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## EFFECT OF ALCOHOL CHAIN LENGTH, CONCENTRATION AND POLARITY ON SEPARATIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING BONDED CYCLODEXTRIN COLUMNS

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### SUMMARY

The effect of alcohol chain length, concentration and polarity on separation in high-performance liquid chromatography using  $\beta$ -cyclodextrin-bonded silica is discussed. The results show that retention times cannot be predicted merely from the polarity of the binary mobile phase. Although organic modifiers with the same physico-chemical properties and from the same solvent group were used, the retention times obtained using binary mobile phases having the same polarity, were different. It was also observed that normal-chain carbon alcohols gave retention times shorter than those obtained with a branched-chain alcohol (*n*-propanol vs. isopropanol), and the longer the alcohol chain the shorter the retention times. A plot of  $\ln k'$  vs. alcohol volume fraction for benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, 1-phenylhexane and 1-phenyloctane gave a linear relationship in methanol, ethanol and propanol (except for 1-phenylhexane). A non-linear relationship was obtained for all the solutes in isopropanol, *tert.*-butanol and 1-butanol, in the alcohol volume fraction studied.

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### INTRODUCTION

Since the introduction of bonded cyclodextrins (CDs) as stable high-performance liquid chromatography (HPLC) stationary phases in 1984 by Armstrong<sup>1</sup>, many applications have been generated which show the potential of these materials<sup>2-6</sup>. These phases have been used for reversed-phase type separations, including polycyclic aromatic hydrocarbons<sup>2</sup>, mycotoxins<sup>2</sup>, quinones<sup>2</sup> cyclic and acyclic nitrosoamines<sup>7</sup>, and for normal phase type separations for mixtures of substituted anilines<sup>8</sup> (positional isomers), and for the separation of chiral, geometrical, optical and positional isomers<sup>5,6</sup> in addition to enantiomers; epimers and conformers<sup>9,10</sup>. Most separations achieved using CD-bonded phases are caused by differential migration of the solutes based on the formation of inclusion complexes by the different solutes entering the CD cavity.

The retention time of a given solute using CD-bonded phases is a function of many factors (elutropic strength of mobile phase, temperature, mobile phase flow-

rate, type and shape of solute, pH and chirality), which determines the formation constant.

Manipulation of the mobile phase composition is undoubtedly the most powerful and the easiest means of adjusting both retention and selectivity in LC. In reversed-phase type separations (inclusion complexes) not only the volume of water can be changed but also the volume and nature of the organic modifier. Tarr *et al.*<sup>11</sup> recently showed the effect of *tert.*-butanol on retention of selected polynuclear aromatic compounds using a  $\beta$ -CD-bonded column. They found a significant effect on retention by adding 1% of *tert.*-butanol to the mobile phase. For example, the retention of 1-methylnaphthalene increased from 7.7 min in 100% water to 13.4 min in *tert.*-butanol-water (1:99), while the retention of 2-methylnaphthalene decreased to 11.0 min in 1% *tert.*-butanol, from 23.0 min in 100% water. A literature survey showed that there is no systematic study on the effect of different alcohols in the range 0–100% in water (where possible) on the retention time and selectivity of solutes using  $\beta$ -CD-bonded columns.

In this study the effect of different alcohols, methanol, ethanol, propanol, isopropanol, butanol and *tert.*-butanol and their content in the mobile phase on retention of several alkylbenzenes was evaluated. Also, the effect of using different alcohol-water mobile phases having the same polarity index on retention, selectivity and resolution were studied.

## EXPERIMENTAL

### Materials

The  $\beta$ -CD column (50.0  $\times$  4.6 mm I.D.) was a gift from Advanced Separation Technologies (Whippany, NJ, U.S.A.). Methanol, isopropanol, propanol, benzene and toluene were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Ethanol (spectroscopic grade) was obtained from NSI Chemicals (Newark, NJ, U.S.A.). 1-Butanol and *tert.*-butanol (99.7%), ethylbenzene (99%), propylbenzene (98%), 1-phenylhexane (97%) and 1-phenyloctane (99%), biphenyl, 2-phenylphenol, diphenylamine and fluorene were purchased from Aldrich (Milwaukee, WI, U.S.A.). Water was deionized glass distilled. All chemicals were used as received without further purification.

### Methods and apparatus

A Hewlett-Packard Model 1090 liquid chromatograph equipped with built-in oven for temperature control, a photodiode array detector, an automatic injector, a strip-chart recorder, a Hewlett-Packard Model 3392A integrator and a Hewlett-Packard Model 85 computer/controller was used. All the alkylbenzenes were dissolved in methanol at concentrations of approximately  $4 \cdot 10^{-4}$  M. Test samples were run in all the different alcohol modifiers at different concentrations with water. Note that of the alcohols selected for this study methanol, ethanol, propanol, isopropanol and *tert.*-butanol are freely miscible in water, while butanol is not. Also, the test solutes may not be retained and will elute with the dead volume in mobile phases with a high content of alcohols. The experiments and the results will, therefore, reflect these two restrictions, where applicable.

During all the experiments, the flow-rate was kept constant at 1 ml/min, and the

temperature at 40°C, unless stated otherwise. Approximately 150–300 column volumes were allowed for column equilibration with each new mobile phase composition. All the retention times of the different solutes in all the different concentrations and types of the alcohol modifiers are an average of three injections of 5  $\mu\text{l}$  each. The value of  $t_0$  was determined by injecting 5  $\mu\text{l}$  of  $10^{-3}$  M sodium nitrite at each mobile phase used.

## RESULTS AND DISCUSSION

Interactions of the solutes in HPLC with bonded-phase CDs and other constituents of the stationary phase sorbents are governed to a large extent by the composition of the mobile phase. The elution volume of a given solute is a function of the formation constant which depends on many factors such as mobile phase composition, temperature, barriers to the formation of inclusion complexes, stabilization energies, etc. Tarr *et al.*<sup>11</sup> proposed that the apparent equilibrium constant may be used to estimate the chromatographic interactions of the solute with both the stationary and the mobile phase, because it expresses the ratio between the solute fraction included (inclusion complex) to the free solute in the mobile phase. Increased mobile phase hydrophobicity is expected to increase the free fraction of the solute resulting in decrease of retention time. Therefore, measuring the retention times of different alkylbenzenes at different concentrations of organic modifiers (alcohols) would be a valuable information which would aid chromatographers in the prediction of solute retention.

### *Effect of methanol content on retention*

There are quite a few articles dealing with the dependence of the capacity ratio on the modifier when reversed-phase columns were used<sup>12–17</sup>. The exact dependence is still a contested issue. The two most frequently used relationships are a linear function:

$$\ln k' = Ax + B$$

and a quadratic function

$$\ln k' = A'x^2 + B'x + C$$

where  $A$ ,  $A'$ ,  $B$ ,  $B'$  and  $C$  are coefficients, the values of which depend on the solute. Recently Geng and Regnier<sup>18,19</sup> detailed a study for the dependence of the coefficients  $A$ ,  $A'$  and  $B'$  on the structure of the solute molecule in reversed-phase systems. They concluded that an increase in the non-polar surface area of a solute would lead to an increase in retention which would require a larger number of solvent molecules for solute displacement. In contrast, increasing the surface area of the solvent will cause a decrease in the number of solvent molecules required for displacement of a particular solute. Figs. 1 and 2 show the variation of the  $\ln k'$  values for seven solutes as a function of the methanol concentration in the range between 0.2 and 0.8 of the fraction of methanol in water. The figures represent the best possible linear fit of the data. The correlation coefficients, slopes and intercepts for the different solutes are

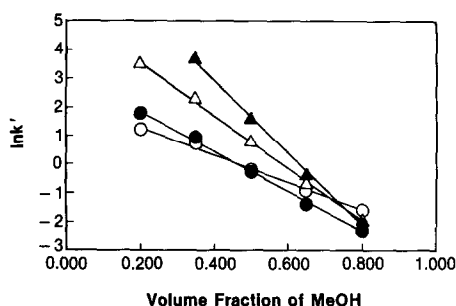
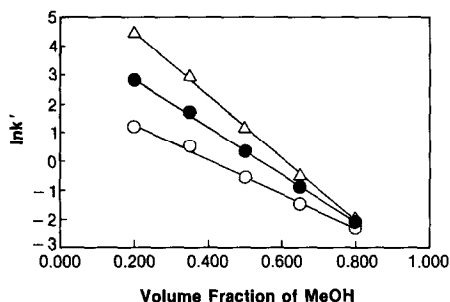


Fig. 1. Variation of retention with the binary mobile phase composition for methanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = toluene;  $\bullet$  = propylbenzene;  $\triangle$  = 1-phenylhexane. The flow-rate was 1.0 ml/min, the temperature was  $40 \pm 0.1^\circ\text{C}$  using a  $\beta$ -CD (cyclobond II) bonded silica gel column, 5  $\mu\text{m}$  spherical ( $50.0 \times 4.6$  mm I.D.). Detection was carried out at 254 nm. MeOH = Methanol.

Fig. 2. Variation of retention with the binary mobile phase composition for methanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = benzene;  $\bullet$  = ethylbenzene;  $\triangle$  = butylbenzene;  $\blacktriangle$  = 1-phenyloctane. Experimental conditions as in Fig. 1. MeOH = Methanol.

shown in Table I. It is observed that the longer the side chain of the benzene ring the greater the decrease in retention as methanol content increases. These results (slope values are increased with solute size) agree with the displacement model postulation of Geng and Regnier<sup>18,19</sup>. However, this sharp decrease in retention is not seen for benzene. According to standard reversed-phase LC theory one should get an orderly change in retention behavior with change in the alkyl chain length as mentioned above. However, benzene behaves as if it was not a member of the homologous series. Since all the solutes examined in this study are small enough to be included in the  $\beta$ -CD cavity, it seems that factors other than size are affecting retention. In fact, benzene seems to be too strongly retained. If it behaves as a normal member of the homologous series it should be less retained than toluene. This means that the binding constant of benzene is greater to  $\beta$ -CD than one might expect. Another interesting observation is that as the percentage of the organic modifier increases, the benzene molecules compete even more effectively for the  $\beta$ -CD relative to other compounds. It is difficult to find the exact answer to this behavior but it may be entropic in nature.

TABLE I

SUMMARY OF RETENTION PARAMETERS FOR BONDED  $\beta$ -CYCLODEXTRIN OF ALKYL-BENZENES, WHEN METHANOL WAS USED AS MODIFIER

| Compound       | Intercept | Slope  | Correlation coefficient (r) |
|----------------|-----------|--------|-----------------------------|
| Benzene        | 2.25      | -4.84  | 0.9973                      |
| Toluene        | 2.50      | -6.03  | 0.9983                      |
| Ethylbenzene   | 3.23      | -6.99  | 0.9989                      |
| Propylbenzene  | 4.47      | -8.18  | 0.9994                      |
| Butylbenzene   | 5.38      | -9.18  | 0.9995                      |
| 1-Phenylhexane | 6.64      | -10.83 | 0.9996                      |
| 1-Phenyloctane | 7.97      | -12.65 | 0.9989                      |

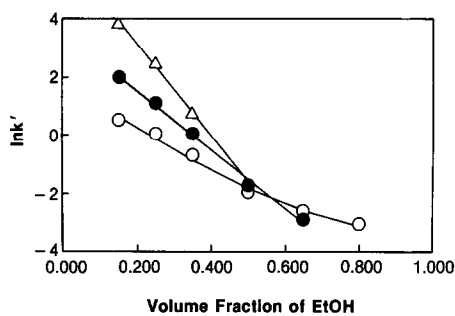
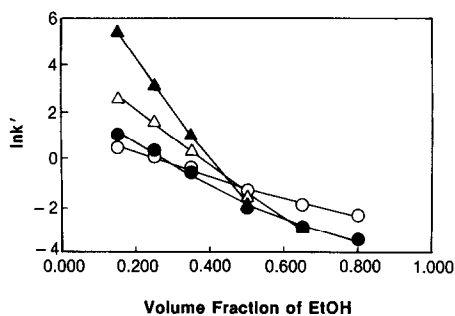


Fig. 3. Variation of retention with the binary mobile phase composition for ethanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = benzene;  $\bullet$  = ethylbenzene  $\triangle$  = butylbenzene  $\blacktriangle$  = 1-phenyloctane. Experimental conditions as in Fig. 1. EtOH = Ethanol.

Fig. 4. Variation of retention with the binary mobile phase composition for ethanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = toluene;  $\bullet$  = propylbenzene;  $\triangle$  = 1-phenylhexane. Experimental conditions as in Fig. 1. EtOH = Ethanol.

The other solutes all have aliphatic groups which would carry them into the CD. This hydrophobic binding to CD may restrict their motion and geometry in the CD cavity (less entropy) which is not helpful for binding<sup>20</sup>. Benzene on the other hand, is free to assume many orientations and positions in the CD cavity (*i.e.*, high entropy).

#### Effect of ethanol content on retention

As mentioned in the introduction several alcohols with different side chain length are used to examine the effect of their content in the mobile phase on retention. The second alcohol is ethanol, which is rarely used in practice for chromatographic separations. Figs. 3 and 4 show the effect of increasing ethanol content in the mobile phase on retention. It was found that the correlation coefficient ( $R$ ) for the linear and quadratic fits between  $\ln k'$  and ethanol volume fraction are very close, as seen from Table II. Most of the values for both linear and quadratic fits are close to or higher than 0.99. For practical considerations it is assumed here to be linear. Several points are worth mentioning here: the slope values (Table II) for the different solutes, show that as the non-polar surface area of the solute increases the retention decreases with

TABLE II

SUMMARY OF RETENTION PARAMETERS FOR BONDED  $\beta$ -CYCLODEXTRIN OF ALKYL-BENZENES, WHEN ETHANOL WAS USED AS MODIFIER

| Compound       | Intercept | Slope  | $r$ (linear fit) | $r$ (quadratic fit) |
|----------------|-----------|--------|------------------|---------------------|
| Benzene        | 1.11      | -4.50  | 0.9932           | 0.9956              |
| Toluene        | 1.33      | -5.87  | 0.9863           | 0.9941              |
| Ethylbenzene   | 2.02      | -7.28  | 0.9882           | 0.9959              |
| Propylbenzene  | 3.52      | -10.00 | 0.9981           | 0.9984              |
| Butylbenzene   | 4.38      | -11.46 | 0.9977           | 0.9982              |
| 1-Phenylhexane | 6.31      | -15.87 | 0.9994           | 0.9996              |
| 1-Phenyloctane | 8.44      | -20.78 | 0.9989           | 1.0000              |

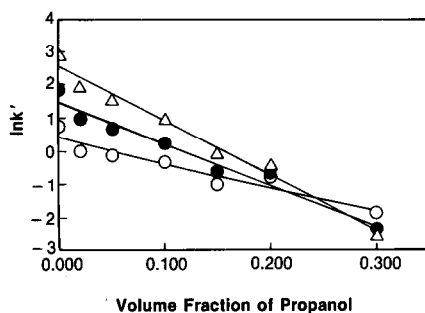
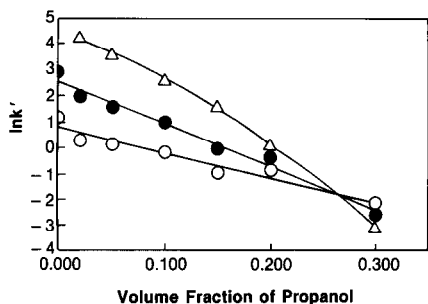


Fig. 5. Variation of retention with the binary mobile phase composition for propanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = benzene;  $\bullet$  = ethylbenzene;  $\triangle$  = butylbenzene. Experimental conditions as in Fig. 1.

Fig. 6. Variation of retention with the binary mobile phase composition for propanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = toluene;  $\bullet$  = propylbenzene;  $\triangle$  = 1-phenylhexane. Experimental conditions as in Fig. 1.

increase in ethanol content. However, for the solutes 1-phenylhexane and 1-phenyloctane there is a noticeable drop in retention as ethanol content increases. For example, the retention time of 1-phenyloctane was close to the dead volume at 35% (v/v) ethanol, while it was 15 min at 25% ethanol. This change in retention for small changes in ethanol content can be explained by the competition of the formation of inclusion complexes between the ethanol molecules and the solutes. It seems that at higher concentrations of ethanol the stabilization energy and the barriers for the formation of inclusion complexes favors the ethanol. The second competing factor is the increase in hydrophobicity of the mobile phase, which causes an increase in the free fraction of solute, a decrease in formation constant and thus decrease in retention.

#### Effect of 1-propanol content on retention

Figs. 5 and 6 show the plots of  $\ln k'$  of the test solutes against the volume content of 1-propanol in the mobile phase. At 30% 1-propanol all the test solutes eluted with the dead volume. The same effect was observed with 80% methanol and 65% ethanol. This is due to the fact that the solubility of the different solutes is much better in the long-chain alcohol, because of the increase in hydrophobicity. Table III

TABLE III

SUMMARY OF RETENTION PARAMETERS FOR BONDED  $\beta$ -CYCLODEXTRIN OF ALKYL-BENZENES, WHEN PROPANOL WAS USED AS MODIFIER

| Compound       | Intercept | Slope  | $r$ (linear fit) | $r$ (quadratic fit) |
|----------------|-----------|--------|------------------|---------------------|
| Benzene        | 0.378     | -7.32  | 0.9480           | 0.9499              |
| Toluene        | 0.757     | -9.59  | 0.9675           | 0.9683              |
| Ethylbenzene   | 1.46      | -12.50 | 0.9818           | 0.9819              |
| Propylbenzene  | 2.56      | -16.62 | 0.9899           | 0.9899              |
| Butylbenzene   | 3.38      | -19.57 | 0.9949           | 0.9963              |
| 1-Phenylhexane | 5.03      | -25.93 | 0.9930           | 0.9996              |

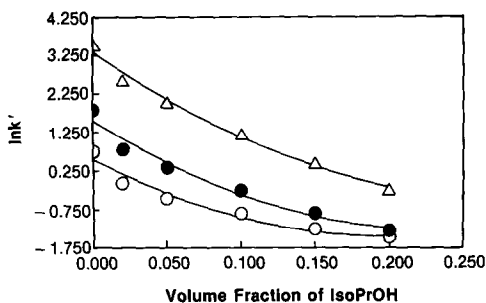


Fig. 7. Variation of retention with the binary mobile phase composition for isopropanol-water mixtures on a  $\beta$ -CD column. Solutes: ○ = benzene; ● = ethylbenzene; △ = butylbenzene. Experimental conditions as in Fig. 1. IsoPrOH = Isopropanol.

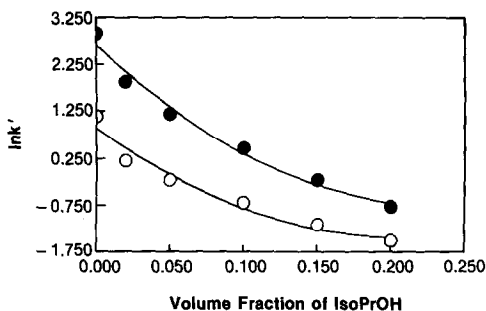


Fig. 8. Variation of retention with the binary mobile phase composition for isopropanol-water mixtures on a  $\beta$ -CD column. Solutes: ○ = toluene; ● = propylbenzene. Experimental conditions as in Fig. 1. IsoPrOH = Isopropanol.

shows that the correlation coefficient values of the linear and quadratic fits are very close. Here also, as in the case of ethanol, a linear fit is assumed. The decrease in retention of the solutes benzene and toluene, over the whole range of propanol content, is moderate relative to the decrease in retention of other solutes as can be seen from the slope values. This is attributed to the relatively free movement of these solutes, in and out of the cavity, because of their small molecular volume, as compared to the size of the CD cavity. The solute 1-phenylhexane, exhibits a more gradual decrease in retention, high slope values than the other solutes.

At this point it is clear that the longer the side chain of the alcohol used the less volume fractions are needed in the mobile phase to obtain the same decrease in retention time using a shorter chain alcohol, *i.e.*, methanol, as discussed earlier. Such behavior was observed using micellar chromatography where water forms the bulk of the mobile phase<sup>21</sup> and reversed-phase chromatography<sup>15,17</sup>.

#### *Effect of isopropyl alcohol content on retention*

It was of interest to examine the effect of the same number of carbon alcohols but with different geometry on the retention of the same solutes. Figs. 7 and 8 give the plots of the behaviour of the  $\ln k'$  against the isopropanol content in the mobile phase. A small change in mobile phase alcohol content caused a sharp decrease in retention times for all the solutes. At 20% isopropanol all the test solutes eluted close to the dead volume. The best fit was found to be a quadratic one, this means that the mechanism of separation (or retention) is a complex function of both the mobile and stationary phases, in 1-propanol it was mostly linear. Overall the retention time of the solutes was shorter in isopropanol than in 1-propanol.

#### *Effect of tert.-butanol content on retention*

Tarr *et al.*<sup>11</sup> found that the retention of some of the polycyclic aromatic hydrocarbons decreased with the addition of 1% butanol to the mobile phase butanol-water (1:99) and increased for others. This small change in the content of alcohol in the mobile phase is not enough to give the chromatographer a clear idea of the

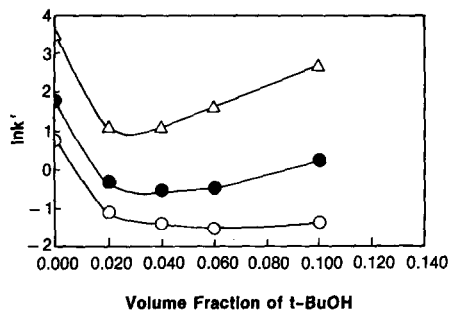


Fig. 9. Variation of retention with the binary mobile phase composition for *tert.*-butanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = benzene;  $\bullet$  = ethylbenzene  $\Delta$  = butylbenzene. Experimental conditions as in Fig. 1. *t*-BuOH = *tert.*-Butanol.

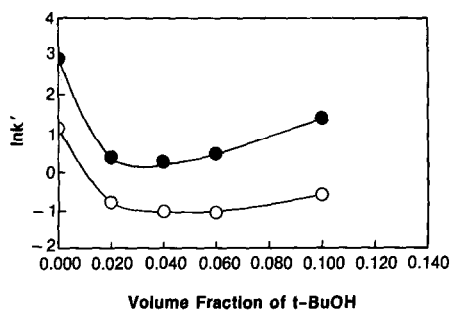
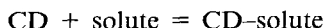


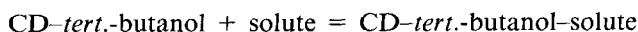
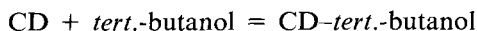
Fig. 10. Variation of retention with the binary mobile phase composition for *tert.*-butanol-water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = toluene;  $\bullet$  = propylbenzene. Experimental conditions as in Fig. 1. *t*-BuOH = *tert.*-Butanol.

behavior of several classes of compounds when higher quantities of *tert.*-butanol is used. Therefore, it was important to examine the effect of higher concentrations of *tert.*-butanol on the retention of the alkylbenzenes and to compare the results to those obtained using *n*-butanol. Figs. 9 and 10, which are the plots of  $\ln k'$  against volume fraction of *tert.*-butanol, show that the retention time is a complex function of the alcohol content. At 2–4% alcohol a decrease in retention time for all the solutes except butylbenzene at 4% is observed. However, the addition of higher percentages of alcohol results in higher retention times, except for benzene and toluene where the change in retention is minimal.

This decrease in retention at the low concentrations of *tert.*-butanol and retention increase at the higher quantities of the alcohol supports the explanation given by Tarr *et al.*<sup>11</sup> and Nelson *et al.*<sup>22</sup>. The presence and type of alcohol have a substantial effect on the fluorescence lifetime of the pyrene-CD inclusion complex systems<sup>22</sup>. This observation indicates that the changes in the  $\alpha$ -CD interactions are not merely a solvent effect due to the bulk presence of alcohol outside the  $\alpha$ -CD cavity. If no local participation of the alcohol molecules with the pyrene-CD complex occurs then a change in the fluorescence lifetime of the complex would not be expected. It was proposed that the increased solvent hydrophobicity and enhancement by coinclusion of alcohols are the two competing factors that influence the retention behavior. So at the low *tert.*-butanol concentrations the equilibrium reaction for the inclusion complex formation is:



and for the higher *tert.*-butanol concentrations is:





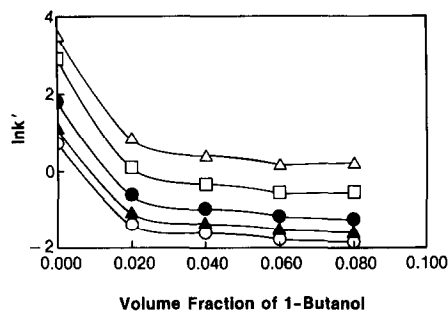


Fig. 11. Variation of retention with the binary mobile phase composition for 1-butanol–water mixtures on a  $\beta$ -CD column. Solutes:  $\circ$  = benzene;  $\blacktriangle$  = toluene;  $\bullet$  = ethylbenzene;  $\square$  = propylbenzene;  $\triangle$  = butylbenzene. Experimental conditions as in Fig. 1.

Deactivation of the amphiphilic hydroxyls of the CD outer surface, causes an increase in the core hydrophobicity.

Fig. 11 gives the behavior of  $\ln k'$  against the volume percentage of 1-butanol. The maximum solubility of 1-butanol in water was 8%. An addition of 2% 1-butanol resulted in a large decrease in retention for all the solutes. Increasing 1-butanol volume fraction from 6 to 8% in the mobile phase caused a minor decrease in retention of the small molecules benzene, toluene and ethylbenzene but a slight increase in retention of the solutes propylbenzene and butylbenzene. The decrease in retention at the low percentages of the alcohol is the same for the two alcohols (1-butanol and *tert.*-butanol). The effect is caused by an increase in the hydrophobicity of the mobile phase.

#### *Effect of different binary alcohol–water mobile phases having the same polarity on separation and selectivity*

In a previous work<sup>23</sup> the effect of mobile phase composition on separation and selectivity using reversed-phase thin-layer chromatography plates and a water–alcohol mobile phase was studied. As an extension to the present study it was of interest to see what effect the different alcohols in the mobile phase would have, if all the mobile phases had the same polarity index. The polarity of each mobile phase was calculated according to Snyder<sup>24</sup> based on the following equation:

$$P' = \Phi_1 P_1 + \Phi_2 P_2$$

where  $P'$  is the polarity of the mixture,  $P_1$  and  $P_2$  are the polarity of the pure solvents and  $\Phi_1$  and  $\Phi_2$  are the volume fractions of the two solvents.

The experiment was run at room temperature, using a 5- $\mu\text{m}$  spherical  $\beta$ -CD column (125  $\times$  4.6 mm I.D.), at a flow-rate of 1 ml/min. The solutes used were 2-phenylphenol diphenylamine, fluorene and biphenyl. The alcohols used in the binary mobile phase mixture were methanol, ethanol, *n*-propanol, isopropanol and *tert.*-butanol. Butanol and isobutanol were not miscible in water at the required volume.

Table IV shows the results obtained with the above mentioned five alcohols. All the mobile phases had a polarity index of approximately 8.94. Several interesting features are clear from examining the results.

TABLE IV  
EFFECT OF ALCOHOL TYPE ON RETENTION IN HPLC USING BINARY ALCOHOL-WATER MOBILE PHASES HAVING THE SAME POLARITY

| Compound       | Methanol-water <sup>a</sup><br>(25:75) |          | Ethanol-water<br>(21:79) |          | Propanol-water<br>(20:80) |          | Isopropanol-water<br>(20:80) |          | tert.-Butanol-water<br>(20:79) |          |
|----------------|--|----------|--------------------------|----------|---------------------------|----------|------------------------------|----------|--------------------------------|----------|
|                | $t_R$                                  | $\alpha$ | $t_R$                    | $\alpha$ | $t_R$                     | $\alpha$ | $t_R$                        | $\alpha$ | $t_R$                          | $\alpha$ |
| 2-Phenylphenol | 10.9                                   | —        | 6.7                      | —        | 2.4                       | —        | 3.2                          | —        | 2.2                            | —        |
| Diphenylamine  | 12.0                                   | 1.10     | 7.8                      | 1.16     | 2.8                       | 1.17     | 3.8                          | 1.19     | 2.5                            | 1.14     |
| Fluorene       | 20.2                                   | 1.68     | 16.1                     | 2.06     | 5.6 <sup>b</sup>          | 1.24     | 12.8 <sup>b</sup>            | 1.68     | 6.1 <sup>b</sup>               | 1.91     |
| Biphenyl       | 36.2                                   | 1.79     | 21.9                     | 1.36     | 4.5                       | 1.61     | 7.6                          | 2.0      | 3.2                            | 1.28     |

<sup>a</sup> All binary mobile phase mixtures have a polarity index of 8.9

<sup>b</sup> Peak reversal in this alcohol.

The selectivity, solute elution order, of the four test compounds is divided into two groups: one in methanol and ethanol, the other in propanol, isopropanol and tertiary butanol, but different than in the first two alcohols. The difference between the two groups is the peak reversal of biphenyl which eluted last in methanol and ethanol, but eluted before fluorene in the second group. A second observation is that although the percent of alcohol decreased in the mobile phase from methanol, ethanol to propanol, the retention times decreased considerably almost 8-fold for biphenyl. Increasing the chain length of the alcohol increases the hydrophobic properties of the alcohol, *i.e.* increasing the chain length increases the solubility of the solutes in the mobile phase and also weakens the formation of the inclusion complex in the CD cavity which results in a decrease in retention time.

A third observation is that when the retention times in *n*-propanol are compared to those in isopropanol better separation and longer retention times are obtained using the isopropanol mobile phase. The explanation for this was given earlier.

A fourth observation is that when the retention times obtained using isopropanol and *tert.*-butanol are compared (isopropanol is not soluble in water at the required level), the retention times decreased with increasing the total number of carbons as was observed with the straight-chain alcohols. A fifth and final observation is that although all the mobile phases (with the five alcohols) had the same polarity, the retention times obtained were different in each case. On the other hand this behavior can be used to an advantage in that when the components of a mixture are not resolved in one alcohol, using a different alcohol may resolve them.

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