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RESEARCH ARTICLE

# Development of a green and efficient methodology for the heterocyclic aromatic amine determination in biomass samples generated from cigarette combustion and tobacco

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## Abstract

A green methodology was developed for the analysis of ten heterocyclic aromatic amines (HAAs) in biomass samples from cigarette combustion such as mainstream smoke, paper ashes, as well as tobacco and paper wraps. The cellulose filter used for sample collection was also evaluated. This strategy was based on ultrasound-assisted extraction (UAE) associated with a solid-phase extraction procedure employing multi-walled carbon nanotubes (MWCNTs-SPE) as a cleanup step followed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Under optimal experimental conditions, the linearity of the method was in the range from 0.08 to 160 ng $cig^{-1}$, with correlation coefficients ($R^2$) higher than 0.991. The limits of detection resulted to be between 0.03 and 0.63 ng $cig^{-1}$. Concentrations of the HAAs in the mainstream smoke were from 5.7 to 145.2 ng $cig^{-1}$ and in paper ashes from 0.1 to 0.6 ng cig $^{-1}$, while in tobacco were between 1.0 and 38.5 ng $cig^{-1}$. Meanwhile, no HAA contribution was observed in the case of paper wraps and the filter used for sample collection. The knowledge of the presence and the concentration levels of the selected HAAs in each cigarette's physical component after its combustion is essential to understand the formation processes and contribution during cigarette burning. Besides, this is the first report about the presence of some HAAs in the proposed samples. Finally, a comparative study was employed to classify the sustainability of several recent approaches for HAA extraction from cigarette combustion samples using *Green Certificate* as a metric tool.

**Keywords** Heterocyclic aromatic amines · UAE-MWCNT-based SPE · Mainstream smoke · Paper ashes · Tobacco · Green Certificate

## Introduction

Tobacco use is associated with the main cause of preventable death (WHO 2013). Cigarette smoke presents more than 8000 chemical hazardous compounds evidencing an acutely complex composition (Rodgman and Perfetti 2016). Particularly, the mainstream cigarette smoke contains a complex mixture of organic and inorganic chemical compounds (Ren et al. 2017; Ticha and Wright 2016), produced through diverse generation mechanisms such as combustion, distillation, pyrolysis, and psychosynthesis (Wu et al. 2015). Besides, temperatures above 400 °C facilitate the formation of hazardous substances such as trace metals, carbon monoxide, nicotine, nitrosamines, polycyclic aromatic hydrocarbons (PAHs), and heterocyclic aromatic amines (HAAs), among others (Cheng et al. 2019; Rodgman and Perfetti 2016; Wang et al. 2016). Hereby, these analytes are incorporated through particles emitted during cigarette combustion into the respiratory tract and the

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surrounding ambient, having a potential impact on human health and environment (Roemer et al. 2016; Stabile et al. 2017).

In this context, heterocyclic aromatic amines (HAAs) are organic substances representing a significant kind of carcinogen in cigarette smoke (Jain 2018; Zhao et al. 2014). These compounds are formed during the heating at high temperatures (above 100 °C) of various materials containing mainly nitrogenous compounds such as wood, biomass, and tobacco (Canales et al. 2018; Capistrano et al. 2017; Dong et al. 2020). Thereabout twenty HAAs have been identified as potent mutagens by the *Ames* mutagenicity test. Based on available data, the International Agency for Research on Cancer (IARC) has recognized some HAAs as possible human carcinogens (IARC 1997). Moreover, the list of 93 Harmful and Potentially Harmful Constituents (HPHCs) in tobacco smoke and other products provided by the US Food and Drug Administration (FDA) has involved various toxic chemicals (FDA 2012). In accordance, the continuous exposition to these substances would induce accumulated genetic alteration with the consequent potential development of several sorts of cancer (Roemer et al. 2016).

A conventional technique considered effective for the extraction of organic compounds from solid samples is ultrasound-assisted extraction (UAE), which presents as an advantage the immediate mass transfer of analytes due to the formation of the high-frequency ultrasonic waves (Banožić et al. 2019). Likewise, cleanup methodologies based on solid-phase extraction (SPE) have been commonly utilized for numerous organic compounds in mainstream smoke and tobacco samples (Zhao et al. 2014; Zhang et al. 2017). This approach is capable of retaining the target analytes, which undesired components (commonly matrix interferences), and then eluting the desired analytes with an adequate extraction solvent (Zhang et al. 2016).

Recently, nanomaterials associated with SPE as multi-walled carbon nanotubes (MWCNTs) have shown great utility due to their sturdy retention/elution abilities (Canales et al. 2020; Yu et al. 2015). This sorbent material is constituted by graphene sheets, which are rolled themselves, demonstrating interactions with many organic compounds. Hence, the MWCNTs are appraised adsorbents to be used in the cleanup stages (Canales et al. 2020; Luo et al. 2016; Yu et al. 2015). In this context, coupled methodologies development is a useful alternative in the reduction/elimination of interferences during cleaning procedures.

Nowadays, little is known about how the physical components of cigarettes such as tobacco, paper wraps, and its ashes contribute to the generation of these hazardous substances during burning. Owing to HAAs' potent toxicity, it is essential to understand the quantitative role of the physical component of cigarettes to the generation of those analytes.

In the present work, a green and simple UAE-MWCNT-based SPE approach coupled to UHPLC-MS/MS analysis was developed for the determination of ten HAAs in biomass samples from cigarette combustion, mainly mainstream smoke, and paper ashes, as well as tobacco, paper wraps and the cellulose filter used for sampling. The influence of diverse variables affecting the HAAs' recoveries in both extraction/cleanup stages was optimized and evaluated. The presence and the concentration levels of the selected analytes in each cigarette's physical component before and after its combustion are essential to understand HAA formation processes during smoking. Also, a comparative discussion was carried out to assess the greenness of the most recent approaches for HAA extraction from biomass burning samples using the analytical available metrics.

## Materials and methods

### Reagents and chemicals

Acetonitrile (ACN), methanol (MeOH), and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). All HAA standards with a purity > 98% were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The following analytical chemical standards of ten HAAs were selected: 2-amino-1,6-dimethylimidazo-[4,5-*b*]-pyridine (DMIP), 2-amino-3-methylimidazo-[4,5-*f*]-quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]-quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo-[4,5-*f*]-quinoxaline (MeIQx), 2-amino-3,4,8trimethylimidazo-[4,5-*f*]-quinoxaline (4,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]-pyridine (PhIP), 3-amino-1,4-dimethyl-5H-pirido-[4,3-*b*]-indole (Trp-P-1), 3-amino-1-methyl-5H-pirido-[4,3-*b*]-indole (Trp-P-2), 2-amino-9H-pyrido-[2,3-*b*]-indole (AαC), and 2-amino-3-methyl-9H-pyrido-[2,3-*b*]-indole (MeAαC). Working standard solutions were prepared daily in ACN/$H_2O$ (1:3) by stepwise dilution of a 5.0 mg $L^{-1}$ stock solution of each HAA. The solutions were maintained at 4 °C, protected from light, and kept in amber flasks.

Formic acid was obtained from Fisher Scientific (Loughborough, UK). Non-modified multi-walled carbon nanotubes (O.D. × I.D. × L, 10 nm ± 1 nm × 4.5 nm ± 0.5 nm × 3 to ~ 6 μm; number of walls, 6–8) were acquired from Sigma-Aldrich. Co. (St. Louis, USA).

### Instrumentation

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer configured with a Z-Spray™ electrospray ionization source (ESI, Waters, Milford, USA). An Acquity™

Ultra-High-Performance LC system (Waters, Milford) equipped with an autosampler injection and pump systems (Waters, Milford, USA) was employed. The autosampler vial tray was maintained at 4 °C. The separation was accomplished using an ACQUITY UPLC® BEH Shield RP18 (Waters, Milford, USA) analytical column (100 × 2.1 mm i.d., 1.7 μm). During the sample pretreatment, an electronic microbalance with a readability of 0.1 mg (Ohaus, model UMX2, Switzerland), an ultrasonic bath (Testlab (model TB-04 TA, Buenos Aires, Argentina)), a centrifuge (U-320R-BOECO, Germany), and a Minipuls 3 peristaltic pump (Gilson (Villiers-Le-Bell, France)) were employed.

### UHPLC-MS/MS analysis

The binary mobile phases consisted of water (A) and acetonitrile (B), both with 28.3 mM of formic acid, which was pumped at 0.25 mL $min^{-1}$. The gradient elution program was previously developed by Canales et al. (2020) and was employed for the chromatographic separation of ten HAAs. The total running time was 5 min. Injection volume was 10 μL and the column temperature was maintained at 30 °C. The selected chromatographic conditions allowed obtaining acceptable peak shapes in short analysis times (Figure S1-Electronic Supplementary Material (ESM)).

Mass spectrometry analyses were performed on a tandem triple quadrupole mass spectrometer equipped with an ESI interface. For each HAA, the interface was operated in a positive mode, and the data were acquired in multiple reaction monitoring modes (MRM) of selected ions at the first (Q1) and third quadrupoles (Q3). The source working 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 $h^{-1}$; and desolvation gas flow rate, 400 L $h^{-1}$. Ultrapure nitrogen was used as cone gas and argon was employed as collision gas at flow 0.18 mL $min^{-1}$, respectively. MRM was used to select the fragmentation patterns for each HAA. Direct infusion (via syringe pump) of standard solutions (0.5 mg $L^{-1}$) into the MS was carried out, solutions were prepared as detailed in the previous section. The product ion scan mass spectra were recorded. MS/MS settings previously developed by the research group were used (Table S1-ESM) (Canales et al. 2020). The MassLynx Mass Spectrometry (Waters, Milford, USA) data acquisition software was used.

### Sampling and sample preparation

Before analysis, cigarettes of five recognized brands were conditioned in an environmental chamber held at 24 ± 2 °C and 60 ± 5% relative humidity for 24 h.

Mainstream smoke generated from cigarette combustion (biomass sample) was collected on cellulose fiber filters using a machine smoking regime (35 mL puff of 2 s duration taken three/min to a butt length of 3 mm beyond the filter overwrap). Under the smoking conditions, individual filter pads collected the smoke condensate from one cigarette. Three replicates per cigarette and six replicates per brand were evaluated. The ashes obtained from taking apart and burning paper wraps were used also as biomass samples. On the other hand, support filters were conditioned before and after being weighed in a climatic chamber provided with a control system for temperature and humidity. Paper wraps and tobacco were carefully separated from the cigarette, and then, the whole paper and 20 mg of tobacco were used for the extraction procedure.

### Sample preparation and UAE-MWCNT-based SPE procedure

In the case of mainstream smoke, one-eighth of the whole filter area (≈ 2.17 $cm^2$) was weighed and used. On the other hand, 20 mg of tobacco and paper ashes from each cigarette were weighted and used for extraction/cleanup procedures. Finally, paper wraps and an adequate portion of the cellulose filter were treated. All samples were transferred into a glass tube, where an adequate extraction solvent volume (2 mL, MeOH) was added. After that, the system was shaken and immersed in an ultrasonic bath for 30 min. The obtained methanolic extract was centrifuged at 3000 rpm for 5 min. Due to the suppression matrix effect observed on the HAA analytical signal, the methanolic extract samples were taken to a final volume of 10 mL with water for a subsequent cleanup stage. Thus, these aqueous solution samples were passed through an SPE cartridge, which contained 30 mg of non-modified MWCNTs, by a peristaltic pump at an optimal loading flow rate of 1 mL $min^{-1}$. The retained analytes were eluted with a mixture of ACN/$H_2O$ (80:20 (v/v)), conditioned with of HCOOH, at an elution flow rate of 0.8 mL $min^{-1}$ up to a final volume of 0.8 mL. Finally, the eluate was collected into a vial for direct analysis by UHPLC-MS/MS. The schematic diagram of UAE-MWCNT-based SPE is shown in Fig. 1.

## Method validation

The quantification and method validation parameters were achieved by spiking the samples under study, mainstream smoke and paper ashes (biomass samples), tobacco, as well as cellulose fiber filter and paper wraps of diverse blond cigarette brands with HAA mix standard solutions at 0.01, 1, 10, 20, 40, 80, and 160 ng $cig^{-1}$ concentration levels for each analyte. Before analysis, all the samples were stirred for 1.0 min and incubated at 25 °C for 3 h.

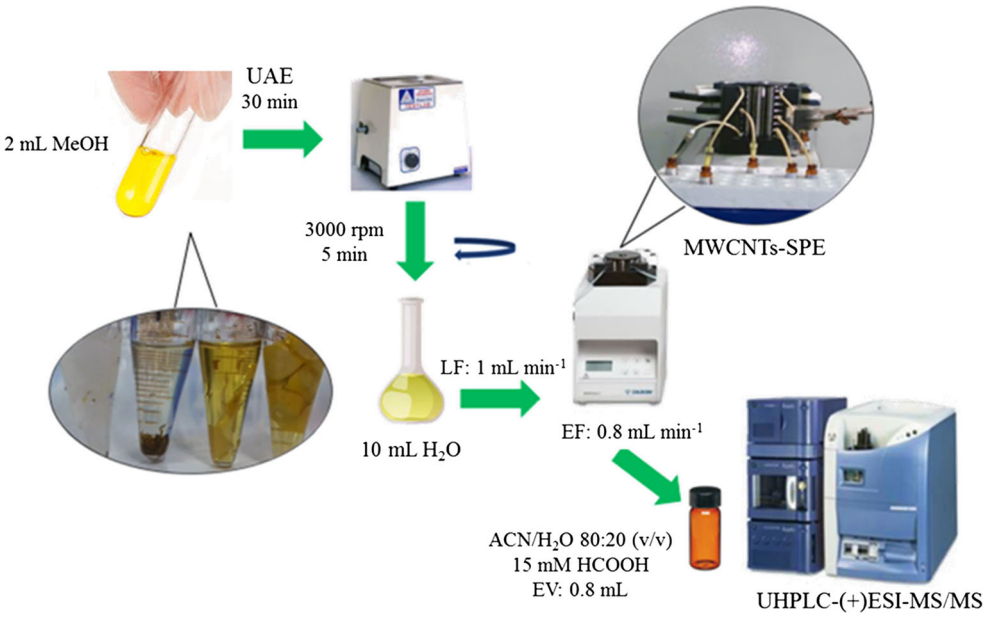


**Fig. 1** Scheme of the experimental UAE-MWCNT-based SPE procedure applied to sample extraction/cleanup of the selected HAAs. LF, loading flow rate; EF, elution solvent flow rate; EV, elution volume

## Linear range

Lineal range (LR) of the calibration curves for spiked biomass samples generated from cigarette combustion and tobacco was attained by the least-squares linear regression analysis of the intensity of signal vs. HAAs concentrations. The linearity of the fitted model agreed with the *F*-test in the working range (values close to the LOQ up to around 160 ng cig $^{-1}$).

## Detection and quantification limits

Limit of detection (LOD) and limit of quantification (LOQ) are defined as performance characteristics in method validation. LOD (ng cig$^{-1}$) and LOQ (ng cig$^{-1}$) allow appointing the smallest level concentration of an analyte that could be reliably measured and quantified by an analytical procedure. Thereby, these terms were calculated following the International Union of Pure and Applied Chemistry (IUPAC) recommendations according to Eqs. (1) and (2) (Uhrovčík 2014).

$$\mathrm{LOD}\left(ng\ cig^{-1}\right) = \frac{3.3\mathrm{S_{y/x}}}{\mathrm{b}}\sqrt{\frac{1}{\mathrm{m}}+\frac{1}{\mathrm{n}}+\frac{\overline{\mathrm{x}}^{2}}{\sum_{\mathrm{i}=1}^{\mathrm{n}}\left(\mathrm{x_I}-\overline{\mathrm{x}}\right)^{2}}} \quad (1)$$

$$\mathrm{LOQ}\left(ng\ cig^{-1}\right) = \frac{10\mathrm{S_{y/x}}}{\mathrm{b}}\sqrt{\frac{1}{\mathrm{m}}+\frac{1}{\mathrm{n}}+\frac{\overline{\mathrm{x}}^{2}}{\sum_{\mathrm{i}=1}^{\mathrm{n}}\left(\mathrm{x_i}-\overline{\mathrm{x}}\right)^{2}}} \quad (2)$$

where $\overline{x}$ corresponds to the mean concentration; $x$ the calibration concentration value; $y$ the experimental response values for the samples; $S_{y/x}$ the residual standard deviation; $b$ the slope of the calibration curve; $m$ the number of replicates per concentration level of the spiked samples; and $n$ the number of concentration levels for spiked samples: i = 1, 2…I.

## Extraction recovery

The extraction recovery (ER (%)) was calculated using Eq. 3 and was used to evaluate the analytical performance in optimal conditions at the concentration levels previously mentioned for all studied HAAs.

$$ER\ (\%) = \frac{C_{found}-C_{real}}{C_{added}} \times 100 \quad (3)$$

where $C_{\mathrm{found}}$ refers to the analyte concentration after adding a known amount of standard to the real sample, $C_{\mathrm{real}}$ denotes the analyte concentration in the real sample, and $C_{\mathrm{added}}$ indicates the known amount of standard that was spiked to the real sample.

## Matrix effects

Frequently matrix interferences might generate suppression or enhancement effects on the signals of the compounds of interest (Cortese et al. 2020). In this study, the matrix effect (ME (%)) was assayed by comparison of the calibration curves slopes ($b$), which were created with analytical standards of HAAs in pure solvent/blank (zero) sample and spiked samples generated from cigarette combustion and tobacco, before applying the MWCNTs-SPE cleanup strategy. The percentage of the quotient of the slopes was employed to calculate the ion signal suppression/enhancement extension (Eq. 4).

$$ME\ (\%) = 100 - \left( \frac{b_{Spiked\ sample}}{b_{Standard\ in\ pure\ solvent/blank\ (zero)\ sample}} \times 100 \right) \quad (4)$$

## Uncertainty evaluation

The data quality is usually evaluated based on its uncertainty. Therefore, the relative uncertainty ($u_r$) analysis allows an understanding of the analytical procedure and its source variations, such as sample quantity used for the determination, recovery ($u_1$), repeatability ($u_2$), analyte concentration ($u_3$), and calibration curve ($u_4$) (Konieczka and Namieśnik 2010); the contribution of all these terms can be calculated through the relative combined uncertainty ($u_{rc}$, dimensionless value) (Eq. 5). On the other hand, the expanded uncertainty (U) is the quantity defining an interval about the result of the determination that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to, in this case, the analytes' concentration. It can be calculated as shown in Eq. 6, where $k$ is the coverage factor, equal to 2—at a 95% confidence level and under normal distribution assumption—and $c$ is the average concentration of the analyte. Also, this uncertainty can be expressed as a percentage value as described in Eq. 7. The relative combined uncertainty and the expanded uncertainty were estimated according to the EURACHEM/CITAC guide and Konieczka (Ellison et al. 2000; Konieczka and Namieśnik 2010).

$$u_{rc} = \sqrt{\left(u_{r(1)} + u_{r(2)} + u_{r(3)} + u_{r(4)}\right)} \quad (5)$$

$$U\left(ng\ cig^{-1}\right) = k \times c \times u_{rc} \quad (6)$$

$$U(\%) = k \times u_{rc} \times 100 \quad (7)$$

## Parameters for the assessment of the Green Certificate

At present, analytical metrics have been employed to evaluate the green character of a sample treatment methodology (Płotka-Wasylka 2018). Armenta proposed the *Green Certificate* as a tool of sustainability. Thus, the green efficiency of the proposed UAE-MWCNT-based SPE was compared with the recently reported methodologies mainly for the determination of HAAs in diverse biomass burning samples (Armenta et al. 2015).

The penalty points (*PP*) are referred to as the main factors for green assessment such as reagent ($PP_R$), waste volume generated ($PP_W$), and energy consumption ($PP_E$). Thus, the *penalty points* were calculated using Eqs. (8) and (9); respectively (Armenta et al. 2015), both are dimensionless values.

$$PP_R = (0.61 \pm 0.05)\ V^{(0.31 \pm 0.02)} \quad (8)$$

$$PP_w = (0.50 \pm 0.08)\ W^{(0.40 \pm 0.02)} \quad (9)$$

where $V$ represents the reagent volume and $W$ denotes the waste volume generated. The $PP_E$ was evaluated concerning the power-hour required in the proposed UAE MWCNT-based SPE (Espino et al. 2018).

# Results and discussion

## Extraction step: UAE conditions

The UAE conditions were previously reported by Canales et al. (2018). Nevertheless, in the present study, this methodology suffered some modifications due to the samples' nature and the support material used during the mainstream smoke collection. To analyze the ultrasound-assisted time influence on the ER (%), different times (5–40 min) were evaluated. Due to its polarity and capability to quantitatively improve the mass transfer of all HAAs from the cellulose fiber filters, MeOH was used as the extractive solvent (2 mL). Time and centrifugation rate (5 min, 3000 rpm) were kept constants. As the ultrasound-assisted time increases, the ER (%) improves in all samples studied (Fig. 2). Considering the obtained results, the optimal UAE time was attained when a 30-min cycle was applied. As can be observed (Fig. 2), DMIP and MeAαC were selected as models because each of them represents the chemical properties of the rest of the analytes based on the HAA classification into polar and non-polar analytes (Dong et al. 2020). When optimizing the conditions, no statistical differences between the selected models and the rest of the compounds of its chemical group were observed.

## Cleanup step: MWCNT-based SPE conditions

In the present study, an important suppression effect of the analytical signal was observed (around 80–100%). Consequently, after the extractive procedure, a MWCNTs-SPE cleanup strategy was applied to minimize it. To achieve compatibility between UAE and MWCNTs-SPE, a simple step was added, which consisted of taking the methanolic extract up to a final volume of 10 mL with water due to this way the aqueous solution improves the retention of the HAAs on the SPE cartridge (Sierra and Morante-Zarcero 2018).

To explore the influence of the aqueous sample volume on the efficiency of the proposed cleaning strategy, the ER (%) for all HAAs were analyzed. For this purpose, different volumes (5, 10, 15, 20, and 25 mL) of aqueous sample solution containing the HAAs were evaluated (Figure S2-ESM). The

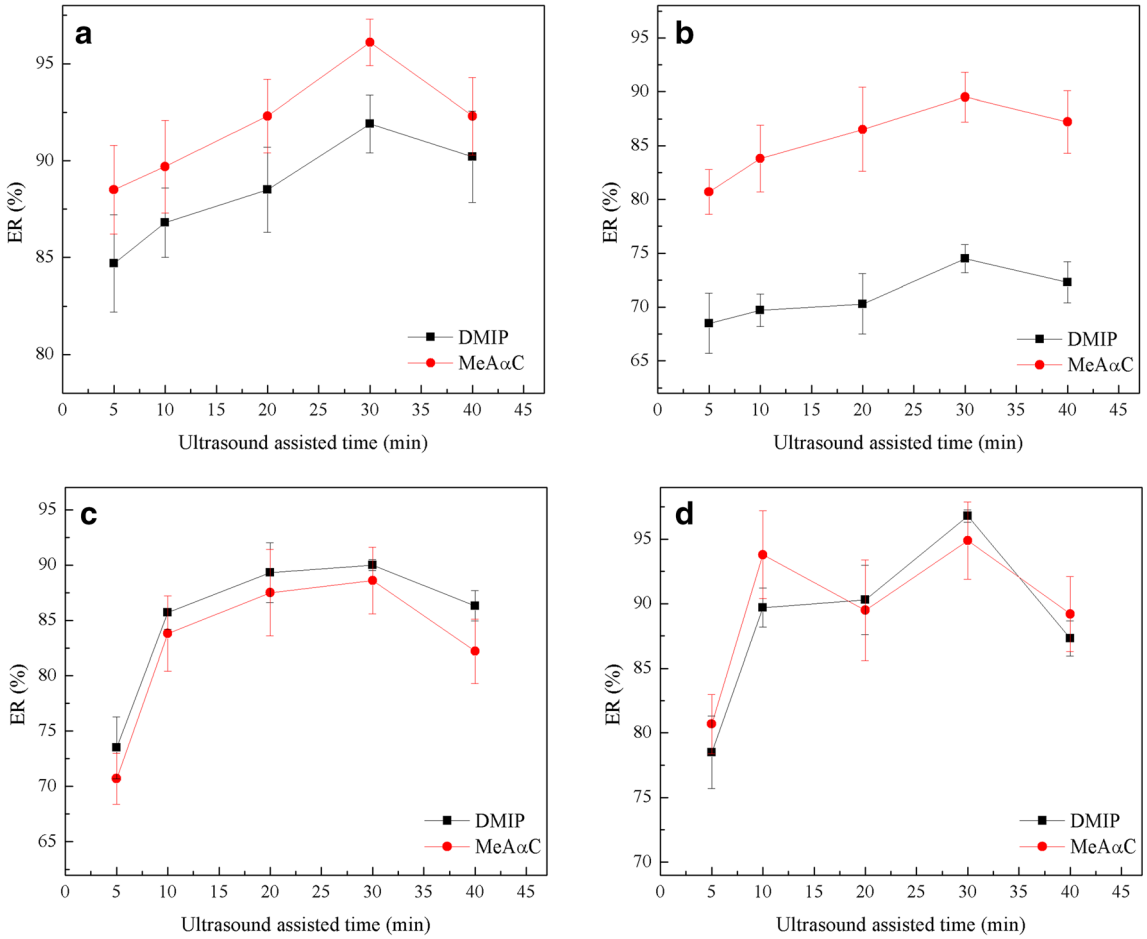


**Fig. 2** Ultrasound-assisted time influence on ER (%) of DMIP and MeAαC as model HAAs. **A** Paper wraps. **B** Paper ashes. **C** Tobacco. **D** Mainstream smoke. Optimal MWCNTs-SPE conditions. UAE conditions: type and volume extractive solvent (MeOH, 2 mL), time, and rate centrifugation (5 min, 3000 rpm)

results demonstrated optimal performance and no significant differences between 10 and 25 mL, but the ER (%) decreased at volumes lower than 10 mL for all the samples. Consequently, a 10-mL sample volume was selected.

Regarding the HAA elution step, some literature reports indicate that solvent mixtures such as ACN/$H_2O$ or MeOH/$H_2O$ containing additives such as formic acid and sodium hydroxide have been employed for efficient elution of HAAs from several SPE cartridges (Zhao et al. 2014; Guiñez et al. 2020; Zhang et al. 2011). In this study and following some previous reports (Guiñez et al. 2020; Canales et al. 2020), an efficient elution—based on the recoveries observed—of the selected ten HAAs with a mixture of ACN/$H_2O$ (80:20 (v/v)), with 15 mM of HCOOH, was observed. The ACN/$H_2O$ mixture resulted to be more efficient than other solvents tested (MeOH, MeOH/$H_2O$) in the elution of both polar and non-polar amines from the SPE cartridge and increased with acid additives. Besides, this mixture of solvents was also compatible with the chromatographic conditions.

## Sample flow rate optimization

The aqueous sample solution was passed through an SPE cartridge containing 30 mg of non-modified MWCNTs as an adsorbent nanomaterial. In this sense, the sample loading flow rate is one of the most important variables to evaluate due to its effect over the analysis of adsorption/elution processes into the cartridge filling. Accordingly, the loading flow rate (0.5–2 mL $min^{-1}$) influence on the ER (%) was considered. The best efficiency yielding extraction values were observed at a loading flow rate of 1 mL $min^{-1}$, as shown in Fig. 3.

## Matrix effect

The corresponding calibration curves from spiked mainstream smoke and paper ashes from cigarette combustion samples and tobacco samples were created. Besides the corresponding curves for paper wraps, cellulose filter fiber, and pure solvent (ACN/H2O 80:20 (v/v) with 15 mM of HCOOH) were obtained.

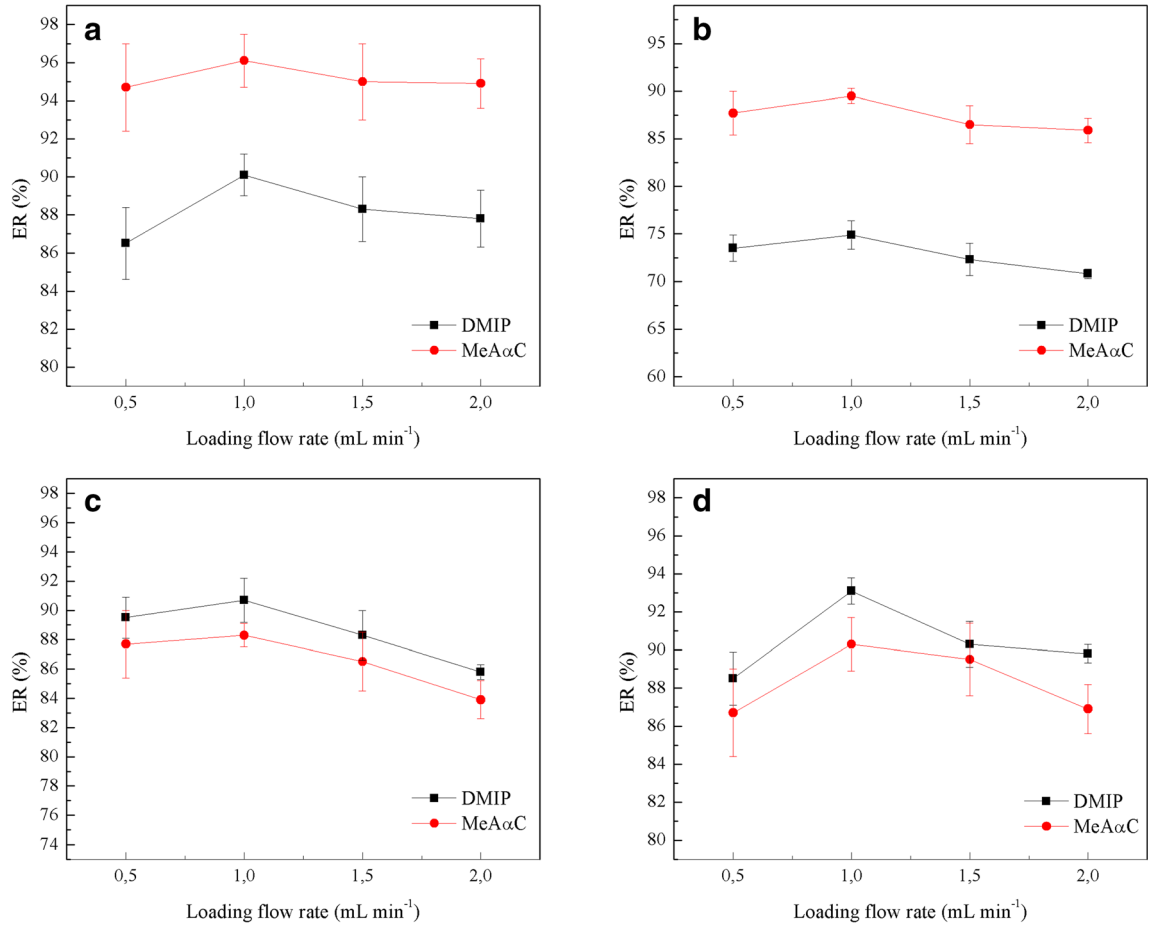


**Fig. 3** Effect of loading flow rate on ER (%) of DMIP and MeAαC as models HAAs. **A** Paper wraps. **B** Paper ashes. **C** Tobacco. **D** Mainstream smoke. Optimal UAE conditions. MWCNTs-SPE conditions: elution solvent flow rate (0.8 mL $min^{-1}$), type and volume of elution solvent (ACN/$H_2O$ (80:20) with 15 mM of HCOOH; 0.8 mL

As can be seen in Fig. 4I, the analytical signals of DMIP (as HAA compound model) were suppressed, to an extent around ~ 80%, mainly for mainstream smoke, paper ashes, and tobacco matrices. The paper wraps and cellulose fiber filter itself did not show a significant matrix effect over the analytical signal of DMIP (shown as an example). After the proposed SPE cleanup step, the matrix effect diminished to values between 10 and 20% (Fig. 4II). This fact can be explained by the effective sample cleanup of the MWCNT-SPE step due to the selective retention/elution of the targeted HAAs in cigarette combustion samples and tobacco. Thus, the cleanup strategy was essential as denoted in Table 1 as an example of a model compound.

## Quantification and method validation parameters

Under optimal conditions, the main figures of merit including LR, correlation efficient ($R^2$), LOD, and LOQ from calibration curves, besides relative standard deviation (RSD (%)), ER (%), and expanded uncertainty (U (%)), were calculated for the proposed UAE-MWCNT-based SPE strategy. The analytical performance was only focused on mainstream smoke, paper ashes (biomass samples), and tobacco since only in these samples the analytes were detected (Table 2).

The calibration curves for the validation of the proposed methodology were created by spiking samples from 0.01 to 160 ng $cig^{-1}$ concentration levels. LR was evaluated through determination coefficients ($R^2$), which were higher than 0.991. The *F*-test demonstrated that linear regressions were statistically acceptable in the working ranges and this model showed the goodness of fit. The obtained LOD values were in the range from 0.08 to 0.63 ng $cig^{-1}$ for mainstream smoke, from 0.03 to 0.26 ng $cig^{-1}$ for paper ashes, and from 0.04 to 0.18 ng $cig^{-1}$ for tobacco. Consequently, the obtained LOQs were in the ranges 0.25 to 1.92 ng $cig^{-1}$, from 0.10 to 0.70 ng $cig^{-1}$, and from 0.13 to 0.54 ng $cig^{-1}$, respectively. Average intraday RSD (%) values were less than 10% in all cases. The ER (%) ranged from 88.7 to 101.6% for mainstream smoke, from 74.9 to 93.8% for paper ashes, and from 78.3 to 90.7% for tobacco. The *U* (%) varied from 1.5 to 10.3% for all HAAs at different concentration levels according to the sample, indicating the satisfactory overall accuracy of the methodology.

**Table 1** Matrix effect study of DMIP (as HAA compound model) for the analysis of spiked samples by applying the proposed methodology

| Matrix | Calibration curve slopes *(b)* before MWCNTs-SPE | Calibration curve slopes *(b)* after MWCNTs-SPE | ME (%)[a] |
|---|---|---|---|
| Pure solvent | 4035.7 | 3935.2 | 2.55 |
| Cellulose fiber filters | 3979.6 | 3856.5 | 3.19 |
| Paper wraps | 3922.6 | 3895.8 | 0.68 |
| Paper ashes | 754.8 | 2872.7 | 73.2 |
| Mainstream smoke | 783.1 | 4092.6 | 80.8 |
| Tobacco | 789.5 | 2755.6 | 71.3 |

[a] Signal suppression extension

From the findings, after applying the UAE MWCNT-based SPE method, it was possible to conclude that the developed analytical method resulted to be sensitive, selective, with adequate recoveries. This satisfactory performance demonstrated that the proposed methodology is robust for its application in the different samples under study. The results are gathered in Table 2.

## Application of the method to real samples

Different collections of cigarette samples generated from the combustion of each cigarette's physical component and its corresponding tobacco content (five blond cigarette brands) were analyzed as described in the "Sample preparation and UAE-MWCNT-based SPE procedure" section.

Nowadays, the presence of some HAAs in the smoke condensate of different cigarettes has been reported. However, there are no analytical methods in the available literature that describe the determination of the ten proposed HAAs (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, DMIP, AαC, MeAαC, Trp-P-1, and Trp-P-2) in biomass burning samples from cigarette combustion, tobacco, and paper wraps. The data of this study revealed that the concentration and distribution of the proposed HAAs were different in the diverse parts of the cigarette; no detectable amounts were found neither in paper wraps nor in the material used for sampling.

In this context, all HAAs were detected in the mainstream smoke with concentration levels higher than the other samples analyzed in this work. The concentration ranges for the analytes ranged from 5.7 to 145.2 ng cig$^{-1}$ (Table 3). Also, differences among cigarette brands were noted (Table S2-ESM). In agreement with other reports, the concentration levels of some HAAs were found in the ng cig$^{-1}$ levels. Particularly, the first HAAs detected in cigarette smoke were AαC and MeAαC at 9–258 ng cig$^{-1}$ (Matsumoto et al. 1981; Yoshida and Matsumoto 1980). Years later, IQ was also quantified at 0.26 ng cig$^{-1}$ (Yamashita et al. 1986). Later, Manabe contributed with important studies to identify PhIP and four other HAAs from the amino carboline group (AαC, MeAαC, Trp-P-1, and Trp-P-2), which were determined at 0.2–43 ng cig$^{-1}$ levels (Manabe et al. 1990a, b; 1991). Also, Kataoka analyzed some polar (IQ and MeIQ) and non-polar (PhIP,

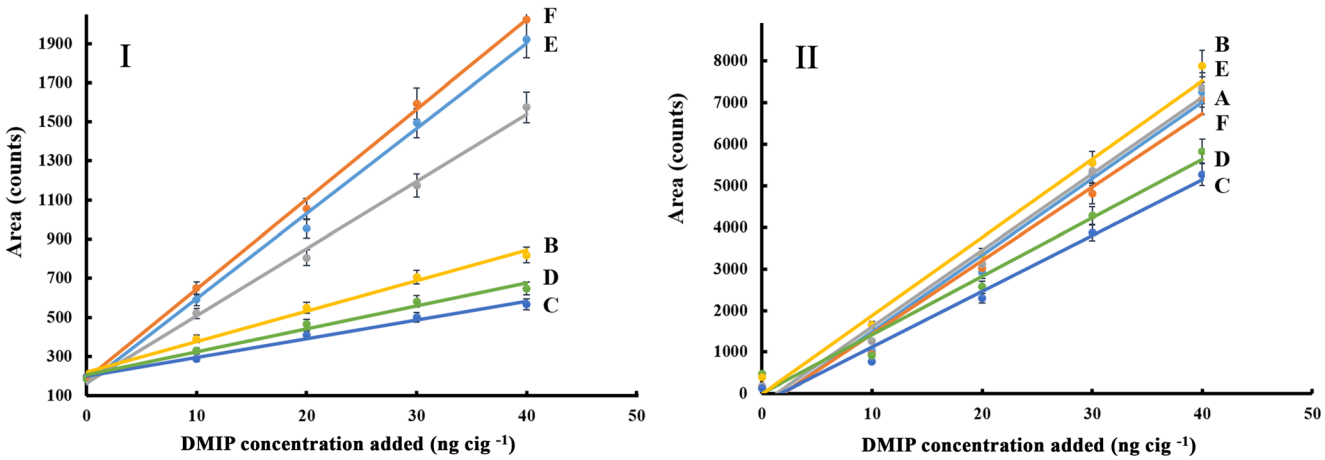


**Fig. 4** Calibration plots from spiked pure solvent (ACN/$H_2O$ 80:20 (v/v) with 15 mM of HCOOH) and spiked matrix samples for DMIP as model HAAs. **I** Before the SPE cleanup step. **II** After the SPE cleanup step. **A** Paper wraps. **B** Paper ashes. **C** Tobacco. **D** Mainstream smoke. **E** Cellulose fiber filter. **F** Pure solvent

**Table 2** Analytical figures of merit of the UAE combined with MWCNTs-SPE strategy followed by UHPLC-MS/MS in detected samples* are depicted

| Figures of merit | IQ | MeIQ | MeIQx | 4,8-DiMeIQx | DMIP | PhIP | Trp-P-1 | Trp-P-2 | AαC | MeAαC |
|---|---|---|---|---|---|---|---|---|---|---|
| Mainstream smoke | | | | | | | | | | |
| $R^2$ | 0.991 | 0.999 | 0.991 | 0.995 | 0.999 | 0.997 | 0.994 | 0.999 | 0.992 | 0.993 |
| LR (ng cig$^{-1}$) | 0.63–80 | 1.38–80 | 1.92–160 | 0.42–160 | 1.30–160 | 1.59–160 | 0.28–80 | 1.23–80 | 0.45–160 | 0.25–160 |
| LOD (ng cig$^{-1}$) | 0.21 | 0.46 | 0.63 | 0.14 | 0.43 | 0.53 | 0.09 | 0.40 | 0.15 | 0.08 |
| LOQ (ng cig$^{-1}$) | 0.63 | 1.38 | 1.92 | 0.42 | 1.30 | 1.59 | 0.28 | 1.23 | 0.45 | 0.25 |
| ER (%)** | 88.7 | 101.6 | 93.2 | 95.8 | 93.1 | 96.7 | 99.0 | 95.1 | 92.1 | 90.3 |
| RSD (%, $n$ = 3) | 4.2 | 5.3 | 6.1 | 7.3 | 6.3 | 4.9 | 7.4 | 8.3 | 4.0 | 4.1 |
| $U$ (%)*** | 8.8 | 4.1 | 4.2 | 3.0 | 6.9 | 9.7 | 6.1 | 8.3 | 9.7 | 2.8 |
| Paper ashes | | | | | | | | | | |
| $R^2$ | 0.999 | 0.991 | 0.993 | 0.997 | 0.996 | 0.998 | 0.999 | 0.996 | 0.997 | 0.992 |
| LR (ng cig$^{-1}$) | 0.49–80 | 0.58–80 | 0.33–160 | 0.79–160 | 0.08–80 | 0.19–80 | 0.12–80 | 0.43–80 | 0.10–160 | 0.42–160 |
| LOD (ng cig$^{-1}$) | 0.16 | 0.19 | 0.11 | 0.26 | 0.03 | 0.06 | 0.04 | 0.14 | 0.03 | 0.14 |
| LOQ (ng cig$^{-1}$) | 0.49 | 0.58 | 0.33 | 0.70 | 0.08 | 0.19 | 0.12 | 0.43 | 0.10 | 0.42 |
| ER (%)** | 78.5 | 89.3 | 81.7 | 85.1 | 74.9 | 89.6 | 85.5 | 84.0 | 93.8 | 89.5 |
| RSD (%, $n$ = 3) | 6.0 | 5.3 | 5.1 | 6.7 | 7.2 | 8.5 | 6.2 | 7.0 | 9.7 | 4.9 |
| $U$ (%)*** | 7.1 | 5.1 | 3.5 | 4.7 | 10.3 | 5.9 | 9.6 | 5.1 | 3.5 | 8.9 |
| Tobacco | | | | | | | | | | |
| $R^2$ | 0.992 | 0.996 | 0.997 | 0.991 | 0.998 | 0.999 | 0.992 | 0.994 | 0.998 | 0.999 |
| LR (ng cig$^{-1}$) | 0.54–80 | 0.30–80 | 0.39–160 | 0.16–160 | 0.15–80 | 0.24–80 | 0.36–80 | 0.13–80 | 0.16–160 | 0.20–160 |
| LOD (ng cig$^{-1}$) | 0.18 | 0.10 | 0.13 | 0.05 | 0.05 | 0.08 | 0.12 | 0.04 | 0.05 | 0.06 |
| LOQ (ng cig$^{-1}$) | 0.54 | 0.30 | 0.39 | 0.16 | 0.15 | 0.24 | 0.36 | 0.13 | 0.16 | 0.20 |
| ER (%)** | 84.7 | 88.7 | 89.9 | 86.9 | 88.3 | 85.9 | 83.7 | 78.3 | 84.1 | 90.7 |
| RSD (%, $n$ = 3) | 5.8 | 5.5 | 6.8 | 7.2 | 6.9 | 9.5 | 8.8 | 4.9 | 4.7 | 6.3 |
| $U$ (%)*** | 3.0 | 1.7 | 1.5 | 1.8 | 2.4 | 6.2 | 9.5 | 6.7 | 4.5 | 5.6 |

* Cellulose filters and paper wraps were not included in the table since the HAAs were not detected in the mentioned samples

** ER (%): the data shown corresponded at a concentration of 10 ng cig$^{-1}$ for IQ, MeIQ, and Trp-P-2 and a concentration of 20 ng cig$^{-1}$ for the rest of the HAAs under study

*** Expanded uncertainty ($k$ = 2)

*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-3,4,8trimethylimidazo-[4,5-f]-quinoxaline, *DMIP* 2-amino-1,6-dimethyl-imidazo-[4,5-b]-pyridine, *PhIP* 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine, *Trp-P1* 3-amino-1,4-dimethyl-5H-pirido-[4,3-b]-indole, *Trp-P-2* 3-amino-1-methyl-5H-pirido-[4,3-b]-indole, *AαC* 2-amino-9H-pyrido-[2,3-b]-indole, *MeAαC* 2-amino-3-methyl-9H-pyrido-[2,3-b]-indole

AαC, and Trp-P1) HAAs in the same run (Kataoka et al. 1998). More recently, Zhang reported the determination of four amino carboline at 1.2–96 ng cig$^{-1}$ levels (Zhang et al. 2011). Besides, Roemer quantified seven HAAs at 0.09–28 ng cig$^{-1}$ as well as an important contribution to the bacterial mutagenesis of these compounds in cigarette smoke was informed (Roemer et al. 2016). As can be seen in the mentioned reports, the HAAs in mainstream smoke present a wide range of concentration levels. The influence of matrix effects and the cleanup strategies were essential for the correct quantification, as well as the smoking regime used.

From paper ashes analysis, eight HAAs were detected and their concentration ranges were from 0.1 to 0.6 ng cig$^{-1}$ (Table 3). This work introduces into the literature the contribution of this type of sample in the overall HAA detection. In addition, it has been demonstrated that paper wraps do not statistically modify these compounds' overall content since no detection of any HAAs was observed in paper wraps or cellulose fiber filters (Table S2-ESM).

Regarding the tobacco analysis, nine HAAs were detected and their concentration levels varied from 1.3 to 38.5 ng cig$^{-1}$ (Table 3). The HAA concentrations determined in tobacco were, for some analytes, drastically lower than those found in the mainstream smoke. In addition, some of the HAAs were only detected in the mainstream smoke, which highlights the importance of biomass burning. As the brands were

**Table 3** Quantitation of ten HAAs in biomass generated from cigarette combustion and tobacco samples of five commercial brands

| HAAs | Mainstream smoke[a,b] | Paper ashes[a,b] | Tobacco[a,b] | Paper wraps | Cellulose fiber filter |
|---|---|---|---|---|---|
| IQ | 9.9 ± 5.4–79.0 ± 5.6 | 0.2 ± 2.3–0.4 ± 2.7 | 5.7 ± 8.2–24.7 ± 7.0 | * | * |
| MeIQ | 15.4 ± 3.4–41.9 ± 2.1 | 0.2 ± 3.2–0.3 ± 3.4 | 2.9 ± 4.3–38.5 ± 7.4 | * | * |
| MeIQx | 5.5 ± 2.8–33.8 ± 2.1 | 0.1 ± 2.3–0.3 ± 3.6 | 5.5 ± 2.8 | * | * |
| 4,8-DiMeIQx | 7.0 ± 5.8–30.8 ± 5.5 | 0.1 ± 2.2–0.3 ± 5.7 | 1.3 ± 2.7–1.5 ± 4.5 | * | * |
| DMIP | 30.9 ± 8.2–60.2 ± 8.2 | * | 11.2 ± 7.5–26.4 ± 5.3 | * | * |
| PhIP | 16.0 ± 2.3–65.4 ± 1.9 | * | 1.0 ± 1.0–3.4 ± 8.3 | * | * |
| Trp-P-1 | 8.5 ± 3.1–31.7 ± 2.6 | 0.1 ± 2.9–0.6 ± 4.8 | 9.2 ± 3.4–31.2 ± 4.8 | * | * |
| Trp-P-2 | 14.4 ± 6.6–43.9 ± 3.1 | 0.1 ± 5.5–0.4 ± 1.8 | 10.8 ± 3.6–23.8 ± 6.2 | * | * |
| AαC | 7.1 ± 4.4–123.0 ± 6.4 | 0.1 ± 5.6–0.4 ± 6.4 | 6.5 ± 6.6 | * | * |
| MeAαC | 12.4 ± 4.6–145.2 ± 5.3 | 0.2 ± 3.1–0.3 ± 7.5 | * | * | * |

[a] (ng cig$^{-1}$ ); [b] mean value ± expanded uncertainty ($U$) for $k = 2$; * < LOD

*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-3,4,8trimethylimidazo-[4,5-f]-quinoxaline, *DMIP* 2-amino-1,6-dimethyl-imidazo-[4,5-b]-pyridine, *PhIP* 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine, *Trp-P1* 3-amino-1,4-dimethyl-5H-pirido-[4,3-b]-indole, *Trp-P-2* 3-amino-1-methyl-5H-pirido-[4,3-b]-indole, *AαC* 2-amino-9H-pyrido-[2,3-b]-indole, *MeAαC* 2-amino-3-methyl-9H-pyrido-[2,3-b]-indole

compared, the highest values were for brand number four, following the trend observed for mainstream smoke (Table S2-ESM).

To the best of our knowledge, the results demonstrated for the *first time* that the target HAAs have been formed (partially or completely) from the combustion of each physical component of the cigarette. The HAA detection in these samples remarks on the novel contribution of this work to literature in terms of the insights of the HAA generation during smoking and the fact that these compounds could be considered as cigarette burning toxic markers. Further, the amounts of the studied analytes in mainstream smoke reveals the potential impact on health also for non-smoking individuals. Additionally, the tobacco analysis evidenced a baseline concentration of these analytes, which could be generated during the treatment or combustion of tobacco leaves. Likewise, the variability in the concentration levels may be an indicator of the tobacco variety in the different brands, as well as the treatment that it receives before the final product elaboration (commercial cigarettes).

As a summary, Table 4 shows a comparison of the analytical performance among the proposed UAE-MWCNT-based SPE method and other related works for the determination of HAAs in biomass samples generated from cigarette combustion and tobacco. As can be observed, the herein proposed multi-analyte

**Table 4** Summary of reported studies for the determination of HAAs in biomass samples generated from cigarette combustion and tobacco

| Sample | Extraction/ cleanup methodology | Separation/ detection technique | Analytes analyzed | Reported concentration values (ng cig$^{-1}$) | [a] LODs/[b]LOQs (ng cig$^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| Mainstream smoke | UAE Oasis® -MCX-SPE | HPLC-MS/MS | AαC MeAαC Trp-P-1 Trp-P-2 | 18.1–76.4 1.8–7.4 1.0–3.4 0.6–4.6 | [a]0.08–0.56 [b]0.26–1.85 | Zhao et al. 2014 |
| Cigarette ashes | UAE-DSLME PUF-SPE | UHPLC-MS/MS | IQ MeIQ 4,8-Di-MeIQx PhIP | 287.1* 87.2* 22.4* 82.2* | [a]3.5–10.7 * [b]9.4–16.9* | Guiñez et al. 2020 |
| Mainstream smoke Tobacco Paper ashes | UAE MWCNTs based-SPE | UHPLC-MS/MS | IQ MeIQ MeIQx 4,8-DiMeIQx PhIP DMIP AαC MeAαC Trp-P-1 Trp-P-2 | 0.2–83.0 0.3–41.9 0.1–33.8 0.1–30.8 1.0–65.4 11.9–62.2 0.1–123.0 0.2–155.2 0.1–31.7 0.1–43.9 | [a]0.03–0.63 [b]0.10–1.92 | This work |

*(ng g$^{-1}$ )

**Table 5** Comparative *Green Certificate* for HAA extraction methods from burning biomass samples

| Extraction technique | Sample | Reagent amount (mL) | $PP_R$ | Hazard ($PP_{RH}$) | Subtotal $PP_R$* | Volume Waste (mL) | $PP_W$ | $PP_E$ | Total PPs | Green Certificate** | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| UAE<br>SPE (Oasis MCX® cartridge) | Mainstream smoke | MeOH (47.5) Ammonia (1) HCl (1) | 2.36<br>0.66<br>0.66 | 6<br>4<br>4 | 19.44 | 95 | 11.41 | 3 | 30.85 | 69.15 "D" | Zhao et al. 2014 |
| UAE-DSLME PUF-SPE | Cigarettes ashes | Acetone (0.5) n-Hexane (1) ACN (3.3) HCOOH (0.15x $10^3$) | 0.52<br>0.66<br>0.98<br>0.03 | 4<br>6<br>4<br>6 | 10.18 | 11.5 | 4.53 | 6 | 20.71 | 79.29 "C" | Guiñez et al. 2020 |
| UAE MWCNTs based-SPE | Mainstream smoke<br>Tobacco Paper ashes | MeOH (2) ACN (0.64) HCOOH (0.15 x $10^3$) | 0.43<br>0.57<br>0.03 | 6<br>4<br>6 | 5.10 | 10 | 4.28 | 6 | 15.38 | 84.62 "B" | This work |

*Subtotal $PP_R = PP_R \times PP_{RH}$ [21] ; ** Green Certificate = 100 - total PPs [21]

procedure is a sensitive, efficient, and selective alternative as compared with other methodologies. Minimum solvent volumes are required making the method also eco-compatible. Additionally, the simple and satisfactory sample treatment makes an adequate option to determine HAAs in cigarette derivatives.

## Assessing the greenness of the methodology

To assess the greenness of the proposed methodology, the *Green Certificate* was calculated as described in the "Parameters for the assessment of the Green Certificate" section. Considering the PPs obtained for each procedure, the UAE-MWCNT-based SPE procedure can be assigned as green (Green Certificate: 84.62). Also, a comparative study was carried out to classify the sustainability of several recent miniaturized approaches for HAAs extraction from diverse biomass burning samples (Table 5).

In this aspect, there is scarce information concerning the HAA composition in these samples. However, some reports informed about the presence of non-polar HAAs, which were extracted from fiber pads with a high MeOH volume as well as some indispensable additives were used during the cleanup strategy, mainly in the case of mainstream smoke (Zhao et al. 2014) (Table 5). On the other hand, Guiñez et al. identified the presence of polar HAAs in cigarette ashes employing a miniaturized technique with various steps, including solvents as n-hexane and acetone (Guiñez et al. 2020) (Table 5). Analyzing the results, the PPs ranged from 15.38 to 30.85, which demonstrates an increase of penalty due to the large volumes and the nature of reagents employed (Zhao et al. 2014). The addition of diverse solvents and/or reagents as well as the number of steps in extraction/cleanup procedures is a disadvantage on sustainability even in miniaturized methodologies. As observed after the analysis, the proposed methodology can be classified as "B" (scale from A to G) and resulted to be greener than the other approaches described. Therefore, the herein presented strategy appears as a sustainable alternative for HAAs extraction and cleanup, reducing waste production and decreasing the impact on the environment.

## Conclusions

A green and novel analytical methodology based on UAE-MWCNT-based SPE associated with UHPLC-MS/MS was developed for the efficient extraction/cleanup of ten HAAs in mainstream smoke and paper ashes samples from cigarette combustion considered as biomass samples, tobacco, paper wraps, and other constituents of the sample collecting device.

This work demonstrated high concentration levels of the HAAs mainly in the mainstream smoke. The contribution from the different parts of the cigarette was verified based on the variation of the concentration levels. Furthermore, some of the amines were detected only after the biomass burning. As compared with previously reported analytical procedures, similar or even lower LODs/LOQs could be reached by the proposed methodology. This literature report provides information about the formation/distribution of these analytes in diverse blond cigarette brands. Future studies are needed to support the herein reported findings to consider the HAAs as potential markers of biomass from cigarette combustion. It is believed that the results of this investigation will supply important information about potential health risks and environmental impact.

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**Authors' contributions** RC performed the experiments, analyzed and interpreted the data, and wrote the manuscript. MG helped to perform the experiments, interpreted the data, and wrote the manuscript. CT introduced the smoker system and how to use it properly. MR supervised the work. SC supervised the work and critically revised the manuscript. All authors read and approved the final manuscript.

**Availability of data and materials** All data generated or analyzed during this study are included in this published article (and its supplementary information files).

## Compliance with ethical standards

**Competing interests** The authors declare that they have no competing interests.

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