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Multiple headspace sampling coupled to a programmed temperature vaporizer – Gas chromatograph-mass spectrometer for the determination of polycyclic aromatic hydrocarbons in water and saliva

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ABSTRACT

Here we describe the development of a multiple headspace sampling methodology coupled to a programmed temperature vaporizer and a gas chromatograph-mass spectrometer (MHS-PTV-GC-MS) for the determination of 13 polycyclic aromatic hydrocarbons (PAHs) which are considered widespread environmental pollutants. Coupling with the PTV in solvent vent mode made it possible to enrich the analytes present in the headspace through multiple extractions and transfers to the injector previous to the chromatographic run. All parameters affecting the headspace generation, extraction, and stepwise transfer to the PTV were optimised. The method was successfully validated using UHQ-water, for thirteen PAHs, with limits of detection (LODs) in the range of 1.0–11 ng L^{-1}. The method was also validated in saliva, with LODs in the range of 1.4–43 ng L^{-1}. Intra- and inter-day repeatability values expressed as relative standard deviation percentage (RSD) were found to be lower or equal to 8.3% and 12.6%, respectively, in water, and 15.2% and 10.0% in saliva. Furthermore, all calibrations presented good linear behaviour (R^2 values >0.98) and fitted to the model according to ANOVA model validation. This method was applied to the quantification of PAHs in 14 saliva samples from 11 subjects, both non-smokers and smokers. PAHs were not detected in saliva from non-smokers and light-smokers above the LODs. However, these compounds could be found in saliva samples from heavy smokers taken right after smoking. A study of the same subjects in saliva samples provided one hour later was also conducted to evaluate the concentration change in time.

1. Introduction

During the last years, polycyclic aromatic hydrocarbons (PAHs) [1], one class of persistent organic pollutants (POP), have drawn great attention, not only from the scientific community but also from general population, due to their high toxicity [2,3]. In fact, the International Agency for Research on Cancer (IARC) has classified some PAHs as human carcinogen and others as probably or possibly carcinogenic to humans [4]. PAHs originate from anthropogenic actions like pyrolysis or incomplete combustion of organic substances such as coal, diesel, oil, wood, garbage or tobacco. Furthermore, they can have natural occurrence during forest fires or volcano eruptions. The introduction of these compounds into the human body can be *via* several ways, being direct ingestion the most usual [5–7], as well as dermal contact or inhalation [8].

Many works can be found for the determination of PAHs in water [9,10], however, considering the hazard that this class of compounds represent to human beings [11], the development of new and better ways to determine their amount and presence in biological samples is of paramount importance [12]. Urine is one of the most available, yet non-invasive, human samples that can be rapidly collected and processed. Many determinations of metabolised and non-metabolised PAHs in this matrix have been published [12,13]. After following an oxidative metabolism, PAHs are transformed into hydroxyl-PAHs (OH-PAHs) and then excreted through urine [14]. Both the concentrations of PAHs metabolites and non-metabolised PAHs are individual dependant. Hence, to overcome this issue, saliva [15] has become a good alternative since it is still a non-invasive and easy to collect sample where non-metabolised PAHs can be determined with less variability among different people. The determination of the concentration of non-metabolised PAHs in saliva allows to estimate the degree of exposure to these compounds that a person has experienced due to several factors,

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such as food ingestion, environmental exposure or smoking.

Only a few examples of PAH determination in saliva can be found in literature [16–20] and most of them require some class of sample preparation or extraction steps, which are time-consuming and/or need the use of large amounts of organic solvents, along with the risk of losing analytes or having lower accuracies due to human error. There is only one reported method [18] which needed no sample treatment, consisting of dipping a glass rod into the sample and directly analysing it using atmospheric pressure solid analysis probe (ASAP). The main drawback of this technique is that there is no chromatographic separation of the analytes so it is not possible to differentiate between isomers, such as pyrene and fluoranthene.

With the aim of developing a method with minimal or no sample treatment which, at the same time, provides a chromatographic separation of different non-metabolised PAHs, we decided to apply a new methodology previously proposed by our group [21], based on the use of a multiple headspace system for the sampling step, coupled to a programmed temperature vaporizer (PTV) as injector into a gas-chromatograph–mass-spectrometer (GC–MS) for separation and determination.

The idea of using headspace for sampling PAHs in saliva can be challenging due to several factors. First, the concentrations this matrix may contain are quite low. For instance, when liquid-liquid extraction-programmed temperature vaporizer-gas chromatography-mass spectrometry (LLE-PTV-GC-MS) was used to analyse PAHs in saliva, the lowest concentrations found in healthy volunteers were between the limits of detection (lower than or equal to 57 ng L^{-1}) and approximately 500 ng L^{-1} [17]. Secondly, the low volatility of these compounds (boiling points ranging from 218 °C for naphthalene to 393 °C for pyrene), together with their octanol/water (Table S1, see Supplementary material) partition coefficients [5,22] make them hard to determine using headspace in aqueous matrices. In order to enhance sensitivity, rather than using conventional headspace analysis, here we propose a multiple headspace sampling (MHS) methodology using a PTV inlet as a cold trap. Thus, instead of incubating the sample during a certain time and then withdrawing the gas phase once to be transferred to the chromatograph injector (conventional HS) the headspace generation step is split (typically 2–8 times) and the withdrawing and transfer to the injector is repeated. Here, a PTV in solvent vent injection mode is required, so as the successive HS gas phase transfers are collected and retained until the chromatographic process starts rendering a single chromatogram. Optimisation of solvent vent injection mode allows analytes to be focused in the liner packing material removing the most volatile compounds, along with the excess of water vapour. In this manner, HS signals can be increased several fold, especially for the heaviest or least volatile and polar analytes, which addresses the limit of detection problematics.

This MHS methodology has a different purpose from that initially developed by Kolb et al. [23] to avoid matrix effects in HS sampling by exhaustive extraction. Instead, it is oriented to enhance sensitivity. As a proof of concept, this new methodology was successfully applied to determine aldehydes in urine samples [21]. In that case, enrichment factors using MHS ranged from 3 to almost 7 times compared to conventional HS. In this work we propose, for the first time, the use of MHS-PTV-GC–MS for the determination of non-metabolised PAHs in water and human saliva. This methodology has been evaluated and applied to saliva samples from different subjects, both non-smokers and smokers.

2. Experimental

2.1. Reagents and standards

Naphthalene (NAP, 99%), 1-methylnaphthalene (1-MNAP, 97%), 2-methylnaphthalene (2-MNAP, 99%), biphenyl (BIP, 99%), 4-phenyltoluene (4-PTOL, 98%), phenanthrene (PHE, 97%), fluoranthene (FLT, 98%), pyrene (PYR, 98%), acenaphthene (ACE, 99%) and fluorene (FLR, 98%) were supplied by Acros Organics (Geel, Belgium). 3-Phenyltoluene (3-PTOL, 95%), acenaphthylene (ACY, 99%), anthracene (ANT, 99%), ethyl acetate (HPLC grade), acetone (HPLC grade) and methanol (HPLC grade) were supplied by Sigma-Aldrich (Steinheim, Germany). Sodium chloride (99.5%) was purchased from Scharlab (Barcelona, Spain). Stock solutions (1000 mg L^{-1}) of each compound were prepared in methanol, except for pyrene, which was prepared in acetone (100 mg L^{-1}). All solutions were stored at 8 °C. Before use, these solutions were left to warm up to room temperature and subsequently diluted to prepare the working solutions for spiking samples. Ultra-high quality water (UHQ, obtained with a Wasserlab Ultramatic water purification system, Noain, Spain) was used. Optimisation studies were done with UHQ-water spiked with 50 μg L^{-1} of each analyte.

2.2. Saliva samples

Saliva samples were obtained from 6 non-smoker (S1–S6) and 5 smoker subjects (2 light smokers (S7, S8) and 3 heavy smokers (S9–S11)) and collected in 10 mL headspace vials. All the samples were collected after at least 1 h without eating, drinking, smoking or brushing their teeth. Additionally, the heavy smoker subjects provided one extra sample each, right after smoking one cigarette. The containers were seal-closed and kept at −20 °C until analysis (typically 24–72 h, except for stability studies). After thawing at room temperature, samples were vortexed at 3000 rpm during 1 min for homogenization prior to use.

For analysis, all saliva samples were treated as follows: in a 10 mL headspace vial, 1.0 mL of saliva was added followed by 4.0 mL of UHQ-water and 2.5 g of NaCl. The vial was hermetically closed and placed in the autosampler tray. The rest of the process was automatically run, using approach 1.

2.3. Instrumental conditions

2.3.1. Headspace

All steps of headspace sampling were automatically performed with a MPS2 Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany).

2.3.1.1. Conventional headspace sampling. A 10 mL headspace vial was heated in the headspace oven (90 °C) and shaken (750 rpm) for 5 min, to generate and equilibrate the headspace. Next, a 2.5 mL gas-tight syringe (set at 120 °C) withdrew 2.5 mL of the gas phase and transferred it to the PTV inlet. Fill speed and injection speed were fixed at 100 μL s^{-1} and 250 μL s^{-1}, respectively. After injection, the hot syringe was automatically purged with N_2 (99.999%, Air Liquid) for 2 min.

2.3.1.2. Multiple headspace sampling. The same conditions of conventional headspace aforementioned were used. However, after headspace generation the gas phase withdrawing and transfer process to the PTV was repeated for 3 more times prior to chromatographic separation (no pause time for re-equilibration was used). After the last injection, the hot syringe was also cleaned purging with N_2 (99.999%, Air Liquid) for 2 min.

2.3.2. Programmed temperature vaporizer

The PTV liner (71 mm × 2 mm I.D., Gerstel CIS-4) was packed with Tenax TA®. One transfer (for conventional HS) or 4 transfers (for multiple HS) of the gas phase into the PTV were performed. Operating in solvent vent mode, split valve remained opened during transfers for 0.45 min (vent flow: 150 mL min^{-1}; vent pressure: 6 psi) and closed between them. The temperature was set at 115 °C. After the last transfer, and using the maximum heating ramp (720 °C min^{-1}), the PTV was heated to 340 °C and then the analytes were injected into the chromatographic system (1 min in splitless injection mode). After injection, the temperature was maintained during 5 min with split valve opened for cleaning, using a split vent purge flow of 150 mL min^{-1}. Finally,

liquid CO_2 was used to reach initial conditions again.

2.3.3. Gas chromatography–mass spectrometry

Analyses of PAHs were performed on a GC–MS instrument (Agilent Technologies, Santa Clara, CA, USA) consisting of an Agilent 7890A series gas chromatograph interfaced to an Agilent 5975C inert XL MSD.

Separation of PAHs was performed on a HP-5MS UI capillary column (30 m × 0.25 mm, 0.25 μm; J&W Scientific, Folsom, CA, USA) using He (99.999% pure; Air Liquide) as carrier gas (flow rate of 2 mL min^{-1}). The column oven temperature program [17] started at 60 °C, held for 0.5 min; then the temperature was risen at 60 °C min^{-1} to 175 °C (held for 0 min) and finally a 45 °C min^{-1} increase was applied up to 325 °C, holding this temperature for 1.5 min. Total chromatographic run time was 8.25 min. The total time needed for the analysis was 16 min: 5 min for HS generation, 4 min for transfers to the PTV (1 min per transfer) and 7 min for GC run.

A quadrupole mass spectrometer detector (electron ionization mode with ionization voltage of 70 eV) was used for detection (ion source temperature: 230 °C; quadrupole temperature: 150 °C; transfer line temperature: 300 °C). Synchronous scan/SIM mode was used for collection of both types of data in each run (solvent delay: 2.5 min). The *m/z* range selected was 35–300 amu. In the selected ion monitoring (SIM) mode, one quantitation and two qualifier ions were monitored for quantitation purposes. Five SIM groups with a dwell time value of 1 ms each, and different *m/z* were employed (Table S1). MSD ChemStation, Ver. E.02.00.493 software from Agilent Technologies was used for data acquisition. NIST_98 (NIST/EPA/NIH Mass Spectral Library, version 2.0) database was used for identification.

3. Results and discussion

Several factors affecting the different steps of the analysis have been optimized: PTV conditions, headspace generation and injection conditions.

For the optimisation studies, unless otherwise stated (changes corresponding to the optimised variable in each of the following sections), the instrumental conditions used were the ones aforementioned in Section 2.3, and using 5 mL of UHQ-water spiked with 50 μg L^{-1} of each analyte.

3.1. Programmed temperature vaporizer conditions

First, different initial and final PTV temperatures were evaluated selecting the solvent injection mode. Regarding initial temperature, three different values were studied: 90 °C, 115 °C, and 130 °C. The results are shown in Fig. S1 (see Supplementary material). Increasing temperature to 130 °C worsened results, especially for the most volatile analytes, with up to 90% signal decrease for naphthalene vs around 20% for heavier analytes. Instead, 115 °C provided the best results for analytes of medium to low polarity and volatility, with only 0 to 15% signal decrease for the most volatile among them (except for naphthalene, whose signal suffers a signal decrease of 40%). As a compromise, 115 °C was chosen as the optimum initial temperature. Supporting information.

Regarding PTV final temperature, a range of temperatures from 290 °C to 340 °C was tested (Tenax TA® maximum working temperature recommended by manufacturer is 350 °C). For the most volatile PAHs, increasing PTV final temperature had little influence on the results (Fig. S2). However, for those heavier analytes such as phenanthrene, anthracene or fluoranthene higher temperatures enhanced the signals, since they were more strongly retained in the Tenax-packed liner. Thus, the highest value tested, without reaching maximum recommended operating temperature, 340 °C, was selected as optimum, in order to maximise liner lifetime. Blanks analysed after injections of samples containing PAHs provided no signals above detection limits, showing that desorption was complete.

Vent flow was evaluated with values ranging from 150 to 200 mL min^{-1} (Fig. S3). For the most volatile PAHs, increasing vent flow produces a diminishing of signal (up to 30% for naphthalene). For the least volatile analytes, such as phenanthrene and anthracene, however, the behaviour is opposite, although the increment is not as noticeable. Thus, 150 mL min^{-1} was chosen as working vent flow.

Injection time also plays a critical role since, especially for the least volatile PAHs, they need more time to be desorbed from the liner. Injection times of 0.5, 1 and 2 min were evaluated. There was a considerable difference between 0.5 and 1 min, but not between 1 and 2 min, as can be observed in Fig. S4. Hence, 1 min was chosen as optimum injection time.

3.2. Headspace sampling optimisation

The effect of different parameters was optimised: ionic strength, sample volume and headspace generation time. Regarding incubation temperature and vial shaking, 90 °C (maximum recommended temperature to generate headspace when using aqueous samples, in order to avoid vial leakage or breaking because of boiling) and 750 rpm (maximum speed permitted by the equipment) were used.

First we evaluated the effect of ionic strength using NaCl to improve the extraction of the analytes from the aqueous phase. In this regard, a comparative study was performed by using two 5 mL UHQ-water samples. One did not have NaCl and the other contained 2.5 g to saturate the aqueous phase. The signal increase was around 5 fold for the most polar analytes such as naphthalene, whereas for heavier and less polar compounds the increment was up to 10–12 fold. Thus, all further samples were analysed after saturation with NaCl.

The volumes of gas (headspace) and sample (liquid) phases are critical for this type of analysis [23]. The ratio β between these volumes and the partition coefficient of each analyte play an important role on the concentration of the compounds in the headspace. To evaluate the effect of the sample volume, 0.5, 1.0, 2.5 and 5.0 mL of UHQ-water samples, spiked at the same PAHs concentrations, were tested. All samples were supersaturated at 50% (w/v) with NaCl (Fig. S5). Results confirmed what it was expected, as described in literature [23], that is, an increase of sample volume (decreasing the β ratio) is related to an increase of concentration in the gas phase. Depending on their partition coefficient, each analyte showed a different behaviour. The most polar PAHs rapidly incremented their concentration in the gas phase as the sample volume raised. However, the least polar analytes such as fluoranthene or pyrene experienced a minor signal growth.

Considering that low concentration of analytes are expected to be found in saliva samples, 5.0 mL was selected as optimum volume in order to maximize the analytical signals without the risk of contaminating the vial septum or the needle of the headspace sampler. Since one of the aims of this work was the determination of PAHs in saliva samples, higher volumes would also add difficulties to sample collection.

For the evaluation of headspace generation time, values from 1.25 to 10 min were tested. Maximum signals were already obtained after 2.5 min for the most volatile compounds and after 5 min for all the others. Thus, 5.0 min was selected as the optimum time for headspace generation (Fig. S6).

3.3. Multiple headspace sampling optimization

For the multiple headspace methodology, we studied two different approaches. First approach would select an initial HS generation time. Next, a determined number of extractions from the gas phase would be successively withdrawn and injected into the PTV. Second approach would consist of splitting the HS generation time in different fractions, withdrawing the sample from the gas phase and transferring it to the PTV after each one. In both approaches the analytes would be retained in the liner between injections, using solvent vent injection mode.

Since 5 min was considered to be the optimum time for conventional headspace generation and equilibration, the next step in our study was,

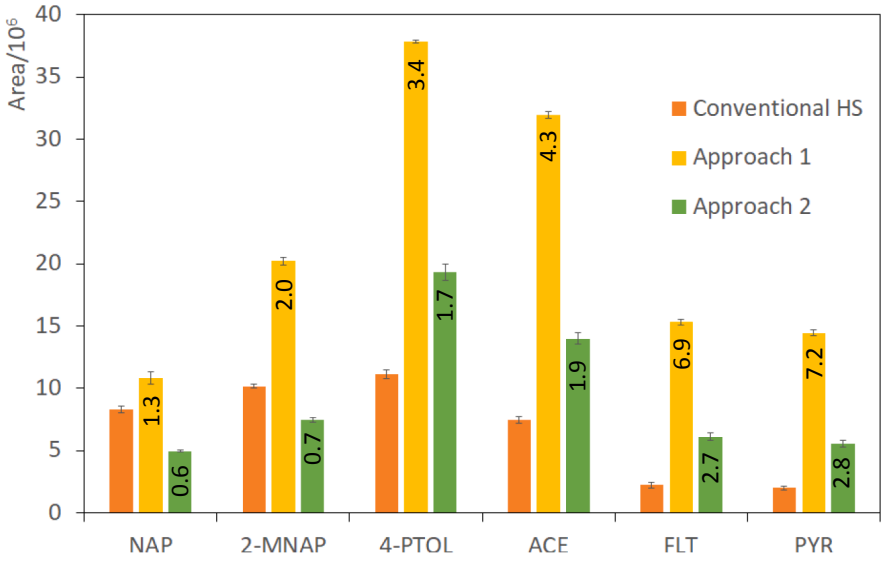

Fig. 1. Signals obtained using conventional headspace (orange), approach 1 (yellow) and approach 2 (green) in UHQ-water. UHQ-water spiked with 50 μg L^{-1} of each analyte was used. Approach 1: 5 min of initial HS generation time followed by 4 extractions from the gas phase withdrawn and injected into the PTV. Approach 2: HS generation split in 4 fractions of 1.25 min each, withdrawing the sample from the gas phase and transferring it to the PTV after each one. The number in the bars represents the enrichment factor. For other instrumental conditions see Section 2.3.

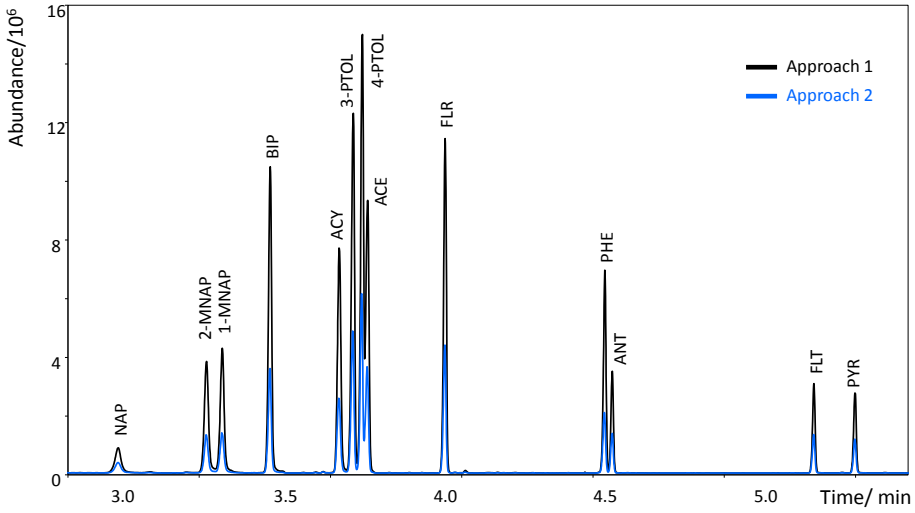

Fig. 2. Total ion chromatogram of approach 1 (black line) and approach 2 (blue line) for the 13 PAHs in UHQ-water. Concentration levels and instrumental conditions as in Fig. 1.

on one hand, to test a single 5-min HS generation time, followed by 2, 4, 6 or 8 gas transfers to the PTV (first approach). On the other hand, to split the 5-min HS generation time into 2, 4, 6 or 8 fractions (of 2.5, 1.25, 0.83 and 0.62 min, respectively), and then perform one transfer to the PTV after each fraction (second approach). In order to evaluate the results, we used the enrichment factors (EF), calculated as the increase of signal area obtained for each analyte in every multi-headspace experiment compared to conventional headspace. The best results for the first approach were obtained with 4 gas transfers, and for the second approach, when the time was split into 4 fractions. Fig. 1 shows these results compared to conventional headspace data.

In both approaches, EFs higher than 1 can be explained because successive extractions and injections from the same vial produce a disturbance in the equilibrium reached, displacing the analytes towards the gas phase. This could be compared to a dynamic headspace generation in steps.

Using the first approach, the least volatile analyte, naphthalene, showed only an EF of 1.3. This can be explained considering that the first extraction withdraws a substantial fraction of the naphthalene present in the sample. Transferences 2–4 still take some coming from the condensed phase, increasing the signal compared to conventional HS, but only a 30% more, taking also into account that the high volatility and polarity of naphthalene makes more difficult its retention in the PTV liner during the solvent vent repeated process. For heavier PAHs, higher EF values were obtained, up to 7.2 for pyrene.

Using the second approach, EFs were lower than those obtained with the first one for all the analytes. Values lower than 1 (no enrichment in comparison with conventional HS) were even found for naphthalene, 2-methylnaphthalene and 1-methylnaphthalene, the most volatile compounds. Thus, the first approach was selected. Fig. 2 shows a comparison of both approaches.

3.4. Matrix effect and saliva sample volume

In order to check whether there exists matrix effect when analysing saliva samples, we compared the signals obtained after spiking 5 mL of UHQ-water and 5 mL of saliva sample using approach 1. For this purpose, we performed this part of the study with five different saliva samples from five different subjects, 3 non-smoker (S1–S3) and 2 light smokers (S7 and S8). The concentrations used to spike each sample were

in the range of 250–1000 ng L^{-1} and differed for each analyte due to their different response (BIP, 3-PTOL, 4-PTOL and FLR: 250 ng L^{-1}; ACY and ACE: 500 ng L^{-1}; 2-MNAP, 1-MNAP, and PHE: 750 ng L^{-1}; NAP, ANT, FLT and PYR: 1000 ng L^{-1}).

Results showed an evident matrix effect (see Fig. S7). Signal for naphthalene in saliva was about a 70% of that in UHQ-water. The effect increased as analyte volatility and polarity diminished reaching around a 4% for pyrene. Among the different saliva samples this effect was much less pronounced. This effect is probably due to the interactions that PAHs may have with different saliva components which eventually affect to the equilibrium between the liquid and the gas phase.

In order to minimise this matrix effect, we evaluated the dilution of the saliva sample. For this purpose, volumes of saliva of 0.75, 1.0 and 2.5 mL, diluted with UHQ-water to a final volume of 5 mL and 5 mL of pure saliva were spiked at the same concentrations as before.

As shown in Fig. S8, the matrix effect was not completely removed by dilution. However, all analytes gave better signals when saliva sample was diluted using 0.75 or 1.0 mL. Comparing these, the best RSD values (from 5.7 to 13.1%) were obtained with the latter. Thus, we decided to use 1 mL of saliva, diluted with 4 mL of UHQ-water to a final volume of 5 mL, as standard conditions for sample preparation.

Since it was not possible to completely eliminate matrix effect, it was decided to use a one-point standard addition method for quantification of samples, which has been proved to be successful for other determinations [24,25] and it is advantageous when only small volumes of sample are available.

3.5. Stability study

To the best of our knowledge, to date, a study of the stability of the concentration of PAHs in saliva samples during several days has not been carried out. In literature there are some examples of stability studies but either they have been performed in a different matrix [26] or with metabolised products such as hydroxylated-PAHs [27].

For this experiment, we combined the contents from two randomly chosen saliva vials of non-smokers' subjects. After spiking (same concentrations as for matrix effect studies), the pooled saliva was aliquoted by 1 mL in 10 mL vials and stored at −20 °C. Three aliquots of the spiked saliva were taken from the freezer at days 0, 1, 3, 7, and 14, and treated as previously described. Selected analytes with low, medium and high boiling points are shown in Fig. S9. As can be seen, there is not significant variance in time for the concentration of PAHs in saliva, which proves their stability for analysis. This allows for enough time to determine the contents of PAHs in saliva samples that have been previously stored correctly. In this way, patients can provide their samples at any time without disturbing the course of the pending analyses being performed at the time, since these samples can be stored and analysed later on.

3.6. Analytical characteristics of the method

The method was evaluated using UHQ-water and saliva (from subject S1) spiked with PAHs at concentrations ranging from LOD to those shown in Table S2 (see Supplementary material). Six calibration levels were measured in triplicate. Extracted quantitation ions from SIM mode chromatograms were used to integrate areas of each analyte. All calibrations presented good linear behaviour (R^2 values >0.98). Furthermore, their validity was checked using ANOVA, and it was observed that they did not exhibit any lack of fit. The limits of detection (LOD) and quantification (LOQ) were calculated using the signal-to-noise ratio criterion, as recommended by ISO 11843-1 [28]. Values of S/N = 3 and S/N = 10 for LOD and LOQ, respectively, were used.

As shown in Table S2, the LODs and LOQs in both matrices were in the ppt range, which is lower than the US-EPA maximum contamination limits (MCL) [29], European Union (EU) limits for human consumption [30], and EU environmental quality standard [31]. LOD values in water

Table 1
Trueness assessment.

Compound	Added concentration (ng L^{-1})	Found concentration (ng L^{-1})																									
		NON-SMOKERS																LIGHT-SMOKERS									
		S1		S2		S3		S4		S5		S6		S7		S8											
NAP	159	170	±	20	170	±	20	180	±	20	180	±	20	160	±	20	160	±	20	155	±	7	170	±	20		
2-MNAP	119	123	±	8	125	±	6	140	±	9	130	±	10	140	±	20	130	±	10	123	±	5	120	±	20		
1-MNAP	119	120	±	10	120	±	10	130	±	10	130	±	10	140	±	20	120	±	20	123	±	9	120	±	10		
BIP	40	40	±	4	41	±	4	46	±	3	45	±	8	50	±	10	40	±	10	45	±	8	42	±	8		
ACY	79	70	±	10	75	±	5	83	±	9	80	±	20	80	±	20	80	±	20	90	±	10	80	±	10		
3-PTOL	39	39	±	5	40	±	4	50	±	10	50	±	10	46	±	9	43	±	7	44	±	9	40	±	6		
4-PTOL	40	39	±	2	41	±	4	49	±	6	44	±	7	46	±	6	42	±	8	45	±	5	37	±	8		
ACE	80	73	±	6	76	±	8	85	±	6	80	±	10	100	±	20	80	±	10	90	±	20	80	±	10		
FLR	40	37	±	3	39	±	5	43	±	2	40	±	10	44	±	8	40	±	6	42	±	7	42	±	6		
PHE	120	107	±	10	110	±	10	140	±	20	136	±	20	130	±	20	120	±	10	130	±	10	130	±	10		
ANT	161	140	±	20	150	±	20	170	±	10	161	±	10	470	±	20	150	±	20	180	±	20	153	±	8		
FLT	159	140	±	20	170	±	20	150	±	20	160	±	10	150	±	20	140	±	20	160	±	10	140	±	20		
PYR	173	170	±	10	160	±	10	170	±	10	180	±	20	170	±	20	160	±	20	180	±	10	160	±	10		

Table 2
PAHs' concentrations found in heavy smoker's saliva samples.

	S9 (ng L^{-1})						S10 (ng L^{-1})						S11 (ng L^{-1})					
	After smoking			1 h later			After smoking			1 h later			After smoking			1 h later		
NAP	700	±	100	150	±	50	1700	±	200	360	±	90	4100	±	400	750	±	40
2-MNAP	420	±	70	101	±	20	800	±	100	310	±	60	1510	±	90	640	±	50
1-MNAP	530	±	80	70	±	30	700	±	100	390	±	60	1380	±	80	600	±	80
BIP	120	±	10		<LOQ		170	±	50	80	±	20	390	±	80	150	±	20
ACY	250	±	50		<LOQ		490	±	60	160	±	30	1300	±	200	270	±	30
3-PTOL	90	±	20		<LOQ			<LOQ			<LOQ		260	±	50	130	±	40
4-PTOL	80	±	20		<LOQ			<LOQ			<LOQ		170	±	30	60	±	30
ACE		<LOQ			<LOQ			<LOQ			<LOQ		170	±	40		<LOQ	
FLR	320	±	50		<LOQ		320	±	40	220	±	40	800	±	180	290	±	70
PHE	260	±	30		<LOQ		290	±	70	190	±	40	400	±	180	310	±	20
ANT		<LOQ			<LOQ			<LOQ			<LOQ			<LOQ			<LOQ	
FLT		<LOQ			<LOQ			<LOQ			<LOQ			<LOQ			<LOQ	
PYR		<LOQ			<LOQ			<LOQ			<LOQ			<LOQ			<LOQ	

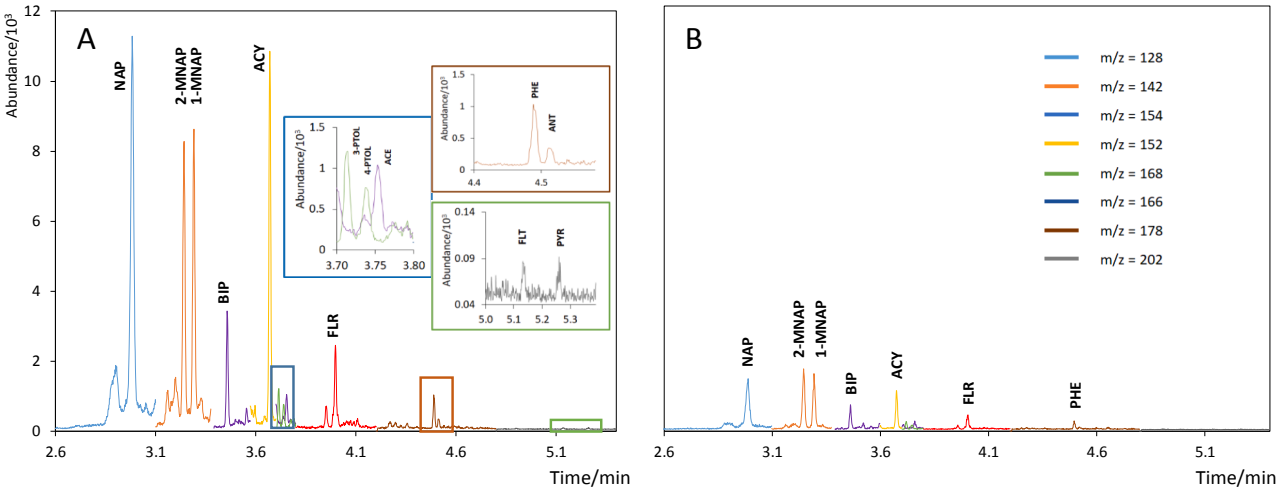

Fig. 3. Chromatograms of saliva samples for S11, (A) after smoking and (B) 1 h later. Extracted ion chromatograms recorded in SIM mode (*m/z* 128, 142, 152, 154, 166, 168, 178 and 202).

were in the range from 1.0 to 11 ng L^{-1}, while in saliva were between 1.4 and 43 ng L^{-1}, with similar values in both water and saliva matrices for most of the analytes. The differences were bigger for the heaviest compounds analysed here.

Intra- and inter-day repeatability studies were performed in UHQ-water and saliva samples, both spiked at low (68.7–687 ng L^{-1}) and high (250–2500 ng L^{-1}) concentrations levels. For intra-day repeatability, the corresponding sample was analysed 10 times on the same day. For inter-day repeatability in UHQ-water, the sample was analysed in triplicate in 5 successive days. In saliva, one sample was first divided into 5 portions and frozen. Then, each day during 5 consecutive days, the sample was prepared as previously described and spiked with the same levels of calibration as for repeatability. Then, each sample was analysed 3 times each day. As shown in Table S2 values for intra- and inter-day repeatability studies, expressed as RSD percentage values, were lower or equal to 8.3% and 12.6% respectively in water, and 15.2% and 10.0% in saliva.

To check accuracy, we performed the analysis of saliva samples from the 6 non-smokers subjects (S1–S6) and the 2 light-smokers (S7–S8). All of them provided signals below the limit of detection for every analyte. Hence, we decided to spike them at known concentrations and use the one-point standard addition protocol to determine PAHs in these samples. Table 1 summarizes the results obtained, showing the actual concentration and the value calculated by the model. As shown, the model predicts the concentration with good accuracy for all the analytes as the confidence intervals include the actual concentration.

3.7. Analysis of heavy smokers' saliva samples

Saliva samples from 3 heavy-smokers (S9–S11, being S11 the heaviest smoker and S9 the lightest of this group) were analysed by using the validated model proposed. Every subject provided two samples: one right after smoking one cigarette and one more, 1 h later, to study concentration changes over time. For these samples, the same one-point standard addition method was applied to predict the PAHs concentrations (Table 2).

The concentration of most of the compounds was higher in S11, while the smallest concentrations were found in S9. It is also noticeable that some compounds present in S9 and S11 such as 3-phenyltoluene and 4-phenyltoluene were found below the LOQ in S10. Next we performed the analysis of the saliva samples collected 1 h after smoking, from the same subjects. As shown, most of the compound concentrations were reduced by half in just 1 h. However, others were reduced about three times like 4-phenyltoluene or fluorene or even up to five times like naphthalene in S11. This proves how PAHs are rapidly removed from saliva. Fig. 3 shows the comparison of chromatograms of samples from the heaviest smoker just after smoking and one hour later.

3.8. Comparison with other works

There are few works in literature dealing with the determination of PAHs in saliva [16–19] and, to the best of our knowledge there is no example of the use of HS directly coupled to GC–MS. Recently, a

Table 3
Comparison with other works.

Method	Sample	PAHs	LOD (ng L^{-1})	Estimated analysis time per sample (min)	Ref.
HS-SDME-LC-UV	Aqueous solution	7	300–2500	167	36
HS-SE-GC–MS	Herbal infusions	10	11–26	286	32
HS-SDME-LC-UV	Aqueous solution	4	40–500	100	37
HS-SPME-GC–IDMS	Urine	13	2.28–22.8*	93	33
HS-SPME-GC-MS	Environmental water	6	1.09–2.46	30**	35
HS-TFME-GC-MS	Urine, industrial water, drinking water	6	80–200	45	34
HS-SPME-GC-MS/MS	Saliva	8	0.7–22.2	90	20
MHS-PTV-GC-MS	Saliva and water	13	4.7–43.2 (saliva) 1.0–58.9 (water)	16	This work

*Limit of quantitation (LOQ); **Only fiber extraction time.
SDME: single drop microextraction; **SE**: sorptive extraction; **SPME**: solid phase micro extraction; **IDMS**: isotope dilution mass spectrometry; **TFME**: thin film microextraction.

publication dealing with the determination of PAHs in saliva was reported [20]. In the work, the authors describe the combination of the HS-SPME and GC–MS/MS in which the analysis time was five times longer than that of the present work.

Furthermore, HS has been used in combination with additional procedures, as preconcentration step for the determination of PAHs in other matrices, such as herbal infusions [32], urine [33,34], or environmental water samples [35]. These works make use of different strategies for this purpose, such as HS-sorptive extraction (HSSE), HS-thin film microextraction (HS-TFME) or the abovementioned HS-solid phase micro extraction (HS-SPME). There are other examples in literature where headspace is used for the determination of PAHs in aqueous matrices using liquid chromatography (LC) instead of GC. Again, HS is combined with other procedures, such as single drop microextraction (HS-SDME) [36,37].

Table 3 summarizes a comparison of these different methodologies with that developed in this work. Using MHS-PTV-GC-MS, it is possible to determine 13 analytes in water and saliva samples with LODs in the same order of magnitude than those reported for GC methods and better than those achieved with LC. Sample throughput is improved, each sample only takes approximately 16 min for a complete analysis, including multi-step headspace sampling, injection and GC–MS run, making it easier to be transferred for a future clinical application. Furthermore, the methodology is simple and reduces the possible human error. Its main drawback is the limitation for heavy PAHs, given by their high boiling points and the affinity for the saliva matrix they can present. Thus, analytes such as benzo(k)fluoranthene (bp: 480 °C) or benzo (a)pyrene (bp: 495 °C), are not volatile enough to pass to the gas phase when performing headspace analysis [4,38,39].

4. Conclusions

We have successfully applied for the first time a multiple headspace coupled to PTV-GC-MS for the determination of PAHs in water and saliva samples. This methodology allows enrichment factors from 1.3 (naphthalene) to 7.2 times (pyrene) for the most and least volatile PAH, respectively, when compared with conventional HS. The method has been evaluated showing good linearity, intra- and inter-day repeatability and accuracy. In terms of sensitivity, LOD values found are in concordance with those found in literature. We have also performed the first stability study of the concentration of PAHs in saliva, proving that, at least for 14 days, this matrix can be stored at −20 °C without affecting the concentration of this type of analytes.

The method is simple and fast, it requires only 16 min per sample and there is no need for extra instrumentation beyond the already required for headspace analysis coupled to a gas chromatograph provided with a PTV injector. In addition, the fact that there is almost no manipulation of the saliva samples, reduces the errors coming from this stage and also the time of analysis, facilitating a future plausible application to clinical analysis. After analysing samples from 11 volunteers (6 non-smokers and 5 smokers), the PAHs were only found above the LOQs in saliva from heavy smokers right after smoking a cigarette or one hour later, in lesser concentrations, showing the evolution of the concentration of these compounds with time. The amounts present in all the other samples analysed were below the LOQs.

CRediT authorship contribution statement

Javier Peña: Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Mª Esther Fernández Laespada:** Conceptualization, Methodology, Supervision, Validation, Writing - original draft, Writing - review & editing. **Carmelo García Pinto:** Conceptualization, Methodology, Supervision, Validation, Writing - review & editing. **José Luis Pérez Pavón:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105822.

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