 8. This may result in comparable interactions with the stationary phase at both pH values.

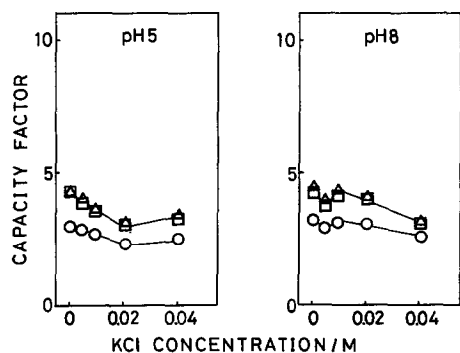


Fig. 5. Dependence of retention of iodoanilines on the KCl concentration. Details as in Fig. 2.

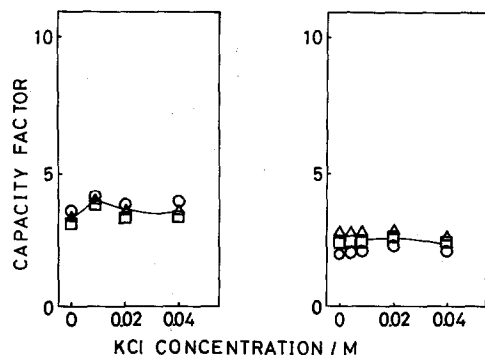


Fig. 6. Dependence of retention of iodoaniline isomers on KCl concentration on poly(vinylbenzoglyme-6)-immobilized silica (right) and polystyrene-immobilized silica (left). Details as in Fig. 3.

Figs. 5 and 6 show the retention dependence of iodoaniline on the potassium chloride concentration in the mobile phase. When poly(vinylbenzo-18-crown-6)-immobilized silica was used as the stationary phase, the retention of iodoaniline was decreased on adding 0.04 *M* potassium chloride to the mobile phases, although the change in the retention was not as drastic as that for the anionic solutes. However, as shown in Fig. 6, on poly(vinylbenzoglyme-6)- or polystyrene-immobilized silica the addition of potassium chloride to the mobile phase hardly afforded any change in the retention times, as was the case with the anionic solutes. With the mobile phase at pH 5, the polar group (amino) of iodonaniline is easily protonated, thus being positively charged. The electrostatic repulsive force between the positive charges of the cationic solutes and the crown-complexed cation on the stationary phase decreases their retention (Fig. 5).

On the poly(vinylbenzo-18-crown-6)-immobilized silica, the change in the retention for the cationic solutes was not greater than that for the anionic solutes. The reason may be explained as follows. The 18-crown-6 moiety may form complexes with the anilinium ion of the cationic solutes in addition to  $K^+$ . The complex formation between the 18-crown-6 moiety and the anilinium ions would enhance the chromatographic retention of the cationic solutes. Probably,  $K^+$  competes with the anilinium

TABLE I

DEGREE OF RETENTION ENHANCEMENT ON ADDITION OF POTASSIUM CHLORIDE TO THE MOBILE PHASE ON POLY(VINYLBENZO-18-CROWN-6)-IMMOBILIZED SILICA

Mobile phase: methanol-water (60:40, v/v) containing 2 *mM* KCl.

pH	Aminobenzoic acid			Nitrophenol			Nitroaniline		
	<i>o</i> -	<i>m</i> -	<i>p</i> -	<i>o</i> -	<i>m</i> -	<i>p</i> -	<i>o</i> -	<i>m</i> -	<i>p</i> -
5	4.23	4.96	2.41	1.24	1.21	1.67	0.90	0.85	0.85
8	6.10	4.49	4.11	4.50	1.52	— <sup>a</sup>	0.97	0.92	0.97

<sup>a</sup> Not eluted within 60 min.

ions on complex formation with the 18-crown-6 moiety on the stationary phase. Thus, the electrostatic repulsion is alleviated by the binding of the anilinium ion to the crown ether moiety of the stationary phase. In contrast, on poly(vinylbenzogllyme-6)- or polystyrene-immobilized silica, the addition of potassium chloride to the mobile phase hardly affects the retention behaviour of the cationic solutes. This is again explained by the fact that the benzogllyme-6 and styrene moieties do not form stable complexes with  $K^+$  (Scheme 2).

Table I summarizes the degree of retention enhancement by adding potassium chloride (2 mM) to the methanol-water (60:40) mobile phase, that is,  $t'_{R_1}/t'_{R_2}$ , where  $t'_{R_1}$  and  $t'_{R_2}$  are the adjusted retention times with and without the salt added to the mobile phase, respectively, on poly(vinylbenzo-18-crown-6)-immobilized silica.

The dependence of the retention times of phenol and benzene, which are difficult to or are not able to proton-dissociate at pH 5, on the potassium chloride concentration in the mobile phase was examined. Even in the chromatography on poly(vinylbenzo-18-crown-6)-immobilized silica, the addition of potassium chloride to the mobile phase did not give any change in the retention as expected. As phenol and benzene are hardly charged, there is very little electrostatic interaction between the solutes and the stationary phase.

When potassium chloride was added to the mobile phase, the retention of aminobenzoic acid increased as described above. However, when lithium chloride was added to the mobile phase, the retention of aminobenzoic acid hardly changed. This is probably due to the fact that the 18-crown-6 moiety cannot form a stable complex with  $Li^+$ . Hence the addition of lithium chloride instead of potassium chloride to the mobile phase hardly affects the chromatographic retention of the polar organic compounds on poly(vinylbenzo-18-crown-6)-immobilized silica.

#### *Separation of isomers of polar organic compounds by reversed-phase liquid chromatography on poly(vinylbenzo-18-crown-6)-immobilized silica*

The isomers of aminobenzoic acid and nitrophenol were separated successfully by reversed-phase liquid chromatography on poly(vinylbenzo-18-crown-6)-immobilized silica.

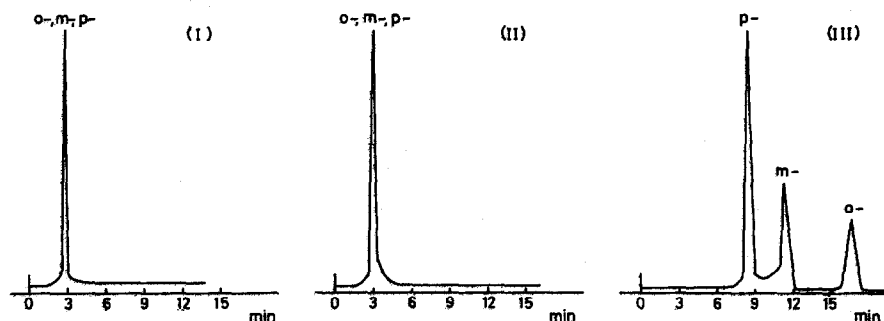


Fig. 7. Comparison of separation of aminobenzoic acid isomers (I) on ODS in methanol-water (pH 5) containing 20 mM KCl (60:40, v/v), (II) on poly(vinylbenzo-18-crown-6)-immobilized silica in methanol-water (pH 5) (60:40, v/v) and (III) on poly(vinylbenzo-18-crown-6)-immobilized silica in methanol-water (pH 5) containing 20 mM KCl (60:40, v/v).



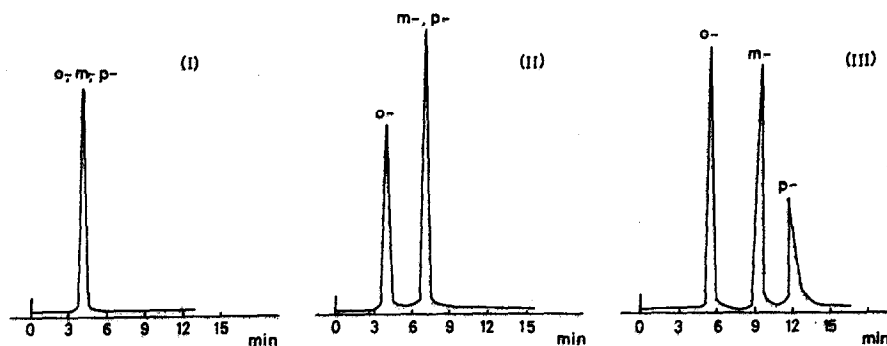


Fig. 8. Comparison of separation of nitrophenol isomers. Details as in Fig. 7.

bilized silica. In conventional reversed-phase liquid chromatography on octadecylsilanized (ODS) silica using methanol–water (pH 5) (60:40) as the mobile phase, the *o*-, *m*- and *p*-aminobenzoic acid isomers were not separated at all. Even on poly(vinylbenzo-18-crown-6)-immobilized silica, the aminobenzoic acid isomers were not separated without the addition of potassium chloride to the mobile phase. However, the addition of 20 mM potassium chloride to the mobile phase improved the chromatographic separation of the aminobenzoic acid isomers drastically, affording a baseline separation, as illustrated in Fig. 7.

In the case of the nitrophenol isomers, chromatography on ODS with methanol–water (pH 5) (60:40) could not separate *o*-, *m*- and *p*-nitrophenol at all (Fig. 8). When poly(vinylbenzo-18-crown-6)-immobilized silica was used as the stationary phase, *o*-nitrophenol could be separated from *p*- and *m*-nitrophenol, but the latter two were still difficult to separate. On addition of 20 mM potassium chloride to the mobile phase, however, *p*-nitrophenol was retained strongly on poly(vinylbenzo-18-crown-6)-immobilized silica. Consequently, the *ortho*, *meta* and *para* isomers were separated successfully, being eluted in the order *ortho* < *meta* < *para*. It is interesting and of great value to compare the poly(crown ether) phase with the well known  $\beta$ -cyclodextrin for the separation of isomers. On the cyclodextrin phase, the isomers of both nitrophenol and aminobenzoic acid are eluted in the order *meta* < *ortho* < *para*. The retention of nitrophenol increases with increasing pH, whereas that of aminobenzoic acid decreases [14]. Therefore, it is apparent that the retention mechanisms of the two stationary phases are different.

Accordingly, poly(crown ether)-immobilized silica is promising as a stationary phase for the reversed-phase liquid chromatography of polar organic compounds, and the chromatographic behaviour can be modified by adjusting not only the composition of the organic–aqueous mobile phase but also the pH and the concentration of  $K^+$ , which forms a stable complex with the crown ether moiety in the stationary phase. This type of chromatography is different from conventional reversed-phase liquid chromatography in that the crown-complexing cation affects the chromatographic separation considerably. The poly(crown ether)-immobilized silica seems to be useful for the separation of other polar organic compounds and might be also applied as a stationary phase for protein separations without denaturation by applying salt concentration gradient elution.

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