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Improved optimization of polycyclic aromatic hydrocarbons (PAHs) mixtures resolution in reversed-phase high-performance liquid chromatography by using factorial design and response surface methodology

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ABSTRACT

A new procedure for optimizing PAHs separation in very complex mixtures by reverse phase high performance (RPLC) is proposed. It is based on changing gradually the experimental conditions all along the chromatographic procedure as a function of the physical properties of the compounds eluted. The temperature and speed flow gradients allowed obtaining the optimum resolution in large chromatographic determinations where PAHs with very different medium polarizability have to be separated. Whereas optimization procedures of RPLC methodologies had always been accomplished regardless of the physico-chemical properties of the target analytes, we found that resolution is highly dependant on the physico-chemical properties of the target analytes. Based on resolution criterion, optimization process for a 16 EPA PAHs mixture was performed on three sets of difficult-to-separate PAHs pairs: acenaphthene–fluorene (for the optimization procedure in the first part of the chromatogram where light PAHs elute), benzo[g,h,i]perylene–dibenzo[a,h]anthracene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene (for the optimization procedure of the second part of the chromatogram where the heavier PAHs elute). Two-level full factorial designs were applied to detect interactions among variables to be optimized: speed flow, temperature of column oven and mobile-phase gradient in the two parts of the studied chromatogram. Experimental data were fitted by multivariate nonlinear regression models and optimum values of speed flow and temperature were obtained through mathematical analysis of the constructed models. An HPLC system equipped with a reversed phase 5 μm C18, 250 mm × 4.6 mm column (with acetonitrile/water mobile phase), a column oven, a binary pump, a photodiode array detector (PDA), and a fluorimetric detector were used in this work. Optimum resolution was achieved operating at 1.0 mL/min in the first part of the chromatogram (until 45 min) and 0.5 mL/min in the second one (from 45 min to the end) and by applying programmed temperature gradient (15 °C until 30 min and progressively increasing temperature until reaching 40 °C at 45 min).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants because of their chemical stability and the multiplicity of the emission sources [1]. They are mainly formed by incomplete combustion of carbon-containing fuels such as wood, coal, garbage, gas, diesel, fat, or tobacco [2,3]. Their carcinogenicity and their immunological damages are often regarded as the most critical effects in humans [4–6] and their quantification and identification in a wide range of environmental matrices has been frequently accomplished by reverse-phase high-performance liquid chromatography (RPLC) with UV or fluorescence detectors [7,8] for high selectivity, precision, and sensitivity [9,10]. In fact, some of the recommended U.S. EPA methods for the analysis of PAHs (i.e. method 550.1 for analysis of PAH in drinking water, Method 610 for analysis of PAH in waste water and method 8310 for analysis of PAH in ground water and solid waste) are based on RPLC analysis with UV/vis and fluorescence detection [9,11]. However, resolution of PAHs by RPLC is considered difficult because of their structural and chemical similarities. Optimization procedure can be avoided applying manufacturers' specifications. Most of the commercial procedures provide maximum efficiency (high throughput) but not necessarily maximum resolution or sensitivity. On the other hand, complex mixtures like soot extracts require high selectivity and high analysis times, although this implies efficiency loss.

So far, overlapping resolution mapping procedure [12], the sequential simplex optimization method [13,14], factorial and response surface methodology [15], and window diagrams [16,17] have been used in order to optimize the separation of PAHs in

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complex mixtures by RPLC. However, some authors have warned against the use of the simplex technique without further studies to determine if the apparent optimum is a local or global optimum [13,18]. In fact, Walters and Deming [16] have stated as early as 1985 that the use of the sequential simplex technique may yield false local optima if the simplex is not large enough. On the other hand, window diagrams seem to be excellent for locating the regions of local and global optima in two or three dimensions but they are model-dependent and somewhat inefficient for multifactor optimizations [16]. In fact this chemometric procedure is often used only for optimizing the gradient or composition of isocratic mobile phase [17].

The optimization of PAHs separation in very complex mixtures by RPLC analysis can be based on different criteria: sensitivity, resolution, cost, robustness, and efficiency [13,14]. The parameters influencing sensitivity, resolution, and efficiency are: column stationary phase (type and percentage of C loaded), diameter of stationary phase particles [19,20], separation temperature [21], type and amount of modifier (gradient), and speed flow [22].

C_{18} stationary phases are usually recommended for PAHs analysis. The diameter of stationary phase particles is fixed and also recommended by manufacturers for PAHs analysis. The modifier is acetonitrile because other solvents have higher viscosity (methanol for example) causing too high pressures inside the column [23]. Thus, only the temperature, initial amount of mobile phase modifier, gradient, and speed flow of mobile phase through the column should be taken into account in order to achieve maximum resolution.

Most of the optimized separation methods found in the literature for the analysis of PAHs mixtures by RPLC involve a variation of the starting and ending compositions of acetonitrile and water, linear gradient time, mobile phase flow rate, column temperature, and holding time of the final mobile phase composition [14]. But so far no paper considered the possibility of carrying out gradual changes of experimental conditions all along the chromatographic run, as proposed in this paper. As a matter of fact, here we introduce a new optimization approach of RPLC methodology based on the physical properties (medium polarizability) of the compound eluted for efficient separation of namely EPA PAHs mixtures by varying speed flow, temperature and gradient gradually during the chromatographic procedure. Application to real samples as complex as kerosene soot extracts provided highly satisfactory results.

2. Experimental

2.1. Material and reagents

An HPLC system from Shimadzu (Shimadzu Corporation, Kyoto, Japan) equipped with a reversed phase, 5 μm C_{18}, 250 mm × 4.6 mm column (Grace Davidson, Belgium), a column oven CTO-20A/20AC Prominence capable of cooling and controlling at the levels mentioned in this work, binary pump, PDA (Photodiode Detector Array) and a fluorimetric detectors were used.

2.1.1. Reagents

Water and acetonitrile Chromasolv plus for HPLC from Sigma–Aldrich, PAHs (16 EPA TLC Polynuclear Aromatic Hydrocabons Mix, 2000 μg/mL in methylene chloride:benzene, 50:50) from Supelco (Supelco Park, Bellefonte, PA, USA). PAHs included in the standard were, in alphabetic order: acenaphthene (Ace); acenaphthylene (Acy); anthracene (Anthr); benz[a]anthracene (B[a]A); benzo[a]pyrene (B[a]P); benzo[b]fluoranthene (B[b]F); benzo[g,h,i]perylene (B[ghi]P); benzo[k]fluoranthene (B[k]F); chrysene (Chry); dibenz[a,h]anthracene (D[a,h]A); fluoranthene (Flura); fluorene (Flu); indeno[1,2,3-cd]pyrene (IP); naphthalene (naph); phenanthrene (Phen); pyrene (Pyr). These 16 PAHs were considered in this paper since more information is available on them than on others. Moreover, they are included in EPA (Environmental Protection Agency, USA) List Priority contaminants, and were identified at the highest concentrations in environmental samples [24].

2.2. Software

Statistical treatment of data was carried out using the Statgraphics Centurion XV (Aux. USA) program package.

3. Results and discussion

The operational parameters affecting resolution, selectivity, sensitivity, and efficiency in HPLC are: (i) the column stationary phase (type and percentage of C loaded), (ii) the column oven temperature, (iii) the type of organic modifier in mobile phase, (iv) the amount of modifier in mobile phase, (v) the speed flow of the mobile phase, and (vi) the diameter of the stationary phase particles. As mentioned in the Introduction, the operational parameters (i) and (vi) are fixed. The analytical column is 25 cm in length and the size of the stationary phase particles is 5 μm, which corresponds to usual combination according to the literature [17]. The organic modifier is acetonitrile because for gradient separations the mixture ACN/H_2O is highly recommended on the basis of low viscosity and miscibility. So the parameters (ii), (iv), and (v) will be the critical factors in our study. Among all the criteria for optimization of PAHs determination by RPLC, resolution is the selected criterion as mentioned in Section 1.

Optimization of PAHs mixtures separation according to resolution criterion is carried out through the evaluation of the following three sets of difficult-to-separate pairs: acenaphthene/fluorene (at the beginning of the chromatogram), dibenzo[a,h]anthracene/benzo[g,h,i]perylene (at the end of the chromatogram) and benzo[g,h,i]perylene/indeno(1,2,3-cd)pyrene (at the end of the chromatogram). Regarding to optimization variables influencing resolution, optimization should be based on the interaction effects rather than a single factor investigation because the effect of one factor depends upon the value of another, that is, the factors interact mathematically [25].

The experimental setup for studying factors interactions and constructing surface response models can be either sequential (e.g. simplex algorithm) or simultaneous (e.g. factorial design). A factorial model was selected for the optimization of RPLC parameters, rather than simplex methods or other search algorithms, because the number of experiments is known in advance, an empirical (polynomial) model can be derived, the statistical significance of the parameters (variables) can be tested, and the optimum may be calculated by differentiation of the model functions constructed [26].

In factorial designs we have "*n*" variables which can be adjusted at fixed levels [26]. Since interactions between variables cannot be ruled out, full factorial designs with several levels for the following variables must be applied:

(i) *Temperature*: This factor could be considered as the overlooked optimization parameter in RPLC [21]. The use of elevated temperatures to improve column efficiency reduces the column pressure drop and separation times. Sub-ambient temperatures have been less commonly used to improve the resolution of difficult-to-separate compounds by increasing band spacing at the expense of poor column efficiency and long separation times [27]. Temperatures between 15 and 40 °C were checked out here.

(ii) *Mobile-phase gradient*: Boolliet and Poole [27] reported that changes in solvent composition are more powerful for optimization than usual changes in temperature [22]. The general trends observed for variation of composition are similar to those observed for variation of temperature, but the magnitude of the changes in the system is much larger when solvent composition is varied.

(iii) It was suggested that composition variation and temperature interact. Consequently, a simultaneous optimization must be done. This would be the most effective strategy for difficult separations [28].

(iv) *Flow rate* (*or speed flow*): Flow rates higher than 1 mL/min were not tested because the pressure inside the column increases too much when the speed flow exceeds this value. Actually, a speed flow of ca. 1.25 mL/min yielded a column pressure of 197 bars, close to the recommended pressure limit for this column (200 bars).

(v) The initial concentration of ACN influences resolution too. But the influence is not too high and the range of initial ACN concentrations is small because:
   (a) small initial concentrations of ACN make the dead volume increase too much and consequently the band spacing between two consecutive peaks gets shorter. Also,
   (b) too high initial concentrations of ACN (higher than 50%) entails constraint mobile-phase gradients in the last part of the chromatogram and consequently worsen the resolution at the end of the chromatogram.

We studied several initial concentrations of ACN (20%, 40% and 50%) and the best results were obtained using 50% ACN. Resolutions of the acenaphthene-fluorene pair obtained under the initial concentrations aforementioned were very similar but the dead volume is lower using an initial concentration of 50% ACN than using other concentrations. Initial concentrations of acetonitrile of ca. 20% yielded with short spacing band and large dead volumes. Therefore, we used an initial concentration of acetonitrile of 50%. On the other hand, the initial concentrations of ACN is limited since high mobile-phase gradients for resolution of the pairs dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene-indeno[1,2,3-cd]pyrene is needed at the end of the chromatogram, preventing initial concentrations of ACN higher than 50%. Application of two-level full factorial design is only possible if linearity condition between resolution and each of the variables to optimize is fulfilled. The experiments shown in Figs. 1–3 were used to test it. As we can see from these figures, linearity condition of resolution with respect to each variable (temperature, speed flow and mobile-phase gradient) is fulfilled.

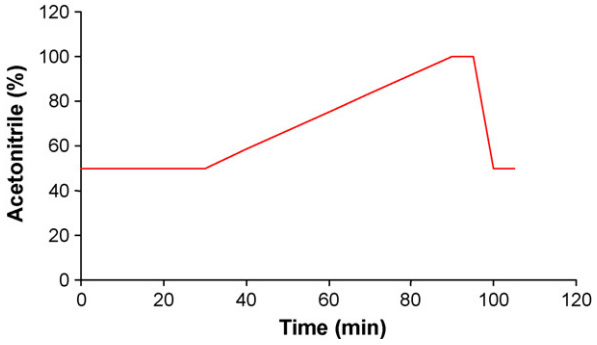

Fig. 1. Graphic representation of the gradient mobile-phase (acetonitrile:water) program selected.

Resolution versus mobile-phase gradient provides the following linear relationship: $y = -0.3885x + 2.2754$ ($R^2 = 0.9922$). Resolution versus speed flow gives the following linear relationship: $y = 0.8611x - 1.1359$ ($R^2 = 0.9955$). Resolution versus temperature leads to the following linear relationship: $y = -0.0519x + 2.7053$ ($R^2 = 0.9943$). Therefore, two-level full factorial designs can be applied. According to this kind of design, to calculate interactions among variables, we only consider two levels for each variable: high (+) and low (−) (Table 1). Consequently,

(i) for the temperature, 15 °C (low level) and 40 °C (high level) are selected,

(ii) for the speed flow, 0.5 mL/min (low level) and 1.0 mL/min (high level) are selected,

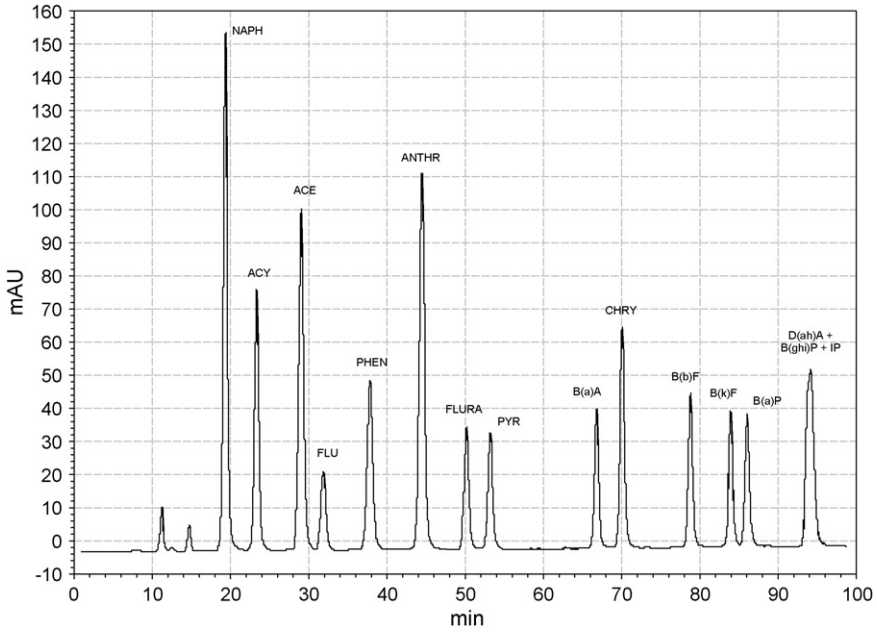

Fig. 2. Chromatogram 1 recorded for a mixture of 16 EPA PAHs in acetonitrile, concentration of PAHs: 3.7 ppm approximately. Volume of injection 20 μL, column oven temperature 15 °C, speed flow 0.5 mL/min. Solvent gradient mobile-phase gradient: 0 until minute 50, then mobile-phase gradient 0.83 (ΔACN%/min). Initial concentration of acetonitrile: 50%.

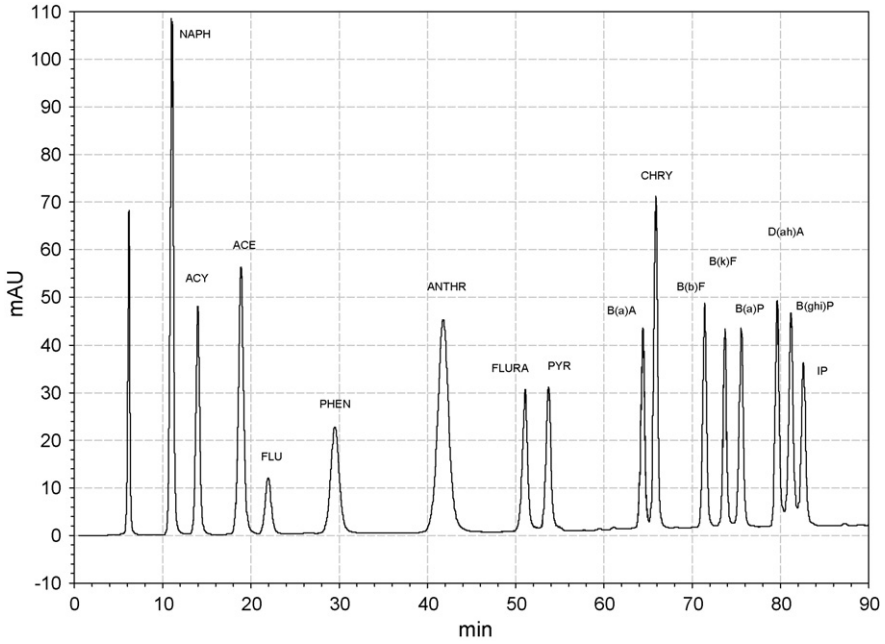

Fig. 3. Chromatogram 2. It was recorded using the following experimental conditions: initial concentration of ACN 50%, speed flow 1.0 mL/min until minute 45, from then until the end, speed flow 0.5 mL/min. Slope of solvent gradient mobile-phase gradient, 0 until minute 30 and then 0.83. Initial temperature: 15 °C, changing about minute 30, reaching 40 °C in minute 45; constant temperature 40 °C from minute 45 until the end. The PDA data are presented.

(iii) for the mobile-phase gradient (ΔACN%/min), 0.00 (low level) and 1.67 (high level) are selected.

Extreme values are the most suitable for this aim [25,29]. With two-level full factorial designs, only 8 experiments are necessary in our case to optimize the speed flow, temperature, and mobile-phase gradient. These experiments are summarized in Table 1.

3.1. Interpretation of the results

Full factorial designs enable to build a multiple linear variable regression model. In our case (2^3 design), the mathematical model (response surface) is:

$$Y = b_0 + b_T X_T + b_F X_F + b_a X_a + b_{T\varphi F} X_T X_F + b_{T\varphi a} X_T X_a + b_{F\varphi a} X_F X_a + b_{T\varphi F\varphi a} X_T X_F X_a \quad (1)$$

where b_0 is the medium value, b_T the temperature effect, b_F the speed flow effect F, b_a the mobile-phase gradient effect, $b_{T\varphi F}$ the interaction coefficient between temperature and flow ($T\varphi F$), $b_{T\varphi a}$ the interaction coefficient between temperature and mobile-phase gradient ($T\varphi a$), $b_{F\varphi a}$ the interaction coefficient between speed flow and mobile-phase gradient ($F\varphi a$), and $b_{T\varphi F\varphi a}$ the interaction coefficient among temperature, speed flow and mobile-phase gradient ($T\varphi F\varphi a$).

These coefficients are calculated from both the experimental matrix X and the response matrix Y. X is a Hadamard matrix (X respects the condition $({}^tXX)^{-1} = (1/n)I_n$ with $n = 8$ in this case), thus the matrix of coefficients B can be easily calculated since $B = (1/n)^tXY$. Applying this formula gives:

$$B = \begin{pmatrix} b_0 \\ b_T \\ b_F \\ b_a \\ b_{T\varphi F} \\ b_{T\varphi a} \\ b_{F\varphi a} \\ b_{T\varphi F\varphi a} \end{pmatrix} = \begin{pmatrix} +1.4263 \\ -0.6763 \\ +0.1088 \\ -0.2313 \\ -0.0487 \\ +0.0963 \\ +0.0863 \\ -0.0088 \end{pmatrix}$$

$$ {}^tB = \begin{pmatrix} b_0 & b_T & b_F & b_a & b_{T\varphi F} & b_{T\varphi a} & b_{F\varphi a} & b_{T\varphi F\varphi a} \end{pmatrix} = \begin{pmatrix} 1.4263 & -0.6763 & 0.1088 & -0.2313 & -0.0487 & 0.0963 & 0.0863 & -0.0088 \end{pmatrix} \quad (2)$$

Table 1
Resolution of acenaphthene–fluorene. Simultaneous optimization of column oven temperature, speed flow and solvent gradient mobile-phase gradient over HPLC in the first part of the chromatogram ($n = 3$) $\sigma_{n-1} = 0.021$.

Column oven temperature (°C)	Speed flow (mL/min)	Mobile-phase gradient (α)	Resolution
15 (−)	0.5 (−)	0.00 (−)	2.35 (y_1)
15 (−)	0.5 (−)	1.67 (+)	1.54 (y_2)
15 (−)	1.0 (+)	0.00 (−)	2.51 (y_3)
15 (−)	1.0 (+)	1.67 (+)	2.01 (y_4)
40 (+)	0.5 (−)	0.00 (−)	0.92 (y_5)
40 (+)	0.5 (−)	1.67 (+)	0.46 (y_6)
40 (+)	1.0 (+)	0.00 (−)	0.85 (y_7)
40 (+)	1.0 (+)	1.67 (+)	0.77 (y_8)

Note: Minus and plus signs mean lower and upper values for each variable, respectively.

i. The mean value $b_0 = 1.4263$ is the mean value in the centre of the domain.
ii. *Effects of two-factor interactions*. The effects $b_{T\varphi F}$ (−0.0487), $b_{T\varphi\alpha}$ (0.0963) and $b_{F\varphi\alpha}$ (0.0863) measure the influence of each combination on the analytical response. The interaction between mobile-phase gradient and speed flow is more important than between temperature and mobile-phase gradient, or between temperature and speed flow. These interactions values are very low. This indicates that the response depends on individual values of variables rather than on combination of variables. For example, decreasing the temperature always provides a better resolution, regardless the values of the other

variables. Therefore, low-temperature combined with high-speed flow increases more the resolution than low-temperature and low-speed flow. The minus sign of the interaction effect between temperature and flow means that optimum resolution is obtained increasing one of the variables whereas the other one is decreased (low-temperature and high-flow provides better resolution than low-temperature and low-speed flow). $b_{T\varphi a}$ (0.0963) means that temperature and mobile-phase gradient barely interact. Thus, changing the speed flow always gives almost the same results regardless the values for the mobile-phase gradient. Finally, the speed flow and mobile-phase gradient do not interact significantly either.

iii. *Effects of three-factor interactions*. The effect among the three variables, namely *T*, *F*, and α is given by $b_{T\varphi F\varphi a}$ (−0.0088). This value indicates how important the interaction among the three variables is. The obtained low value means that no significant interaction exists among the 3 variables aforementioned.

iv. *Main effects*. Considering no-interactions (only main effects for temperature, speed flow, or mobile-phase gradient flow), the best condition seems to be low-temperature, high-speed flow and slow mobile-phase gradient. However, this interpretation must be modified as a function of interactions among variables. In fact it is recommended to consider results of interaction tests and further, main effect test because conclusions depend on the existence of interactions [30].

Introducing numerical coefficients (3) in expression (1), the following mathematical relationship is deduced:

$$Y = 1.4263 - 0.6763X_T + 0.1088X_F - 0.2313X_a - 0.0487X_TX_F + 0.0963X_TX_a + 0.0863X_FX_a - 0.0088X_TX_FX_a \quad (3)$$

We can also construct mathematical models with decode values. Assuming linear relationship between codified values X_T, X_F, X_a and decode values *T*, *F*, and *a*, we obtain:

$$Y = 2.9103 - 0.0480T + 0.5960F - 0.7712a - 0.0184TF + 0.0067Ta + 0.3207Fa - 0.0034TFa \quad (4)$$

where *Y* is the resolution, *T* is the temperature in °C, *F* is the speed flow in mL/min, and a is the mobile-phase gradient.

Regarding the mobile-phase gradient, we can say that this parameter can be optimized separately because its interaction with the other variables is negligible. mobile-phase gradient and speed flow can be optimized separately too, due to their negligible interactions with other variables.

An isocratic mobile-phase during the first 30 min provides us with the best resolution. Thus, considering the optimum mobile-phase gradient in the first part of the chromatogram, 0 and computing partial derivative of (4) with respect to the temperature yields to a negative value for the speed flow, this has no physical sense. Partial derivative of (4) with respect to speed flow yields a value of temperature of ca. 32.4 °C. Obviously, these values do not provide a maximum resolution. In fact the response surface (4) does not have a maximum, only extreme values for as low-temperature as possible and as high-speed flow as possible. Since the resolution obtained for 1 mL/min and 15 °C is good enough, these will be the selected values.

In the second part of the chromatogram the optimal conditions are different. Actually higher temperatures and low-speed flow seem to provide better resolution. Interactions among variables must be investigated again and the conditions optimized. Optimization in this part of the chromatogram was performed through the separation of the two following difficult-to-separate pairs of PAHs, dibenzo[a,h]anthracene-benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene. Resolutions appearing in Table 2 were calculated for different mobile-phase gradients (ΔACN%/min, increment of acetonitrile percentage per minute) and two different levels of temperature and speed flow. We can see that for the last part of the chromatogram, low mobile-phase gradients, high-temperatures, and low-speed flow favour better resolution.

Apparently, there are interactions between the mobile-phase gradient in the first part of the chromatogram (first 30 min of chromatogram) and the second part. A mobile-phase gradient equal to 0.00 in the first part provides the best resolution in the second part for dibenzo[a,h]anthracene–benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene. Temperature and speed flow do not seem to interact too much. The calculation of interactions will provide the definitive conclusions. Here the experimental matrix X' does not respect the Hadamard criterion since there are 3 different values for the first and the second slope. So the matrix of coefficients has to be

Table 2
Resolution table for the pairs dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene for calculating interactions by factorial design. Simultaneous optimization of column oven, speed flow, and mobile-phase gradient in the last part of the chromatogram ($n = 3$) $\sigma_{n-1} = 0.021$.

Resolution (1)		Column oven temperature (°C)	Speed flow (mL/min)	Slope (α) first part of chromatogram	Slope (α) second part of chromatogram
(1)	(2)				
0.20	3.77	15 (−)	0.5 (−)	0.25 (−) conc. initial ACN 50%	1.00 (+)
1.63	1.63	40 (+)			
0.40	3.45	15 (−)	1.0 (+)		
0.46	1.68	40 (+)			
0.00	0.00	15 (−)	0.5 (−)	0.50 (+) conc. initial ACN 50%	0.70 (−)
1.71	1.70	40 (+)			
0.60	3.11	15 (−)	1.0 (+)		
0.91	2.35	40 (+)			
0.18	3.79	15 (−)	0.5 (−)	0.50 (+) conc. initial ACN 50%	1.00 (+)
1.50	1.93	40 (+)			
0.34	3.75	15 (−)	1.0 (+)		
0.48	2.00	40 (+)			
0.19	3.50	15 (−)	0.5 (−)	0.00 (−) conc. initial ACN 50%	0.83 (−)
1.77	1.90	40 (+)			
0.31	3.39	15 (−)	1.0 (+)		
0.20	1.62	40 (+)			

Note: (1) Resolution for dibenzo[a,h]anthracene and benzo[g,h,i]perylene and (2) for benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene.

calculated as:

$$B' = ({}^tX'X')^{-1}\,{}^tX'Y \tag{5}$$

where Y is the response matrix (resolution matrix) and B' the coefficients of the multivariable regression model (6):

$$\begin{aligned} Y \;=\; & b_0 + b_T X_T + b_F X_F + b_{a1} X_{a1} + b_{a2} X_{a2} + b_{T\varphi F} X_T X_F + b_{T\varphi a1} X_T X_{a1} + b_{T\varphi a2} X_T X_{a2} \\ & + b_{F\varphi a1} X_F X_{a1} + b_{F\varphi a2} X_F X_{a2} + b_{a1\varphi a2} X_{a1} X_{a2} + b_{T\varphi F\varphi a1} X_T X_F X_{a1} + b_{T\varphi F\varphi a2} X_T X_F X_{a2} \\ & + b_{T\varphi a1\varphi a2} X_T X_{a1} X_{a2} + b_{F\varphi a1\varphi a2} X_F X_{a1} X_{a2} + b_{T\varphi F\varphi a1\varphi a2} X_T X_F X_{a1} X_{a2} \end{aligned} \tag{6}$$

Eq. (5) gives for the dibenzo[a,h]anthracene and benzo[g,h,i]perylene pairs:

$$B' = \begin{pmatrix} b_0 \\ b_T \\ b_F \\ b_{a1} \\ b_{a2} \\ b_{T\varphi F} \\ b_{T\varphi a1} \\ b_{T\varphi a2} \\ b_{F\varphi a1} \\ b_{F\varphi a2} \\ b_{a1\varphi a2} \\ b_{T\varphi F\varphi a1} \\ b_{T\varphi F\varphi a2} \\ b_{T\varphi a1\varphi a2} \\ b_{F\varphi a1\varphi a2} \\ b_{T\varphi F\varphi a1\varphi a2} \end{pmatrix} = \begin{pmatrix} +0.6773 \\ +0.4020 \\ -0.2421 \\ +0.0427 \\ +0.0002 \\ -0.3706 \\ +0.0330 \\ -0.0295 \\ +0.1096 \\ -0.0004 \\ -0.0902 \\ +0.0481 \\ +0.0281 \\ -0.0405 \\ -0.0821 \\ -0.0006 \end{pmatrix} \tag{7}$$

Then the resolution can be calculated with Eq. (8) (codified values X_T, X_F, X_{a1} and X_{a2}) or with Eq. (9) (decode values T, F, a_1 and a_2; linear relationship between codified and decoded values):

$$\begin{aligned} Y \;=\; & 0.6773 + 0.4020X_T - 0.2421X_F + 0.0427X_{a1} + 0.0002X_{a2} - 0.3706X_TX_F + 0.0330X_TX_{a1} \\ & - 0.0295X_TX_{a2} + 0.1096X_FX_{a1} - 0.0004X_FX_{a2} - 0.0902X_{a1}X_{a2} + 0.0481X_TX_FX_{a1} \\ & + 0.0281X_TX_FX_{a2} - 0.0405X_TX_{a1}X_{a2} - 0.0821X_FX_{a1}X_{a2} - 0.0006X_TX_FX_{a1}X_{a2} \end{aligned} \tag{8}$$

$$\begin{aligned} Y = & -1.9449 + 0.1640T + 1.9165F - 5.6329a_1 + 0.0698a_2 - 0.1860TF \\ & + 0.0346Ta_1 - 0.0400Ta_2 + 7.3842Fa_1 + 0.4955Fa_2 + 6.4330a_1a_2 \\ & + 0.0659TFa_1 + 0.0612TFa_2 - 0.0826Ta_1a_2 - 8.6165Fa_1a_2 - 0.0051TFa_1a_2 \end{aligned} \tag{9}$$

As can be seen from Eqs. (8) and (9) there are interactions between the mobile-phase gradient 1 and the mobile-phase gradient 2, among speed flow and mobile-phase gradients 1 and 2, and between speed flow and mobile-phase gradient 1. Considering again a mobile-phase gradient equal to 0 in the first part of the chromatogram ($a_1 = 0$) due to the reasons aforementioned and a mobile-phase gradient in the second part equal to 0.83, since the best results are obtained with this value. Partial derivative of expression (9) with respect to temperature, making it equal to zero and solving it, yields to $X_F \approx 1$ mL/min. Partial derivative of relationship (9) with respect to speed flow, making equal to zero the expression obtained and solving it, drives to a temperature of 17 °C. Of course, these values define one local minimum of the function. In fact (8) and (9) have no maximum, and resolution increases as speed flow diminishes and temperature increases. Therefore, for practical reasons, a maximum value of temperature (40 °C) and a minimum value of speed flow (0.5 mL/min) in the range studied will be selected.

The following program of gradient is recommended. The first section is isocratic (mobile-phase gradient 0.00) from 0 to 30 min and then a mobile-phase gradient equal to 0.83 (ΔACN%/min) is established until 90 min (Fig. 1). Finally we carry out a washing step by using 100% of acetonitrile. The last step is a column re-equilibration with a mixture 50% acetonitrile/50% H_2O during several minutes. Isocratic gradient in the first step of the chromatogram provides the best resolution for the pair acenaphthene–fluorene and facilitates the selection of higher mobile-phase gradients in the second part of the chromatogram, necessary to achieve 100% ACN and total elution of the largest PAHs. Therefore, we selected a slope of 0.00 in the first part of the chromatogram and a higher slope in the second part, 0.83. The temperature will be 15 °C during the first 30 min and 40 °C in the second part of the chromatogram. Although higher temperatures are recommended for increasing the resolution in the second part of the chromatogram, increasing temperature from 15 °C until temperatures above 40 °C and then cooling the column oven again for starting a new chromatographic run requires very long times, so we decided to come to a compromise between practical efficiency and resolution by selecting 40 °C as final temperature.

Only the last six chromatographic peaks need high temperatures and low-speed flow. As can be seen from Fig. 2, the pairs benzo[a]anthracene-chrysene, dibenzo[a,h]anthracene–benzo[g,h,i]perylene, and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene are poorly separated under low temperatures (15 °C). Separation of these compounds requires high temperatures. Increased resolution without broadening of peaks in the last part of the chromatogram was obtained under conditions established in chromatogram 2 (Fig. 3). Temperature was increased from 15 °C in the first part of the chromatogram until 40 °C in the second part (from 45 min until the end). The time at which 40 °C was reached is 45 min and the mode gradient was chosen considering technical limitations. In fact, the column oven cannot increase the column temperature immediately but in a gradient mode.

So far, we can draw the conclusion that interactions between mobile-phase gradient in the first part of the chromatogram and speed flow, and interactions between temperature and speed flow strongly influence the resolution in the last part of the chromatogram. We may wonder if resolution in the second part of the chromatogram depends on other experimental conditions established in the first part of the chromatogram. Are there interactions between experimental conditions in the first part of the chromatogram and the experimental conditions in the second part of the chromatogram? To answer this question we use another factorial design (Table 3). The results of the interaction study between the conditions in the first part of the chromatogram and the resolution in the last part ($C_1\varphi C_2$) are:

For the pair dibenzo[a,h]anthracene–benzo[g,h,i]perylene:

$$Y = 1.7675 + 0.0075X_{T1} - 0.1275X_{F1} - 0.0375X_{T1}X_{F1} \tag{10}$$

For the pair benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene:

$$Y = 1.5775 + 0.0625X_{T1} - 0.0375X_{F1} - 0.0225X_{T1}X_{F1} \tag{11}$$

where Y is the resolution, X_{T1} is the temperature during the first part of the chromatogram, X_{F1} is the speed flow during the first part of the chromatogram.

A multiple factor Anova test was applied for stating if the resolution in the second part of the chromatogram is influ-

Table 3
Resolution table for the pair dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene for calculating interactions between experimental conditions in the first part of the chromatogram and in the last part of the chromatogram by using factorial design. Column oven temperature and speed flow were selected at two levels ($n = 3$) $\sigma_{n-1} = 0.021$. Combination of solvent mobile-phase gradients: 0.00/0.83.

Experimental condition in the first 30 min	Experimental conditions in the last 60 min (30–90′)	Resolution	
15 °C/1.0 mL/min	40 °C/0.5 mL/min	1.67	1.50
15 °C/0.5 mL/min	40 °C/0.5 mL/min	1.85	1.53
40 °C/0.5 mL/min	40 °C/0.5 mL/min	1.94	1.70
40 °C/1.0 mL/min	40 °C/0.5 mL/min	1.61	1.58

Note: Resolution for the pairs dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene. $n = 3$.

Table 4
ANOVA table for analysing resolution of the pair dibenzo[a,h]anthracene–benzo[g,h,i]perylene—sum of squares type III.

Source of variance	Sum of squares	DF	Mean square	*F*	Probability
Main effects					
A: Temperature	0.000225	1	0.000225	0.04	0.8743
B: Speed flow	0.065025	1	0.065025	11.56	0.1821
Residual	0.005625	1	0.005625		
Total (corrected)	0.070875	3			

Note: All the *F* are based on the mean squares residual error. DF stands for degrees of freedom.

enced by the experimental conditions used in the first part. This procedure performs the variance analysis of resolution by using several factors and it states the factors influencing significantly resolution. Moreover, it checks for possible significant interactions among the factors studied. A multiple factor Anova test was applied for stating if resolution in the second part of the chromatogram is influenced by experimental conditions used in the first part of the chromatographic run. For both of PAHs pairs (dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene) the variable to be explained is resolution, the explanatory variables are temperature and speed flow and the number of experiments is 4. For the pair dibenzo[a,h]anthracene–benzo[g,h,i]perylene (Table 4). Probability values check statistical significance for each factor. Since no probability value is lower than 0.05, no factor has significant effect on resolution at 95.0% significance for the pair benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene (Table 5). By using Anova test, the variance observed is partitioned into components or factors. Since the sum of squares of type III has been selected, the contribution of each factor is measured after elimination of the effects of the others. Probability values check the statistic significance of each factor. Since no probability value is lower than 0.05, no factor is significant on resolution for a significance level of 95%. Therefore, the results indicate that the experimental conditions in the first part of the chromatogram has no influence on the dibenzo[a,h]anthracene–benzo[g,h,i]perylene resolution neither on the benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene resolution.

Table 5
ANOVA table for analysing resolution of the pair benzo[g,h,i]perylene–indeno(123,cd)pyrene—sum of squares type III.

Source of variance	Sum of squares	DF	Mean square	*F*	Probability
Main effects					
A: temperature	0.015625	1	0.015625	7.72	0.2200
B: speed flow	0.005625	1	0.005625	2.78	0.3440
Residual	0.002025	1	0.002025		
Total (corrected)	0.023275	3			

Note: All the *F* are based on the mean squares residual error. DF stands for degrees of freedom.

The experimental conditions finally selected are the following:

The slopes and program of solvent gradient are: a slope 0.00 from 0 min until 30 min, then a slope 0.83 from 30 min until 90 min, and finally 100% ACN from 90 min until 100 min and 7 min of column re-equilibration (50%ACN/50%H_2O). In the first part of the chromatogram, the best mobile-phase gradient was 0.00 due to several reasons:

i. It is the best one for good resolution of acenaphthene and fluorene in the first part of the chromatogram.
ii. It allows using a long mobile-phase gradient in the second part of the chromatogram where gradient is necessary for efficient elution of all heavier PAHs.

In the first part of chromatogram, the combination of 15 °C and 1 mL/min provides the best resolution. The best experimental conditions (in the range analyzed) for maximum resolution in the second part of the chromatogram are 40 °C and 0.5 mL/min. Moreover, temperature and speed flow influence the resolution of other compounds in the last part, not only dibenzo(a,h)anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene resolution. In fact, benzo[a]anthracene and chrysene can be separated with good resolution by using a progressive increment of temperature along the chromatographic procedure, starting with 15 °C and increasing the temperature until 40 °C (from 30 min until 45 min approximately). But it is difficult-to-separate them by keeping a temperature constant. Although according to literature, the need of establishing experimental conditions in the first part the chromatogram different from those selected in the second part of the chromatogram for proper elution of the compounds studied, might seem to be a consequence of water content in silica-C_{18} particles [31], as there are not interactions between gradient and temperature or between gradient and speed flow, the results aforementioned are probably due to variation of physical properties of compounds eluted.

As we can see from Figs. 3 and 4 (chromatograms 2 and 3), although the combination 15 °C and 1 mL/min (the best one for the first part of the chromatogram) with 40 °C and 0.5 mL/min (the best one in the last part of the chromatogram) provides quite good resolution for dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene (1.61/1.58) (chromatogram 5), improved sensitivity could be achieved by fixing the speed flow at 0.5 mL/min all along the chromatogram.

Under the conditions specified for chromatogram 3 (Fig. 4), we obtain the following advantages:

Good resolution over all the time range of the chromatogram was obtained. So, we can separate acenaphthene and fluorene and the last three peaks (dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene). *Good sensitivity* was obtained: the intensity and sharpness of peaks are very good and higher than in chromatogram 3 in all the cases. The Anthracene peak suffers a slight broadening due to the change in temperature and speed flow in the established time program (Fig. 5). Under these conditions the efficiency of the column diminishes a bit since the first peak of interest, naphthalene, appears at minute 20, but an outstanding sensitivity is observed (comparison between the signal for anthracene in chromatogram 2 and 3). To optimize the resolution of the pair benz(a)anthracene-chrysene (about minute 67), temperature increase can be delayed until minute 70. At minute 70, temperature must be 40 °C according to our calculated interactions among variables.

Usually, RPLC optimisation procedures for 16 EPA PAHs mixtures separation are based on criteria different of those used in this paper. As a matter of fact, optimization is usually uses efficiency

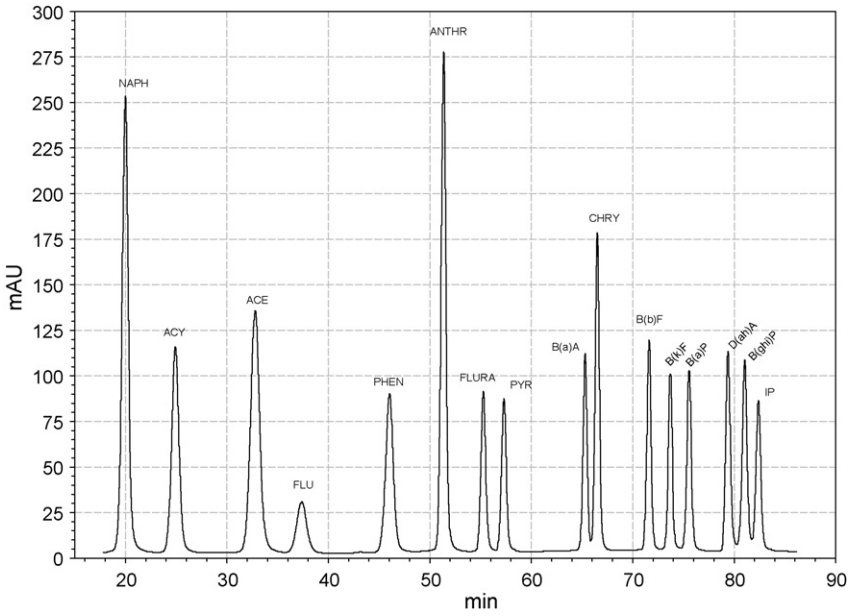

Fig. 4. Chromatogram 3, recorded under the following experimental conditions: initial concentrations of ACN, 50%. Speed flow during all the chromatogram 0.5 mL/min. Column oven temperature: 15 °C from minute 0 until minute 45, then 40 °C from minute 45 until the end. Slope: 0 until minute 30, then 0.83 (Δ%ACN/min) until minute 90. Order of elution: the same than in former chromatograms.

(by minimisation of elution times) or reduced-solvent consumption as criteria. Although shorter analysis times than ours (about 25 min in most cases) were obtained [14,32,33], the resolution was worse than here and the sensitivities [14,33] were lower than ours since probably temperature was not considered for optimizing the chromatographic procedure [33].

Modern tendencies point out to RPLC procedure optimization based on resolution criterion [34] or selectivity and sensitivity criteria [17]. Thus, Dorthe et al. [34] accomplished the optimization of the liquid chromatographic analytical procedure for PAHs mixtures separation by experimental design. The procedure developed allowed a highly satisfactory quantification of 6 PAHs in lipids. The resolution achieved was highly satisfactory and analysis time similar to that achieved in our paper (about 70 min) but the number of PAHs separated was only 6, as aforementioned [34]. Kuppithayanant and colleagues [14] carried out the optimization of the RPLC separation of the 16 EPA PAHs by simplex based on selectivity criteria. These authors achieved an efficient resolution of all the PAHs in only 36 min, but their sensitivities are poorer than ours and their RSD higher. Also, it is worthy to mention that figures in the paper by [17] do not clarify the resolution achieved. In fact, peaks of fluorene and acenaphthene seem to be too small compared to those of the other PAHs present in the mixture and it seems almost impossible drawing definitive conclusions about the resolution of some difficult-to-separate pairs (as acenaphthene and fluorene). Height of acenaphthene and fluorene peaks should be higher and similar to those of the rest of compounds in the chromatogram for drawing conclusions about peaks resolution because sometimes very small peaks seem to be resolved but actually they are not when their concentrations increase. Although we both used a PDA-UV detector and variable wavelength program, it is known that sensitivity is highly dependant on the type of detector used which makes

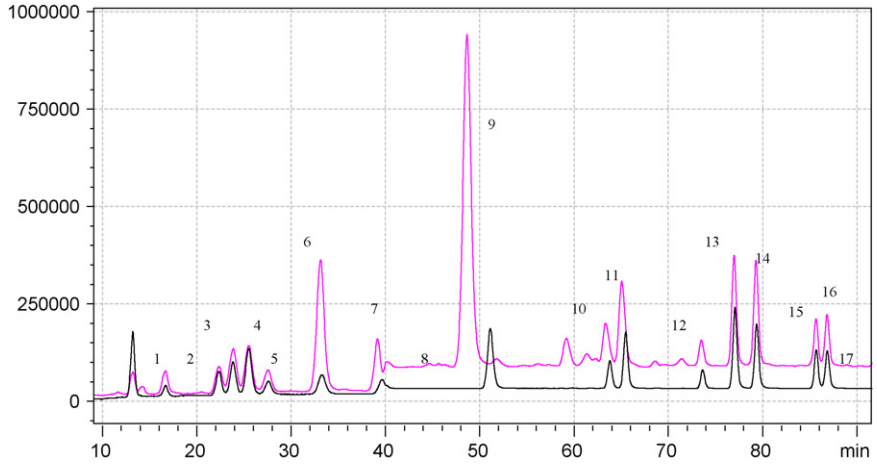

Fig. 5. Chromatograms of kerosene soot extracts from open diffusion flame (pink line), and of the 17 PAHs standard mixture at concentration of 4.65 ppb (black line). The fluorescence data are presented: (1) naphthalene, (2) Methyl-naphthalene, (3) acenaphthylene, (4) acenaphthene, (5) fluorene, (6) phenanthrene, (7) anthracene, (8) fluoranthene, (9) pyrene, (10) chrysene, (11) benz[a]anthracene, (12) benzo[a]pyrene, (13) benzo[b]fluoranthene, (14) benzo[k]fluoranthene, (15) benzo[ghi]perylene, (16) dibenzo[ah]anthracene and (17) indeno(1,2,3-cd)pyrene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

comparison much more difficult. But if comparison between their detection limits and ours have to be addressed, we could conclude the following:

(i) whereas they achieved a detection limit of 40 ng/ml for fluoranthene, we achieved a detection limit of 10.3 ng/ml for this PAH,
(ii) whereas they obtained a detection limit of 10 ng/ml for anthracene, we obtained a detection limit of 5.8 ng/ml for the same compound,
(iii) the differences are similar for indeno[1,2,3-cd]pyrene,
(iv) regarding RSD, we obtained values in the range 2–5% whereas values in the range 1.6–13% were obtained in Ref. [17]. Furthermore, whereas Kuppithayanant and colleagues [17] used modifiers (methanol or isopropanol), which is a serious inconvenience because of their toxicity and consequences on standard deviations of measurements; we did not add any modifier. Regarding analysis time, although they achieved the complete separation of PAHs in only 36 min as aforementioned, we considered that for very complex mixtures this is a serious inconvenience because there is not peak spacing enough to detect and separate other aromatic species in the real samples.

The most outstanding contribution of our work is that optimization procedure in reverse-phase high-performance liquid chromatography should be carried out taking into account physical properties of each target analyte. So far, all the papers published considered the same experimental conditions all along the chromatographic procedure regardless of the target analyte. Thus, when resolution of PAHs mixtures are accomplished, the same conditions are fixed for the dibenzo[ah]perylene–benzo[ghi]perylene pair separation and for the resolution of acenaphthene-fluorene. However, the physical properties of benzo[ghi]perylene are different from those of acenaphthene and consequently its retention in the analytical column requires different experimental conditions for proper elution. As a matter of fact, it should be emphasized that tailing in chromatographic procedures could be a consequence of using indiscriminately the same conditions for resolving the acenaphthene–fluorene pair as those for resolving the dibenzo[ah]anthracene–benzo[ghi]perylene pair.

3.2. Application to the analysis of a real sample of kerosene soot

A sample of 4 mg of kerosene soot was collected, extracted by ultrasound probe during 4 min (4 s on, 4 s off), filtered through a Wheatman filter and purified through a C_{18} column. The chromatogram obtained is shown in Fig. 5. As we can see, long times are needed for analysing EPA PAHs in kerosene soot from open diffusion flames, where a lot of other aromatic compounds are found besides the 16 EPA PAHs. Not overlapping or co-eluting compounds are obtained in that case and perfect match between standard mixture chromatogram and real sample chromatogram was obtained.

4. Conclusions

Chromatograms of the 16 EPA PAHs can be divided into two parts: the first 8 compounds and the last 8 ones. In the first part of the chromatogram (the first 8 PAHs), we observed that temperature and speed flow are not interfering variables. Temperature and mobile-phase gradient do not interfere either and the same conclusion could be drawn for speed flow and mobile-phase gradient. Consequently, temperature, speed flow and mobile-phase gradient can be optimized separately in the first part of the chromatogram.

Regarding interactions between the mobile-phase gradient selected in the first part of the chromatogram and those of the second part of the chromatogram, the mobile-phase gradient employed in the first part of the chromatogram does not seem to interfere significantly with that selected in the second part. Therefore, the mobile-phase gradients can be selected separately in each part of the chromatogram. Other experimental conditions fixed in the first part of the chromatogram do not interfere with experimental conditions applied in the second part of the chromatogram. In the first part of the chromatogram, optimum resolution was achieved for the combination of 15 °C and 1 mL/min. Very good resolutions were obtained for a slope of 0.00 (isocratic mode) over the first 30 min of running. Unlike what happened in the first part of the chromatogram, in the second part of the chromatogram it could be observed that temperature and speed flow are interfering variables. For the pair dibenzo[a,h]anthracene–benzo[g,h,i]perylene, a low-speed flow (0.5 mL/min) provided a very good resolution when combined with high temperatures (40 °C) whereas very poor results were observed when 0.5 mL/min was combined with low temperatures (15 °C). As a consequence, these variables should be optimized simultaneously.

Also, it was concluded that the slope of the mobile-phase gradient is not a critical parameter. In fact, different slopes provide very similar results and the analysis of the interactions confirms this statement. Therefore, we could select a mobile-phase gradient equal to 0.00 for the first part of the chromatogram and 0.83 for the last one. Higher mobile-phase gradient are not convenient because it must be maintained as long as possible. Of course, a slope of 0.00 is not convenient because with this mobile-phase gradient we can never achieve 100% of acetonitrile. Finally, if we select a very small mobile-phase gradient during long time, the broadening of peaks may be too high, worsening the resolution.

In summary, the combination 15 °C and 1 mL/min (in the first part of the chromatogram) turned out to be the best for the resolution of acenaphthene–fluorene, whereas in the last part of the chromatogram, the combination of 40 °C and 0.5 mL/min provides the best resolution for the pairs dibenzo[a,h]anthracene–benzo[g,h,i]perylene and benzo[g,h,i]perylene–indeno[1,2,3-cd]pyrene although better sensitivity can be reached by using programmed increasing temperature and 0.5 mL/min all along the chromatographic run.

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