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Multi-response optimization of a green solid-phase extraction for the analysis of heterocyclic aromatic amines in environmental samples

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Complete List of Authors:	Canales, Romina; Universidad Nacional de San Luis, Chemistry Mariño Repizo, Leonardo; Consejo Nacional de Investigaciones Cientificas y Tecnicas Reta, Mario; National University of La Plata Cerutti, Soledad; Universidad Nacional de San Luis, Chemistry

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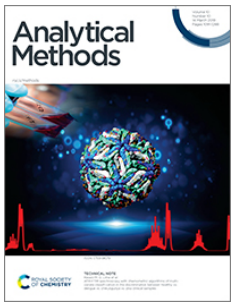

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Associate Editor of Analytical Methods
Prof. Jailson de Andrade

Dear Professor Jailson de Andrade,

On behalf of the other authors, I would like to submit our manuscript entitled *"Multi-response optimization of a green solid-phase extraction for the analysis of heterocyclic aromatic amines in environmental samples",* for its consideration and publication in Analytical Methods. Besides, we would like to inform you that this manuscript and all the included information is unpublished elsewhere.

Our manuscript describes a green extraction methodology based on the use of MWCNTs-SPE prior to liquid chromatography and tandem mass spectrometry for the quantitative analysis of heterocyclic aromatic amines of environmental concern in surface water samples. The methodology was optimized with the employment of experimental designs, which provided to greening the approach.

The figures of merit demonstrated satisfactory results compatible with the concentration levels of the compounds in the samples and comparable, and even better, than other studies reported in the literature.

To the best of our knowledge, this is the first time that a MWCNTs-SPE method is applied for sample clean up and quantitative extraction of HAAs in natural water samples. The extraction/separation and determination approach demonstrated advantages such as sensitivity, selectivity, precision, low cost, reduced solvent consumption –low toxicity-, simplicity and rapidity. Moreover, a comparative study was applied in order to assess the greenness of approaches for the determination of heterocyclic aromatic amines in surface water using the available metrics.

I appreciate so much for your consideration.

Best Regards,

Dr. Soledad Cerutti

Multi-response optimization of a green solid-phase extraction for the analysis of heterocyclic aromatic amines in environmental samples

Romina Canales[a], Leonardo Mariño-Repizo[a], Mario Reta[b], Soledad Cerutti[a,*]

[a] *Instituto de Química de San Luis, Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de San Luis. Facultad de Química, Bioquímica y Farmacia Bloque III, Avda. Ejército de los Andes 950, San Luis, Argentina CP: 5700.*

[b] *Laboratorio de Investigación y Desarrollo de Métodos Analíticos (LIDMA), División Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (B1900AJL) 1900 La Plata, Argentina.*

*Corresponding author:

E-mail address: E-mail: ecerutti@gmail.com (S. Cerutti)

Phone: +54-0266-4520300 (*1311)

Abstract

A multi-response optimization of a green and efficient solid phase extraction (SPE) sample treatment using non-modified multi-walled carbon nanotubes combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed for the quantification of heterocyclic aromatic amines (HAAs) in river and reservoir surface water samples. The proposed methodology was evaluated with the employment of experimental designs, which provided to greening the approach. Ultra-trace amounts of HAAs were retained into the SPE cartridge. Then, these analytes were removed from the carbon nanotubes with 0.8 mL of a mixture of acetonitrile/water with 0.1 % of formic acid. Under the optimal conditions, linearity was achieved for concentration levels ranging from 0.20 μg L^{-1} to 500 μg L^{-1}, with regression coefficients (R^2) from 0.990 to 0.998. Limits of detection varying from 0.06 μg L^{-1} and 0.23 μg L^{-1} were attained, the relative standard deviations ($n=3$) varied from 1.7 to 6.4, and extraction recoveries ranged from 90.6 % to 106.0 % for all the analytes. Results of the presence of HAAs found in the river samples demonstrated levels from 0.16 μg L^{-1} to 0.53 μg L^{-1}; meanwhile, in the reservoir, the levels were higher, from 0.37 μg L^{-1} to 0.93 μg L^{-1}. Finally, a comparative discussion was applied in order to assess the greenness of approaches for the determination of heterocyclic aromatic amines in surface water using the available metrics.

Keywords: Heterocyclic aromatic amines; Solid-phase extraction; Multi-walled carbon nanotubes; Green certificate.

1. Introduction

One of the principal exposure sources to heterocyclic aromatic amines (HAAs) to human health appears to come from the environment.[1,2] The International Agency for Research on Cancer (IARC) has reported the mutagenicity of some HAAs and recommends to decrease their exposure.[3]

These compounds have been assorted as *aminoimidazoazaarenes* (AIAs), which are formed at about 150 °C; and *amino-carbolines*, which are generated at temperatures about 300 °C.[4] Such HAAs contain a distinctive aromatic nucleus conjugated with one or more nitrogen atoms and as well as an exocyclic amino group. [5]

Since negative effects on health and occurrence in the environment, the detection of HAAs in matrices such as protein foods, cigarette smoke, and cigarette and forest fire ashes; among others, has been performed and reported. [2,6-7] In consequence, HAAs may be distributed into the environment from the airborne particles, which are capable to transfer into the atmosphere causing pollution in the different environmental compartments, including air, soil, sediment, and water indeed. [1] Some HAAs have been detected in diverse water samples including surface water, mostly from rivers and reservoirs. [8,9] Due to many countries drinking water is obtained from rivers or reservoirs, which might be affected by pollution of HAAs from wastewater, treatment-plant effluents, as well as several anthropogenic contaminants activities, serious human health issues might be promoted by HAAs contaminated water consumption.

Numerous harmful compounds including HAAs, currently are non-regulated in surface water, therefore they usually are non-considered or removed from water treatment. Neither, such analytes are included in the routine analysis of surface water destined for drinking water. In this sense, HAAs incidence in drinking water due to an inappropriate water

purification decrease significantly its quality. To assess HAAs contamination levels in surface water, suitable monitoring based on capable and affordable methodologies to detect the low levels of these analytes in drinking, river, and reservoir samples is necessary. [10]

Nowadays, analytical methodologies pointed toward to fulfill contaminants ultra-trace determination have been focused on the ability to isolate a wide variety of pollutants in water. [11-13] Particularly, due to an expected low level of concentrations of HAAs (~ng L^{-1}) and the matrix complexity of surface water samples, adequate sample pretreatment procedures are required for the effective extraction of these compounds and diminishing the matrix effect from.

Owing to the feasibility of the solid-phase extraction (SPE) approaches, a methodology based on this focus includes conditioning of an appropriate sorbent material able to retain/release targeted analytes, washing away undesired components (commonly related to matrix effect), and eluting the desired analytes with an optimal extractive and compatible solvent with the detection system. [14,15] In this sense, SPE shows significant advantages over other conventional approaches such as suitable recoveries, concentration with higher enrichment factors, relatively rapid analyte isolation, and relatively fewer organic solvent consumption. [14,15]

Although, currently there are available a broad range of sorbent materials and applications of themselves employed in SPE, carbon nanotubes (CNTs) have been considered an interesting material due to their physicochemical properties, especially their significant skills regarding retention/elution of several analytes might be contained in environmental samples, including surface water. [16,17] Furthermore, it has been reported that CNTs surface has demonstrated suitable strong interactions to extract/concentrate many organic pollutants in environmental water samples. [17]

With the purpose to achieve the optimal conditions of the variables influencing the SPE extraction methodology, statistical and multi-variable approaches such as the design of experiments (DOE) have been currently applied to diminish laboratory supplies and time according to the green chemistry principles, reducing the amount of reagents during the optimization. The DOE approach allows understanding of the system's performance and how the variables (factors), and their interactions, affect the response. [18,19]

Similarly, green analytical chemistry (GAQ) has incorporated sustainable development values to the total analytical process.[20] Currently, in order to develop greener sample treatments, metrics tools have been applied to estimate the greenness of analytical methodology. Thus, the *Green Certificate* proposed by Armenta and co-workers is a metric scale that comprises parameters such as reagents toxicity and amount, waste generated, and energy consumption in the extraction analytical procedure.[21] This tool allows taking into account the analytical sustainability of the proposed methodology.

Thus, the present research proposes the development of a simple and green methodology based on SPE-packed cartridge containing multi-walled carbon nanotubes (MWCNTs) for the extraction and enrichment of HAAs, in river and reservoir water samples, previous to the analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The critical parameters involved in retention-elution efficiency were fully evaluated by a multivariate strategy named Response Surface Methodology (RSM). Furthermore, the analytical performance was studied and validated, as well as, a comparative analysis was performed to assess the greenness of the approaches intended to HAAs determination in environmental samples.

2. Material and methods

2.1. Chemicals and reagents

The *AIAs* analytical standards employed in this study were: IQ: 2-amino-3-methylimidazo-[4,5-*f*]-quinoline, MeIQ: 2-amino-3,4-dimethylimidazo-[4,5-*f*]-quinoline, MeIQx: 2-Amino-3,8-dimethyl-imidazo-[4,5-*f*]-quinoxaline, 4,8-DiMeIQx: 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]-quinoxaline, whereas that, the *amino-indole* standards were: DMIP: 2-amino-1,6-dimethyl-imidazo-[4,5-*b*]-pyridine, and PhIP: 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine. Likewise, the *amino-carbolines* analytical standards used were: Trp-P-1: 3-amino-1,4-dimethyl-5H-pyrido-[4,3-*b*]-indole; Trp-P-2: 3-amino-1-methyl-5H-pyrido-[4,3-*b*]-indole; AαC: 2-amino-9H-pyrido-[2,3-*b*]-indole; and MeAαC: 2-amino-3-methyl-9H-pyrido-[2,3-*b*]-indole. All of them were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada).

Optima® LC-MS grade acetonitrile (ACN), ultra-pure water and HCOOH were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Non-modified multi-walled carbon nanotubes (O.D. x I.D. x L: 10 nm ± 1 nm x 4.5 nm ± 0.5 nm x 3-~6 µm; number of walls: 6-8) were acquired from Sigma-Aldrich. Co., (St Louis, USA). Appropriate dilutions of a 5.0 mg L^{-1} in ACN/H_2O (1:3) stock of the HAAs were prepared daily, and were stored in screw-capped amber glass tubes at 4 °C and kept in the dark. The quantification and further assays were carried out by the analyte´s additions on the real matrices.

2.2. Instrumentation

HAAs determination was performed on an Acquity™ Ultra High-Performance LC system (Waters, Milford, USA) equipped with a binary pump and an autosampler system

(Waters, Milford, USA). The LC system was combined with a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer, and a Z-Spray™ electrospray ionization source (Waters, Milford, USA). An ACQUITY UPLC® BEH Shield RP18 analytical column (100 × 2.1 mm i.d., 1.7 µm, Waters, Milford, USA) was utilized for the chromatographic separation. An electronic microbalance with a readability of 0.1 mg (Ohaus, Switzerland), and a Minipuls 3 peristaltic pump (Gilson, Villiers-Le-Bell, France) were also employed.

2.3. Sampling and sample preparation

Surface water samples were gathered from the *Cosquin River* (31°13′00″S, 64°29′00″O) and the *San Roque Reservoir* (31°22′41″S, 64°28′10″O and 600 m a.s.l.), located in Cordoba Province, Argentina between December 2016 and January 2017 (summer season in the Southern Hemisphere). One of the main effluents of the *San Roque Reservoir* is the *Cosquin River*, which flows through the Punilla Valley (a tourist region). These bodies of water are the principal source of drinking water in the region and are frequently impacted promoting untreated sewage discharges. [22] The collected surface water samples were maintained in dark glass containers at 4 °C until analysis.

2.4. SPE packed cartridge preparation

The lab-made SPE-packed cartridge was developed using commercially available, non-modified MWCNTs. Thirty milligrams of MWCNTs bulk material was carefully introduced into a polypropylene pipette tip (50 mm length) and it was stuffed with glass wool at both ends to avoid material and fluid leaks during sample and eluents flow.

2.5. MWCNTs-SPE procedure

Initially, the SPE-cartridge was conditioned with 5 mL of ACN followed by 5 mL of ultra-pure water. For the SPE procedure, an aliquot of 50 mL of ultra-pure water spiked at 5 μg L^{-1} of a mixture of all HAAs standards was passed through the cartridge at an optimal loading flow rate (3 mL min^{-1}) by a peristaltic pump. Then, the HAAs retained on the MWCNTs-SPE were eluted with a 0.8 mL mixture of ACN/H_2O (80:20 (v/v)), with 15 mM of HCOOH, at an elution flow rate of 0.8 mL min^{-1}. Finally, the HAAs-enriched eluate was collected into a glass vial for analysis. The schematic procedure is shown in **Fig. 1.**

2.6. MWCNTs-SPE: experimental designs and optimization

The principal factors affecting the MWCNTs-SPE procedure were evaluated. Thus, two full factorial designs (2^k) currently. The analyzed factors were ACN/H_2O elution mixture, organic solvent modifier concentration, elution flow rate, and elution volume. For the experimental runs, 50 mL of the spiked surface water sample (5 μg L^{-1} of each targeted HAA) was evaluated.

A fist factorial design was applied based on 19 total runs (*k=4*): 16 runs and 3 central points. The selected-variables were included in the optimization due to their pertinence in the MWCNTs-SPE procedure. Later, a second factorial design was employed considering only the significant variables, which lead to a total of 13 runs (k=3), including 8 runs and 5 central points. These experimental systems were used to assess and select significant factors and their experimental region. The extractive recovery percentage (ER (%)) of targeted HAAs, calculates as shown in **Eq. 1,** were used to evaluate the analytical performance and response in DOE-optimization.

$$ER\% = \frac{\text{Peak Area}_{\text{Spiked water sample}}}{\text{Peak Area}_{\text{Standard in pure solvent}}} \times 100 \quad \textbf{(1)}$$

Moreover, significant experimental factors were employed to build a central composite design (CCD) to find out the optimal analytical conditions for all the responses. This experimental design was analyzed based on 13 runs, 2k+2k+Cp = 4+4+5, which were in agreement with the combinations of the selected independent variables. A single block rotatable design (α = 1.414), including five central points, was built. In each assay, the ER (%) values of the targeted analytes were examined. Additionally, the desirability function was used to select the optimal experimental conditions evaluated in the CCD according to the RSM. [19] All proposed DOEs were evaluated using Design Expert 8.0.0 (Stat-Ease, Inc., Minneapolis, USA).

2.7 UHPLC-MS/MS analysis

The analysis was performed using a binary mobile phase composed of a variable proportion of water (A) and acetonitrile (B), both with 0.1 % (v/v) of HCOOH, which was delivered at 0.25 mL min^{-1}. A gradient elution started at 90 % A, which was held during 0.5 min, afterward 3.5 min gradient to 25 % A, such composition was kept for 3.5 min. Finally, the system was returned to the initial in 0.2 min gradient, where it was held for 0.8 min. The column temperature was kept constant at 30 °C.

The source operational conditions were as follows: capillary voltage, 2.7 kV; extractor voltage, 1.0 kV; source temperature, 150 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 L h^{-1}; desolvation gas flow rate, 400 L h^{-1}. Ultrapure nitrogen and argon were used in the ionization source and collision gases; respectively. For each HAA, the interface was operated in a positive mode and the data were acquired in a multiple reaction

monitoring mode (MRM). To select the fragmentation patterns, each analyte solution at a concentration level of 0.5 mg L^{-1} was injected via direct infusion (using a syringe pump) into the MS. The product ion scan mass spectra were recorded. The retention time (RT) and MS/MS settings for each compound are summarized in **Table 1**. Representative chromatograms for all HAAs under study are depicted in **Fig. 2**.

2.8. Parameters for the greenness assessment of the sample treatment

As stated above, the "*Green Certificate*" is based on the idea of an analytical eco-scale to evaluate how much green a sample treatment is. Thereby, the green efficiency of the proposed MWCNTs-SPE sample treatment for HAAs was compared against currently reported methodologies for the analysis of HAAs in surface water.

The parameters included in the green assessment were defined as penalty points (*PP*) for reagent volume (PP_{RV}), waste volume generated (PP_W), and for energy consumption (PP_E). Where *V* represents the reagent volume and *W* refers to the waste volume produced. The *PP* were calculated using **Eqs. (2)** and **(3)**; respectively. [21]

$$PP_{RV} = 0.61 \pm 0.05\, V^{(0.31 \pm 0.02)} \quad \textbf{(2)}$$

$$PP_w = 0.50 \pm 0.08\, W^{(0.40 \pm 0.02)} \quad \textbf{(3)}$$

While PP_E was calculated considering the power-hour involved in the proposed SPE. Less or equal than 0.1 kWh per sample involves 1 *PP*, from 0,1 to 1.5 kWh per sample refers 2 *PP*, and more than 1.5 kWh per sample, 3 *PP*.[23] Moreover, *PP* were calculated per sample analysis, thus the total penalty points of the MWCNTs-SPE procedure were defined as PP_S.

3. Method validation

The development of an analytical method involves a validation stage to ensure reliability, reproducibility, and accuracy. The figures of merit calculated in the present methodological development, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), selectivity, and inter-day precision expressed as the relative standard deviations (RSD (%)), are summarized in **Table 2**.

The MWCNTs-SPE performance was assayed by extracting spiked surface water samples from river and reservoir water. Spiked samples at three concentration levels (3 replicates at 0.5, 1, and 2 or 5 μg L^{-1}) were studied to evaluate the analytical performance mentioned. The herein proposed method showed proper linearity, with regression coefficients (R^2) in the range of 0.990 to 0.998. The linearity of the fitted model agreed with the *F*-test.

Furthermore, the LOD and LOQ were calculated following the International Union of Pure and Applied Chemistry (IUPAC) recommendations according to **Eqs. 4** and **5.** [24]

$$\mathrm{LOD} = \frac{3.3 S_{y/x}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2}} \quad \textbf{(4)}$$

$$\mathrm{LOQ} = \frac{10 S_{y/x}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2}} \quad \textbf{(5)}$$

The LODs values ranged from 0.06 μg L^{-1} to 0.23 μg L^{-1} and the LOQs values from 0.17 μg L^{-1} to 0.69 μg L^{-1}. The RSDs (%) varied from 1.7 % to 6.4 % for all the HAAs under study. The enrichment factor (EF) values were calculated for all the analytes, which were in a range from 59 to 63 folds.

The accuracy of the analytical method was assessed based on the Recovery (%) of the HAAs from the real samples treated according to Section 2.5. The Recovery (%) values for the studied HAAs varied from 91.6 % to 105.3 %, as shown in **Table 2.**

As known, ion suppression/enhancement occasioned by matrix effect continues being a foremost concern in LC-ESI-MS/MS analysis.[25] Thus, matrix effect was examined by comparison of the calibration curves slopes (*b*), which were created with calibrants of all HAAs in pure solvent and spiked river and reservoir surface water samples. The percentage of the quotient of the slopes was applied to quantify the ion signal suppression/enhancement extension (SSE (%)) (**Eq. 6)**.

$$\text{SSE (\%)} = 100 - \left(\frac{\text{b}_{\text{Spiked water sample}}}{\text{b}_{\text{Standard in pure solvent}}} \times 100\right) \qquad \textbf{(6)}$$

From the findings, after applying the MWCNTs-SPE method, a non-significant matrix effect was observed for the HAAs under study. This fact might be explained by the sample clean-up effect of the MWCNTs-SPE step due to a selective retention/elution of the targeted HAAs in both river and reservoir water samples. In concordance with the results mentioned above, precision, recoveries and detection limits of the developed analytical methodology were compatible with the HAAs trace levels present in aqueous samples. Since non-effect of the matrix on HAAs signal, **Eq. 7** was employed for the calculation of Recovery (%) in the samples under study, at the same spiking level.

$$\text{Recovery \%} = \frac{\text{Peak Area}_{\text{Spiked water sample}} - \text{Peak Area}_{\text{Water sample}}}{\textit{Peak}\ \text{Area}_{\text{Standard in pure solvent}}} \times 100 \qquad \textbf{(7)}$$

4. Results and discussion

4.1. Extraction procedure. Preliminary studies

To evaluate the overall extraction efficiency based on the retention/elution of the HAAs using MWCNTs-SPE, previous experiments intended to assay the sample volume and the loading flow rate. As a result, the MWCNTs-SPE device prepared as mentioned in Section 2.4 was used to efficiently load a 50 mL volume of water at a 3 mL min^{-1} flow rate. Under these conditions, experimental designs were built for the extraction/clean-up strategy optimization.

4.2. MWCNTs-based SPE optimization

4.2.1. Selection of significant factors and experimental region

In a screening phase, two full factorial designs were built to determine the main variables (factors) with a significant influence on the MWCNTs-SPE procedure. In concordance with the experimental design, low, central, and high levels of the variables were designated as (-), (0), and (+), respectively. A two-level-four-factors (2^4) full factorial design consisting of 16 runs and 3 central points was performed in order to determinate the influence of the following four variables: (A): ACN percentage in elution mixture, (B): HCOOH concentration as organic modifier concentration, (C) elution flow rate, and (D): eluent volume. The experimental region for the selected variables at minimum, maximum, and central point levels were as follow: (A): 40, 60 and 50 % of ACN; (B): 0.004, 0.016 and 0.08 mM of HCOOH; (C): 0.15, 0.50 and 0.33 mL min^{-1} as elution flow rate; and (D):500, 1000, and 750 μL as eluent volume. The ER (%) values of all targeted-HAA of spiking surface

water samples (5 μg L^{-1}) were analyzed. In **Table S1 (ESI)**, the experimental matrix and the obtained responses of the primary screening step are detailed.

The performance of the suggested model was evaluated by ANOVA assumptions of the mutagenic IQ (polar) and Trp-P-2 (less-polar) model analytes, and the results are outlined in **Tables S2 and S3** (ESI). The influential factors were defined considering the Pareto charts. **Fig. 3, 1A** and **1B** illustrate Pareto charts for IQ and Trp-P-2; respectively. The analysis of the variables on the ten responses allowed to conclude that the only factor with no significant influence on the MWCNTs-SPE methodology was the elution solvent volume (D).

Therefore, a second two-levels-three-factors (2^3) full factorial design of 8 runs and 5 central points were needed. The following variables and ranges were considered: (A): 40, 80 and 60 % of ACN; (B): 1.6, 15.9 and 8.7 mM of formic acid; and (C): 0.2, 0.8 and 0.5 mL min^{-1} as elution flow rate. The obtained responses are summarized in **Tables S3 and S4 (ESI).**

The results for the analysis of variance were examined (ANOVA test results for IQ and Trp-P-2 are shown in **Table S5, and S6 (ESI)**; respectively). Under the obtained Pareto chart for the second factorial design, only two out of three factors, A and B, were observed as statistically significant for the MWCNTs-SPE methodology (**Fig. 3, 2A** (IQ) and **2B (**Trp-P-2)). Besides, the lack of fit (not significant) and curvature (significant) suggested that the selected experimental region for the mentioned factors was optimal to assay the suitable conditions and to obtain the optimal responses using the proposed approach.

4.2.2. Multi-response optimization

As mentioned previously, only two factors showed a significant effect on the MWCNTs-SPE procedure. Consequently, a second-order design was carried out. A multi-response optimization was achieved with a CCD of 13 runs, (2^k+2k+Cp = 4+4+5) based on combinations of the selected variables. The following variables (and ranges) in the CCD design were studied: (A): 26.4 - 93.6 % of ACN, and (B): 0.0 (no formic acid addition) - 21.0 mM HCOOH. The alpha value used in the design was compatible with the rotatable distribution of the predictive variance. [19] The whole experimental combinations and their ERs (%) are listed in **Table S7 (ESI).**

The model coefficients of the CCD were computed by backward multiple regression and validated by ANOVA. Outliers and influential points were removed or evaluated using the Cook's distance, differences between betas test (DFBETAS) and fitted test (DFFITS). As mentioned above, ANOVA assumptions, the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2adj) were assessed as well. The values of R^2 and R^2 *adj* indicated a suitable relationship between the experimental data and the fitted model (this information is listed in **Table S8 (ESI).** All responses were optimized using the desirability function, which implicates the modification of each expected response variable to a desirability value that varies from zero (undesirable response) to one (optimal/expected response). Once the function is defined for each experimental response, a general function is obtained and represents the integral desirability function. Such function usually is determined as the weighted geometric average of the individual desirability functions.[26] Thereby, the desirability function for the targeted-HAAs response optimization along with its maximization (ER (%)), employing the proposed MWCNTs-SPE approach, were determined. As a result, the experimental conditions corresponding to a maximum of the desirability function (D=0.917) were as follow ACN/H_2O (80:20 (v/v)) elution mixture

composition and HCOOH concentration (15 mM). Consequently, the desirability values under optimal conditions are detailed in **Fig. 4**, resulting in a combined desirability value of 0.917. The obtained results were corroborated and compared with the theoretical ones.

4.2.3. Application to real samples

To evaluate the efficiency of the proposed MWCNTs-SPE, river and reservoir water samples were collected as described in Section 2.3. The findings of the analysis indicated that the levels of the HAAs and their distribution were different in both types of sample. Seven out of ten HAAs were detected in the *Cosquin River*, among them, the mutagenic MeIQ, MeIQx, 4,8-DiMeIQx, Trp-P-1, Trp-P-2, AαC, and MeAαC. Additionally to these compounds, IQ and PhIP, were quantified in *San Roque Reservoir*, while DMIP resulted to be below the detection limits. Concentration levels of all HAAs found at *Cosquin River* ranged from 0.21 μg L^{-1} (Trp-P-1) to 0.56 μg L^{-1} (MeIQ), meanwhile in the *San Roque Reservoir* varied from 0.37 μg L^{-1} (AαC) to 0.93 μg L^{-1} (Trp-P-1) as shown in **Table 3**. As described by other authors, the occurrence of these harmful compounds may be explained by shedding from sewage effluents and human waste into surface water. [27-32] As mentioned above, water surface from the *Cosquin Rive*r and the *San Roque Reservoir* have been affected by untreated sewage effluents, which might be one of the main sources of the occurrence of HAAs in these surface waters. The slight higher concentration levels in the *San Roque Reservoir* is consistent with more stagnant water than the *Cosquin River* and, besides, with the presence of important tourist activity around this area, increasing sewage effluents shedding into the surface water.

The proposed methodology is comparable, and even better in its analytical performance, to other SPE approaches reported in the literature (**Table 4)**. The use of a simple

clean-up strategy as the MWCNTs-SPE procedure allowed decreasing the matrix effect and analysis time. Thus, the proposed methodology included the fast and efficient analysis of ten harmful HAAs in surface water intended to human consumption. It is important to notice as shown in **Table 4**, that no literature reports have been found about the presence of HAAs in water surface samples, particularly for the mutagenic AIAs group, i.e. IQ, MeIQ, MeIQx, and 4,8-DiMeIQx. Thus, AIAs and polar HAAs, such as IQ, MeIQ, MeIQx, as well as the imidazo-pyridine compound DMIP, were retained and eluted from the MWCNTs sorbent-based SPE as sample treatment for the analysis of surface water. In other words, the MWCNTs system provided an efficient extraction and concentration of the targeted analytes with different polarities.

A recent study reported by Basheer, [32] describes an approach based on a μ-SPE device intended for the extraction of seven HAAs from water samples, associated with liquid chromatography with fluorescence detection (LC-FD). Although such μ-SPE device allowed an effective recovery of the targeted analytes, the total chromatographic run comprised 30 min for seven HAAs. [32] On the other hand, LOD and LOQ values for the ten HAAs herein reported were compatible with concentration levels found in the analyzed water samples. In this sense, in order to compare the LOD and LOQ values informed for this methodology to others, it is required a harmonized criteria and to obtain comparable figures of merit. In this sense, the signal-to-noise (S/N) approach was employed by Basheer to calculate LOD and LOQ, while in the proposed study, the approach based on the IUPAC's recommendations was employed.

4.2.4 Assessing the greenness of the methodology

A green evaluation was performed for the proposed MWCNTs-SPE sample treatment. Thus, a comprehensive assessment was encompassed for the *PPs* calculation considering only the extraction or sample treatment step. Since this crucial stage in the analysis flow usually requires extractive reagents in an appropriate amount, their reduction might decrease the negative impact on the environment. As can be seen in **Table 5**, the *Green Certificate* (i.e. 83.70) for the proposed MWCNTs-SPE was stated as "B" (scale from A to G, being A the greenest one and G the less-green one), [21] and greener than other methodologies reported before. The main source of contribution for total of penalty points were the sample volume and MWCNTs sorbent (> 50 %) and 30 % of the penalty points were promoted by regents, i.e. ACN and HCOOH. Although the assessment of the penalty points for the MWCNTs sorbent into SPE packed cartridges was calculated per sample, the cartridges were reused until 10 fold (i.e including samples and calibrants solutions/spiked samples). Also, in order to ensure the yield of MWCNTs-SPE packed cartridges before disposing, both in terms of sample/analyte carryover and targeted analytes retention/elution from the sorbent, were evaluated. In this sense, the benefit in diminishing sorbent amount, might reduce waste production, a lower impact on the environment, and a low-cost analysis.

On the other hand, some selected reports intended to HAAs determination in water samples based on the use of SPE were listed in **Table 5**. Blue-rayon hanging method has been used as HAAs extraction procedure. However, in order to achieve the HAAs desorption from Blue-rayon, a relatively higher amount of several regents (harmful solvents) have been used. Likewise, SPE-based approaches (Strata-X® SPE cartridge), [31] greener methodologies with a higher value of Green Certificate, were obtained due to the lower amount of solvents used during extraction/elution of the analytes (**Table 5**). However, the inconvenience of a

large volume of sample employed (i.e. waste production) increased the penalty point value. Although, the μ-SPE device described by Basheer [32] (**Table 5**) for HAAs determination in water resulted to be sorted as "B class" (Green Certificate: 81.49), only informed amounts of reagents used in the sample treatment were considered for the calculation of the penalty points. However, during the preparation of the μ-SPE device, the authors described such a device was conditioned and stored employing unknown amounts of MeOH and further sonication step, increasing the reagent amount and energy consumption, thus a likely diminution of the greenness of the methodology.

5. Conclusions

A rapid and efficient MWCNTs-SPE methodology was successfully developed and applied for the analytical determination of targeted HAAs in river and reservoir waters. Through a multivariate strategy, optimization of the proposed methodology was achieved, attaining a rapid, simple, sensitive, and green sample treatment approach. Besides, MWCNTs application demonstrated a suitable retention capacity on the extraction of ten HAAs targeted analytes under study and the effective elimination of matrix effects. Besides, the analytical methodology complied with the main points of Green Chemistry, through the low consumption of organic solvents and simplicity, turned the whole procedure in an environmentally friendly tool for analysis. Also, it is remarkable bearing in mind whether the influence of the risk associated with amount and reagent during sample preparation is lessening or replaced for a less-toxic one, it might allow decreasing both waste production and diminishes the exposure to harmful compounds, such as HAAs during the analysis.

Furthermore, this is the first report about the occurrence of both polar and less-polar HAAs in river and reservoir surface water samples. The findings provided information about water quality and will promote further studies to address HAAs health and environmental impacts. This work might add important information on the quality of *Cosquin River* and *San Roque Reservoir* and could also contribute to give a useful analytical methodology to determinate HAAs in real samples from different sources.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. Scheme of the experimental MWCNTs-SPE procedure applied for sample clean-up, extraction and enrichment of the selected HAAs. A: ACN/H_2O elution mixture; B: Organic modifier concentration; C: Elution solvent flow rate; D: Elution volume; SV: Sample volume; LF: Loading flow rate; P.P: Peristaltic Pump; I: loading sample step; II: eluting sample step.

Fig. 2. Chromatograms of the HAAs determined by UHPLC-(+)ESI-MS/MS: (**A**) DMIP (RT: 1.23 min); (**B**) IQ (RT: 1.62 min); (C) MeIQ (RT: 1.93 min); (**D**) MeIQx (RT: 2.13 min); (**E**) 4,8-DiMeIQx (RT: 2.54 min); (**F**) PhIP (RT: 2.80 min); (**G**) Trp-P-1 (RT: 2.94 min); (**H**) AαC (RT: 3.12 min); (**I**) Trp-P-2 (RT: 3.50 min); (**J**) MeAαC (RT: 3.59 min).

Fig 3. A 2^4 full factorial design (1): A: ACN/H_2O elution mixture; B: HCOOH concentration; C: elution solvent flow rate. 1A): Pareto chart for IQ and 1B): Pareto chart for Trp-P-2. A 2^3 full factorial design (2): A: ACN/H_2O elution mixture; B: HCOOH concentration. 2A): Pareto chart for IQ and 2B): Pareto chart for Trp-P-2.

Fig. 4. Values obtained from the desirability function for each compound considering the variables under study. Combined desirability of the ten selected analytes. A: ACN/H_2O elution mixture; B: HCOOH concentration; R: response (R1-DMIP; R2-IQ; R3-MeIQ; R4-MeIQx; R5-4.8-DiMeIQx; R6-PhIP; R7-AαC; R8-MeAαC; R9-Trp-P-1; R10-Trp-P-2).

Highlights

- A MWCNTs-based SPE strategy followed by LC-MS/MS was developed.
- Experimental extraction conditions were chemometrically optimized.
- Trace levels of HAAs in surface waters were determined.
- Comparative analysis using green metrics tools was applied.

Table 1. Optimized chromatographic retention times and MRM experimental conditions for (+)ESI-MS/MS determination.

Analyte (MW)	RT (min)	Precursor ion (*m/z*)	Cone (V)	Quantification		Confirmation	
				Product ion (*m/z*)	Collision (V)	Product ion (*m/z*)	Collision (V)
DMIP (162)	1.23	163	38	-	20	148	20
IQ (198)	1.62	199	25	154	32	184	32
MeIQ (212)	1.93	213	35	145	26	198	26
MeIQx (213)	2.13	214	33	173	31	199	31
4,8DiMeIQx (227)	2.54	228	44	187	26	213	26
PhIP (224)	2.80	224	45	183	30	210	30
Trp-P-1 (211)	2.94	212	35	195	19	168	19
AαC (183)	3.12	184	25	167	25	140	25
Trp-P-2 (197)	3.50	198	27	181	30	154*	30
MeAαC (197)	3.59	198	25	181	25	154/129*	25

RT: retention time. **m/z* 154 was used as confirmation ion for Trp-P-2 and MeAαC

Table 2. Linearity (R^2), lineal range (LR), detection (LOD) and quantification (LOQ) limits, percentage relative standard deviation (RSD (%)) and Recovery (%) of the MWCNTs-SPE following by UHPLC-MS/MS method.

Compound	R^2	LR (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	EF	RSD (%) (*n=3*)	Recovery (%) (*n=3*)
DMIP	0.998	0.25 - 3.00	0.08	0.25	64	5.5	101.5±3.8
IQ	0.997	0.65 - 2.00	0.21	0.65	60	2.9	98.0±2.6
MeIQ	0.994	0.70 - 2.00	0.23	0.69	61	6.4	94.0±2.4
MeIQx	0.995	0.25 - 2.00	0.06	0.19	60	3.6	97.1±1.4
4,8-DiMeIQx	0.990	0.30 - 5.00	0.09	0.28	59	3.8	96.6±2.3
PhIP	0.997	0.25 - 2.00	0.07	0.22	61	3.9	98.5±0.5
Trp-P-1	0.998	0.25 - 5.00	0.08	0.22	62	3.8	99.3±1.1
AαC	0.996	0.20 - 2.00	0.06	0.17	61	1.7	98.0±1.2
Trp-P-2	0.997	0.25 - 2.00	0.08	0.25	61	3.5	98.7±2.2
MeAαC	0.995	0.37 - 2.00	0.12	0.37	63	6.3	101.7±3.2

EF: enrichment factor. Inter-day precision expressed as RSD (%).

Table 3. Occurrence concentration levels of HAAs in river and reservoir surface water samples.

	Cosquin River	*San Roque Reservoir*
HAAs	**Concentration ($\mu g\ L^{-1}$)**	**Concentration ($\mu g\ L^{-1}$)**
DMIP	ND	ND
IQ	ND	<LOQ
MeIQ	0.56	0.92
MeIQx	0.24	0.71
4,8-DiMeIQx	<LOQ	0.62
PhIP	ND	0.56
Trp-P-1	0.32	0.93
AαC	0.21	0.37
Trp-P-2	0.51	0.76
MeAαC	<LOQ	0.56

ND: not detected; LOQ: limit of quantification

Table 4. Summary of reported studies for the analysis of HAAs in surface water

Sample Source Surface Water	Sampling method-Sample Preparation	Detection Method	HAAs analyzed	Reported Concentration Values	LOD	*Ref.*
River water Yodo River - Japan	Blue-rayon hanging method	HPLC-Electrochemical detector (MeIQx) and FD (Trp-P-1, Trp-P-2 and PhIP)	MeIQx PhIP Trp-P-1 Trp-P-2	ND-365 ng g^{-1} BRE ND-118 ng g^{-1} BRE ND-530 ng g^{-1} BRE ND-840 ng g^{-1} BRE	NM	27
River water Yodo River - Japan	Blue-rayon hanging method	HPLC-UV and MS	Trp-P-2	8-9 ng L^{-1} (trapped by blue-rayon resin)	NM	28
River water Danube River (Vienna) Austria	Blue-rayon hanging method; XAD-2 hexane, acetone	GC-MS and GC-NPD	AαC IQ Trp-P-1	0.44±0.44 ng g^{-1} BRE 1.78±0.17 ng g^{-1} BRE 0.14±0.02 ng g^{-1} BRE	NM	29
River water North Kyusyu - Japan	Blue-rayon hanging method	HPLC-Fluorescence Detection	Trp-P-1 Trp-P-2	ND - 6 ng g^{-1} BRE 4 - 13 ng g^{-1} BRE	NM	30
Tap water (Ciudad Real) and river water (Segovia) - Spain	SPE (Strata-X® cartridge)	HPLC-amperometric detection at glassy carbon electrode modified with multiwall carbon nanotubes	AαC Harman MeAαC Nor-Harman Trp-P-1 Trp-P-2	ND	4.0 µg L^{-1} 8.0 µg L^{-1} 7.0 µg L^{-1} 4.0 µg L^{-1} 6.0 µg L^{-1} 3.0 µg L^{-1}	31
Seawater Treatment plant Saudi Arabia	Portable pump coupled with µ-SPE (alumina) for on-site extraction method	HPLC-FD	AαC Harman Nor-Harman PhIP Trp-P-1 Trp-P-2	ND 0.07 µg L^{-1} ND 0.13 µg L^{-1} ND	0.014 µg L^{-1} 0.019 µg L^{-1} 0.021 µg L^{-1} 0.026 µg L^{-1} 0.007 µg L^{-1} 0.004 µg L^{-1}	32
Surface water samples (Argentina)	**SPE (MWCNTs)**	**UHPLC-MS/MS**	**IQ** **MeIQ** **MeIQx** **4,8-DiMeIQx** **DMIP** **PhIP** **Trp-P-1** **Trp-P-2** **AαC** **MeAαC**	**ND-0.48 µg L^{-1}** **0.56-0.92 µg L^{-1}** **0.24-0.71 µg L^{-1}** **0.16-0.62 µg L^{-1}** **ND** **ND-0.56 µg L^{-1}** **0.32-0.93 µg L^{-1}** **0.51-0.76 µg L^{-1}** **0.21-0.37 µg L^{-1}** **0.35-0.56 µg L^{-1}**	**0.21 µg L^{-1}** **0.23** **0.06** **0.09** **0.08** **0.07** **0.08** **0.08** **0.06** **0.12**	**This work**

BRE: Blue-Rayon extract Equivalent; ND: Not Detected; NA: Not Applied; NM: Not Mentioned.

Table 5. Comparative *Green Certificate* for HAAs extraction methods from natural water samples.

Extraction Technique	Sample volume (mL)	Reagent amount (mL)	PP_{RV}	Hazard-(PP_{RH})	Subtotal PP_R*	PP_W	PP_E	Total PPs	Green Certificate**	*Ref.*
Blue-rayon hanging method	1000	MeOH (156.8) Ammonia (3.2)	3.49 0.97	6 4	24.86	34.86	3	62.72	37.28 "F"	27
	-	MeOH (98) Ammonia (2)	2.99 0.83	6 4	21.30	6.59	1	28.89	71.11 "C"	28
	25	*n*-Hexane (100) Acetone (100) MeOH (0.2) DMF (0.01) Ethylacetate (0.02)	3.01 3.01 0.38 1.14 0.18	6 4 6 6 4	34.06	16.56	2	52.62	47.38 "E"	29
	-	MeOH (156.8) Ammonia (3.2)	3.49 0.97	6 4	24.86	1.58	4	30.44	69.56 "C"	30
SPE (Strata-X® cartridge)	10	MeOH (6.7) ACN (1.5)	1.24 0.75	6 4	10.44	4.15	3	17.59	82.41 "B"	31
μ-SPE	100	MeOH (0.4) ACN (0.1)	0.48 0.31	6 4	4.17	11.34	3	18.51	81.49 "B"	32
MWCNTs-SPE	**50**	**ACN (5.6)** **HCOOH (0.05)**	**1.17** **0.05**	**4** **6**	**5.00**	**8.30**	**3**	**16.30**	**83.70** **"B"**	**This study**

* Subtotal $PP_R = PP_{RV} \times PP_{RH}$ [21] ** Green certificate= 100 - Total PPs [21]

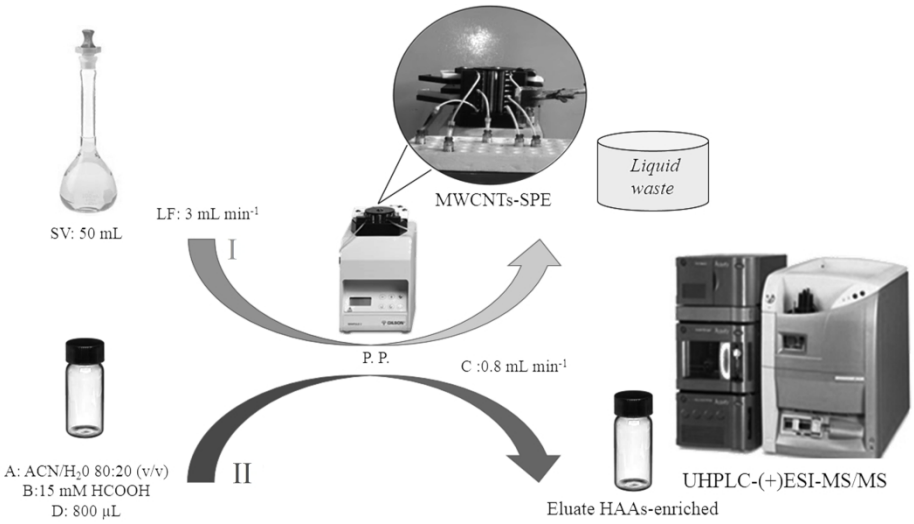

Fig. 1. Scheme of the experimental MWCNTs-SPE procedure applied for sample clean-up, extraction and enrichment of the selected HAAs. A: ACN/H2O elution mixture; B: Organic modifier concentration; C: Elution solvent flow rate; D: Elution volume; SV: Sample volume; LF: Loading flow rate; P.P: Peristaltic Pump; I: loading sample step; II: eluting sample step.

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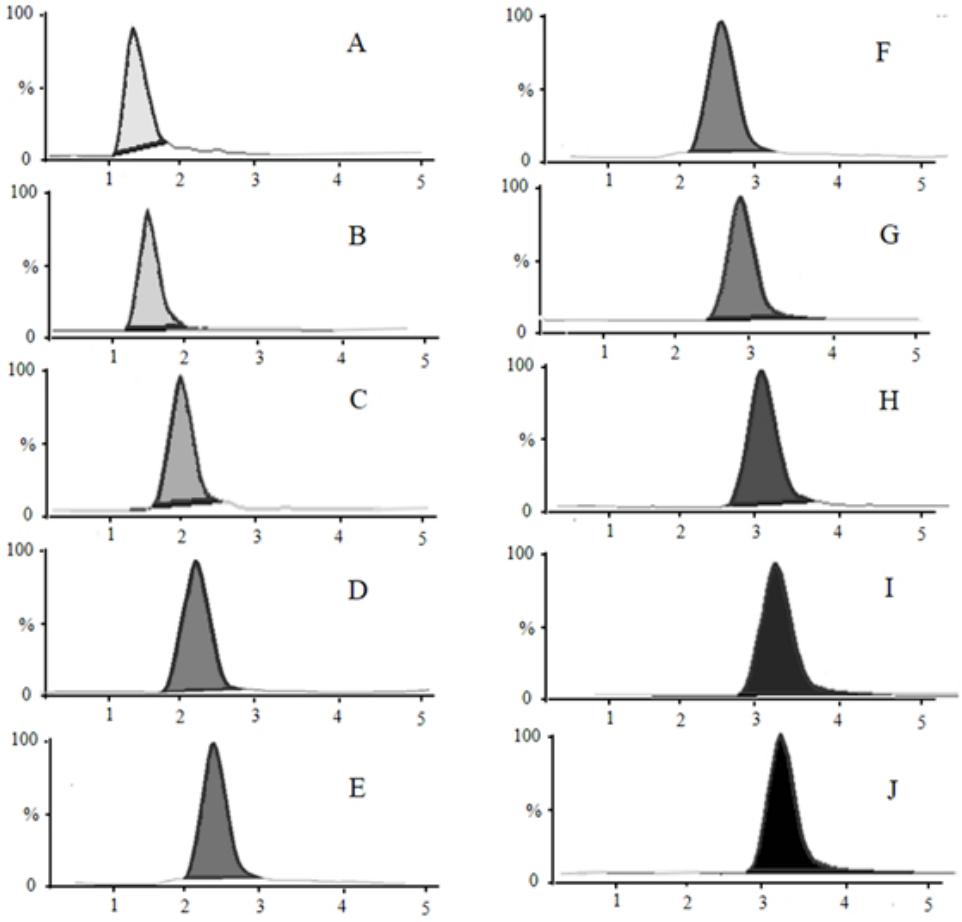

Fig. 2. Chromatograms of the HAAs determined by UHPLC-(+)ESI-MS/MS: (A) DMIP (RT: 1.23 min); (B) IQ (RT: 1.62 min); (C) MeIQ (RT: 1.93 min); (D) MeIQx (RT: 2.13 min); (E) 4,8-DiMeIQx (RT: 2.54 min); (F) PhIP (RT: 2.80 min); (G) Trp-P-1 (RT: 2.94 min); (H) AαC (RT: 3.12 min); (I) Trp-P-2 (RT: 3.50 min); (J) MeAαC (RT: 3.59 min).

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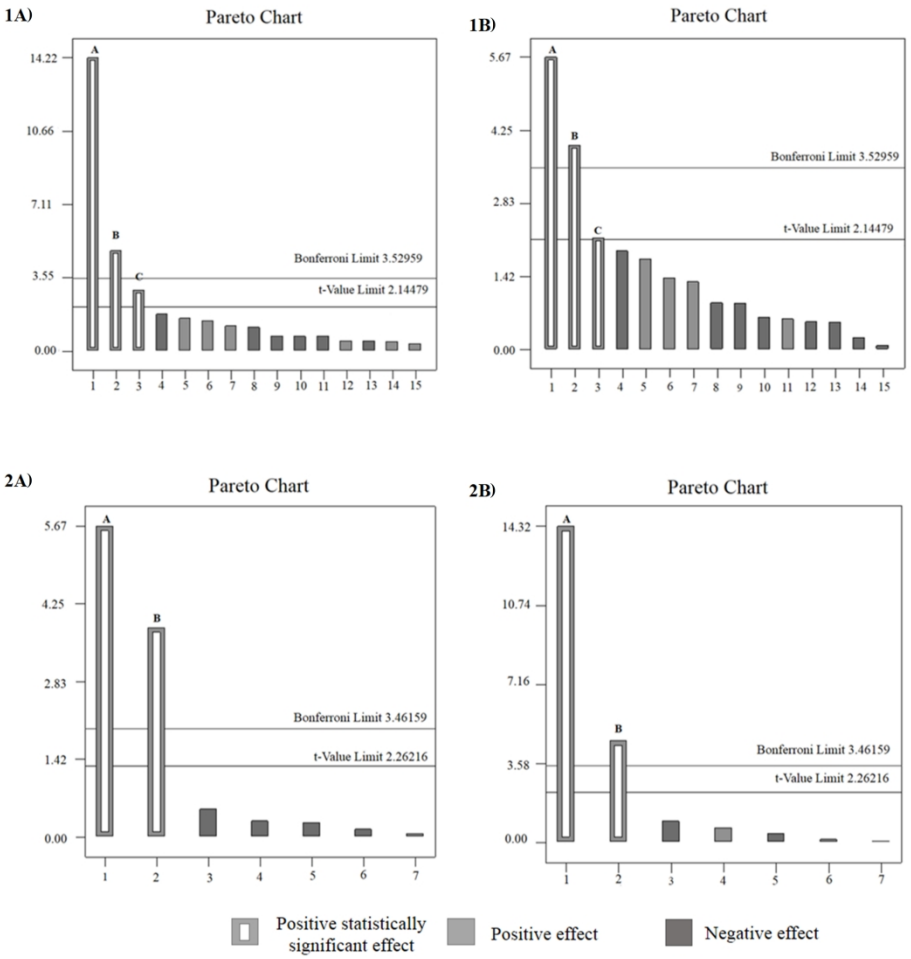

Fig 3. A 24 full factorial design (1): A: ACN/H2O elution mixture; B: HCOOH concentration; C: elution solvent flow rate. 1A): Pareto chart for IQ and 1B): Pareto chart for Trp-P-2.
A 23 full factorial design (2): A: ACN/H2O elution mixture; B: HCOOH concentration. 2A): Pareto chart for IQ and 2B): Pareto chart for Trp-P-2.

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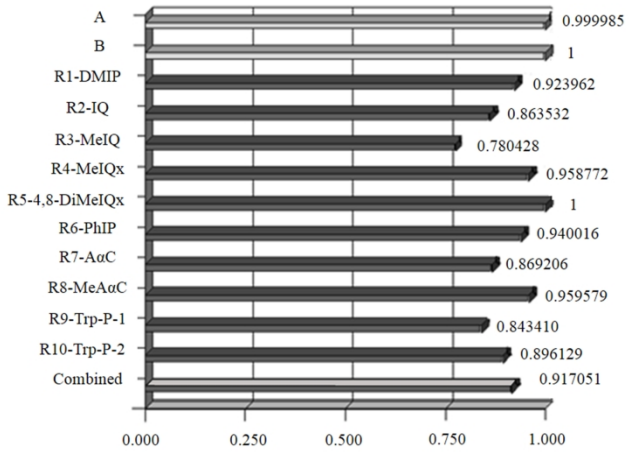

Fig. 4. Values obtained from the desirability function for each compound considering the variables under study. Combined desirability of the ten selected analytes. A: ACN/H2O elution mixture; B: HCOOH concentration; R: response (R1-DMIP; R2-IQ; R3-MeIQ; R4-MeIQx; R5-4.8-DiMeIQx; R6-PhIP; R7-AαC; R8-MeAαC; R9-Trp-P-1; R10-Trp-P-2).

338x190mm (300 x 300 DPI)

- Electronic Supplementary Information -

Multi-response optimization of a green solid-phase extraction for the analysis of heterocyclic aromatic amines in environmental samples

Romina Canales[a], Leonardo Mariño-Repizo[a], Mario Reta[b], Soledad Cerutti[a,*]

[a] *Instituto de Química de San Luis, Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de San Luis. Facultad de Química, Bioquímica y Farmacia Bloque III, Avda. Ejército de los Andes 950, San Luis, Argentina CP: 5700.*

[b] *Laboratorio de Investigación y Desarrollo de Métodos Analíticos (LIDMA), División Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (B1900AJL) 1900 La Plata, Argentina.*

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*Corresponding author:

E-mail address: E-mail: ecerutti@gmail.com (S. Cerutti)

Phone: +54-0266-4520300 (*1311)

Table S1. Experiments and responses of the HAAs in the 2^4 full factorial design.

Run	A	B	C	D	ERs (%)									
	ACN (%)	HCOOH (mM)	Elution flow rate (mL min^{-1})	Elution volume (µL)	DMIP	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP	Trp-P1	AαC	MeAαC	Trp-P2
1	50	0.008	0.33	750	73.1	75.9	78.3	73.7	76.6	82.4	72.3	85.9	78.9	73.1
2	60	0.016	0.15	500	93	90.8	92.5	88.04	88.4	87.9	82.7	87.5	89.9	82.5
3	60	0.004	0.15	1000	80.7	82.5	82.2	79	80.8	79.9	69.4	79.5	77.2	71.9
4	60	0.004	0.50	1000	88.9	90.8	91.3	82.3	86.9	74.7	77.9	69.9	74.2	70.1
5	40	0.016	0.50	1000	63.1	67.6	60.5	75.1	65.7	57.6	74.9	66.3	69.8	72.6
6	60	0.004	0.50	500	85.9	82.6	84.5	82.7	80.3	80.3	70.9	82.3	65.3	68.4
7	50	0.008	0.33	750	75.9	71.3	77.2	79.1	73.9	78.8	79.3	84.2	81.1	78.5
8	60	0.004	0.15	500	82.1	83.1	86.9	80.2	82.5	71	66.1	73.5	73.8	82.7
9	40	0.004	0.50	1000	58	55.7	56.8	62.5	53.8	58.6	70.7	60.1	71.2	53.5
10	40	0.004	0.50	500	56.1	53.9	42.7	54.3	56.2	50.1	59.1	56.4	47.5	69.2
11	40	0.016	0.15	500	56.9	54.9	59.2	53.8	51.8	47.1	52.8	41.7	54.8	51.8
12	60	0.016	0.50	1000	94.1	97.1	96.4	98.5	92.9	97.2	95.5	96.5	92.7	91.1
13	50	0.008	0.33	750	72.9	76.2	72.9	75.27	77.9	75.6	76.6	78.5	73.9	80.5
14	40	0.004	0.15	1000	38.1	42.9	37.8	44.02	52.7	61.4	38.2	50.4	50.5	41.9
15	40	0.016	0.15	1000	62.1	68.2	61.1	70.5	70.2	68.4	86.8	61.2	65.8	70.6
16	60	0.016	0.50	500	92	92.5	97.4	95.74	94.2	99.9	93.1	97.06	92.8	95
17	60	0.016	0.15	1000	82.1	86.5	85.9	84.4	84.1	75.1	76.1	80.8	87.9	83.7
18	40	0.016	0.50	500	60.1	63.4	59.7	58.7	66.3	69.5	81.9	72.6	70.5	72.1
19	40	0.04	0.15	500	30.2	41.1	36.7	38.4	34.1	38.5	30.5	36.7	35.58	40.7

Table S2. ANOVA test results obtained from the first (2^4) full factorial design for IQ response (model analyte).

Source	Sum of Squares	*df*[a]	Mean Square	F_{value}[b]	P_{value}[c]	Prob> *F*
Model	4834.67	3	1611.56	78.19	<0.0001	*significant*
A	4166.70	1	4166.70	202.1	<0.0001	
B	488.41	1	488.41	23.70	<0.0002	
C	179.56	1	179.56	8.71	<0.0105	
Curvature	14.15	1	14.15	0.69	0.4212	*not significant*
Residual	288.55	14	20.61			
Lack of Fit	273.47	12	22.79	3.02	0.2754	*not significant*
Pure error	15.09	2	7.54			
Cor total	5137.38	18				

[a] Degrees of freedom; [b] Test for comparing model variance with residual (error) variance; [c] Probability of seeing the observed *F* value if the null hypothesis is true.

Table S3. ANOVA test results obtained from the first full (2^4) factorial design for Trp-P-2 response (model analyte).

Source	Sum of Squares	*df*[a]	Mean Square	F_{value}[b]	P_{value}[c]	Prob> *F*
Model	3059.53	3	1019.84	17.52	<0.0001	*significant*
A	1870.56	1	1870.56	32.14	<0.0001	
B	915.06	1	915.06	15.72	<0.0001	
C	273.90	1	273.90	4.71	0.0478	
Curvature	142.26	1	142.26	2.44	0.1403	*not significant*
Residual	814.86	14	58.20			
Lack of Fit	785.55	12	65.46	4.47	0.1973	*not significant*
Pure error	29.31	2	14.65			
Cor total	4016.65	18				

[a] Degrees of freedom; [b] Test for comparing model variance with residual (error) variance; [c] Probability of seeing the observed *F* value if the null hypothesis is true.

Table S4. Experiments and responses of the HAAs in the 2^3 full factorial design.

Run	A	B	C	ERs (%)									
	ACN (%)	HCOOH (mM)	Elution flow rate (mL min^{-1})	DMIP	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP	Trp-P-1	AαC	MeAαC	Trp-P-2
1	80.00	0.06	0.80	99.337	95.38	92.29	98.04	96.79	100.21	90.32	97.25	96.1	93.5
2	60.00	0.03	0.50	80.167	83.02	80.04	89.38	86.95	91.06	80.18	88.09	86.8	81.74
3	80.00	0.00	0.80	85.558	80.75	82.89	93.32	85.87	95.65	81.8	88.09	82.21	84.53
4	40.00	0.06	0.20	72.6896	73.96	68.05	76.05	80.21	80.1	67.1	92.42	76.55	70.1
5	40.00	0.06	0.80	76.524	72.89	65.08	74.76	75.82	79.17	63.8	78.97	72.7	68.83
6	60.00	0.03	0.50	77.501	82.12	76.45	90.18	93.9	89.08	78.6	87.55	80.99	78.16
7	40.00	0.00	0.80	62.89	54.2	63.01	66.27	68.9	72.61	60.6	68.57	60.89	62.57
8	80.00	0.00	0.20	88.412	82.93	85.85	90.52	84.72	86.51	79.3	90.49	84.46	86.94
9	80.00	0.06	0.20	96.611	98.27	91.14	99.34	99.22	101.4	93.8	98.1	95.86	95.66
10	60.00	0.03	0.50	79.4029	90.58	84.48	93.87	85.2	93.57	86.6	89.08	89.8	85.1
11	60.00	0.03	0.50	76.912	76.55	84.92	91.58	94.13	92.16	87.1	87.89	78.96	86
12	40.00	0.00	0.20	63.1233	53.4	64.79	62.27	70.39	70.59	56.9	69.6	63.5	63.19
13	60.00	0.03	0.50	78.757	81.15	79.76	82.02	81.12	89.54	89.6	86.74	89.9	80.64

Table S5. ANOVA test results for the IQ response obtained in the second (2^3) full factorial design.

Source	Sum of Squares	df [a]	Mean Square	F_{value} [b]	P_{value} [c]	Prob> *F*
Model	1921.96	2	960.98	71.50	<0.0001	*significant*
A	1323.04	1	1323.04	98.44	<0.0001	
B	598.93	1	598.93	44.56	<0.0002	
Curvature	118.72	1	118.72	8.83	0.0157	*significant*
Residual	120.97	9	13.44			
Lack of Fit	18.21	5	3.64	0.14	0.2754	*not significant*
Pure error	102.76	4	25.69			
Cor total	2161.65	12				

[a] Degrees of freedom; [b] Test for comparing model variance with residual (error) variance; [c] Probability of seeing the observed *F* value if the null hypothesis is true.

Table S6. ANOVA test results for Trp-P-2 response obtained of the second (2^3) full factorial design.

Source	Sum of Squares	df[a]	Mean Square	F_{value}[b]	P_{value}[c]	Prob> *F*
Model	1269.60	2	634.80	113.08	<0.0001	*significant*
A	1150.56	1	1150.56	204.95	<0.0001	
B	119.04	1	119.04	21.21	<0.0002	
Curvature	53.32	1	53.32	9.50	0.0131	*significant*
Residual	50.52	9	5.61			
Lack of Fit	8.79	5	1.76	0.17	0.9613	*not significant*
Pure error	41.73	4	10.43			
Cor total	1373.45	12				

[a] Degrees of freedom; [b] Test for comparing model variance with residual (error) variance; [c] Probability of seeing the observed *F* value if the null hypothesis is true.

Table S7. Experiments and responses of the HAAs in the DCC.

A	B	ERs (%)									
ACN (%)	**HCOOH (mM)**	**DMIP**	**IQ**	**MeIQ**	**MeIQx**	**4,8-DiMeIQx**	**PhIP**	**AαC**	**MeAαC**	**Trp-P-1**	**Trp-P-2**
80.0	15.9	98.4	95.6	90.5	93.7	97.1	97.3	98.5	98.2	96.7	94.6
60.0	8.5	83.6	88.2	80.5	83.5	91.3	92.3	95.5	93.8	97.9	96.6
40.0	1.1	60.5	66.1	62.4	66.9	68.2	98.2	93.3	82.2	91.4	99.8
60.0	0.0	62.6	57.8	64.7	67.5	63.9	87.0	81.5	64.2	85.6	86.8
60.0	8.5	80.9	84.7	80.2	86.9	80.9	81.7	84.1	82.2	85.5	81.8
24.6	8.5	72.2	77.8	74.5	78.8	73.1	72.5	76.7	72.9	76.6	78.2
60.0	8.5	89.9	81.9	83.0	85.8	89.2	95.0	96.1	98.9	92.1	93.3
60.0	8.5	81.3	81.8	87.8	83.6	92.8	94.6	92.7	81.6	86.0	84.2
60.0	21.0	87.1	83.6	85.8	83.6	91.5	96.1	96.1	95.9	97.5	94.5
40.0	15.9	75.5	94.8	90.8	85.6	91.4	87.7	89.4	75.4	88.8	82.0
80.0	1.1	78.6	86.7	81.9	79.3	73.3	43.4	59.0	71.6	63.5	51.6
60.0	8.5	82.5	84.4	88.9	82.0	87.4	66.2	77.4	85.8	83.1	79.9

Table S8. CCD fitting models for HAAs analysis in surface water samples.

Response	Model	R^2	R^2adj	Transformation	Significant terms (*x*)	ANOVA *p*-value [a]	
						Model	Lack of fit
R1-DMIP	Quadratic	0.9235	0.8980	None*	A - B - B^2	<0.0001	0.5854
R2-IQ	*	0.8804	0.8207	*	A -B - AB - B^2	<0.0001	0.0650
R3-MeIQ	*	0.9268	0.8902	*	A -B - AB - B^2	<0.0001	0.8091
R4-MeIQx	*	0.9525	0.9367	*	A - B - B^2	<0.0001	0.4592
R5-4,8-DiMeIQx	*	0.9030	0.8707	*	A - B - B^2	<0.0001	0.6998
R6-PhIP	*	0.7670	0.6505	*	A -B - AB - A^2	<0.0120	0.7833
R7-AαC	*	0.8553	0.7830	*	A -B - AB - A^2	<0.019	0.8326
R8- MeAαC	*	0.7002	0.6002	*	A -B - AB - A^2	<0.0287	0.6070
R9-Trp-P-1	*	0.8852	0.8278	*	A -B - AB - A2	<0.0001	0.7340
R10-Trp-P-2	*	0.8716	0.8074	*	A -B - AB - A^2	<0.0012	0.8282

A: ACN (%) in ACN/H_2O mixture elution; B: HCOOH concentration; [a] *p*-values less than 0.050 indicate significance; * applied to each Response

Table S9. Analytical yields of HAAs in surface water samples by MWCNTs-SPE as clean-up and preconcentration strategy.

Surface water sample			*Cosquin River*					*San Roque Reservoir*		
HAAs	**Sample Concentration (µg L-1)**	**Added (µg L^{-1})**	**Found (µg L^{-1})**	**Recovery (%)**	**RSD (%) *n=3***	**Sample Concentration (µg L^{-1})**	**Added (µg L^{-1})**	**Found (µg L^{-1})**	**Recovery (%)**	**RSD (%) *n=3***
DMIP	N.D.*	0.0	-	-	-	*	0.0	-	-	-
		0.5	0.51	102.0	2.6		0.5	0.53	106.0	3.7
		1.0	1.05	105.0	3.8		1.0	0.98	98.0	3.1
		2.0	1.95	97.5	5.2		2.0	2.00	100.0	3.4
IQ	*	0.0	-	-	-	0.48[a]	0.0	0.48[a]	-	-
		0.5	0.48[a]	96.0	6.7		0.5	0.49	98.9	8.3
		1.0	0.97	97.0	8.9		1.0	1.40	94.6	4.3
		2.0	2.02	101.0	6.0		2.0	2.47	99.6	6.2
MeIQ	0.56	0.0	0.56	-	-	0.92	0.0	0.92	-	-
		0.5	1.03	97.2	5.1		0.5	1.43	100.7	2.9
		1.0	1.46	93.6	5.8		1.0	1.92	100.0	6.4
		2.0	1.85	92.5	1.8		2.0	1.97	98.5	4.2
MeIQx	0.24	0.0	0.24	-	-	0.71	0.0	0.71	-	-
		0.5	0.71	95.9	6.9		0.5	1.19	98.3	3.0
		1.0	1.20	96.8	2.5		1.0	1.70	99.4	6.1
		2.0	1.97	98.5	5.6		2.0	1.99	99.5	5.0
4,8-DiMeIQx	0.16[a]	0.0	0.16	-	-	0.62	0.0	0.62	-	-
		0.3	0.63	94.7	5.3		0.5	1.12	100.0	5.9
		1.0	1.11	95.8	2.6		1.0	1.64	101.2	6.2
		5.0	4.96	99.2	3.7		5.0	4.99	99.8	4.8
PhIP	*	0.0	-	-	-	0.56	0.0	0.56	-	-
		0.5	0.49	98.0	2.82		0.5	0.96	90.6	6.3
		1.0	0.99	99.0	7.20		1.0	1.45	92.9	5.1
		2.0	1.97	98.5	3.00		2.0	1.99	99.5	8.1
Trp-P-1	0.32	0.0	0.32	-	-	0.93	0.0	0.93	-	-
		0.5	0.81	98.8	6.8		0.5	1.46	102.1	4.8
		1.0	1.30	98.5	3.4		1.0	2.02	104.7	2.6
		5.0	5.03	100.6	2.7		5.0	5.29	105.8	7.7
AαC	0.21	0.0	0.21	-	-	0.37	0.0	0.37	-	-
		0.5	0.69	97.2	5.2		0.5	0.88	101.1	6.4
		1.0	1.18	97.5	6.9		1.0	1.34	97.8	4.0
		2.0	1.99	99.5	7.4		2.0	1.97	98.5	4.9
Trp-P-2	0.51	0.0	0.51	-	-	0.76	0.0	0.76	-	-
		0.5	0.98	97.0	5.3		0.5	1.20	95.2	4.8
		1.0	1.48	98.0	4.3		1.0	1.74	98.8	2.3
		2.0	2.03	101.5	2.4		2.0	1.95	97.5	4.8
MeAαC	0.35[a]	0.0	0.35	-	-	0.56	0.0	0.56	-	-
		0.5	0.86	101.2	5.9		0.5	1.04	98.1	4.7
		1.0	1.42	105.2	4.8		1.0	1.59	101.9	6.3
		2.0	1.98	99.0	3.7		2.0	1.98	99.0	2.9

N.D.*: not detected; [a] <LOQ.