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Effect of temperature on retention time reproducibility and on the use of programmable fluorescence detection of fifteen polycyclic aromatic hydrocarbons

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Abstract

The effect of temperature on the retention of 15 polycyclic aromatic hydrocarbons (PAHs), included in the US Environmental Protection Agency method 610, on polymeric octadecylsilyl (ODS) high-performance liquid chromatography phases is described. Indeno[1,2,3-*cd*]pyrene, dibenz[*a*,*h*]anthracene and benzo[*ghi*]perylene are especially prone to change their retention on polymeric ODS phases with temperature. Using temperature control, ($\Delta T \leq 0.1$°C) we have reached good retention time reproducibility: R.S.D. better than 0.07% ($n = 10$) for all the 15 PAHs. In practice this means that the retention time of indeno[1,2,3-*cd*]pyrene varies within 2 s. For the multiple-wavelength shift fluorescence detection retention time reproducibility is an absolute prerequisite.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during incomplete combustion or pyrolysis of organic material. The main sources of PAHs are exhaust fumes of industrial and private furnaces, car exhausts and tobacco smoke. Some working environments and tobacco smoke are the main sources of exposure. The wide occurrence and carcinogenicity of these compounds make them serious organic pollutants, which should be detected even in minute quantities. Therefore more sensitive and accurate measurement methods using high-performance liquid chromatographic (HPLC) separation and multiple-wavelength shift fluorescence detection have been developed for the determination of PAHs.

In the early 1980s the United States Environmental Protection Agency (EPA) published method 610 for the determination of polycyclic aromatic hydrocarbons in municipal and industrial discharges [1]. The method describes the analysis of 16 PAHs and afterwards the application of these compounds for the measurement has been expanded and they are now commonly detected from other kind of matrixes, e.g. from air samples. In the EPA method only one common excitation and emission wavelength is used for the detection of all the 15 fluorescent PAHs with HPLC. Recently more sensitive methods with over 10 wavelength pairs have been introduced, with these methods each compound or closely eluting group of compounds is detected

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with specific excitation and emission wavelengths [2–5].

An essential prerequisite for the multiple-wavelength shift fluorescence detection is the exact control of the retention times of the eluting compounds. The effect of temperature on the retention of PAHs was noted over 15 years ago [6], yet some publications emphasize the length and reproducibility of stabilisation cycles for reproducible retention times [7] or the disadvantageous effect of non-polar solvents residues in the sample matrix [4]. These parameters have some influence on the retention times but we have found the temperature dependence to be the most serious. It is also well recognized among people working with phase technology and the theoretical interpretation of the retention mechanism [8,9] but still the phenomenon has not been properly discussed with an eye to the use of wavelength-programmed fluorescence detection. Only in a recent review article there is a short discussion of the importance of this subject [10].

Much work has been done to understand the retention process in reversed-phase liquid chromatography [11–13] but still the practise is ahead of the fundamental understanding of the physical basis of this separation method. Simple bulk-phase partitioning based on an octanol–water system cannot explain the selectivity of polymeric phases. We have compared the behavior of high-density polymeric ODS phases consisting of oligomeric C_{18} units [14] to other phases containing rigid structures such as cyclodextrins, which are used both in liquid and gas chromatography even for chiral separation. The significant retention time dependence on temperature exists in both liquid chromatography phases [15].

2. Experimental

The chromatographic instrument consists of a Merck–Hitachi L-6200A HPLC pump (Hitachi, Tokyo, Japan), Merck–Hitachi AS-4000A autosampler (Hitachi) and Merck–Hitachi F-1080 fluorescence detector (Hitachi). Both mixing chambers and pulse damper were removed from the pump.

The fluorescence detection wavelength program is presented in Table 1. Slit widths for both excitation and emission wavelengths were 15 nm. Processor output was 1000 mV, photomultiplier voltage at medium setting and time constant 2.0 s.

Solvents were degassed with helium using a laboratory-made degasser consisting of two flow meters and solvent filters, through which helium was purged. For the first 15 min the flow was about 40 ml/min, then the purge was lowered to 1 ml/min so that fine bubbles were flowing without disturbing the liquid surface. With slow helium purging the eluents are ready to use after about 24 h. The purging was maintained during analytical runs.

The column was thermostated to 23.7°C with a Merck T-6300 column oven (Merck, Darmstadt, Germany). As this oven type does not include a cooling unit it was constructed using 8 mm O.D. copper tube, 1 m of which was bent in two 80 mm O.D. spirals. The tube was cooled with water using a flow of 2 l/min. When ambient temperature is over 18°C the cooling unit should be used, otherwise the oven can maintain the required temperature. The temperature inside the column oven was measured using a Philips (France) PM 2513 digital multimeter.

A ChromSpher PAH (Chrompack, Middelburg, Netherlands) column, 100 mm × 3 mm I.D., was used for separation of PAHs, the typical particle size of this polymeric column is 5 μm. The Chrompack catalogue number for this column is 28286. The other column tested was LiChroCART 250-4 LiChrospher PAH (Merck), 244 mm × 4.0 mm I.D.

The solvent gradient started with premixed 40% acetonitrile in water, which was changed linearly to 100% acetonitrile during 16 min, pure acetonitrile was run after that for 13 min. The flow-rate was 0.3 ml/min. In order to wash and equilibrate the column the flow was increased to 1.0 ml/min during the next 2 min, pure acetonitrile was then pumped through the column for 4 min, after which the eluent was changed to 40% acetonitrile during 2 min. Acetonitrile (40%)

Table 1
Fluorescence detection program for 15 EPA PAHs including exitation (E_x) and emission (E_m) wavelengths and quantitation limits for the method

Multiple-wavelength shift program for fluorescence detection			Quantitation limit: pg injected	Reproducibility retention time (R.S.D., %; $n = 10$)
Compound	E_x (nm)	E_m (nm)		
Naph	267	328	10	0.07
Ace	270	315	10	0.03
Flu			10	0.04
Phen	242	380	10	0.05
Anth	250	420	5	0.03
Flt	240	420	10	0.05
Pyr			10	0.04
BaA	260	385	10	0.03
Chrys			10	0.03
BbFlt	292	431	10	0.04
BkFlt	297	410	5	0.05
BaP	250	405	10	0.04
DBahA	288	395	10	0.05
BghiPer	291	411	10	0.04
In123cdPyr	294	499	10	0.05

Retention time reproducibility for ChromSpher PAH column under gradient conditions ($n = 10$, $\Delta T < 0.1$°C) is given as relative standard deviation (R.S.D.) percentages. Abbreviations: Naph = naphthalene; Ace = acenaphthene; Flu = fluorene; Phen = phenanthrene; Anth = anthracene; Flt = fluoranthene; Pyr = pyrene; BaA = benz[*a*]anthracene; Chrys = chrysene; BbFlt = benzo[*b*]fluoranthene; BkFlt = benzo[*k*]fluoranthene; BaP = benzo[*a*]pyrene; DBahA = dibenz[*a*,*h*]anthracene; BghiPer = benzo[*ghi*]perylene; In123cdPyr = indeno[1,2,3-*cd*]pyrene.

was run for 5 min; after that time the flow was decreased to 0.3 ml/min during 2 min. The gradient program is presented in Table 2. Premixed acetonitrile was prepared by measuring first the appropriate volume of water to a 2.5-l bottle and then adding a measured volume of acetonitrile to get the 40% acetonitrile mixture.

Table 2
Solvent and flow program for gradient elution of 15 EPA PAHs

Time (min)	Eluent A: 40% acetonitrile (%)	Eluent B: 100% acetonitrile (%)	Flow (ml/min)
0.0	100	0	0.300
16.0	0	100	0.300
29.0	0	100	0.300
31.0	0	100	1.000
35.0	0	100	1.000
37.0	100	0	1.000
42.0	100	0	1.000
44.0	100	0	0.300

The washing bottle of the autosampler was also filled with 40% acetonitrile in water.

Isocratic separations were done using premixed 78% acetonitrile which was also used in the autosampler washing bottle. The flow-rate during isocratic runs was 1.0 ml/min for both columns. Acetonitrile from two manufacturers was used: LiChrosolv, gradient grade (Merck) and Rathburn HPLC grade S (Rathburn Chemicals, Walkerburn, UK).

Chromatograms and data were handled using Hitachi HPLC Manager Model D-6000 chromatography data station software. The HPLC pump and autosampler were also operated under this software.

3. Results and discussion

In 1982 the EPA published method 610 concerning 16 polycyclic aromatic hydrocarbons to be quantified in effluent waters in the USA (Fig.

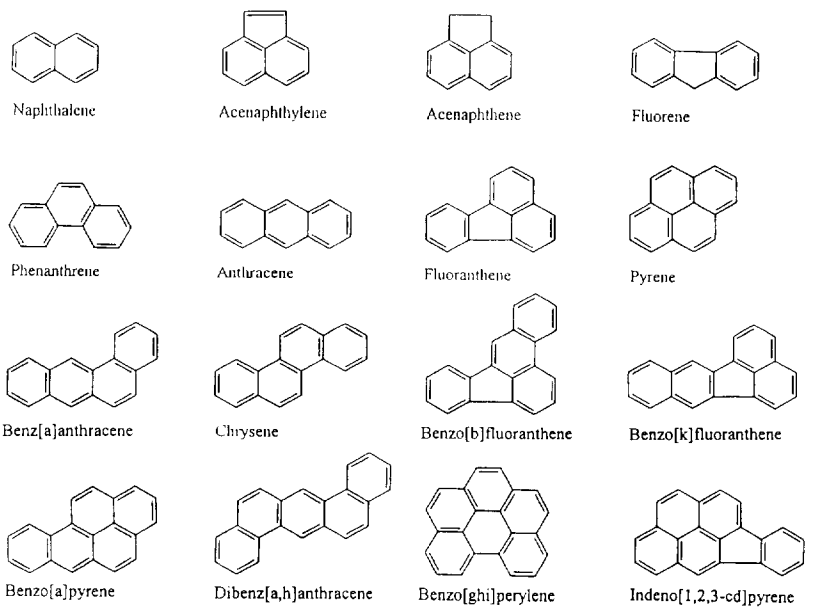

Fig. 1. Structures of the 16 polycyclic aromatic hydrocarbons included in the EPA method 610.

1). Of these 16 PAHs all except acenaphthylene are fluorescent and so the HPLC method described in the EPA paper uses both UV and fluorescence detection of the target compounds. The fluorescence detection, which in this case has been estimated to be about 100 times more sensitive than UV detection, offers the most sensitive detection method for the remaining 15 PAHs.

For the most selective measurement the fluorescence detection can be optimized by using specific excitation and emission wavelengths for each polyaromatic compound (Table 1). As a consequence interference from the sample matrix decreases and "cleaner" chromatograms can be obtained making the interpretation of the results more reliable (Fig. 2). Although specific detection is used, some extra peaks from the sample matrix can exist, together with dissimilarity of columns, even from the same manufacturer; these will determine the final timing of wavelength shifts.

Also lower quantitation limits are achieved with multiple-wavelength shift detection, using e.g. 12 excitation and emission wavelength pairs, than with conventional methods utilizing 3–6 pairs (Fig. 3). The detection limits are about 10 times lower than the quantitation limits presented in Table 1. For phenanthrene and anthracene we are not using the optimum emission wavelengths in order to avoid extra dilutions of samples. In the air samples we are concerned with, phenanthrene is often present in such amounts that dilution would otherwise be necessary and anthracene is especially fluorescent when specific wavelengths are used.

The timing of wavelength shifts must be adjusted with the elution of target compounds. As the wavelength shifts are increased column temperature control during chromatographic separation becomes more important, because the retention of PAHs on polymeric C_{18} ODS columns is highly dependent on temperature (Fig. 4). During gradient elution temperature has the strongest effect on the retention of dibenz[*a*,*h*]anthracene, benzo[*ghi*]perylene and indeno[1,2,3-*cd*]pyrene. Of the 15 fluorescent EPA PAHs these are the last three compounds eluting with pure acetonitrile after the solvent gradient. In most HPLC separations using fluorescence detection indeno[1,2,3-*cd*]pyrene, which has a higher emission wavelength than the

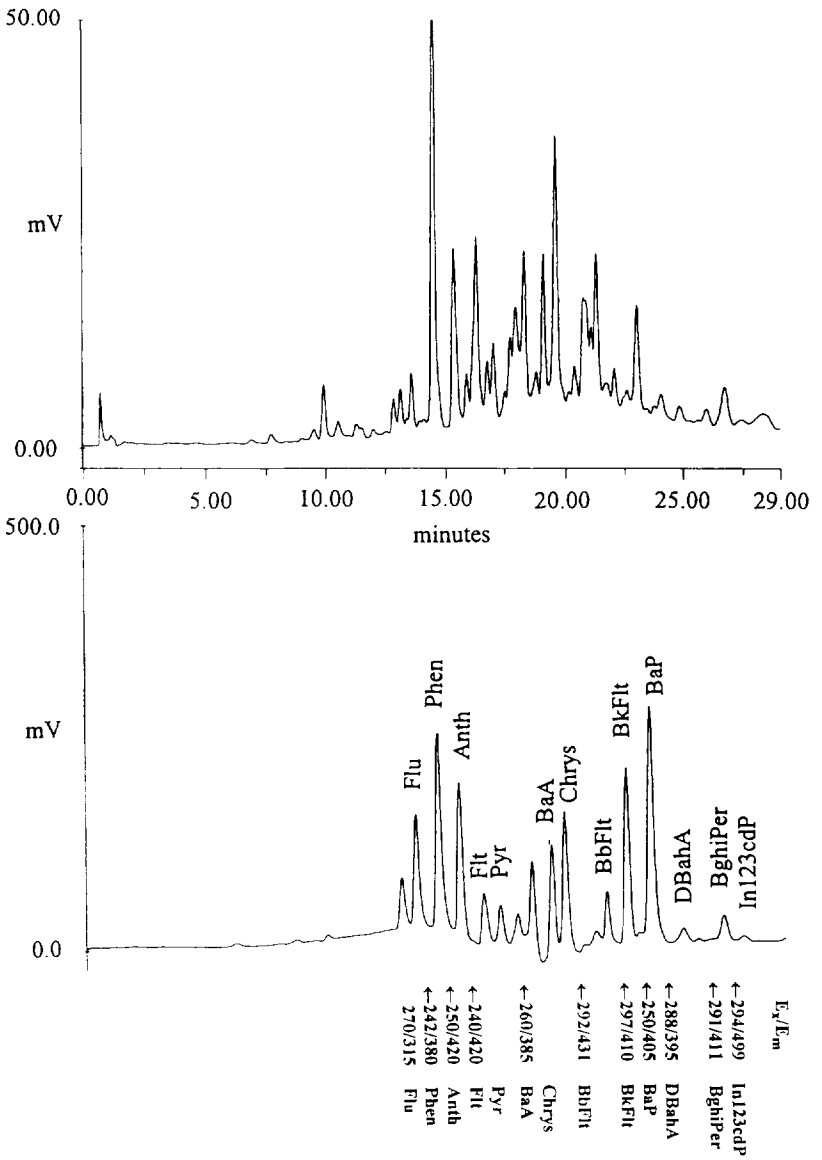

Fig. 2. Typical chromatograms of a dust sample, collected from the air of a coke plant. Upper chromatogram with UV detection ($\lambda = 254$ nm) and lower with fluorescence detection using multiple-wavelength shifts shown below. The sample was a thousand times diluted for fluorescence detection.

two compounds eluting before it, is measured using at least the specific emission wavelength. At a stable temperature ($\Delta T \leq 0.1$°C) we have reached good retention time reproducibility: R.S.D. better than 0.07% ($n = 10$) for all the 15 PAHs (Table 1). In practice this means that the retention time of indeno[1,2,3-*cd*]pyrene varies within 2 s, making the use of specific wavelengths possible.

The retention of the first seven PAHs: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene, eluting

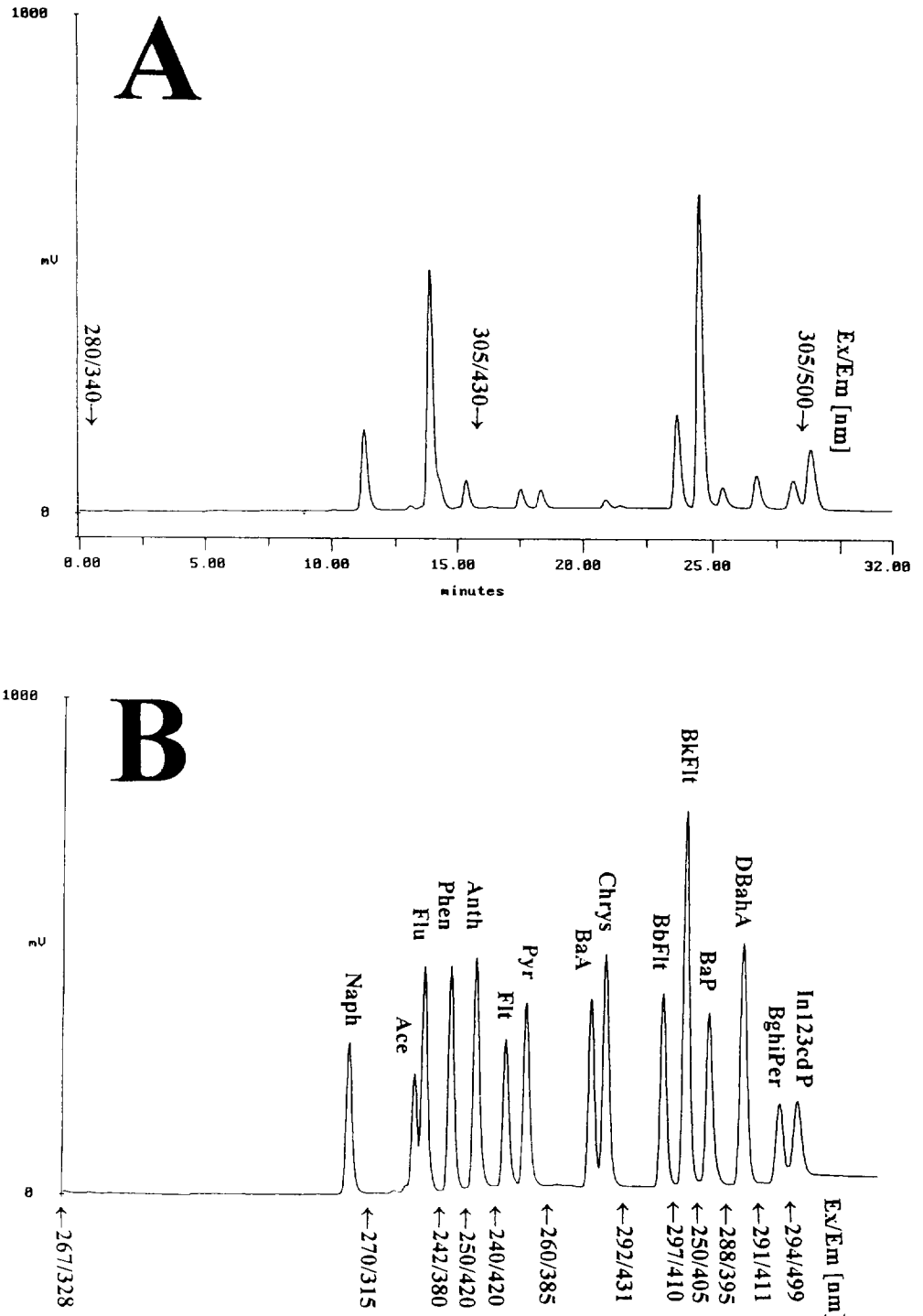

Fig. 3. Detection of the 15 EPA PAHs with a fluorescence detector using 3 (A) and 12 (B) wavelength shifts. The same standard mixture was used for both measurements.

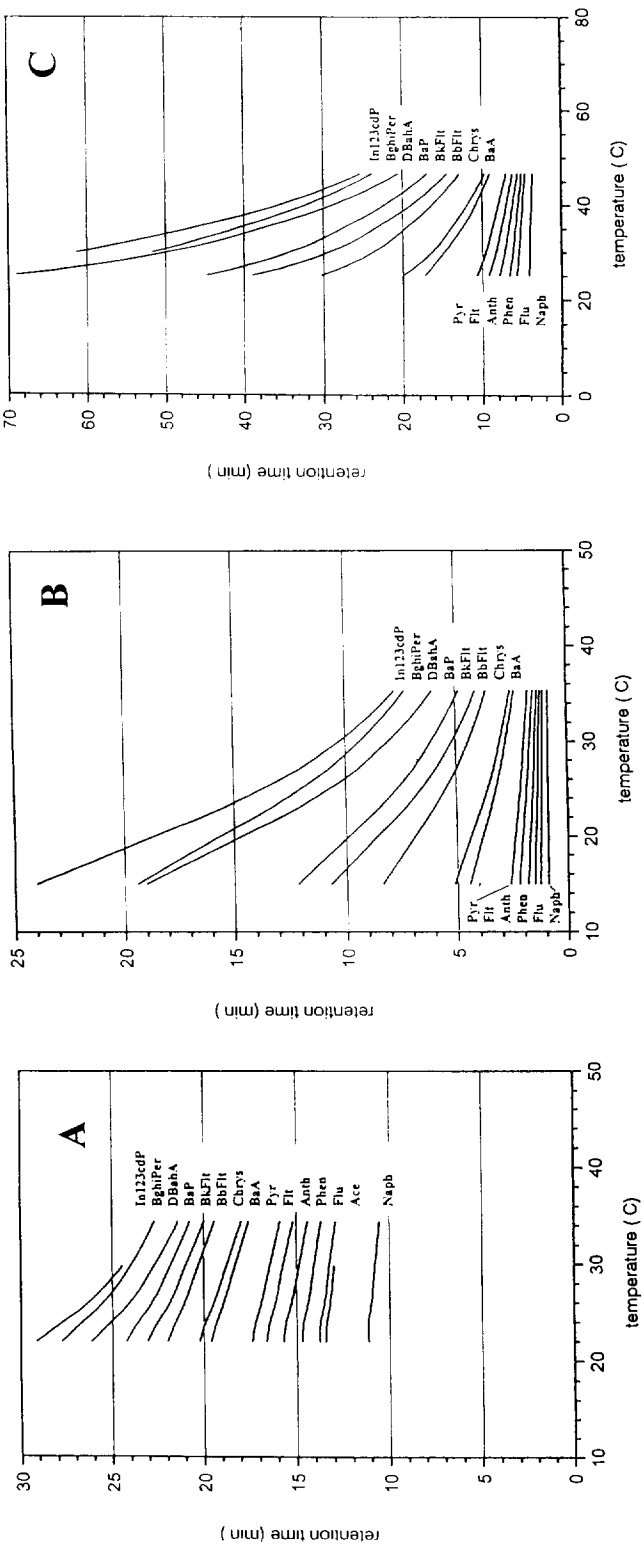

Fig. 4. Retention time versus temperature for EPA PAHs. (A) Gradient elution with ChromSpher PAH column, (B) same column and 78% acetonitrile as eluent, (C) LiChrospher PAH column and same eluent as in (B). When isocratic elution is used fluorene and acenaphthene are coeluted.

during the solvent gradient, are not so much effected by the temperature changes. In most cases the ambient temperature is stable enough for the multiple-wavelength detection of these compounds. However, the next five compounds: benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene and benzo[*a*]pyrene are sensitive to temperature variations. Benz[*a*]anthracene and chrysene are quite easy to detect as they are a group separated from other compounds with a time interval long enough for wavelength changes to be used even if there were temperature fluctuations (Fig. 3). Fortunately, common excitation and emission wavelengths for the measurement of these two compounds can be found. Adjusting the solvent gradient to get about 1 min difference in the retention times of sequentially eluting compounds, or group of compounds, is essential for multiple-wavelength detection and also allows slight temperature deviations ($\Delta T < 0.2$°C) without disturbing the detection.

The retention temperature dependence should give some insight into the molecular mechanism of solute uptake. During isocratic elution this relation was measured for two columns both containing polymeric ODS phase (Fig. 4). Under these conditions the temperature effect is even more pronounced as compared to gradient elution.

The temperature dependence can be interpreted using Van 't Hoff plots of ln k' vs. $1/T$ (Fig. 5). The lack of offset in Van 't Hoff plots suggests that no phase transitions occur over an temperature interval from 15 to 35°C, i.e. there is no change in the way in which the solute interacts with the stationary phase. On account of that, the retention time dependence on temperature cannot be explained by changes in phase morphology.

Solute retention in cyclodextrin phases, which also has been used for separation of PAHs, has many common features with retention on polymeric liquid chromatography phases [16]. According to the slot model, developed by Wise and Sander [17], during elution in polymeric phases PAHs are enclosed into slots between hydrocarbon sheets formed by C_{18} chains which

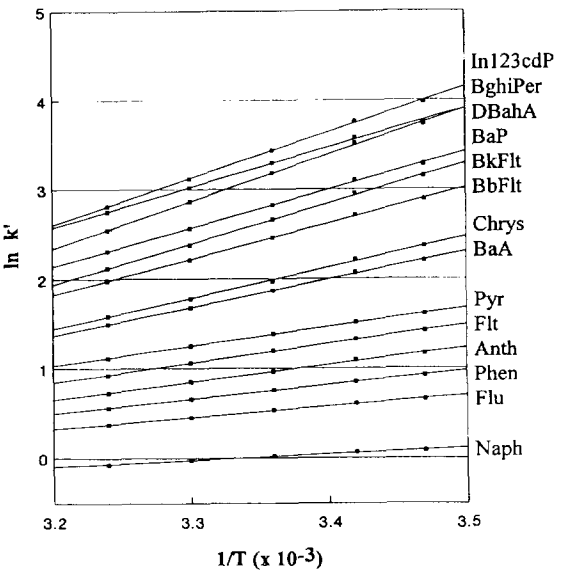

Fig. 5. Van 't Hoff plot, logarithm of capacity factor k' versus reciprocal of temperature $1/T$ (K) for ChromSpher PAH column, 78% acetonitrile as eluent; $r^2 \geq 0.995$ for all lines when the equation $y = a + bx$ is fitted to the data points.

results in retention (Fig. 6). This simple "lock and key" principle was also used in host–guest chemistry, although solvophobic forces, Van der Waals forces and hydrogen bond formation are nowadays recognized [18,19]. As with polymeric stationary phases when the temperature is increased the binding of the solute in the cyclodextrin cavity decreases rapidly as the short distance forces are exceeded [15]. Partition, based on oil–water partition coefficients, is a quite unlike retention mechanism in this context; the increase of organic solvent would make the separation of isomeric PAHs on polymeric phases worse, but this is not the case, in fact the large aromatic ring structures benzo[*ghi*]perylene and indeno[1,2,3-*cd*]pyrene are separated with pure acetonitrile.

4. Conclusions

Fluorescence detection with specific excitation and emission wavelengths is the most selective and sensitive detection method for the 15 fluorescent EPA PAHs used with HPLC sepa-

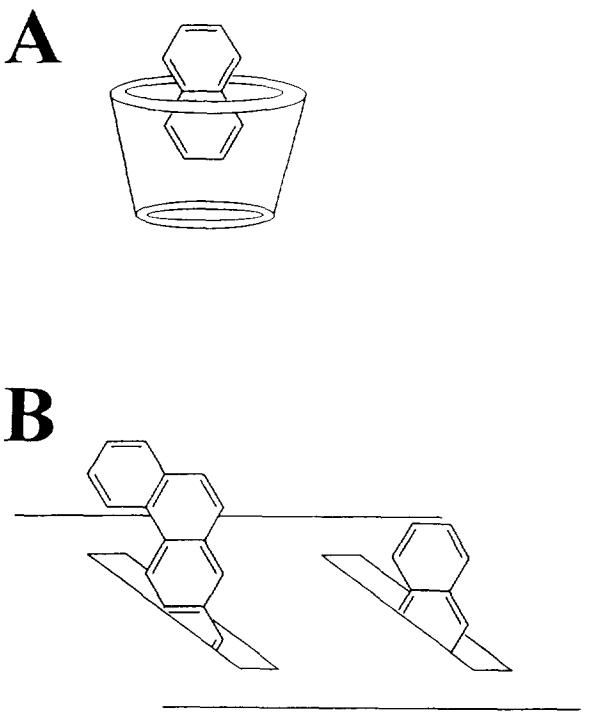

Fig. 6. Retention mechanisms used to explain solute retention on HPLC phases. (A) Inclusion complex formation between solute and cyclodextrin molecule and (B) slot model used by Wise and Sander.

ration. Before wavelength programming can be used, one must be aware of the need to take good care of a stable temperature during separation of solutes. When the temperature fluctuation is within ± 0.05°C the 12 wavelength pair program, described in this paper, could be used with success. However it must be emphasized, that this result concerns columns with a selectivity similar to that of the ChromSpher PAH column under gradient conditions. With a more selective column or under isocratic elution the temperature dependence is more pronounced. Maintaining a good temperature control and using a little slower solvent gradient one can use even 14 wavelength pairs for detection. Namely fluoranthene and pyrene, like benz[*a*]anthracene and chrysene, which are now detected using common excitation and emission wavelength pairs, could then be detected using specific wavelengths for each compound. When the separation mechanism is concerned the ODS phases, used for separation of PAHs, have been compared to liquid crystal phases in gas chromatography. The polymeric ODS phases still have much similarity with cyclodextrin phases, the marked temperature effect on retention being one of the common features.

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