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Retention of benzo[*a*]pyrene on cyclodextrin-bonded phases

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ABSTRACT

The retention of polynuclear aromatic hydrocarbons by cyclodextrin-bonded phases is complex. It consists of inclusion formation, the mechanism which confers the unique retention properties to this phase, an ionic component due to the external hydroxyl groups and a reversed-phase like component. This complex retention mechanism allows the resolution of compounds that would otherwise prove difficult to resolve on conventional normal or reversed phases.

The relative effect of each of these mechanisms on retention is determined by the column temperature and by the type and percentage of the mobile phase organic modifier. In this study the effect that the alteration of these parameters has on the retention of benzo[*a*]pyrene is reported. The multi-mode retention mechanism is clearly shown to be in effect.

INTRODUCTION

Cyclodextrins are polymers consisting of helical 1,4-α-glucoside monomers. The molecule is shaped as a torus with major and minor openings. The minor opening is surrounded by primary hydroxyl groups which are bound to the silica support by a hydrocarbon linkage. The larger major opening is surrounded by secondary hydroxyl groups which are responsible for the external hydrophilic environment. In contrast, the internal environment is hydrophobic and responsible for the formation of cyclodextrin–analyte inclusion complexes. This occurs if the analyte is of the correct size and charge distribution[1].

Three cyclodextrins have been successfully bound to a 5-μm spherical silica base. These have been named α-, β- and γ-cyclodextrins and consist of six, seven and eight glucose monomers, respectively. The internal diameters are 0.57, 0.78 and 0.95 nm and in all three cases the torus depth is around 0.78 nm (ref. 2). Both the α- and β-cyclodextrins are available in an acetylated form to reduce possible ionic interactions. The size of benzo[*a*]pyrene has been calculated as 0.88 nm across the long axis suggesting that while the α-cyclodextrin will not form an inclusion complex, both the β and γ will. This has been confirmed by Woodberry *et al.*[2], where benzo[*a*]pyrene was

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shown not to form an inclusion complex with the α-cyclodextrin, a 1:1 host–analyte ratio was obtained with the β-cyclodextrin and a 1:2 host–analyte ratio with the γ-cyclodextrin. It was proposed that in the γ-cyclodextrin benzo[*a*]pyrene was able to stack allowing two molecules to enter the cavity simultaneously.

The cyclodextrins are a relatively new bonded phase and have been successfully used for the separation of a number of chiral compounds such as benzo[*a*]- and benzo[*e*]pyrene[3], dansyl-D- and -L-leucine[1], and *ortho*-, *para*- and *meta*-nitro-anilines[1]. Mycotoxins, polynuclear aromatic hydrocarbons, quinones and heterocyclic compounds have all been resolved on these phases highlighting the resolving power of these columns[4]. These separations have been achieved due to the ability of the phase to form a stable inclusion complex with the analyte. The stability of the complex depends on the charge distribution, and the shape and size of the molecule forming the complex. There must be a hydrophobic region within the molecule that is the correct size to enter the cavity, the complex stability may be enhanced by the presence of external polar groups that can interact with secondary hydroxyls. If the analyte is too large it will be unable to enter the cavity and no inclusion derived retention will occur, although retention may still occur by an ionic interaction mechanism. In this mode the column acts as a high-density diol column. In the β-cyclodextrin (acetylated) column the secondary hydroxyls have been acetylated thus reducing the possibility of ionic interactions. If the analyte is too small it will enter the cavity but the stability of the complex is poor and the retention will be low. It is also possible to use non-polar solvents such as hexane with these phases, in this case no inclusion occurs and retention is due purely to external adsorption.

In this paper we have investigated the effect of temperature and the type and concentration of mobile phase modifier on the retention of the probe molecule benzo[*a*]pyrene. Benzo[*a*]pyrene has been used in this study as it is known to enter, and thus be retained, by both the β- and γ-cyclodextrins and also due to its environmental importance. In a recent study by Olsson *et al.*[5] the retention of a range of polynuclear aromatic hydrocarbons by monomeric and polymeric C_{18}-bonded phases was not found to be as high as that of conventional reversed phases, the authors did suggest that the cyclodextrin-bonded phases may offer significant advantages where the separation of polynuclear aromatic hydrocarbons of different molecular weights is required. The authors of this paper have reported a multidimensional high-performance liquid chromatographic (HPLC) configuration with both C_{18} and cyclodextrin columns that was used to realise the determination of low levels of benzo[*a*]pyrene in aviation and diesel fuel after direct injection of the fuel[6]. Three different mechanisms of retention have been identified, inclusion derived retention, normal-phase type retention and a novel retention mechanism limited to a number of solvents.

EXPERIMENTAL

Materials

Benzo[*a*]pyrene was obtained from BDH (Poole, U.K.). Chromatographic solvents were also obtained from BDH and were of HiPerSolv grade except for the propan-1-ol which was of Aristar grade. The propan-2-ol was obtained from May and Baker (Dagenham, U.K.). The water used in this study was distilled and stored in glass. The α-cyclodextrin, β-cyclodextrin (acetylated) and γ-cyclodextrin columns (250 ×

4.6 mm I.D.) were packed by Astec (U.S.A.) supplied by Technicol (Stockport, U.K.) Standard benzo[*a*]pyrene solutions were made up in acetonitrile and stored in the dark to avoid photo-induced degradation. A concentration of 400 ng ml^{-1} benzo[*a*]pyrene was used, equivalent to an injection of 8 ng.

Equipment

All chromatography was carried out using either system A (retention measurement of benzo[*a*]pyrene) or system B (determination of the retention time of an unretained compound (t_0).

System A. A Waters series 6000 HPLC pump was used, the mobile phase being generated by a modified Micrometeritic gradient former. The flow-rate used was 1.0 ml min^{-1} for all solvents except for propan-1-ol and propan-2-ol containing mixtures, when, due to pressure limitations, a flow-rate of 0.5 ml min^{-1} was selected. All solvents were filtered through a 2-μm Millipore filter under negative pressure and continuously degassed with helium. A Rheodyne, Model 7125 syringe loading injection valve with a 20-μl sample loop was used to introduce the sample. A Perkin-Elmer series 3000 fluorescence detector was used to monitor the eluent. The excitation and emission wavelengths were 254 nm and 420 nm, respectively. Excitation and emission slits were 5 nm. The columns were maintained at the desired temperature using a Grant Instruments (Cambridge, U.K.), Model SE10 water bath.

System B. A Kontron Instruments 420 LC pump was used for this study, with the mobile phases continuously degassed with helium. The columns under investigation were thermostated in a Perkin-Elmer LC-65T column oven. A house-built conductivity cell, consisting of a 1/16 × 0.043 in. through-hole cross-coupler which had been drilled to allow the positioning of a pair of 1-mm diameter gold disc electrodes in the flow stream, was also maintained at constant temperature in the oven. The conductivity of the column effluent was monitored using an Orion Research 101 conductivity meter. The procedure described by Hinze *et al.*[7] was adapted to estimate t_0. In order to promote a significant change in conductivity due to the probe alcohols, potassium chloride was added to the methanol–water (50:50, v/v) and pure water phases to a concentration of 0.05 *M*. In this way, a decrease in conductivity was measured. For the acetonitrile phase, no electrolyte was added and the probe peaks gave an increase in conductivity. Determinations were made at column temperatures of 25 and 50°C.

During the study both a Hewlett-Packard 3390A integrator and a Midas Chromatographic Data Station, Comus Instruments (Hull, U.K.) were used to determine the retention times and a Goerz BBC SE 120 chart recorder was used to record the chromatograms. When not in use the columns were equilibrated with 100% methanol.

RESULTS AND DISCUSSION

In this study five commonly used reversed-phase solvents and one normal-phase solvent were used to study the retention of the polynuclear aromatic benzo[*a*]pyrene on the β-, β-(acetylated) and γ-cyclodextrin-bonded phases.

When studying the retention of species on stationary phases under different mobile phase conditions, it is beneficial to utilise the parameter of phase capacity ratio,

k', in the description. This relies on obtaining an accurate estimate of the retention time of an unretained spcies, t_0. For most stationary phases, there are recommended probes that yield this information. For cyclodextrin-bonded phases, however, it is not so straightforward.

The method reported by Hinze *et al.*[7] was used in this study in order to obtain an estimate of t_0. Plots of retention times of the probe alcohols *vs.* their formation binding constants for β-cyclodextrin complexation are extrapolated to zero binding constant to yield the retention time equivalent to the column void volume. Table I gives a summary of estimates of t_0 in terms of column void volume for a selection of mobile phases at the two temperatures studied, using the probes methanol, ethanol and propanol. It is interesting to note that in methanol and water mobile phases, the elution order at both temperatures is methanol, ethanol and propanol; whilst in 100% acetonitrile, the elution order is completely reversed. This indicates the complexity of the cyclodextrin retention mechanism and stresses the difficulty in obtaining a realistic value for t_0. Also, for mobile phases consisting of either 100% water or 100% acetonitrile, the data are not linear, such that an exponential rather than a linear curve fit was more appropriate for the extrapolation. In view of these results, it was decided to quote the actual retention time of benzo[*a*]pyrene for a given mobile phase and temperature rather than the preferred parameter of phase capacity ratio.

Fig. 1 shows a three dimensional surface plot of the retention time on a β-cyclodextrin column in response to changes in the temperature and percentage of methanol. The steep peak between 40 and 60% methanol and 20 and 40°C (shaded) corresponds to the region in which inclusion is promoted. At higher temperatures and methanol concentrations a plateau is seen which corresponds to very low levels of retention. The temperature dependence of the inclusion complex is shown with little retention occurring above 60°C.

Similar surface plots have been obtained with both the β-cyclodextrin (acetylated) and γ-cyclodextrin columns. In contrast Fig. 2 shows the surface plot for the β-cyclodextrin column in response to changes in acetonitrile concentration and temperature. The steep inclusion peak is clearly present as is a ridge seen at high acetonitrile concentrations between 80 and 100% (shaded). The ridge shows only slight temperature dependence. Again, similar results are obtained with the other two bonded phases when using acetonitrile as the organic phase modifier. Tetrahydrofuran

TABLE I

ESTIMATED VOID VOLUME RETENTION TIME (t_0) FOR THE β-CYCLODEXTRIN COLUMN OBTAINED AT DIFFERENT OPERATING CONDITIONS

All flow-rates 1.0 ml min^{-1}.

Mobile phase	*Temperature (°C)*	*Estimated t_0 (min)*
Water (0.05 *M* potassium chloride)	25	3.23
Methanol–water (50:50) (0.05 M Potassium chloride)	25	2.95
100% acetonitrile	25	3.58
Water (0.05 *M* potassium chloride)	50	3.28
Methanol–water (50:50) (0.05 M Potassium chloride)	50	3.02

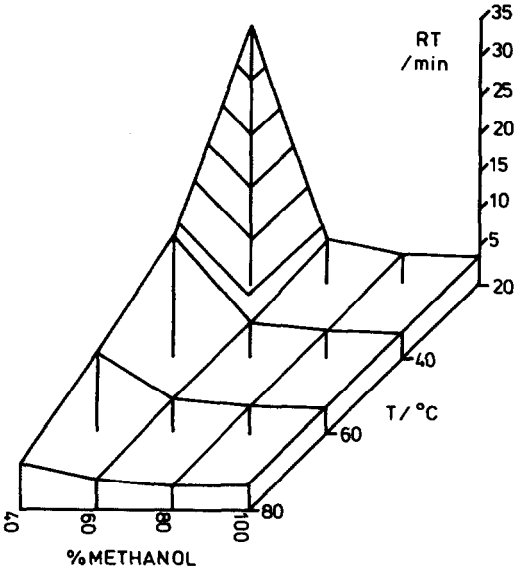

Fig. 1. Three-dimensional surface plot showing the retention time (RT) response with alterations in the column temperature and the percentage methanol in the mobile phase. Column: β-cyclodextrin; flow-rate: 1 ml min^{-1}; 8 ng benzo[*a*]pyrene injected.

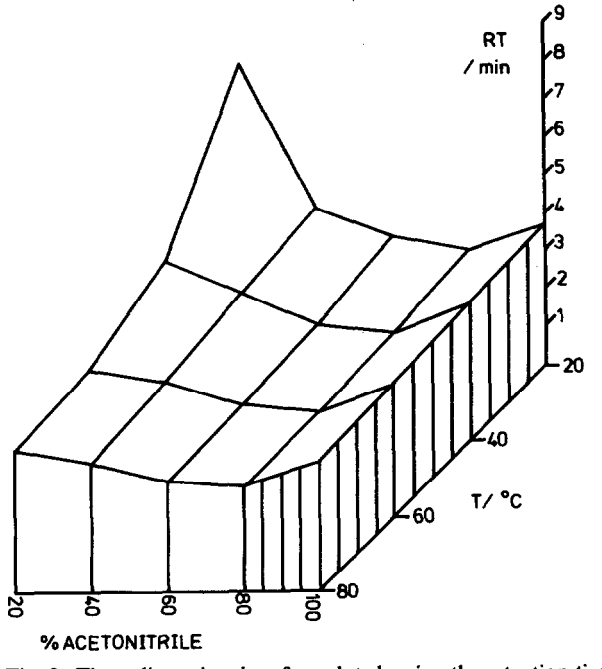

Fig. 2. Three-dimensional surface plot showing the retention time (RT) response with alterations in the column temperature and the percentage acetonitrile in the mobile phase. Column: β-cyclodextrin; flow-rate: 1 ml min^{-1}; 8 ng benzo[*a*]pyrene injected.

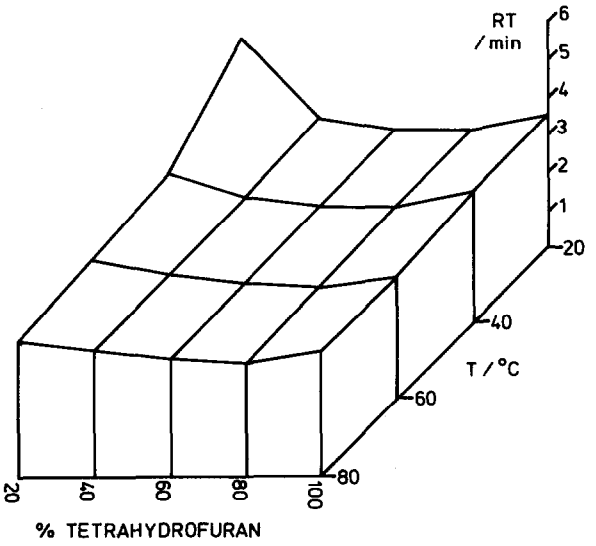

Fig. 3. Three-dimensional surface plot showing the retention time (RT) response with alterations in column temperature and the percentage of tetrahydrofuran in the mobile phase. Column: β-cyclodextrin; flow-rate: 1 ml min^{-1}; 8 ng benzo[*a*]pyrene injected.

(Fig. 3) gave similar results to those obtained using acetonitrile although the size of both the inclusion peak and retention ridge were reduced. In Figs. 4 and 5 the retention times with varying acetonitrile concentrations at 20°C and 80°C, respectively, are compared, all three bonded phases respond in a similar way. From these figures it is possible to isolate three regions on the graph; a steep portion on the left, shown to be temperature dependent, and correspond to inclusion complex formation, a shallow

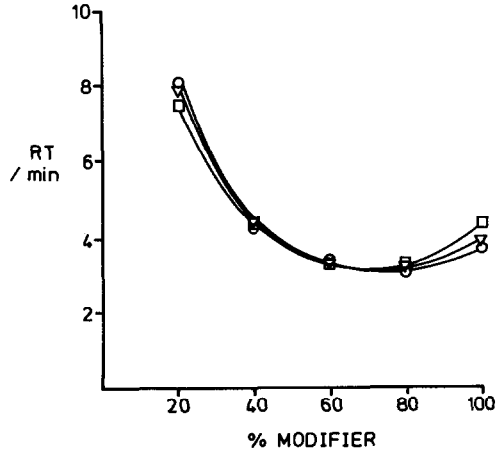

Fig. 4. Comparison of the retention time (RT) of benzo[*a*]pyrene on the three different bonded phases at 20°C with variations in the percentage of acetonitrile in the mobile phase; flow-rate: 1 ml min^{-1}. □ = β-cyclodextrin; ▽ = β-cyclodextrin (acetylated); and ○ = γ-cyclodextrin.

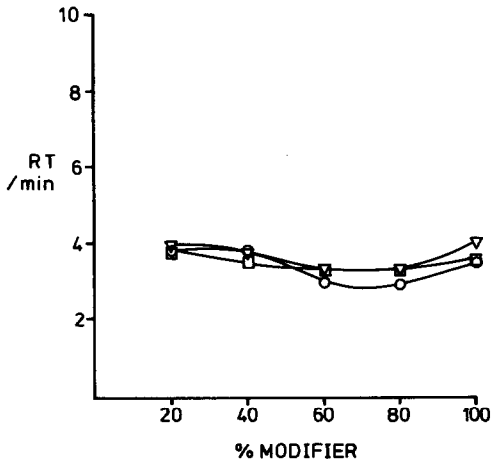

Fig. 5. Comparison of the retention time (RT) of benzo[*a*]pyrene on the three different bonded phases at 80°C with variations in the percentage of acetonitrile in the mobile phase; flow-rate: 1 ml min^{-1}. □ = β-cyclodextrin; ▽ = β-cyclodextrin (acetylated); and ○ = γ-cyclodextrin.

plateau with minimum retention times, and a high percentage organic modifier derived retention increase that is temperature independent. The occurance of a minimum, as seen in Fig. 4 (and also Fig. 6), is similar to that described by Han and Armstrong[8], who studied the influence of percentage acetonitrile on the capacity factor of some dansyl amino acids separated using a β-cyclodextrin phase.

Fig. 6 shows the variation in retention time for benzo[*a*]pyrene at 20°C, when

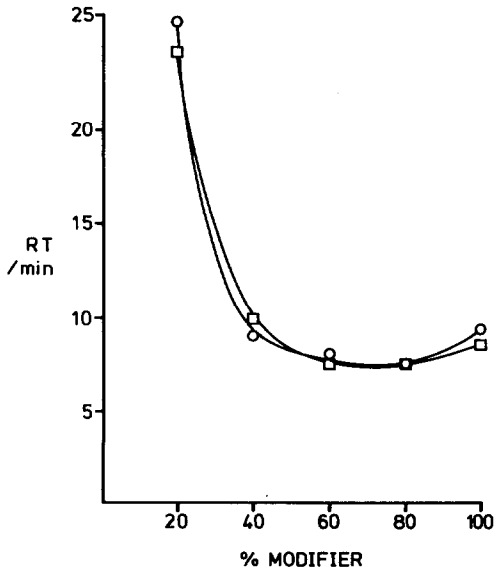

Fig. 6. Retention time (RT) of benzo[*a*]pyrene on the β-cyclodextrin (acetylated) column at 20°C with propan-1-ol (□) and propan-2-ol (○) as the organic modifiers; flow-rate: 0.5 ml min^{-1}.

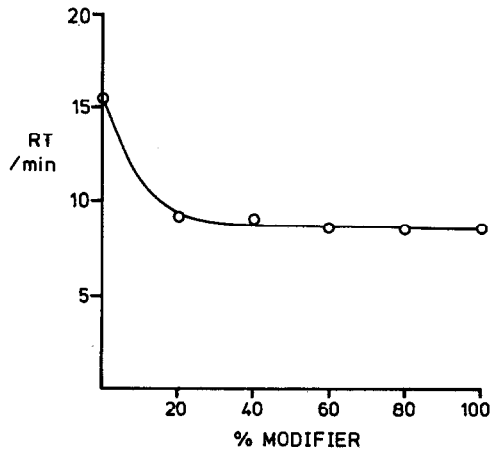

Fig. 7. Retention time (RT) of benzo[*a*]pyrene on the β-cyclodextrin (acetylated) column in the normal phase. From 100% hexane to 100% propan-2-ol (modifier); flow-rate: 0.5 ml min^{-1}.

propan-1-ol or propan-2-ol is used as the organic phase modifier. The effect obtained is more similar to acetonitrile than to methanol. In the final figure (Fig. 7) the retention of benzo[*a*]pyrene in the normal-phase mode is shown. In the normal-phase hexane was used as the bulk solvent with propan-2-ol as the modifier. The flow-rate used to obtain the data reported in Figs. 6 and 7 was 0.5 ml min^{-1} due to pressure limitations caused by the high viscosity of propan-1-ol and propan-2-ol. It is clear from these figures that retention can occur in both the normal and reversed phases, highlighting the complex retention mechanism of the bonded phases.

The results have clearly outlined the dual retention mechanism of the β-, β-(acetylated) and γ-cyclodextrin bonded phases. In the reversed phase mode the retention is achieved by two separate mechanisms that can be classified as temperature dependent and temperature independent. The temperature dependent retention occurs due to the formation of an inclusion complex between the cyclodextrin torus and the analyte. This is the normal retention mode for this type of bonded phase, and is affected by temperature. At higher temperatures due to increased molecular vibration the formation of the complex is suppressed, complete dissociation occurs at between 60 and 70°C. This mechanism is responsible for the stereo selectivity of the bonded phase. At low polarities the mobile phase organic modifier forms a more stable complex in the cavity than can be formed by the analyte and so little retention, by this mechanism, occurs.

The temperature independent mechanism is more complex. The following explanation, based on our data has been proposed. In polar organic phases (*i.e.* up to 60% acetonitrile) the cyclodextrin cavity is open and analyte inclusion, and so retention, can occur if the shape and charge requirements are met. In addition, the tori form a tight coat over the silica base as the hydrocarbon linkage between the torus and the silica particle will be constricted due to the high polarity. A similar effect occurs in bonded reversed phases such as octadecyl silanol and octa silanol and is responsible for the alteration in retention with mobile phase polarity. At higher organic modifier percentages (between 60 and 80% acetonitrile) the formation of the inclusion complex

is almost totally suppressed. The tori coat, although slightly relaxed does not significantly alter the retention. This is reflected in the plateau in retention seen between these percentages; over this range the retention time is not affected by the mobile phase polarity. At lower polarities, (*i.e.* above 80% acetonitrile) the formation of the inclusion complex is fully suppressed as the cyclodextrin cavity is completely occupied by the mobile phase. Therefore the inclusion complex formation cannot lead to the increased retention at these polarities. This is confirmed as the effect is temperature independent. It is proposed that further relaxation of the silica support–cyclodextrin linkage leads to a reduction in the coat density so allowing the analyte to pass around and behind the torus and so be retained by a reversed phase, or ionic interaction type of mechanism. Ionic interactions with the cyclodextrin, leading to increased retention have been ruled out as the β-cyclodextrin (acetylated) column gives an almost identical surface plot to that of the β-cyclodextrin column. The phenomenon is seen most clearly with acetonitrile, and to a lesser extent with tetrahydrofuran, propan-1-ol and propan-2-ol. The temperature independent retention increase is not present when methanol is used as the organic modifier. This can be explained when the relative polarities of the solvents are taken into account. Methanol has a greater polarity than either acetonitrile or tetrahydrofuran and so at even 100% methanol the polarity has not fallen to a value capable of promoting linkage relaxation. Tetrahydrofuran, although capable of forming solutions with a similar polarity to 100% acetonitrile, does not produce the effect to a similar degree. This is probably due to the higher dipole moment of acetonitrile producing a more profound relaxation induced retention. Propan-1-ol and propan-2-ol, although in the same solvent group as methanol have significantly lower polarities, closer to acetonitrile than to methanol, and so are likely to cause the relaxation induced retention in high concentration organic mobile phases.

In this study only benzo[*a*]pyrene has been closely studied although similar results have been noted with pyrene and benzo[*a*]anthracene suggesting that this phenomenon may been seen with a wide range of compounds. benzo[*a*]pyrene and other polynuclear aromatic hydrocarbons have been shown to form stable inclusion complexes with both the β- and γ-cyclodextrin-bonded phases[4].

While the increase in retention by the temperature independent process reported here is small, when compared to that achieved by complexation, it is believed that this phenomenon should be closely studied. The production of packing materials, processing "reversed-phase like" retention mechanisms in organic mobile phases could be coupled, without difficulty, to normal and size-exclusion columns without any solvent incompatibility problems. Such a ability would signicantly aid the analyst attempting the separation of complex matrices within a multicolumn separation scheme. Further studies are to be undertaken to probe more closely the nature of this novel and useful bonded phase. It is planned to study the relaxation induced effect more closely, this will include the synthesis of bonded phases with structural characteristics similar to those of the cyclodextrin molecule but without the ability to form the inclusion complexes. Other compound groups are also to be studied to determine how widespread this effect is.

CONCLUSIONS

The retention mechanism of benzo[*a*]pyrene on β-cyclodextrin-, β-cyclodextrin-(acetylated) and γ-cyclodextrin-bonded-phase columns is complex consisting of at least three distinct retention mechanisms. Certain solvents such as acetonitrile may cause some relaxation in the link binding the cyclodextrin to the silica support. This effect results in a broad minimum retention time at acetonitrile concentrations between 60 and 80%. At lower concentrations retention due to inclusion occurs, while at higher concentrations, and thus lower polarities, retention, possibly due to tori coat relaxation occurs. Although the degree of relaxation induced retention is small when compared to inclusion related retention, it is still significant and may possibly be used to finely adjust the retention of a specific analyte with respect to interferences; thus allowing the specific analysis of an analyte in a complex mixture. Because of the problems associated with the estimation of the t_0 the k' values should be treated with caution when referring to cyclodextrin stationary phases and the conditions under which they were obtained must be explicitly reported.

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