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Reversed-phase liquid chromatographic measurement of the influence of a co-modifier functional group on the retention behavior of the β-cyclodextrin–pyrene complex

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ABSTRACT

Reversed-phase liquid chromatography was used to determine the stoichiometry and formation constants of the complex of β-cyclodextrin with pyrene in the presence of several secondary modifiers in a 59% (v/v) methanol in water mobile phase. All secondary modifiers contained a bulky and hydrophobic *tert.*-butyl moiety attached to functional groups of varying polarity and heteroatom composition. Although pyrene exhibits no interaction with β-cyclodextrin in a 59% (v/v) methanol in water solvent alone, the addition of a co-modifier resulted in dramatically short retention times. The stoichiometry of the β-cyclodextrin–pyrene was determined to be predominantly 2:1. Formation constants were estimated to be approximately $10^4 M^{-2}$. Adding functionality and increasing chain length in co-modifiers resulted in a corresponding enhancement of formation constants and reduction of capacity factors. The effects of pH on the equilibrium and the applications of co-modifier effects in environmental analysis are discussed.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides that form inclusion complexes with a variety of organic guest molecules [1,2]. The use of CDs as mobile phase modifiers in reversed-phase liquid chromatography has been widely documented [3–9]. The fixed cavity sizes of the cyclodextrins impart a high degree of selectivity upon the separation, since the elution time of a given analyte depends directly on the strength and stoichiometry of its complex with cyclodextrin [10,11].

The impact of polynuclear aromatic hydrocarbons (PAHs) on the environment has been well established [12–15]. As a result, significant effort has been focussed towards characterizing the behavior of such compounds in aqueous and non-aqueous media [16]. Complexation of PAHs with cyclodextrins has been widely studied using a variety of spectroscopic techniques, including absorbance and fluorescence [17–20]. Although these methods are highly sensitive and have relatively short analysis time, their use is limited to the determination of analytes that undergo a significant change in their spectroscopic properties upon inclusion in the CD cavity. Several studies have utilized reversed-phase HPLC as an alternative analytical technique to investigate the complexation of PAHs with cyclodextrins [21–

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24]. An example of a common PAH is pyrene, which has been shown to form a strong 2:1 (β-CD–pyrene) complex with β-CD [25]. Recently, Anigbogu et al. [26] investigated the retention behavior of β-CD:pyrene complexes in the presence of alcohol comodifiers using reversed-phase HPLC. The retention time of pyrene in a methanol-water solvent system was found to be relatively unchanged in the presence of increasing amounts of β-cyclodextrin. The addition of 1% *tert.*-butanol or cyclopentanol as a secondary modifier, however, caused a dramatic reduction in the capacity factor of pyrene. This effect has been attributed to the formation of a ternary β-CD–pyrene–alcohol complex. A later study of apparent formation constants determined that the alcohol strengthens the β-CD–pyrene complex [27].

The effect of an appropriate secondary modifier on the β-CD–PAH complex can have significant environmental applications in selective extractions of non-aqueous solutes in a complex mixture. This study is a comparison of several secondary modifiers in an effort to characterize these types of ternary complexes, which may improve upon existing methodologies in environmental analysis. The selection of modifiers was based upon the assumption that the bulky and hydrophobic *tert.*-butyl group in *tert.*-butanol is partially included in the β-CD cavity, with the hydroxyl group hydrogen bonding with the primary and secondary hydroxyl groups located on the periphery of the cyclodextrin. This orientation of the alcohol has previously been proposed by Muñoz de la Peiia *et al.* [28] in their investigation of the β-CD–pyrene–*tert.*-butanol system in aqueous media. The nature and polarity of the functional group on the *tert.*-butyl may therefore have a significant effect upon the strength and extent of the hydrogen bonding occurring at the periphery of the cyclodextrin. The comodifiers used in this study contain the *tert.*-butyl (tBu) moiety attached to functional groups of varying polarity and heteroatom composition.

EXPERIMENTAL

Apparatus

The chromatographic apparatus used in this study has been described elsewhere [26].

Reagents

HPLC-grade methanol and water were purchased from B&J (Baxter, McGraw Park, IL, USA) and Fisher (Fair Lawn, NJ, USA), respectively. Pyrene (99+%) and the secondary organic modifiers, tBu-OH, tBu-acetate, tBu-formate, tBu-carbamate, tBu-carbazate, tBu-formate, tBu-acetate, N(tBu-carbonyl)glycine and N(tBu-hydroxy)carbamate were all purchased from Aldrich (Milwaukee, WI, USA) and were used as received. The β-CD used in this study was provided by American Maize Products (Hammond, IN, USA) and was recrystallized twice from deionized water before use. The potassium nitrite used for determining the void volume of the column was purchased from Mallinckrodt (Paris, KY, USA).

Procedure for the liquid chromatographic runs

The procedure for the chromatographic runs was similar to that previously described by Anigbogu *et al.* [26]. The mobile phase consisted of a mixture of 59% (v/v) methanol, 39% (v/v) water mixture, and 0.2 mol% (approximately 0.075 *M*) of secondary modifier.

RESULTS AND DISCUSSION

Effect of secondary organic modifier on the capacity factor of pyrene

Anigbogu *et al.* [26] recently reported the effects of the addition of *tert.*-butanol as a secondary modifier on the retention time of pyrene in the presence of β-CD in a water–methanol system. The capacity factor of pyrene was found to be remarkably reduced upon the addition of the alcohol. This was attributed to the formation of a ternary β-CD–pyrene–alcohol complex, which has been extensively investigated in aqueous media by researchers using fluorescence and proton NMR analyses [28]. The hydrophobicity of the *tert.*-butyl moiety of the

alcohol would make it more likely for it to be associated with the interior of the cyclodextrin cavity. **Muñoz** de la **Peña** et ***al.*** **[28]** suggested two possible configurations for the **β-CD–**pyrene-alcohol complex, both of which involve the hydrogen bonding interaction of the OH group of the alcohol with the hydroxyl groups lining the periphery of the cyclodextrin. As a result, changing the size and polarity of the functional group of the ***tert.*-butyl** modifier would cause a corresponding change in the formation of the ternary complex.

Table I provides a list of the modifiers used in this study. The purpose of selecting these particular ***tert.*-butyl** compounds was to systematically vary the polarity, chain size and heteroatom composition of the functional group in an effort to determine the influence of these variables on the retention of the **β-CD–pyrene** complex under reversed phase conditions. ***tert.*-Butanol** has been used as a reference to compare the extent of the effect of the various comodifiers on the retention time of pyrene.

Fig. la shows the effect of comodifiers I, II, III and IV on the capacity factor of the **β-CD–**pyrene complex. As expected, the addition of ***tert.*-butanol** (I) resulted in an overall decrease in the capacity factor of pyrene (Fig. la), which is in agreement with previous results **[26]**. It is interesting to note, however, that unlike the other secondary modifiers, ***tert.*-butyl formate** (II) and **N(*tert.*-butoxycarbonyl)glycine** (VIII) appear to significantly influence the capacity factor (k_0') of the **uncomplexed** pyrene (Fig. lb). The factors that may have contributed to this

TABLE I

SECONDARY MODIFIERS

tert.-Butyl alcohol (I)	$(CH_3)_3C–O–H$
tert.-Butyl formate (II)	$(CH_3)_3C–O–CO–H$
tert.-Butyl acetate (III)	$(CH_3)_3C–O–CO–CH_3$
tert.-Butyl carbamate (IV)	$(CH_3)_3C–O–CO–NH_2$
tert.-Butyl carbazate (V)	$(CH_3)_3C–O–CO–NH–NH_2$
tert.-Butyl (N-hydroxy)-carbamate (VI)	$(CH_3)_3C–O–CO–NH–OH$
N(*tert.*-Butoxycarbonyl)-glycine (VII)	$(CH_3)_3C–O–CO–NH–CH_2–COOH$

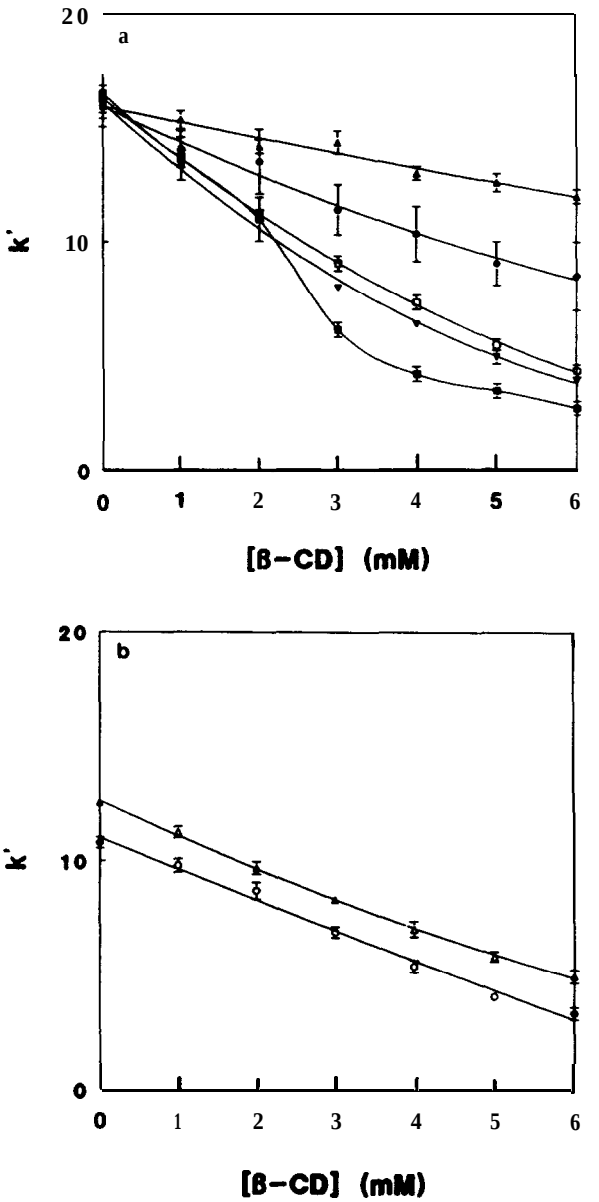

Fig. 1. Effect of β-CD concentration on the capacity factor of pyrene in the presence of different secondary modifiers. (a) A = *tert.*-Butyl alcohol (I); ● = *tert.*-butyl acetate (III); □ = *tert.*-butyl carbazate (V); ▼ = *tert.*-butyl carbamate (IV); ● = *tert.*-butyl N-(hydroxy)carbamate (VI). (b) A = *tert.*-Butyl formate (II); 0 = N(*tert.*-butoxycarbonyl)glycine (VII).

apparent anomaly and the effects on the calculated K_f values are discussed later.

The effect of replacing the hydrogen in ***tert.*-**

butyl formate (II) with a methyl group may be seen in the *tert.*-butyl acetate (III) data in Fig. la. In this case, the co-modifier has no observable effect upon the retention time of free pyrene .

The retention times of the β-CD–pyrene complex, however, are significantly reduced in the presence of *tert.*-butyl acetate (III) compared to *tert.*-butyl alcohol (I). This suggests that the ester is involved in the formation of the inclusion complex in a way such that the pyrene molecule is further protected from interaction with the C_{18} mobile phase. This may be due to hydrogen bond formation between the carbonyl group of the co-modifier and the hydroxyl groups lining the periphery of the cyclodextrin.

Further evidence of the formation of hydrogen bonds can be seen when the methyl group on *tert.*-butyl acetate (III) is replaced by an amino group in *tert.*-butyl carbamate (IV). Fig. la shows that the addition of *tert.*-butyl carbamate as a secondary modifier to a methanol-water solvent results in a dramatic decrease in the capacity factors of the β-CD–pyrene complex. This suggests that the interaction of IV with the inclusion complex is stronger than that of III, which indicates that the amino group is either directly or indirectly involved in complex formation. Aqueous phase spectroscopic studies of the ternary β-CD–pyrene–amine species have revealed the formation of charge transfer complexes between the amine and pyrene, which would involve the nitrogen being in close proximity to the pyrene molecule [29,30]. Several of these investigations, however, assumed a 1:1 association ratio for the β-CD–pyrene complex. This stoichiometry would allow less water to be excluded from the cavity, causing it to be more polar, thereby making it more likely for the polar amino group to penetrate the cavity and interact with the pyrene. Recent studies in our laboratory have determined, however, that the stoichiometry between β-CD and pyrene is 2:1 (2 cyclodextrin molecules encapsulating 1 pyrene molecule) [25,28]. This configuration results in a larger amount of water being displaced from the cavity, making the cavity more hydrophobic. Taking into consideration the space and polarity restrictions of the cyclodextrin cavity, it seems more likely that the non-polar and bulky *tert.*-butyl group is included, with the carbonyl and amino groups protruding out into relatively aqueous microenvironment. The hydrogen bonding interactions are expected to be stronger for *tert.*-butyl carbamate (IV) compared to *tert.*-butyl acetate (III) due to the presence of the amino group in former, which may explain the enhanced reduction of pyrene retention times that occur upon its addition.

Fig. 1 also depicts the effect of changing both the chain size as well as the polarity of the functional group attached to the *tert.*-butyl moiety of the secondary modifier upon the retention times of the β-CD–pyrene complex. Addition of another amino group to *tert.*-butyl carbamate (IV) produces no significant change, as can be seen in the data obtained in the presence of *tert.*-butyl carbazate (V). When compared to *tert.*-butanol, however, the effect of *tert.*-butyl carbazate is very pronounced. Retention times of free pyrene undergo a significant decrease in the presence of N (*tert.*-butoxycarbonyl)glycine (VI), in which the second amino group of V is replaced by a hydroxyl group.

Determination of the stoichiometry of the β-CD–pyrene complex in the presence of secondary modifiers

Consider the reaction between pyrene (P) and β-CD

$$P + n(\beta\text{-CD})_m \rightleftharpoons [(\beta\text{-CD})_n\text{–P}]_m \quad (1)$$

where the subscript *m* denotes the concentration in the mobile phase. Armstrong et *al.* [4] determined the relationship between the capacity factor of the probe and equilibrium concentration of β-CD in a water-primary organic modifier mobile phase to be as follows:

$$1/k' = 1/k'_0 + K_f[\beta\text{-CD}]^n_m/k'_0 \quad (2)$$

where *k'* is the capacity factor of the probe; k'_0 is the capacity factor of the probe in the absence of β-CD; K_f is the formation constant for the &CD-probe complex, and $[\beta\text{-CD}]_m$ is the equilibrium concentration of β-CD. Assuming a correct stoichiometry between β-CD and the guest, a plot of 1 *lk' versus* $[\beta\text{-CD}]^n_m$ would be

linear with slope K_f/k'_0 and intercept $1/k'_0$. The equilibrium concentration of β-CD can be determined by the following equation

$$[\beta\text{-CD}]_m = [\beta\text{-CD}]/(1 + K_{m1}[M_1] + K_{m2}[M_2]) \quad (3)$$

where M_1 and M_2 are the primary (e.g. methanol) and secondary (e.g. *tert.*-butyl carbamate) modifiers, respectively, and K_{m1} and K_{m2} are the formation constants for β-CD–M_1 and β-CD–M_2 complexes, respectively. Assuming negligible interaction between the modifiers and β-CD, the initial concentration of β-CD equals its equilibrium concentration. Initial &CD concentrations were used to determine stoichiometries and formation constants for the β-CD–pyrene complex in the presence of the various modifiers.

Fig. 2 shows a plot of l/k' vs. [β-CD] for pyrene in a water-methanol system with 0.20 mol% tBu-carbamate. Assuming a 1:1 stoichiometry between β-CD and pyrene, a curvilinear fit is obtained. Assuming that two cyclodextrins associate with every pyrene molecule, *i.e.*, $n = 2$, the plot of $1/k'$ vs. [P-CD], gives a linear fit, indicating that the stoichiometry between the species is predominantly 2: 1 (β-CD–pyrene). Similar results were obtained when different comodifiers were used. The correlation coefficients for both 1: 1 and 2:1 fits for all the comodifiers are reported in Table II. These values indicate that the predominant stoichiometry is 2:1 in the presence of all the secondary modifiers used in this study. This is consistent with previous studies of β-CD–pyrene complexes [25,27], which reported the formation of a 2:1 complex between β-CD and pyrene.

Determination of formation constants for the β-CD–pyrene complex in the presence of different secondary modifiers

The equilibrium constant of the β-CD–pyrene complex with various co-modifiers quantifies the strength and stability that may be conferred upon this inclusion complex due to the presence of the secondary component. This information can have significant impact upon method development procedures using cyclodextrins in pharmaceutical and analytical applications. Apparent formation constants for the β-CD–pyrene complex in the presence of the various co-modifiers were determined by utilizing eqn. 2. The slopes and intercepts of the linear plots obtained by assuming a 2:1 pyrene–β-CD stoi-

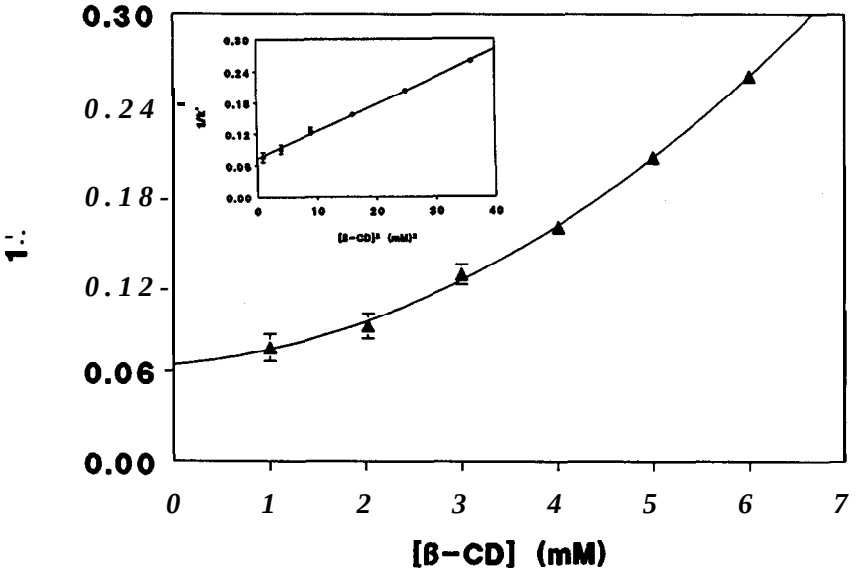

Fig. 2. Plot of $1/k'$ *vs.* $[\beta\text{-CD}]_m^n$ for pyrene in the presence of 0.075 *M tert.*-butyl carbamate assuming a (A) 1:1 ($n = 1$) and (●) 2:1 ($n = 2$) (inset) β-CD–pyrene stoichiometry.

TABLE II

CORRELATION COEFFICIENT VALUES FOR THE PLOTS OF $1/k'$ VS. [β-CD] IN THE PRESENCE OF DIFFERENT SECONDARY MODIFIERS

Co-Modifier	1:1 Stoichiometry	2: 1 Stoichiometry
tert.-Butyl alcohol (I)	0.973	0.991
tert.-Butyl formate (II)	0.989	0.998
tert.-Butyl acetate (III)	0.981	0.990
tert.-Butyl carbamate (IV)	0.984	0.998
tert.-Butyl carbazate (V)	0.976	0.998
N(*tert.*-Butyl hydroxy)carbamate (VI)	0.981	0.997
N(*tert.*-Butoxycarbonyl)glycine (VII)	0.971	0.997

chiometry were used to estimate the K_f values. Table III lists the apparent K_f values calculated for the β-CD–pyrene complex in the presence of the different co-modifiers used in this study.

Previous studies utilizing β-CD in a methanol–waster mobile phase have shown that pyrene and other polyaromatic hydrocarbons interact more with the C_{18} stationary phase and are not eluted off the column in significant quantities [31,32]. As a result, the association constant of the pyrene–β-CD complex under these conditions is negligible. Introducing a secondary modifier like *tert.*-butyl alcohol was found to enhance the interaction between the solute and the β-CD mobile phase, suggesting the formation of a ternary complex, and subsequently it became possible to estimate a value for the formation constant of the pyrene–β-CD complex. The value of the apparent formation constant in the presence of *tert.*-butyl alcohol at 59% methanol calculated here is along the same order of magnitude as that reported by Anigbogu *et* al. [27].

TABLE III

APPARENT FORMATION CONSTANTS FOR THE β-CD–PYRENE COMPLEX IN THE PRESENCE OF SEVERAL SECONDARY MODIFIERS

Co-modifier	K_f (M^{-2}) ($\times 10^4$)
tert.-Butyl alcohol (I)	1.3 ± 0.2
tert.-Butyl formate (II)	2.9 ± 0.2
tert.-Butyl acetate (III)	1.4 ± 0.4
tert.-Butyl carbamate (IV)	1.4 ± 0.1
tert.-Butyl carbazate (V)	1.5 ± 0.1
N(*tert.*-Butyl hydroxy)carbamate (VI)	1.6 ± 0.2
N(*tert.*-Butoxycarbonyl)glycine (VII)	1.7 ± 0.3

A general increasing trend in K_f is observed in Table III as the hydioxyl group in *tert.*-butyl alcohol is replaced by ester groups of varying size and polarity. Close examination shows that there is a direct correlation between the K_f values in Table III and the slopes of the k_0' *versus* [β-CD] plots (Fig. 1) for the majority of the co-modifiers except for the *tert.*-butyl formate and the N(*tert.*-butoxycarbonyl)glycine data. An explanation is given later.

The addition of *tert.*-butyl acetate (III) as a secondary modifier results in a slightly higher formation constant for the pyrene–β-CD complex. This value is comparable to that estimated in the presence of *tert.*-butyl carbamate (IV). In contrast to I, both III and IV have longer chains and contain carbonyl groups, which may contribute to the stability of the ternary complex by forming hydrogen bonds with the hydroxyl groups located on the periphery of the β-CD torus. From the value obtained for *tert.*-butyl carbazate (V), it can be seen that increasing the chain length by the addition of another amino group to *tert.*-butyl carbamate produces a more significant increase in the formation constant value. Interestingly, in this case, keeping the chain length comparable but substituting the second amino group in V for a hydroxyl group in N(*tert.*-butyl hydroxy)carbamate (VI) results in a corresponding increase in the formation constant. Elongating the chain as well as adding another carbonyl group in N(*tert.*-butoxycarbonyl)glycine (VII) further enhances the value of the binding constant for the pyrene–β-CD complex.

The anomalous behavior exhibited by *tert.*-

butyl formate (II) and N(*tert.*-butoxycarbonyl)glycine (VII) may be attributable to the acidity of these two modifiers. The addition of either of these compounds resulted in a drastic decrease in the pH of the mobile phase (Fig. 3) to values as low as 2.2. It was previously noted that, unlike the other co-modifiers, II and VII caused a significant decrease in the capacity factor k_0' of the uncomplexed pyrene. The reasons for the marked decrease in retention time of uncomplexed pyrene in the presence of these co-modifiers are not exactly known. It should be noted, however, that the lower pH limit generally recommended for the operation of C_{18} columns is 3.5. Below this pH, the column could undergo undesirable changes including stripping of the stationary phase. However, the response of the column used here was successfully restored to normal after regeneration by washing with methanol and water. It then follows that the observed effects were due to changes in the retention characteristics of the column under acidic conditions.

The method of determination of formation constants (K_f) employed here [which involves the division of the slope by the intercept (eqn. 2)] assumes that the intercept is relatively constant while the slope varies with the experimental conditions. Fig. 1 confirms the changing slopes and the relatively unchanging intercept for the majority of the modifiers. Under these circumstances, there is a direct correlation between the slope and the calculated K_f value. In the case of co-modifiers II and VII, the lower intercept values compared to those for the other co-modifiers, resulted in higher K_f values which, in turn, do not correlate with the slope of the k_0' *versus* [β-CD] plots. Consequently, the formation constant values calculated for *tert.*-butyl formate (II) and N(*tert.*-butoxycarbonyl)glycine (VII) cannot be compared to those estimated for the other modifiers. This observation suggests that the K_f values determined by the RPLC technique are only comparable when the solutes of interest exhibit similar k_0' values, a point already alluded to by Anigbogu *et* al. [26].

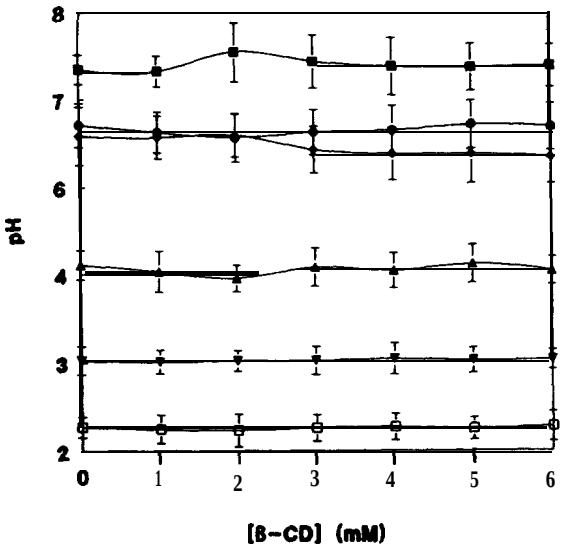

Fig. 3. Variation of pH with increasing β-CD concentration in the presence of several secondary modifiers. ◆ = *tert.*-Butyl alcohol (I); A = *tert.*-butyl acetate (III); ■ = *tert.*-butyl carbazate (V); ● = *tert.*-butyl carbamate (IV); ▼ = N(*tert.*-butoxycarbonyl)glycine (VII); □ = *tert.*-butyl formate (II).

Although the nature of the forces contributing to the formation of cyclodextrin inclusion complexes is not fully understood, it is fair to assume that the driving force for the process is in a large part due to hydrophobic interactions, with some stability conferred upon the complex by hydrogen bond formation. In this particular case, several factors could influence the formation of the pyrene–β-CD complex. As expected, the presence of methanol as a primary organic modifier in the bulk aqueous solvent has been shown to adversely affect the formation constant of the complex [9]. This may be attributed to the competitive equilibrium which causes the pyrene to partition between the relatively hydrophobic bulk medium and the cyclodextrin cavity. In reversed-phase HPLC, the competition for pyrene is three-fold: between the cyclodextrin, the solvent and the stationary phase. Previous studies in our laboratory have revealed that in this case, pyrene is retained on the C_{18} column, suggesting that the formation constant for the pyrene–β-CD complex is negligible under these circumstances [26]. The addition of a small amount of secondary modifier was found to dramatically affect the retention behavior of pyrene, and formation constant values for its complex with β-CD at several methanol concen-

trations have been reported [27]. The enhancement of equilibrium constants due to the secondary modifier may be attributed to the formation of a more stable ternary complex. The stability of ternary cyclodextrin complexes may be a result of more polar water molecules being excluded from the cavity to accommodate the non-polar *tert.*-butyl group of the secondary modifier. Also, the formation of hydrogen bonds between the heteroatom containing functional groups of the co-modifier and the hydroxyl groups of the cyclodextrin may play a role in conferring stability upon the inclusion complex. This could be a plausible explanation for the enhancement of formation constants observed upon increasing functionality of *tert.* -butyl modifiers.

Effect of pH upon the retention characteristics of the β-CD–pyrene complex in the presence of secondary modifiers

Although ternary complex formation may be the primary reason for the low retention times observed, the addition of a secondary modifier may also change the pH of the bulk mobile phase, which may contribute to the affinity of pyrene for the solvent. Fig. 3 depicts the variation in pH with increasing amounts of β-CD for several of the modifiers. As expected, the differences in pH range from 3.0 to 7.5, depending upon the number of acid or amino groups present. Changing the pH of the methanol–water-p-CD bulk mobile phase in the absence of secondary modifiers did not significantly affect the retention of pyrene, which did not elute off the column. This indicates that the pH difference is not a major contributing factor to the retention characteristics observed in the presence of the co-modifiers. It is also interesting to note in Fig. 3 that varying the β-CD concentration does not affect the pH of the mobile phase for each co-modifier used, suggesting that the significant change in pyrene retention is due to some type of complex formation rather than pH effects.

Influence of secondary modifier concentration on the capacity factor of pyrene

Fig. 4 shows the effect of increasing modifier concentration on the capacity factor of pyrene

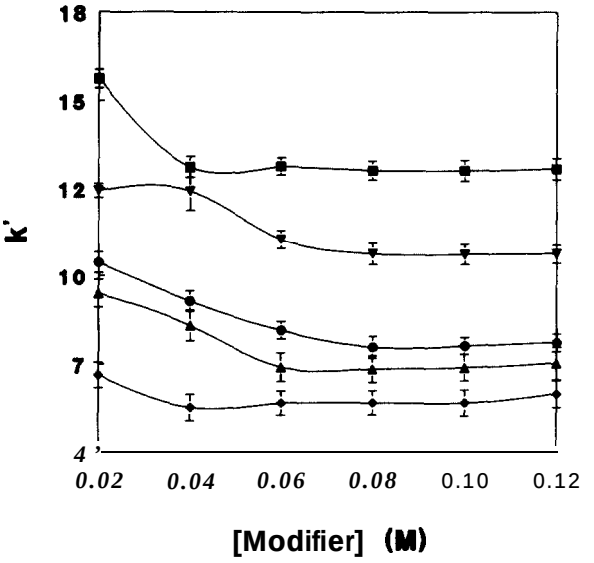

Fig. 4. Plot of capacity factor vs. secondary modifier concentration optimum co-modifier concentration. ■ = *tert.*-Butyl alcohol (I); ▼ = *tert.*-butyl acetate (III); ● = *tert.*-butyl carbazate (V); A = *tert.*-butyl carbamate (IV); ◆ = N(*tert.*-butoxycarbonyl)glycine (VII).

for several of the compounds used in this study. It is evident that the greatest variation in retention characteristics occurs in the 0.01-0.06 *M* range for most of the modifiers. It may be inferred from this observation that at approximately 0.06 *M*, all the binding sites for the co-modifier on the inclusion complex are saturated. Thus, the addition of greater amounts of the secondary modifier are not used in ternary complex formation, and consequently do not affect the capacity factor.

CONCLUSIONS

The nature and polarity of the functional group attached to the *tert.*-butyl moiety of the secondary modifier appears to significantly affect the equilibrium of pyrene between the C_{18} stationary phase and the methanol-water-&CD mobile phase. Since under these reversed-phase chromatographic conditions, CD inclusion complexes do not appear to form in the absence of a co-modifier, these compounds can play an important role in the cyclodextrin-aided extraction of environmentally significant PAHs from contaminated sites. In addition to facilitating remov-

al of PAHs, the use of secondary modifiers with added functionality may reduce analysis time, which would improve the practicality of employing cyclodextrins in industrial applications.

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