

Direct large-volume injection analysis of polycyclic aromatic hydrocarbons in water

Ainhoa Rubio-Clemente,^{1,2,3,*} Edwin L. Chica,⁴ Gustavo A. Peñuela,²

Edited by

Juan Carlos Salcedo-Reyes

(salcedo.juan@javeriana.edu.co)

1. Universidad Católica de Murcia UCAM, Facultad de Ciencias de la Salud, Avenida de los Jerónimos, 135, Guadalupe-Murcia, Spain.

2. Universidad de Antioquia UdeA, Facultad de Ingeniería, Sede de Investigaciones Universitarias (SIU), Grupo de Diagnóstico y Control de la Contaminación (GDCCON), Calle 70, No. 52-21, Medellín, Colombia.

3. Tecnológico de Antioquia–Institución Universitaria TdeA, Facultad de Ingeniería, Calle 78b No. 72A-220, Medellín, Colombia.

4. Universidad de Antioquia UdeA, Facultad de Ingeniería, Departamento de Ingeniería Mecánica, Calle 70, No. 52-21, Medellín, Colombia.

* ainhoarubioclem@gmail.com

Received: 07-10-2017

Accepted: 19-04-2018

Published on line: 05-06-2018

Citation: Rubio-Clemente A, Chica EL, Gustavo, Peñuela A. Direct large-volume injection analysis of polycyclic aromatic hydrocarbons in water, *Universitas Scientiarum*, 23 (2): 171-189, 2018. doi: 10.11144/Javeriana.SC23-2.dlvi

Funding:

Colombian Institute of Science and Technology (COL.CIENCIAS) and the Research Vice-rectory of Universidad de Antioquia.

Electronic supplementary material: N.A.



Abstract

Due to the health risks for both humans and living beings caused by polycyclic aromatic hydrocarbons (PAHs), the monitoring of these compounds in environmental matrices is mandatory. This work proposes an analytical method for analyzing anthracene (AN) and benzo[a]pyrene (BaP), two of the most representative PAHs, at ultra-trace concentrations in water, employing direct injection of large volumes of samples coupled with reversed-phase high-performance liquid chromatography. For this purpose, principal component analysis was used to examine the behavior of AN and BaP within the chromatographic system. Results showed that AN and BaP chromatographic behavior can be described by three models representing their identification, the quantification of AN and that of BaP, respectively. The factors affecting the obtained models, such as the injection volume, column temperature, flow rate, strength of the mobile phase, and the excitation and emission wavelengths, were examined and optimized by means of design of experiments. Finally, the analytical method was validated, obtaining promising limits of detection and quantification. The developed analytical method was demonstrated to be useful for a sensitive analysis of the target analytes in relatively clean natural water matrices.

Keywords: anthracene; benzo[a]pyrene; design of experiments; matrix constituents; principal component analysis; ultra-trace level

Introduction

Aqueous resources pollution is an issue of current special concern. A number of pollutants can be found in water, including polycyclic aromatic hydrocarbons (PAHs) (Tian *et al.* 2012; Rubio *et al.*, 2013; Rubio-Clemente *et al.* 2014a, 2015). These compounds constitute a group of organic pollutants formed of two or more fused benzene rings containing mainly carbon and hydrogen (Rubio-Clemente *et al.* 2014b; Dos Santos *et al.* 2018). PAHs are ubiquitous compounds in the environment (Alves *et al.* 2017; Segura

et al. 2017). They come from anthropogenic sources, such as fossil fuel combustion, metal smelting processes and food smoking, among other human activities; and can be found in the atmosphere, soil, water and even in living beings because of their bioaccumulative properties throughout the food chain (Chizhova *et al.* 2013; Menezes *et al.* 2013; Santos *et al.* 2017).

The main concern related to the presence of PAHs in the environment is ascribed to their toxic potential, such as anthracene (AN), which has exhibited a high acute phototoxicity, and carcinogenic, mutagenic and teratogenic characteristics, like benzo[a]pyrene (BaP) (Rubio-Clemente *et al.* 2014b). In this regard, these compounds are subjected to be monitored by national and international regulations (Directive 2013; Ribeiro *et al.* 2015); therefore, the adoption of an analytical method aiming at their determination is required (Rubio-Clemente *et al.* 2017).

Due to their hydrophobicity, PAHs are poorly soluble in water, being present at ultra-trace levels in the range of ng/L or $\mu\text{g/L}$; fact that limits PAH identification and quantification in aqueous matrices (Rubio-Clemente *et al.* 2017). Recently, several analytical techniques have been reported (Nawaz *et al.* 2014; Petridis *et al.* 2014; Ahmadvand *et al.* 2015; Khodae *et al.* 2016). However, they use previous separation and pre-concentration procedures, being PAH analysis a tedious process. Additionally, separation and pre-concentration techniques might contaminate the sample to be analyzed and produce losses of analytes; especially when multistep procedures are performed (Buczyńska *et al.* 2014; Anumol *et al.* 2015; Boix *et al.* 2015). Consequently, large-volume injection techniques are proposed to be used as alternative procedures (Boix *et al.* 2015). Sample large-volume injection techniques can also be used with reversed-phase high-performance liquid chromatography (RP-HPLC) and gas chromatography (GC), and be combined with fluorescence detector (FLD) or diode array detector, and even with mass spectrometry, finding out accurate and repeatable results within a short period of analysis, without incurring high costs, neither the contamination of the sample nor the loss of the target analytes.

On the other hand, during the development of new analytical methods, one-factor-at-a-time techniques are commonly used. However, the evaluation of several factors influencing the chromatographic system by analyzing the effect of one single parameter at a time can be an expensive task, and valuable information about the analyzed factors can be missed (Trably *et al.* 2004; Hanrahan & Lu 2006, Andrade-Eiroa *et al.* 2010; Rubio-Clemente *et al.* 2017). In this regard, multivariate statistical approaches can overcome these drawbacks by using principal component analysis (PCA) and design of experiments (DOE). PCA is a multivariate analytical tool that can be

used to reduce a set of original variables and to extract a small number of latent factors, also called principal components (PCs), which allow the analysis of the relationships among the observed variables (Machala *et al.* 2001; Golobočanin *et al.* 2004). In turn, DOE can be used to determine the most influential factors within the considered experimental system (Ferreira *et al.* 2007; Dejaegher & Vander 2009, 2011).

Under this scenario, this work is focused on analyzing AN and BaP chromatographic behavior in order to optimize the system for the simultaneous analysis of the analytes of interest at ultra-trace levels in aqueous samples by means of RP-HPLC coupled with FLD and using PCA and DOE under different experimental conditions. In addition, the validation of the optimized experimental chromatographic conditions was carried out using different natural water matrices.

Materials and methods

Reagents and solutions

Anthracene (AN, 99 %) and benzo[a]pyrene (BaP, 98 %) analytical certified standards from Dr. Ehrenstorfer (Ausgurg, Germany) and gradient-grade acetonitrile purchased from Merck (Darmstadt, Germany) were used without further purification. Deionized water with a resistivity of 18.2 M Ω and obtained from a Millipore purification system (Bedford, USA) was also employed.

Stock standard solutions of AN and BaP were prepared in acetonitrile at a concentration of 1000 mg/L. The working solutions used during the PCA and DOE were prepared by spiking deionized water with a small aliquot of AN and BaP for obtaining a final concentration of 20 μ g/L and 2 μ g/L, respectively.

The calibration curves were built within a range of 75 - 3000 ng/L for AN and of 30 - 3000 ng/L for BaP, using diluted standard solutions and 10 % of acetonitrile (v/v) so that the target PAH adsorption on the walls of the vials is prevented (Martinez *et al.* 2004).

Analytical methods

AN and BaP were identified and quantified in aqueous samples with an Agilent HPLC system series 1100/1200 (Palo Alto, USA) equipped with a G1322a vacuum degasser unit, a G1311a quaternary pump, a G1321a multiwavelength fluorescence detector, a G1316a column oven, and a G1329a autosampler. The

column was a 5 μm Kinetex core-shell technology C18 (150 x 4.6 mm i.d.) from Phenomenex (Torrance, USA). Unless otherwise mentioned, elution was carried out under isocratic conditions using a mobile phase composed of acetonitrile and deionized water (90:10, v/v), a flow rate of 1 mL/min, a column temperature of 35 °C, a sample injection volume of 100 μL , an emission wavelength of 416 nm and excitation wavelengths of 254 nm from 0 to 3.20 min and 267 nm from 3.21 to 5 min. OpenLab CDS Chemstation software (Agilent, Palo Alto, USA) was used for chromatographic data analysis.

Statistical analysis

PCA was used to examine the behavior of AN and BaP within the chromatographic system under several experimental conditions. The objective of PCA consists of building k lineal combinations (Y_k) of the considered (X_p) variables containing the major variability, being a the associated coefficients. The lineal combinations can be expressed as Eq. 1-3.

$$Y_1 = a_{11}x_1 + a_{12}X_2 + \cdots + a_{1p}X_p \quad (1)$$

$$Y_2 = a_{21}x_1 + a_{22}X_2 + \cdots + a_{2p}X_p \quad (2)$$

$$Y_k = a_{k1}x_1 + a_{k2}X_2 + \cdots + a_{kp}X_p \quad (3)$$

The first PC (Y_1) refers to the lineal combination of the response variables with the maximal variability. The second PC (Y_2) is the lineal combination with the second major variability that is not correlated with the first PC. The variability grouped by the following PC (Y_k) is decreased up to a non-statistical significant variability.

Additionally, a fractional factorial DOE was employed to find the optimal operating conditions that allow for the simultaneous identification and quantification of the target analytes.

Statgraphics Centurion XVII (Statpoint, Warrenton, USA) was used for the statistical treatment of the experimental data.

Results and discussion

Chromatographic behavior using principal component analysis

Taking into account the different factors that influence the separation of compounds in a chromatographic system for an accurate identification and quantification, the strength of the mobile phase, which was evaluated in

terms of acetonitrile percentage, was selected to be analyzed. The injection volume is another parameter to be considered, particularly when determining compounds in the range of ng/L, as it is the case, since the injection of large volumes of samples may derive in the increase of the number of molecules and, therefore, in the increase of the detector analytical response. The excitation and emission wavelengths also play a main role in the quantification of compounds that exhibit an excitation and relaxation behavior under ultraviolet radiation, such as PAHs. Consequently, these two factors were also taken into consideration. Moreover, the flow rate of the mobile phase and the temperature of the column were demonstrated to influence the chromatographic system; that is, the determination of analytes due to the correlation between their elution and the pressure of the system. Thus, six factors affecting the chromatographic system were initially considered, and varied within different operational ranges. In **Table 1** the factors and ranges used are listed. These ranges were selected according to different investigations (Bourdat-Deschamps *et al.* 2007; Lucio-Gutierrez *et al.* 2008; Andrade-Eiroa *et al.* 2010), the chromatographic expertise of the authors and previous experimental runs.

Among the different responses to be measured related to the identification and quantification of organic compounds, the retention time of AN and BaP, as well as the resolution between these two compounds, were selected for identification purposes. Since identification is not enough, in terms of regulation accomplishment, the counts of area and height of AN and BaP were also considered for quantification purposes. In order to find out the optimal experimental conditions for AN and BaP analysis in water at ultra-trace concentrations, PCA was firstly conducted to correlate the different responses selected and, therefore, to obtain those models describing the chromatographic behavior of AN and BaP. However, it is highlighted that prior to any further statistical analysis, normality of the response variables was checked. All the measured variables were verified to follow a normal distribution by using Kolmogorov-Smirnov test, with the exception of AN area and AN height. Consequently, these variables were treated and log transformations of AN area and AN height were obtained for assuring normality assumption.

Subsequently, the response variables were subjected to PCA. It was found that the first three PCs explained more than 97 % of the total variability among the seven considered response variables. These results were confirmed by the scree plot represented in Fig. 1, The scree plot displays the number of principal components versus their corresponding eigenvalues. This kind of plot indicates in a graphical way the number of PCs to be retained based on the size of their eigenvalues. The ideal pattern is a steep curve that is gradually smoothed up to a straight line, as represented by the blue line in the figure.

Table 1. Factors and levels tested for the considered responses.

FACTOR (UNIT)	LEVEL
Injection volume (μL)	50 - 100
Strength of the mobile phase (%)	70 - 90
Excitation wavelength (nm)	230 - 280
Emission wavelength (nm)	408 - 424
Flow rate (mL/min)	1 - 1.5
Column temperature ($^{\circ}\text{C}$)	25 - 35

The number of principal components suggested to be selected corresponds to those components with eigenvalues higher than 1; that is, the components that remain above the horizontal red line. In **Table 2**, the estimated values of the coefficients for each extracted principal component are shown.

From **Table 2**, it can be observed that the coefficients having the main weights (weight > 0.5) in PC 1 are the retention times of AN and BaP, and the resolution between AN and BaP; that is, all the response variables related to the elution of the target analytes. In this regard, PC 1 can be representative of the identification index of AN and BaP. Concerning PC 2, the main coefficients are the log area of AN and the log height of AN. In turn, for PC 3, the coefficients representing the area and height of BaP are the highest ones. Therefore, PC 2 and PC 3 might be indicative of the behavior of AN and BaP, respectively, in terms of the peak area and height; that is, PC 2 and PC 3 represent the quantification index of AN and BaP, respectively. PCA tool has also been used for developing retention models in liquid chromatography and standard fingerprints, among other uses (Nikitas *et al.* 2012; Qi *et al.* 2017).

Optimization using design of experiments

Once the identification and quantification of AN and BaP were described by these indices, corresponding to PC 1, PC 2 and PC 3, the factors statistically significant for each component were examined and the experimental conditions were optimized by using DOE; particularly, a fractional factorial

Table 2. Coefficient estimated values contained in the considered principal components.

COEFFICIENT	PC 1	PC 2	PC 3
Retention time of AN	0.5453	0.0422	0.1432
Retention time of BaP	0.5573	0.0511	0.0965
Area of BaP	0.0405	-0.3385	0.6593
Height of BaP	-0.2635	-0.2974	0.5825
Resolution between AN and BaP	0.5323	0.1019	0.1420
Log Area of AN	-0.12	0.6288	0.3016
Log Height of AN	-0.1526	0.6219	0.2916

design due to the high number of factors considered initially. A total of 16 runs plus 5 central points were executed within the selected operating ranges, and analysis of variance (ANOVA) test was performed for each chromatographic index. For a confidence interval of 95 %, it was observed that the block effect was not significant for the chromatographic system under the tested experimental conditions. Concerning PC 1, representing the identification index of AN and BaP, it was evidenced to be influenced negatively by the flow rate and the strength, in terms of acetonitrile content of the mobile phase. This means that as the flow rate is increased, the analytes elute faster and the resolution is, subsequently, decreased. This inversely proportional linear relationship between resolution and flow rate was also observed by Andrade-Eiroa *et al.* (2010) while optimizing the separation of the pairs dibenzo[a,h]anthracene-benzo[g, h, i]perylene and benzo[g, h, i]perylene-indeno[1, 2, 3-cd]pyrene. Similar reasoning can be withdrawn when considering the percentage of organic solvent in the mobile phase. An increase of the mobile phase strength leads to AN and BaP are eluted more rapidly, decreasing their retention times by the stationary phase of the chromatographic column, which results in a decrease of resolution between these two organic compounds. From these two factors, the flow rate was found to exert a higher influence in the identification index of both pollutants, since it has a coefficient associated of -2.1767 in comparison with the coefficient linked to the acetonitrile percentage (-0.1774) of the mobile phase.

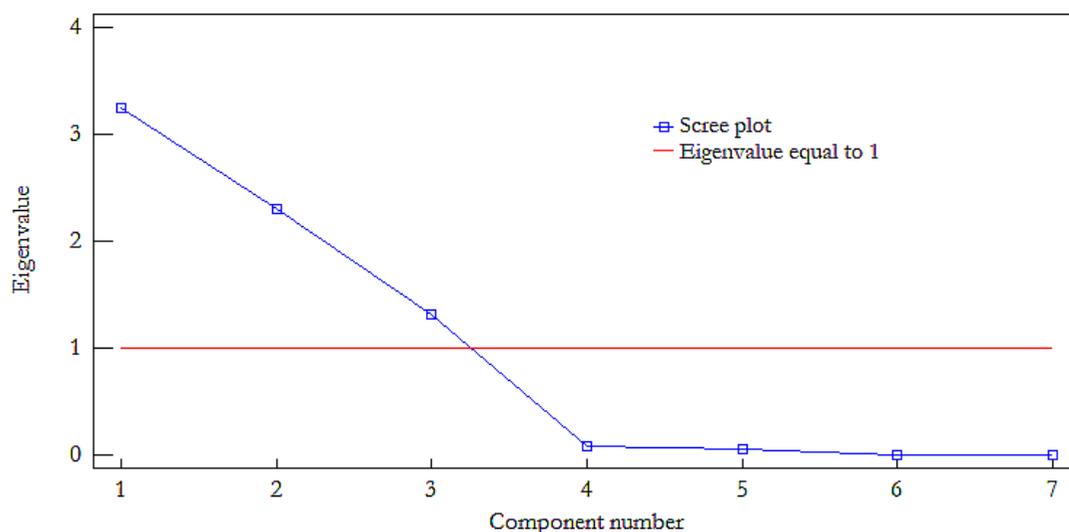


Figure 1. Scree plot of the considered responses. Operating conditions: [anthracene]₀ = 20 $\mu\text{g/L}$; [benzo[a]pyrene]₀ = 2 $\mu\text{g/L}$; injection volume = 50-100 μL ; strength of the mobile phase = 70-90 %; excitation wavelength = 230-280 nm; emission wavelength = 408-424 nm; flow rate = 1-1.5 mL/min; column temperature = 25-35 $^{\circ}\text{C}$.

With respect to PC 2, representing AN quantification index, the excitation wavelength was observed to develop a major role in AN area and height with a weight of -0.0542 . This fact indicates that a decrease of the excitation wavelength results in an increase of the index describing AN quantification and, therefore, an increase of the log area and of the log height of AN. Thus, AN area is increased as well as AN height, improving AN signal detected by the FLD.

Finally, concerning PC 3, it was found that it is statistically affected by the injection volume by a weight of 0.0286 . Thus, when the injection volume is increased, the amount of BaP molecules eluting is correspondingly increased with the subsequent augmentation of the area and height of BaP chromatographic peak. Additionally, for a significant level equal to 0.05 , the flow rate and the excitation wavelength exerted a negative (-2.1898) and positive (0.0190) influence, respectively. On the one hand, an increase of the flow rate of the mobile phase leads to a decrease of the area of BaP peak. This can be explained from the fastest elution of the analyte molecules, reducing BaP band and, therefore, decreasing the dimensions of BaP peak. On the other hand, an increase of the excitation wavelength results in an increase of the area and height of BaP. It must be noted that, despite the

non-statistically significance of the excitation wavelength for $\alpha = 0.05$, it was considered in the BaP quantification index because its p-value was close to 0.05 ($p\text{-value} = 0.0772$).

In **Fig. 2**, the described magnitudes and signs of the selected factors, both the statistically and non-statistically significant ones, for the three PCs are represented through the main effect plots.

The models built describing the three chromatographic indeces representing the chromatographic behavior of AN and BaP under the experimental conditions tested with p-values lower than 0.05, corresponding to 0.0000, 0.0000 and 0.0085, respectively, are described by Eq. 4-6.

$$I_i = 16.9144 - 2.1767 * FR - 0.1774 * S \quad (4)$$

$$I_{ii} = 13.8225 - 0.0542 * EW \quad (5)$$

$$I_{iii} = -4.2625 - 2.1898 * FR + 0.0286 * IV + 0.0190 * EW \quad (6)$$

where I_i is the AN and BaP identification index, I_{ii} is the AN quantification index and I_{iii} is the BaP quantification index. In turn, FR , S , EW and IV represent the flow rate, strength of the mobile phase, excitation wavelength and the injection volume of the sample, respectively.

By optimizing all the principal components obtained simultaneously using multicriteria decision approach, the optimal chromatographic conditions with a desirability of 83.52 % were 1.0 ml/min, 90 %, 100 μ L, 230 nm, 409 nm and 25 $^{\circ}$ C for the flow rate, acetonitrile content of the mobile phase, injection volume, excitation and emission wavelengths and the column temperature, respectively. However, it is worldwide known that the absorption and fluorescence emission capacities of a substance depend on the substance itself. Additionally, absorption and fluorescence properties of the particularly tested compounds do not follow a linear relationship with the wavelengths used, since their absorption molar coefficients and absorption spectra vary with the single excitation wavelength (Rubio-Clemente *et al.* 2017). For example, in the case of AN, the absorption coefficient at 254 nm is $\log \varepsilon_{254} = 4.9$ in ethanol. For BaP, it has a value of $\log \varepsilon_{254} = 4.6$ in ethanol (Zsila *et al.* 2006; Jones, 1947). BaP absorption spectrum is characterized by several bands of varied intensity; a first one in the range between 245 and 305 nm, higher than 254 nm; and a second one from 320 to 410 nm (Thomas & Burgess, 2007). Moreover, AN absorption spectrum has a distinguished band around 254 nm (Thomas & Burgess, 2007). In this regard, the optimization procedure used in this work is limited when the studied system is influenced by the quadratic effects of the considered factors, as it is the case for these parameters.

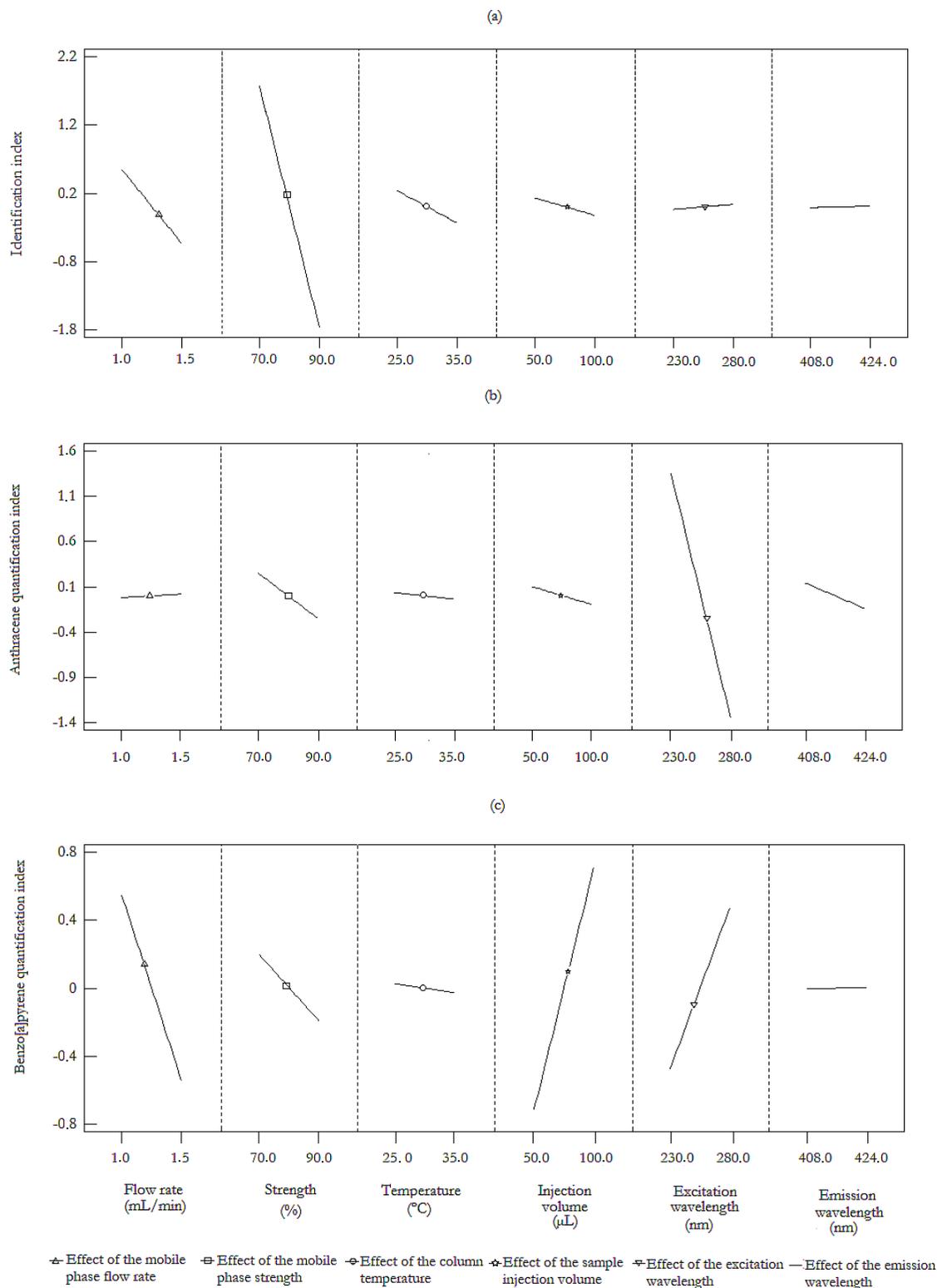


Figure 2. Main effect plots for the identification index (a), anthracene quantification index (b) and benzo[a]pyrene quantification index (c). Operating conditions: [anthracene]₀ = 20 μg/L; [benzo[a]pyrene]₀ = 2 μg/L; injection volume = 50-100 μL; strength of the mobile phase = 70-90 %; excitation wavelength = 230-280 nm; emission wavelength = 408-424 nm; flow rate = 1-1.5 mL/min; column temperature = 25-35 °C.

Therefore, a minacious study was subsequently performed to find out the excitation and emission optimal wavelengths of AN and BaP. It was found that 416 nm was the optimal emission wavelength within the selected initial range for both of the examined analytes. The excitation wavelength was fixed at 254 nm and 267 nm during AN and BaP elution time, respectively.

On the other hand, taking into account that the column temperature was not statistically significant for the models built and considering that the analysis time can be reduced by augmenting the temperature of the column oven, reducing also the pressures in the system and improving the column efficiency, as reported by Andrade-Eiroa et al. (2010), the optimal column temperature was kept at 35 °C.

Validation

Under the optimized conditions, the proposed analytical method was validated. Good linearity values and low limits of quantification and detection of 75 and 5.54 ng/L for AN, and 30 and 4.26 ng/L for BaP were obtained. Additionally, intraday and interday precisions lower than 2 and 11 %, respectively, were found for the high, medium and low levels tested. Accuracy was also verified and relative standard deviations (RSD) lower than 10 % were evidenced. Furthermore, the analysis of AN and BaP in different matrices of real natural water gave satisfactory recoveries (RSD < 13 %).

Conclusions

The results of the present study indicated that the chromatographic behavior of the selected PAHs under the experimental conditions tested may be explained by PCA using three indeces describing the elution of AN and BaP, the peak shape of AN and that of BaP, representing the former one and the latter ones the identification and the quantification of the target compounds, respectively. It was demonstrated that the identification index of the target compounds under the experimental domain studied here was defined by the flow rate and the strength of the mobile phase. Concerning the AN quantification index, the excitation wavelength was found to develop a main role. Finally, the BaP quantification index, as expected, was also influenced by the excitation wavelength; however, the injection volume and the flow rate were observed to exert also a main function.

The optimal operating conditions found using DOE that maximizing the indices referred above were 1 mL/min, 90 %, 35 °C, 100 µL, and 416 nm for the flow rate, acetonitrile content of the mobile phase, column temperature, injection volume and the emission wavelength, respectively. The optimal excitation wavelengths were 254 nm and 267 nm for AN and BaP. The developed and validated method showed to be suitable for the identification

and quantification of AN and BaP at ultra-trace levels in relatively clean natural water by direct injection in only 5 min of analysis.

Acknowledgements

This work was supported by the Colombian Institute of Science and Technology (COLCIENCIAS) and the Research Vice-rectory of Universidad de Antioquia.

Conflicts of interest

The authors state that their sole interest in the results of this research is scientific.

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Análisis de inyección directa en gran volumen de hidrocarburos aromáticos policíclicos en agua

Resumen. Los hidrocarburos aromáticos policíclicos (HAPs) causan problemas en la salud de los seres humanos y seres vivos, por lo que se requiere un monitoreo de estos compuestos en matrices ambientales. Este trabajo propone un método analítico para analizar el antraceno (AN) y el benzopireno (BAP), los hidrocarburos más representativos en concentraciones de ultra trazas en el agua, empleando inyección directa en grandes volúmenes en muestras acopladas a la fase inversa con cromatografía líquida de alto rendimiento. Por tal razón, se utilizó el análisis de componentes principales para examinar el comportamiento de AN y BAP en el sistema cromatográfico. Los resultados mostraron que el comportamiento cromatográfico de AN y BAP podría describirse por medio de tres modelos que representan su identificación, la cuantificación de AN y de BAP, respectivamente. Se examinaron los factores que afectan a los modelos obtenidos, como el volumen de inyección, la temperatura de la columna, la tasa de flujo, la fuerza de la fase móvil, y las longitudes de las ondas de excitación y emisión, y se optimizaron mediante el diseño de experimentos. Finalmente, se validó el método analítico, obteniendo límites de detección y cuantificación. Se demostró que el método analítico desarrollado fue útil para el análisis sensible de los analitos en matrices de agua natural relativamente limpia.

Palabras clave: antraceno; benzopireno; diseño de experimentos; componentes matriciales; análisis de componentes principales; ultra trazas.

Análise de injeção direta de grande volume de hidrocarbonos aromáticos policíclicos em água

Resumo Devido aos riscos para a saúde tanto para humanos como para os seres vivos em geral causados pelos hidrocarbonos aromáticos policíclicos (HAPs), o monitoramento de estes compostos em matrizes ambientais é prioritário. Este trabalho propõem um método analítico para analisar antraceno (AN) e benzo[α]pireno (BaP), dois dos hidrocarbonos mais representativos, em concentrações de ultra traços em água, empregando injeções diretas de grandes volumes de amostra acoplada a cromatografia líquida de alta eficiência em fase reversa. Usando Análises por Componentes Principais e desenho experimental, foram avaliados os efeitos de diversos fatores que afetam o sistema cromatográfico, tais como o volume de injeção, a temperatura da coluna, fluxo, força da fase móvel e comprimentos de onda de excitação e emissão. Os resultados demonstraram que o comportamento cromatográfico de AN e BaP pode ser descrito por meio de três que representam sua identificação, quantificação de AN e de BaP, respectivamente. Os resultados mostraram que o comportamento cromatográfico de NA e BAP poderia ser descrito por meio de três modelos que representam sua identificação, a quantificação de NA e de BAP, respectivamente. Examinaram-se os fatores que afetam aos modelos obtidos, como o volume de injeção, a temperatura da coluna, a taxa de fluxo, a força da fase móvel, e as longitudes das ondas de excitação e emissão, e se otimizaram mediante o desenho experimental. Finalmente, se validou o m todo analítico, obtendo os limites de detecção e quantificação. O método analítico desenvolvido demonstrou ser útil para uma análise sensível para os compostos de interesse em matrizes de água natural relativamente limpas.

Palabras clave: antraceno; benzopireno; desenho experimental; componentes de matriz; análise de componentes principais; ultra traços.

Ainhoa Rubio-Clemente

She is an Environmental Engineer by University of Salamanca. She has a MSc. and she is a junior researcher. She has research experiences in the field of water pollution decontamination and drinking water production using conventional and advanced treatment processes. Additionally, she has been involved in developing several analytical methods.

Edwin L. Chica

He is a Mechanical Engineer by University of Antioquia. Currently he is a MSc. PhD. associate professor and researcher at University of Antioquia, heading the Research Group 'Energía Alternativa'. He has research experiences in the field of renewable energy production and water decontamination and purification using conventional and advanced treatment processes, regarding design, scaling and performance purposes.

Gustavo A. Peñuela

He is a Chemist by the National University. He is the director of the Research Group 'Diagnóstico y Control de la Contaminación' (GDICON) at University of Antioquia. He has large experience in the field of water, soil and air pollution. Additionally, he has conducted a number of researches in developing analytical methods for several purposes.