ELEVATED TEMPERATURE REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF POLICYCLIC AROMATIC HYDROCARBONS ON ODS-SILICA PHASE

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Abstract : Reversed-phase high performance liquid chromatography (RP-HPLC) was used at various temperatures (room temperature to 70ºC) to separate Polycyclic aromatic hydrocarbons (PAHs) on silica ODS using proportions of organic modifier in the mobile phase. The selectivity and retention patterns of four PAHs (naphthalene, fluorene phenanthrene and fluoranthene) were investigated. Successful separations with high column efficiencies were achieved at high column temperatures. Selected separation conditions for all PAHs compounds at elevated temperature were: mobile phase: acetonitrile-water: 40-60 v/v, column: Hypersil ODS, 5µ (4.6mm I.D. x 100mm), flow rate: 2.5 mL/min, UV wavelength at 254 nm.

KEYWORDS: Polycyclic Aromatic Hydrocarbons (PAHs), high temperature HPLC, silica column

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Introduction

High-temperature liquid chromatography (HTLC) has limited use in the laboratory due to instrument and column limitations. New instrumentation is now available that allows operation at temperature of up to 200ºC with mobile phase preheating to eliminate thermal mismatch (Schrenker, 1981; Smith and Burgess, 1997; Yang *et al.*, 2002; Marin *et al.*, 2004). This development has generated interest in utilizing high-temperatures in separation work on a more routine basis. Column selection, however, is still rather limited; no stationary phases other than those based on zirconia have been used at this extreme temperature for routine work (Carr and Li, 1997; Smith, 2002). Stationary phases based on graphitic carbon, rigid polystyrene-divinylbenzene polymeric particles and polydentate silica phases should be stable at much higher temperatures than the traditional limits of 50 or 60ºC. One of the most intriguing aspects of HTLC is the ability to perform temperature programming (Melander and Horvath, 1980).

The advantages of using elevated temperatures in HPLC analysis are well documental in the literature (Smith, 1988; Smith and Burgess, 1997; Openheim and Grushka, 2002; Bidlingmeyer and Henderson, 2004). HTLC offers several distinct advantages to the separation process. Back pressure is reduced as the temperature is increased, allowing the use of stationary phases with smaller particle sizes for increased efficiency. The analyst can also operate at higher flow rates because of lower back pressure. The Van Deemter curve "flattens out" as a result of increased diffusion rates within the stationary phase and the mobile phase as the temperature is increased. This allows operation at flow rates that are many times the optimal velocity without sacrificing efficiency that is found at ambient temperature. The net result is faster and more efficient separation.

By far, silica is still the most popular substrate used to manufacture RP stationary phases. Silica has a high mechanical strength that enables its use under high pressure conditions encountered in HPLC. Furthermore, this substrate does not swell or shrink when exposed to organic solvents. Finally, its production and bonding chemistry is well understood and can be performed in much different morphology and is also reproducible. For this reason, silica seems the perfect starting material for the manufacturing of (bonded) phases for HPLC. In the last few decades, many researchers have suggested different approaches for the synthesis of silica-based RP stationary phases and also to improve their chemical and thermal stability. Recently, the preparation and properties of different types of RP stationary phases were extensively discussed and reviewed by Nawrocki *et al.* (2004).

Another approach to improve the chemical stability of silica substrates and subsequently the RP stationary phases has been described by Neue. In addition, Collins and coworkers described that titanium grafting or zirconization of silica substrates may also substantially improve the chemical stability under neutral and high pH conditions (Neue, 1997; Collins *et al.*, 2002).

In a later study, Kirkland investigated a number of RP C-18 phases under non-recycling conditions using freshly prepared eluents at pH 10 (Kirkland *et al.*, 2002). Neue (1997) have discussed another interesting concept for the preparation of alkyl-modified silicas of substantially improved chemical stability.

Polycyclic aromatic hydrocarbons (PAHs) are a class of diverse organic compounds containing two or more fused aromatic rings of the carbon and hydrocarbon atoms. PAHs is one of the typical persistent organic compounds (POPs) featured in regional and global cycling. PAHs are emitted mainly into the atmosphere and have been detected far from their source. Because of their low vapor pressures, compounds with five or more aromatic rings exist mainly adsorbed to airborne particulate matter, such as fly ash and soot. PAHs are adsorbed strongly to the organic fraction of sediments and soils. Therefore, it can be concluded that sediments and soils are considered as the main sinks for PAHs in the environment and PAHs with four or more aromatic rings are persistent in the environment. In order to minimize or prevent adverse effects of POPs, many studies illustrated the fate of POPs in natural environments. In the past 20 years, numerous important researches focused on transport of POPs in multimedia environment, e.g., between soil/water etc (Eschenbach *et al.*, 1994).

Experimental

Reagents and chemicals

HPLC grade solvent acetonitrile was obtained from Scharlau AC0340 (Barcelona, Spain) and methanol solvent was obtained from HyperSolv, BDH Laboratory, EC No. 200-659-6 (England). The polycyclic aromatic hydrocarbons (naphthalene, fluorene (EC No. 2016955), phenanthrene (EC No. 2015815) and fluoranthene (EC No. 2059124) were sourced from Fluka Chemika, Sigma-Aldrich Chemic, Steinheim, (Switzerland). Double-distilled deionized water of at least 18 MΩ was purified by Nano ultra pure water system (Barnstead, USA).

Chromatographic conditions

The high temperature HPLC systems consisted of a conventional HPLC system coupled with a column oven of a Shimadzu GC-8A Gas Chromatograph (Shimadzu Kyoto, Japan). HPLC separations were carried out using Waters 515 HPLC pump (Mildford, USA) for mobile phase delivery. Samples

were injected into the system using a 25μL loop for sample introduction. Analyte peaks were detected using a Shimadzu SPD- 6A UV detector (Kyoto, Japan) and were recorded on a Waters 746 Data Module integrator (Mildford, USA).

A 30 cm length of stainless-steel tubing was placed in the oven between the injection valve and the column as pre-heating coil. The column used in this research was packed with silica (Hypersil ODS, 5μm) (4.6 mm I.D. x 100 mm, Zorbax HPLC column, Agilent Technologies). The column and the preheating coils were placed together in the oven. A thermometer (Zecol, England) with temperature range of 20ºC-360ºC was placed inside the oven to measure the exact oven temperature.

For high temperature operations, column effluent was cooled by ice water before it reached the detector. This procedure stabilizes the baseline reading and avoids possible damage to the detector. In order to prevent the mobile phase from boiling when passed through the detector, an aluminium tubing of 0.1 mm I.D. was placed at the outlet of the detector to serve as a restriction tubing to maintain a constant back pressure in the detector cell. Mobile phase used in this research was degassed using a vacuum-ultrasonic degassing procedure. Mobile phase was degassed in the ultrasonic bath (NEY 300 Ultrasonic, USA) and solvent peak was used instead of uracil as the column void volume marker.

Procedure

Samples of four PAHs (naphthalene, fluorene, phenanthrene and fluoranthene) were dissolved in the acetonitrile and were kept in the freezer as shown in Table 1. Mixture solutions were prepared by mixing the specific quantities of analytes from each stock solution. The prepared mixture solution was injected in triplicate onto the column.

Separations were carried out using different compositions (acetonitrile-water: 70-30; 60-40; 50-50; 40-60, v/v). The eluent flow rate was 1.0 mL/min and sample injection volume was 1μL. UV detection of analytes for the comparison study was at 254 nm.

Results and Discussion

Figure 3-5 illustrates the Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) separations of PAHs at elevated temperatures of 40-70ºC with different mobile phase compositions at a flow rate 2.5 ml/min on silica column. In each separation the analytes were eluted with good peak shapes. The separation improved considerably as the temperature was increased from 40ºC to 70ºC. The retention factor were calculated (Table 2). For each compounds studied, there was marked decrease in the retention factors with increasing temperature from 40ºC to 70ºC with 10ºC increments using different propositions of acetonitrile (Table 2). It was shown that a 1 % increase in acetonitrile concentration has the same effect as an approximately 4ºC increase in column temperature in controlling solute retention. This is in agreement with previous work by Bowermaster and McNair. With acetonitrile-water (50-50) eluent at 40ºC, the PAHs compounds were eluted within 14 minutes, while at 70ºC the analysis time for the test compounds were reduced by about 5 minutes. It was observed that the retention factor for all PAHs compounds studied were inversely proportional (Bowermaster and McNair, 1984).

According to Smith, very rapidly eluted peaks usually give poorer efficiencies because they are affected more significantly by extra column band-broadening factors, whose influence is virtually constant for all peaks. Besides, very narrow peaks are more prone to error in the measurement of the peak width. Hence, small differences in efficiencies should not be regarded as significant (Smith,

1988). Recent reviews by Yang *et al.* have extensively discussed the temperature effects on column efficiency by increasing the temperature from 100ºC to 120ºC, the diffusivity is further increased and even better mass transfer results (Yang *et al.*, 2002). In another study, McCalley reported that working under controlled, temperature programming conditions is a strong tool to adjust and to improve retention time and selectivity (McCalley, 2000).

Greibrokk and Andersen reported that temperature programming in the range of approximately 25-125ºC in miniaturized RP columns produced positive influence of temperature elevation on the reduction of analysis time, the improvement of peak shapes and the achievement of higher efficiencies (Greibrokk and Andersen, 2003).

Resolution and column efficiency at flow rate; 2.5 ml/min investigated were summarized (Table 3). It was observed that, a time of approximately 43 minutes was required to separate all the test compounds at 70ºC and with mobile phase composition, acetonitrile-water; 30-70 (v/v). From the chromatograms illustrated (Figure 3-5), it is obvious that the peak shapes for separations using low composition of organic modifier coupled with elevated flow rate and elevated temperature are acceptable.

Retention factors of the test compounds as a function of temperature ranging from 40ºC to 70ºC with 10ºC increments using proportions of organic modifier. At 70ºC, the analysis times for test compounds were reduced around 4 minutes. Separation has been successfully carried out at elevated flow rate coupled with elevated temperature, using composition acetonitrile-water; 30:70 (v/v). At elevated temperature the eluent viscosity decreases and eluent velocity increases. The relationship between the separation factors (k) with different proportions of organic modifier in mobile phase for PAHs compounds can be seen more clearly by creating two-dimensional plots (Figure 1). These graphs showed that organic modifiers play a very important role in activating the surface area of the stationary phase, increase the solubility of the solutes in mobile phase and reduces total analysis time.

For each of the compound studied, there was a systematic decrease in resolution and separation factors with the increase in temperature. For example, the resolution and separation factor values for phenanthrene/fluorene combination at 40ºC using acetonitrile-water 40:60 (v/v) were 26.03 and 1.25 respectively. Further increase in temperature of up to 70ºC decreased the resolution and separation factor values to 6.99 and 1.21 respectively. The resolutions for each combination of components were almost always greater than 1.0, hence, sufficient for accurate quantification. In general, the R_s values and separation factor values were inversely proportional to the percentage of organic modifier used in the mobile phase at all of the temperature used. The variation of column efficiency increased significantly with the elution sequence.

It was observed that column efficiency increased as a function of mobile phase composition for PAHs (Figure 2 (a) and (b)). This indicated that the theoretical plate number, *N*, was inversely proportional to the percentages of organic modifier in the mobile phase. It was observed that the column efficiency (*N*/m) markedly increased when a mobile phase with a low organic modifier was used.

Working under controlled temperature programming conditions is a strong tool to adjust and to improve retention and selectivity. In addition, higher temperatures also decrease eluent viscosity allowing significantly higher linear velocities. The retention times can also be drastically reduced. Concomitantly at higher temperature, the diffusivity of analytes in the mobile and stationary phases increased, resulting in better efficiencies and improved peak shapes. Combining these latter effects, higher temperatures in RPLC may provide better resolution and faster analysis (Table 3). In addition, at higher analysis temperature lesser amounts of organic modifiers in eluent are needed to achieve the same separation. In turn, this contributes to reduce the use of organic solvent and hence contribute to the appearance of "greener" laboratories.

Conclusions

The separations of polycyclic aromatic hydrocarbons (PAHs) using HPLC separation technique at elevated temperature have been examined. In this work, the relationship between retention factor, k; separation factor, α; theoretical number of separation plates per column length, N/m and the resolution, Rs with different column temperatures were analysed and demonstrated. It was observed that a better separation mechanism was achieved during the high operating temperature. The overall column efficiencies at high operating temperatures were higher relative to those at ambient operating temperature conditions. High temperature operation in RP-HPLC provides the opportunity to reduce the quantity of organic solvent used in mixed organic-water mobile phase, decreases total analysis time, and increases analyte mass transfer rates.

Acknowledgements

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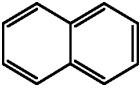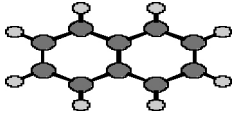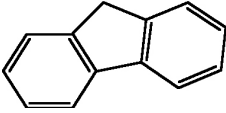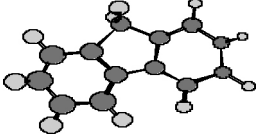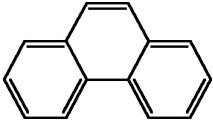Table 1: Properties of four polycyclic aromatic hydrocarbons (PAHs)
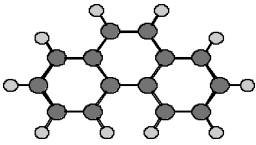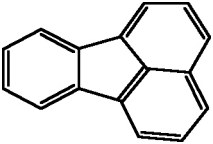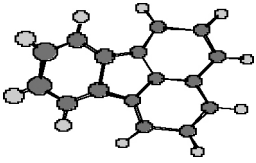
Compound	Formula Structure	Molecular Weight	3D Molecular Structure
Napthalene	$C_{10}H_8$	128	
Fluorene	$C_{13}H_{10}$	166	
Phenanthrene	$C_{14}H_{10}$	178	
Fluoranthene	$C_{16}H_{10}$	202	

Table 2: Retention factor of PAHs as a function of temperature using different proportion of organic modifier on silica column at flow rate 2.5 mL/min

Compounds	Mobile Phase Composition (v/v)	Retention time, t_r				Retention Factor, k			
		Column Temperature (°C)							
		40	50	60	70	40	50	60	70
Napthalene	50:50	4.32	3.25	2.53	2.25	7.86 (5.07)	7.47 (5.70)	5.50 (0.13)	5.00 (1.44)
	40:60	9.5	7.12	6.01	5.07	20.88 (1.41)	17.74 (0.23)	14.77 (0.90)	12.51 (2.98)
	30:70	19.41	14.15	10.41	8.5	59.10 (0.10)	48.65 (0.12)	40.47 (0.30)	23.08 (0.70)
Fluorene	50:50	7.59	5.67	4.17	3.59	14.61 (4.91)	13.76 (4.85)	9.72 (0.25)	8.560 (1.55)
	40:60	21.52	15.64	12.69	10.4	48.59 (1.72)	40.16 (0.52)	32.29 (0.14)	26.73 (4.31)
	30:70	35.65	37.09	26.04	20.47	109.372(0.10)	129.14(0.15)	102.73(0.10)	56.99 (0.25)
Phenanthrene	50:50	8.75	6.52	4.73	4.03	17.01 (4.72)	15.97 (4.77)	11.18 (0.10)	9.77 (0.12)
	40:60	26.71	19.19	15.34	12.47	60.55 (1.64)	49.50 (0.56)	39.26 (0.13)	32.24 (3.89)
	30:70	53.85	48.27	33.29	25.67	165.71 (0.43)	168.36 (0.00)	131.63(0.33)	71.71 (0.60)
Fluoranthene	50:50	12.46	9.09	6.45	5.39	24.64 (4.77)	22.68 (4.55)	15.6 (0.36)	13.39 (1.49)
	40:60	43.08	30.15	23.71	18.77	98.25 (1.38)	78.34 (0.17)	61.22 (0.22)	49.05 (0.57)
	30-70	-	-	56.79	42.23	-	-	225.26(0.11)	118.62(0.21)

(R.S.D. %) was based on triplicate injection.
- = not eluted within 60 minutes.

Table 3: Resolution and separation factor values as a function of temperature ranging from

Compounds	ACN–Water (v/v)	Resolution, R_s (R.S.D%)				Separation Factor, a (R.S.D%)			
		Column Temperature (ºC)							
		40	50	60	70	40	50	60	70
Fluorene	50:50	15.48 (4.05)	14.91 (3.03)	14.26 (0.57)	13.33 (0.46)	1.85 (0.80)	1.84 (2.58)	1.77 (0.12)	1.72 (3.22)
Napthalene	40:60	45.04 (4.79)	23.31 (0.35)	30.48 (3.12)	31.46 (0.19)	2.33 (0.30)	2.19 (2.17)	2.19 (0.25)	2.14 (1.51)
	30:70	46.22 (0.31)	87.08 (1.23)	72.02 (0.93)	75.51 (0.31)	1.85 (0.28)	2.66 (0.50)	2.54 (1.10)	2.47 (1.70)
Phenantrene	50:50	4.60 (0.23)	3.94 (2.78)	3.85 (1.07)	3.33 (4.24)	1.17 (0.54)	1.16 (0.24)	1.15 (0.18)	1.14 (0.50)
Fluorene	40:60	26.03 (5.81)	8.38 (0.29)	7.96 (4.57)	6.99 (0.35)	1.25 (0.60)	1.22 (0.92)	1.22 (0.25)	1.21 (0.35)
	30:70	61.48 (0.14)	80.07 (0.76)	39.96 (1.98)	32.89 (0.46)	1.52 (1.90)	1.30 (1.08)	1.28 (0.44)	1.26 (0.56)
Fluorathene	50:50	11.20 (3.59)	9.79 (3.63)	9.38 (0.58)	8.42 (3.74)	1.14 (1.64)	1.42 (0.65)	1.40 (0.35)	1.37 (1.13)
Phenantrene	40:60	74.72 (7.10)	42.08 (0.15)	13.04 (0.21)	11.16 (0.10)	1.62 (0.26)	1.56 (0.27)	1.56 (0.27)	1.52 (0.19)
	30:70	-	-	89.36 (1.40)	86.26 (0.69)	-	-	1.71 (0.82)	1.65 (1.70)

40ºC to 70ºC using different mobile phase composition at flow rate 2.5mL/min.
(R.S.D. %) was based on triplicate injection.
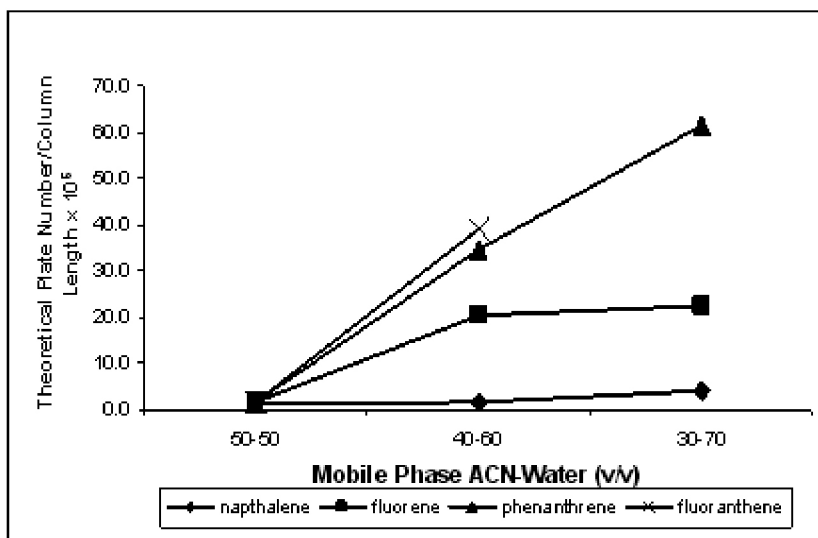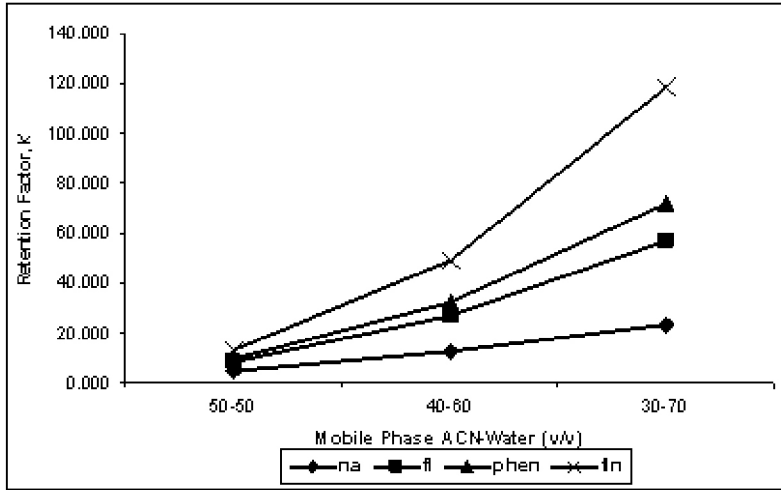- = not eluted within 60 minutes.

Figure 1: Variation of retention factor (k') porpotions of organic modifier acetonitrile in the mobile phase for PAHs compounds
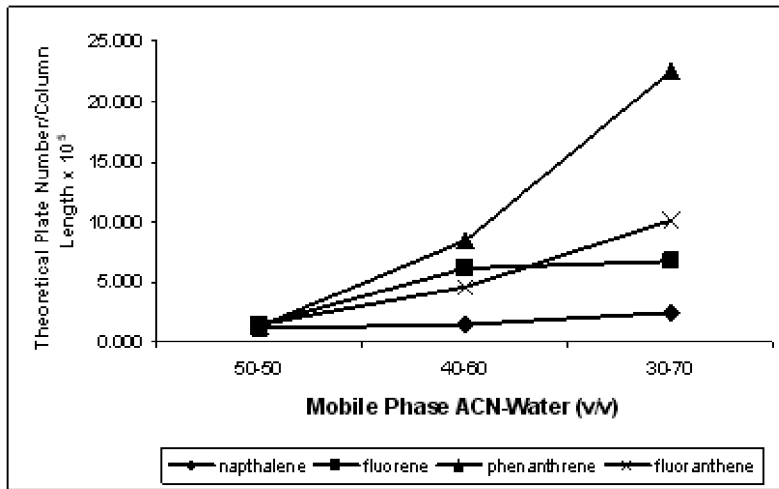
(a)

(b)

Figure 2: Variation of column efficiency with different porpotions of organic modifier in mobile phase for PAHs at (a) 40ºC, (b) 70ºC.
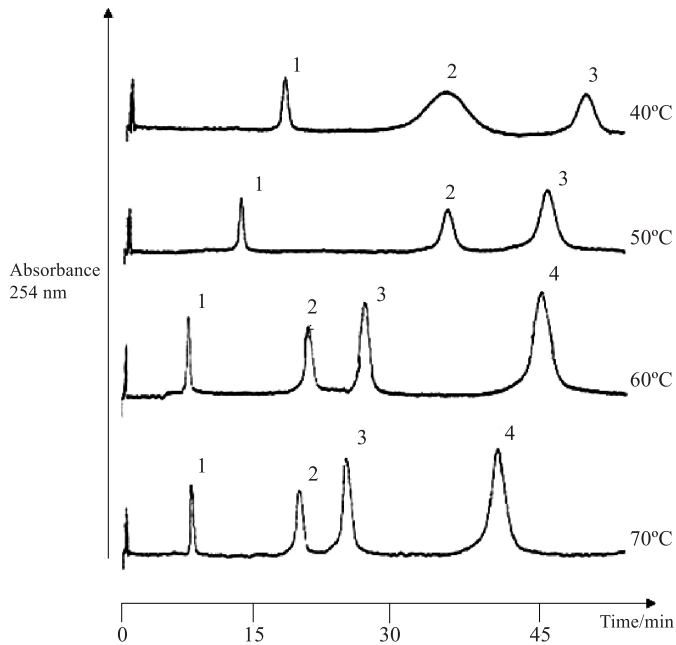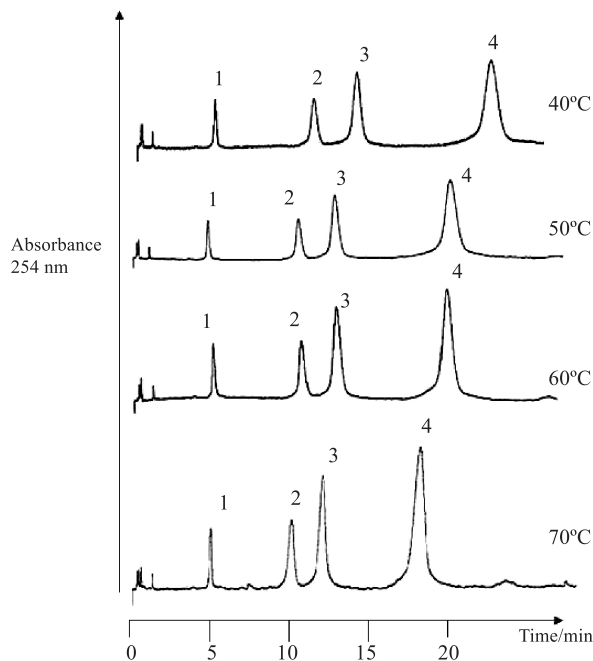
Figure 3: Separation of PAHs on ODS-silica column (100 mm x 4.6 mm I.D.) Chromatographic condition: mobile phase, acetonitrile-water 30:70 (v/v); Flow rate: 2.5 ,l/min; temperature: 40ºC - 70ºC; detection: UV at 254 nm. Peaks: 1-napthalene, 2-fluorene, 3-phenanthene, 4-fluoranthene

Figure 4: Separation of PAHs on ODS-silica column (100 mm x 4.6 mm I.D.) Chromatographic condition: mobile phase, acetonitrile-water 40:60 (v/v); Flow rate: 2.5 ,l/min; temperature: 40ºC - 70ºC; detection: absorbance at 254 nm. Peaks: 1-napthalene, 2-fluorene, 3-phenanthene, 4-fluoranthene
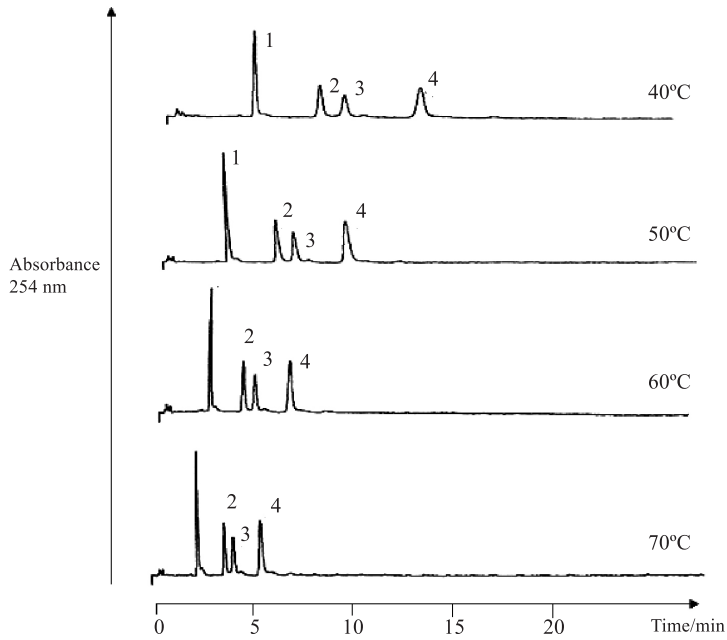
Figure 5: Separation of PAHs on ODS-silica column (100 mm x 4.6 mm I.D.) Chromatographic condition: mobile phase, acetonitrile-water 50:50 (v/v); Flow rate: 2.5 ,l/min; temperature: 40ºC - 70ºC; detection: UV absorbance at 254 nm. Peaks: 1-napthalene, 2-fluorene, 3-phenanthene, 4-fluoranthene