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Headspace-single drop microextraction (HS-SDME) in combination with high-performance liquid chromatography (HPLC) to evaluate the content of alkyl- and methoxy-phenolic compounds in biomass smoke

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ABSTRACT

The content of ten phenolic compounds present in four different biomass smoke materials: rock rose (*Cistus monpelienisis*), prickly pear (*Opuntia ficus indica*), pine needles (*Pinus canariensis*), and almonds skin (*Prunus dulcis*), have been evaluated. The sampling method mainly consisted of a trap alkaline solution to solubilize the phenols, and was optimized by an experimental design. Average sampling efficiencies of 78.1% and an average precision value of 10.6% (as relative standard deviation, RSD), were obtained for the selected group of phenols. The trapped phenolates were further analyzed by a headspace-single drop microextraction (HS-SDME) procedure, in combination with high-performance liquid chromatography (HPLC) with UV detection. The optimum variables for the HS-SDME method were: 1-decanol as extractant solvent, 3.5 μL of microdrop volume, 2 mL of sample volume, a pH value of 2, saturation of NaCl, an extraction temperature of 60 °C, and an extraction time of 25 min. The optimized HS-SDME method presented detection limits ranging from 0.35 to 5.8 $\mu g\,mL^{-1}$, RSD values ranging from 0.7 to 7.4%, and an average relative recovery (RR) of 99.8% and an average standard deviation of 5.2. The average content of phenolic compounds in the biomass materials studied were 70, 161, 206 and 252 $mg\,kg^{-1}$ of biomass for prickly pear, almonds skin, rock rose, and pine needles, respectively. The main components of the smokes were vanillin, phenol and methoxyphenols, in all smoking materials studied.

1. Introduction

Food smoking belongs to one of the oldest food technologies. Nowadays, it is widely used not only to give special organoleptic profiles to foods, but also as a way to inactivate the actions of enzymes and microorganisms [1]. The foods most commonly smoked are meat, fish, shellfish, and cheese [2,3]. Smoke is traditionally generated utilizing different kind of biomass [2]. The visible smoke is mainly formed by low volatile components altogether with soot particles. The remaining volatile components in the biomass smoke distribute between the gas and disperse phase. The composition of biomass smoke and the extent to which the food absorbs smoke components depends on several factors: type of biomass used, temperature of the smoking process, amount of oxygen present during the smoke generation, moisture content of biomass, among others [1,4].

Several studies have reported an extensive list of organic compounds found in biomass smoke [5,6]. Among these compounds, phenols and methoxyphenols are components of great importance for the smoke flavor, preservation of foods, and antioxidant effects [7–11].

Gas-chromatography (GC) is commonly used to determine phenols, but a derivatization procedure is usually necessary [12]. High-performance liquid chromatography (HPLC) is also common for the analysis of phenols [13–16], and it avoids the tedious derivatization step.

Given the fact that phenolic compounds are volatile or semi-volatile, losses during sampling and analysis are almost unavoidable. Several sampling systems have been designed to analyze phenolic compounds in air or biomass smoke [17–19], but they are not always useful for high volumes of sampling. A relatively simpler sampling system is described in US Environmental Protection Agency (EPA) Method TO-8 [20], and was designed for the determination of phenol and methylphenols in high volumes of ambient air. Phenols are trapped as phenolates in two impingers, each one containing an aqueous solution of sodium hydroxide. Afterwards, the impinger solution is adjusted to pH < 4 and the phenols are determined by HPLC. However, it is evident that the matrix of ambient air is less complex than that of the biomass smoke.

Hence, the analysis of biomass smoke requires further treatments of the sample. Liquid–liquid extraction, Soxhlet extraction [21], and solid-phase extraction [22], are common techniques for

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sample cleanup and preconcentration of phenols. These methods can be hazardous to human health as they often use large amounts of organic solvents, which are damaging to the environment. New analytical methods are aimed to eliminate, or at least minimize, the organic solvent consumption in sample preparation. Among them, solid-phase microextraction (SPME) [23,24] and liquid-phase microextraction (LPME) [25,26] can be highlighted. SPME has shown to be enormously efficient in different environmental applications. In spite of the high enrichment factors achieved with SPME, the technique mainly suffers from carry over between extractions, and the fiber lifetime is limited. LPME can overcome these disadvantages: the memory effect between extractions is negligible, it requires very simple and inexpensive devices, and it demands little organic solvent consumption while maintaining high enrichment factors. There are two main modes of LPME sampling: direct-LPME and headspace-LPME (HS-LPME). HS-LPME is more suitable for the extraction of volatile and semi-volatile organic compounds. Headspace-single drop microextraction (HS-SDME), a variant of HS-LPME in which a micro-drop of extractive solvent is suspended at the tip of a micro-syringe, has been widely used for volatile and semi-volatile organic compounds analysis [27,28], but not applied yet to the analysis of phenolic compounds in biomass smoke.

Artisanal smoking processes of foods are extensively used in the Canary Islands, Spain. The smoking is conducted by allowing the direct contact of the smoke with the food. Because the fuels used in the smoking process are different types of biomass, a high content of phenolic compounds can be present in the smokes. The determination of these compounds results quite important, for their above mentioned role in the special organoleptic profiles of the smoked food generated, and for their bacteriostatic and antioxidant properties.

In this sense, the main aim of the present work is the optimization of the sampling and analysis of ten phenolic compounds in biomass smoke from traditional food smoking (specifically cheese) of the Canary Islands. The sampling system used is an adaptation of the method described in US EPA TO-8 for ambient air [20] and was optimized to maximize its efficiency by using a chemometric approach. The analysis of phenolic compounds was further carried out using an optimized HS-SDME-HPLC-UV procedure. To our knowledge, there are no published methods based on HS-SDME to determine alkyl- and methoxy-phenolic compounds in biomass smoke.

2. Experimental

2.1. Reagents and materials

The phenolic compounds used in this study were: 2-ethylphenol (2-EP), 3-ethylphenol (3-EP), supplied by Dr. Ehrenstorfer (Reference Materials, Augsburg, Germany) with 99.4% purity; *orto*-Cresol (*o*-Cr), *meta*-Cresol (*m*-Cr), and *para*-Cresol (*p*-Cr), supplied by Sigma–Aldrich Chemie (Steinheim, Germany) with a purity of 99%; phenol (P), supplied by Merck (Darmstadt, Germany) with a purity of 99.5%; vanillin (Va) supplied by Fluka Chemie (Buchs, Switzerland) with a purity of 98%; and 3-methoxyphenol (3-MeP), 2,6-dimethoxyphenol or syringol (2,6-DMeP), and eugenol (Eu), also supplied by Fluka Chemie but with a purity of 97%. Methanol of 99.9% purity, supplied by Sigma–Aldrich Chimie, was used to prepare individual standard solutions of these phenols at concentrations of 2000 mg L^{-1}. These standard solutions were kept at 4 °C and protected from light.

Other reagents used for the sampling and analysis of these phenolic compounds were: sodium hydroxide of 99% purity, supplied by Scharlau-Chemie (Barcelona, Spain); sodium chloride of 99.5% purity, supplied by Sigma–Aldrich Chemie; hydrogen chloride at 37% (v/v), supplied by Scharlau-Chemie; and 1-decanol with 99% purity, supplied by Sigma–Aldrich Chemie.

Deionized water (18.2 mΩ cm^{-1}) was obtained through a Milli-Q gradient A10 system (Millipore, Watford, UK). Mobile phases for the high-performance liquid chromatograph (HPLC) were composed of acetonitrile of HPLC grade (99.9% purity), supplied by Merck; and a buffer solution formed by anhydride sodium acetate with 99% purity, supplied by Sigma–Aldrich Chemie, and acetic acid with 99.8% purity, supplied by Merck. Mobile phases were filtered through membrane disks of 0.45 μm from Durapore® (Millipore), before being pumped through the chromatographic equipment.

The biomass materials used for the combustion procedures were rock rose (*Cistus monpelienisis*), prickly pear (*Opuntia ficus indica*), pine needles (*Pinus canariensis*), and almonds skin (*Prunus dulcis*), and can be observed in Fig. 1(A). Almonds skin materials were obtained from a local cheese factory, which uses such material as the smoke source. The rest of biomass materials were directly obtained in Tenerife Island forest with permission. They were used as obtained, but using scissors with pine needles, a hammer for almonds skin, and a small metal sandpaper tool for rock rose and prickly pear, to decrease the sampling size.

2.2. Equipment

The sampling system used for obtaining the smokes generated by the combustion of the biomass materials is schematically shown in Fig. 1(B). It is formed by a grid metallic biomass sample container, followed by two impingers which contains, under optimum conditions, 16 mL of sodium hydroxide 0.1 N, and silica gel, respectively. The smoke generated by the uncontrolled combustion of the biomass materials (to imitate the traditional smoking procedure) is pumped through the system by a portable pump Bravo H supplied by TCR Tecora (Milan, Italy). The pump also incorporates time, flow and pressure controllers. In artisanal smoking, the cheese is placed in a drum or in a kiln where smoking is conducted. The common distance between the cheese and the fire is 40–100 cm (and so 60 cm is used in our sampling device). Smoke temperatures in the vicinity of the cheese range between 20 and 55 °C, with the median temperature of the smoke being between 22 and 39 °C [21].

The system used for the headspace-single drop microextraction (HS-SDME) procedure is illustrated in Fig. 1(C). It is formed by a 25 μL syringe Hamilton-Bonaduz (Schweiz, Switzerland), designed for HPLC, and a thermostatic bath Comfort CB 8-30 (Heto, Allerod, Denmark) filled with silicone oil. Temperature was measured with a calibrated thermometer 638 Pt from Crison (Barcelona, Spain). Working vials of 4 mL were supplied by Supelco (Bellefonte, USA), equipped with polypropylene caps of 13 mm diameter and PTFE/silicone septa, also supplied by Supelco.

The system used to evaluate the sampling efficiency is shown in Fig. 1(D). It is formed by U-shaped glass tube placed right before the sodium hydroxide impinger. The tube is located inside a thermostatic bath heated at 90 °C to favor the volatilization of a standard mixture of phenols, which is spiked in the tube.

The HPLC equipment was supplied by Varian (Palo Alto, USA), equipped with a gradient solvent system Prostar 230 (Varian), a 20 μL injection Rheodyne valve, and a diode array detector Prostar 330 (Varian). The control of the HPLC system and the treatment of the data were carried out with the Star Chromatography Workstation V. 6.20 software (Varian). The analytical column was a Microsorb-MV 100-5 C18 (150 mm × 4.6 mm I.D.), supplied by Varian, and protected with a 2 cm Pelliguard™ LC-18 precolumn, supplied by Supelco.

The statistical treatment of the data was carried out using the Statgraphics Plus software for Windows V 5.1 (Statistical Graphics, Rockville, USA).

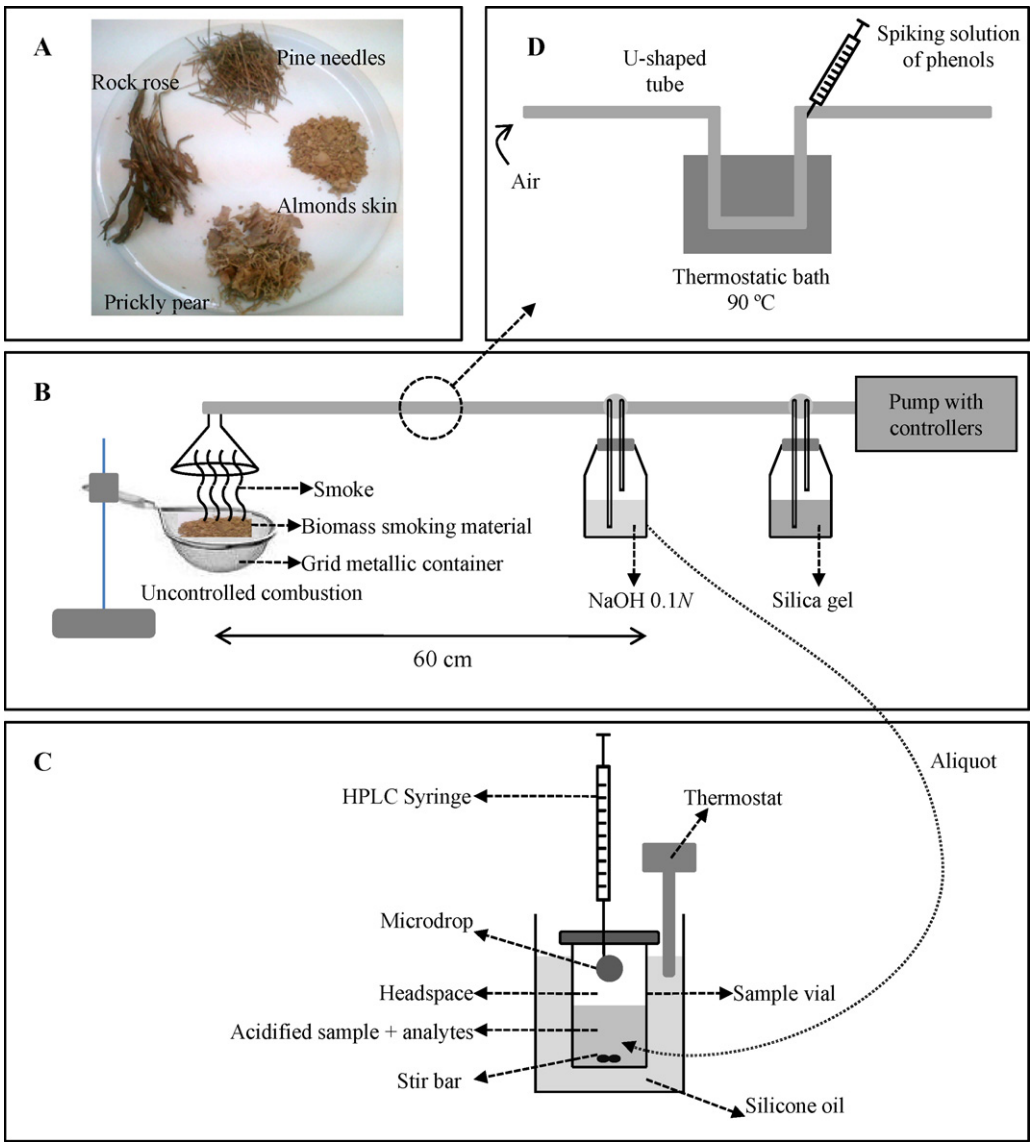

Fig. 1. Schematic diagram of (A) biomass materials used, (B) sampling system used to trap the smokes of the biomass materials studied, (C) headspace-single drop microextraction procedure to analyze the extracted analytes, and (D) system used to evaluate the sampling efficiency.

2.3. Procedures

The HPLC separation of the studied phenols has been conducted using a binary mixture of mobile phases formed by a buffer solution of acetic acid:sodium acetate at a pH value of 4.60–4.80 (phase A) and acetonitrile (phase B). The initial conditions were 85% of A, which were kept during 10 min, then linearly decreased to 80% of A in 10 min, and then linearly decreased to 50% of A in another 10 min, being kept at 50% of A during 5 more minutes The total chromatographic time was lower than 35 min. The flow was 1 mL min^{-1}, and the wavelength of the detector was fixed at 274 nm.

HS-SDME was conducted, under optimum conditions, using sample vials filled with 2 mL of NaOH 0.1 N (coming or not from the biomass smoke sampling device). 0.7 g of NaCl was then added to the sample solution, as well as few drops of concentrated hydrochloric acid, and the sample vial was closed with the cap. Samples coming from the biomass smoke sampling device were not spiked with standard solutions of phenols. After stirring for few seconds, the sample vial is placed in the silicone oil thermostatic bath at 60 °C. Afterwards, the HPLC syringe is introduced into the sample vial, and a 3.5 μL microdrop of 1-decanol is formed at the tip of the syringe in the headspace of the vial. The drop was exposed to the headspace during 25 min, and then retracted into the syringe. The syringe was then completed to 20 μL with methanol and further injected into the HPLC system without any additional clean-up step.

The sampling of the biomass smoke was carried out, under optimum conditions, placing 20 g of biomass material in the grid metallic container. The combustion of the biomass is then conducted, without controlling the process (as it happens in real smoking processes of Canary cheeses). The generated smoke during the combustion is pumped through the sampling device at a flow of 1 mL min^{-1} during 25 min. Then, the sodium hydroxide solution is kept at 4 °C protected from light for a time period always lower than 24 h, to avoid losses of phenols (trapped as phenolates). The sampling system, including the polypropylene tubes, was scrupulously cleaned after each combustion procedure to avoid inter-samples contamination. The cleaning used water with soap, then water, and then acetone, followed by drying with compressed air.

The sampling extraction efficiency was evaluated spiking 200 μL of a known solution of phenols dissolved in acetonitrile in the

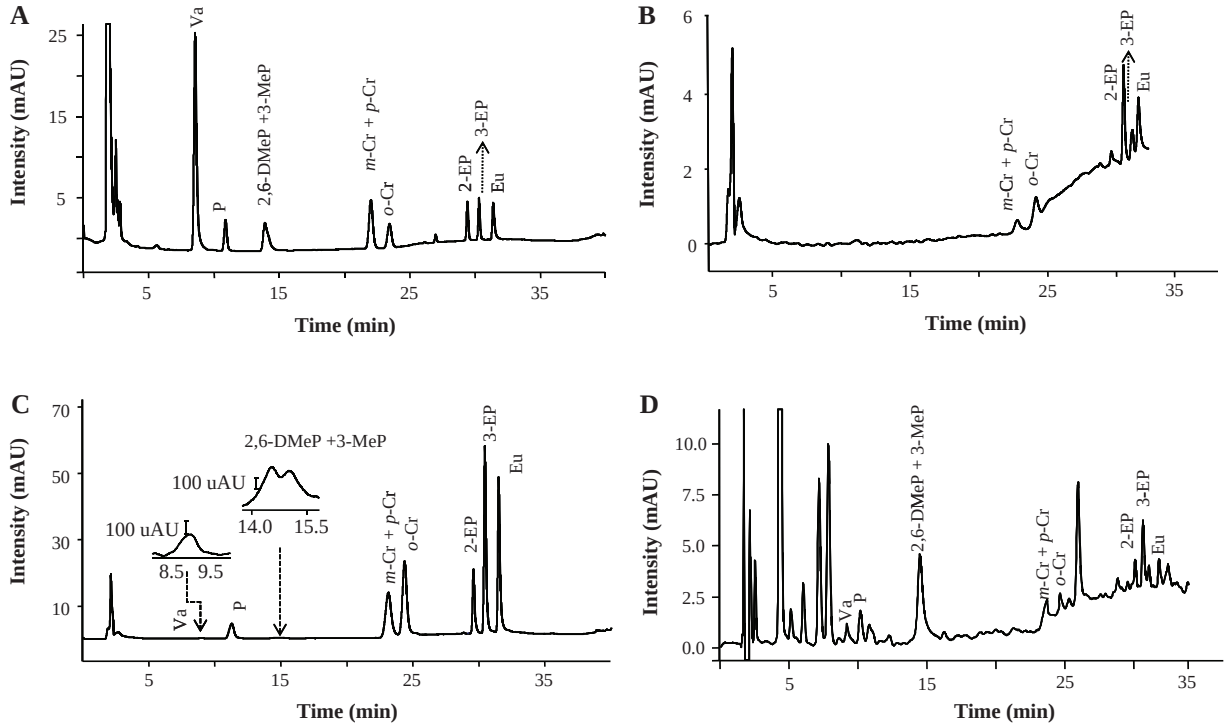

Fig. 2. Chromatograms of: (A) standard mixture of phenols injected in the HPLC without any previous sampling or extraction procedure. The concentration values of the phenols in the chromatogram are 24 μg mL^{-1} for the pair *m*-Cr + *p*-Cr, 5 μg mL^{-1} for Va, and 4 μg mL^{-1} for the rest of phenols; (B) aqueous solution spiked with phenols at concentrations of 22 μg mL^{-1} for the pair *m*-Cr + *p*-Cr, 10 μg mL^{-1} for Va, 8.0 μg mL^{-1} for 3-MeP, 6.4 μg mL^{-1} for P, and 1.6 μg mL^{-1} for the rest of phenols, without adjusting the ionic strength, and subjected to HS-SDME; (C) aqueous solution spiked with phenols at the same concentration of (B) but using saturation of NaCl followed by HS-SDME; and (D) pine needles sample combusted, sampled and analyzed under optimum conditions.

U-shaped tube, followed by switching on the pump. All the rest of experimental conditions are the same as above mentioned.

3. Results and discussion

3.1. Chromatographic separation of phenols

HPLC is a common technique to determine phenols [14–16], and it was also selected for this study. The optimum chromatographic conditions for the selected group of phenols are described in Section 2.3. Fig. 2(A) shows a chromatogram obtained in direct liquid injection mode under the optimum chromatographic conditions. It can be observed an acceptable separation for the phenols except for the pairs: 2,6-DMeP + 3-MeP, and *m*-Cr + *p*-Cr. The reproducibility of the retention times presented relative standard deviation (RSD) values oscillating between 0.1 and 2.8% during the whole experimental study.

Calibration curves of the HPLC-UV method were obtained by plotting the peak area (counts) *versus* the concentration of the phenol (μg mL^{-1}) injected in the HPLC. Linear regressions were obtained with R^2 values higher than 0.990. The calibrations ranges were from 3 to 24 μg mL^{-1} for Va, 2-EP, 3-EP and Eu; from 1 to 5 μg mL^{-1} for the pair *m*-Cr + *p*-Cr, and *o*-Cr; from 4 to 14 μg mL^{-1} for P; and from 10 to 60 μg mL^{-1} for the pair 2,6-DMeP + 3-MeP. The sensitivity of the chromatographic method, which can be evaluated by the slope of the calibrations, was higher for vainillin (slope ~ 4.8×10^5), and lower for the ethylphenols (slopes ~ 7.8×10^3 and 8.2×10^3 for 2-EP and 3-EP, respectively). The chromatographic precision was calculated with five independent determinations of a standard solution of phenols at an intermediate level of each calibration range, and the obtained RSD values ranged from 4.6% for *o*-Cr to 10% for the pair *m*-Cr + *p*-Cr.

3.2. Optimization of the HS-SDME procedure

Many variables can affect the extraction efficiency in HS-SDME [28]. Among them it can be cited: nature of analytes to be extracted, nature of selected extractant solvent, size of the microdrop, ionic strength and pH of the aqueous solution containing the analytes, working temperature, extraction time, sample volume as well as sample headspace volume.

An ideal extractant solvent for HS-SDME must possess the following properties: high extraction efficiency of analytes of interest, adequate boiling point to avoid losses by evaporation when heating, an optimum viscosity to ensure the formation of a drop at the top of the syringe while ensuring its handling, and ability to maintain the drop stability during long periods of extraction time. Moreover, the extractant solvent must later be compatible with the chromatographic system to be used, and it should not interfere with the detection of analytes (in terms of absorption or co-elution). Given the fact that HPLC is going to be used in this study, 1-octanol, 1-decanol, and the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BMIm-PF_6) were tested as extractant solvents. 1-Decanol was finally selected for ensuring adequate size of the microdrop during long periods of time, while being compatible with HPLC-UV. The drop size volume of 1-decanol was optimized by maximizing its size while ensuring its stability during periods of time higher than 30 min. 3.5 μL was the volume of the microdrop finally selected, which is within the range usually employed in SDME-HPLC applications [29,30]. The high viscosity of 1-decanol favors the stability of the drop for long periods of time.

In this study, the sample volume was fixed to 2 mL in order to have an adequate headspace volume to introduce the syringe and form the microdrop. The rest of experimental variables were studied using a factor-by-factor optimization.

The pH of the aqueous solution is an important parameter in SDME when the analytes to be extracted are in ionic form. Given the fact that only the neutral species are going to be present in the headspace to be efficiently extracted by 1-decanol, it is necessary to adjust the pH of the solution to ensure the neutral form of the phenols studied. Attending to the p*K*a values of the studied phenols, it results convenient to use pH values lower than 5. Conde et al. have shown that there are no significant changes in the extraction efficiency of a similar group phenols by HS-SPME when pH values were varied between 2 and 7 [31]. In this sense, the pH value was fixed at a value of 2.

It is also well-known that increases in the ionic strength of the solution can decrease the solubility of phenols in water and therefore help in increasing their concentration in the gas phase [32]. This effect was evaluated by modifying the ionic strength of the aqueous solution with NaCl contents between 0 and saturation. The HS-SDME was conducted using an aqueous pH value of 2, a microdrop size of 3.5 μL of 1-decanol, an extraction temperature of 60 °C, and an extraction time of 25 min. Fig. 2(B) shows the chromatogram obtained when the HS-SDME procedure is carried out without addition of NaCl to the aqueous solution, and Fig. 2(C) shows the chromatogram obtained with saturation of NaCl. The superior performance of the method when using high ionic strength is quite obvious, resulting in peak areas increases between 395 and 5604%. Given these results, the ionic strength was fixed using saturation of NaCl (0.7 g of NaCl in 2 mL) in the rest of the experiments.

The extraction temperature affects both the kinetics and thermodynamics of the headspace extraction procedures of volatile and semivolatile compounds [33]. In general, increases in the temperature are accompanied by higher Henry constants and diffusion coefficients, which result convenient in a HS extraction. Nevertheless, the sorption process on the drop is exothermic, and so higher temperatures also decrease the partition coefficients of the analytes from the aqueous phase to the organic phase of the drop [34]. Furthermore, the stability of the drop at the tip of the micro-syringe can be also compromised at higher temperatures. The extraction temperature in HS-SDME cannot be studied isolated, without considering the extraction time. For instance, the utilization of longer extraction times at higher temperatures make difficult to stabilize the drop. The equilibration time in SDME determines the maximum amount of analyte that can be extracted by the drop, but it is not necessary to work under equilibration conditions as long as the timing is carefully controlled [35]. In fact, it is convenient to use a relatively short extraction time, which ensures acceptable extraction efficiency and limits of detection. Studies were therefore conducted varying the extraction time between 5 and 25 min (to ensure drop stability) with temperatures varying from room temperature to 60 °C. Higher temperatures were not adequate to ensure the 1-decanol drop stability. The rest of experimental conditions were a pH value of 2, saturation of NaCl, and a microdrop size of 3.5 μL of 1-decanol. The obtained results showed that for all studied phenols, with the exception of methoxyphenols, there were increases in the extraction efficiency when increasing the extraction time in the working range of temperatures studied. Hence, the extraction time selected was 25 min. With respect to the extraction temperature, the most adequate one to work with was 60 °C, independently of the extraction time used. Fig. 3 shows the effect of extraction time and temperature for 3-EP and the pair 2,6-DMeP + 3-MeP, as representative examples.

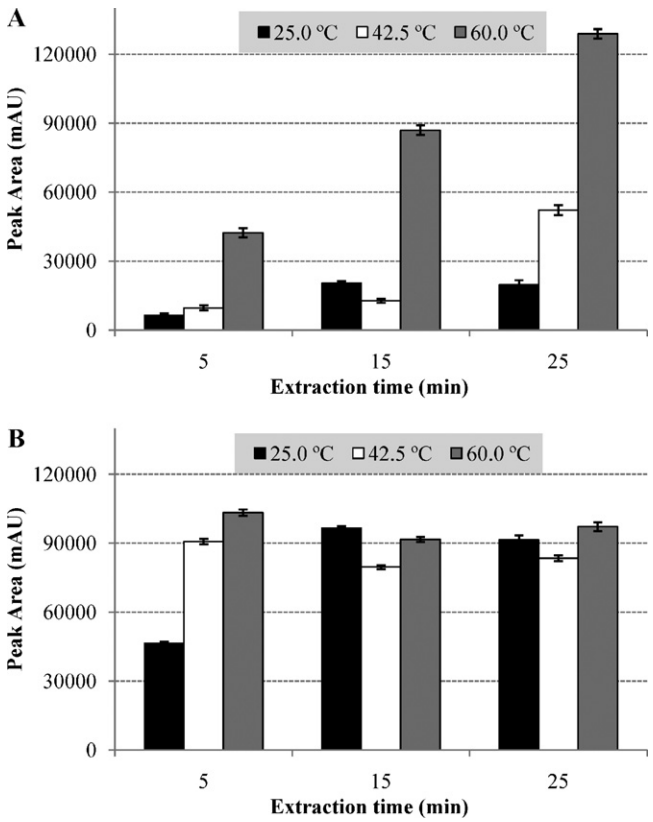

Fig. 3. Effect of extraction time and temperature on HS-SDME extraction efficiency (expressed as peak-area), measured by triplicate, for representative phenols: (A) 3-EP and (B) 2,6-DMeP + 3-MeP. Rest of conditions as described in the text.

3.3. Quality parameters of the HS-SDME-HPLC-UV procedure

The analytical performance of the already optimized HS-SDME-HPLC-UV procedure is shown in Table 1. Calibration curves were obtained after applying the HS-SDME-HPLC-UV method to aqueous samples spiked in the working range shown in the table, using seven calibration levels. Correlation coefficients (R^2) ranging from 0.990 to 0.996 were obtained, which must be highlighted considering that they are not exclusively related to the chromatographic method. Precision was evaluated by applying the optimized HS-SDME-HPLC-UV method to an aqueous standard during three different days, three times each day. The obtained RSD values, also included in Table 1, oscillate from 0.7% for 2-EP to 7.4% for *o*-Cr. Given the fact that SDME is not an exhaustive extraction method, the utilization of the relative recovery is an appropriate tool to evaluate the extraction performance. The relative recovery (RR) is obtained from the following equation:

$$\text{RR}\ (\%) = 100\frac{C_{\text{found}}}{C_{\text{initial}}} \quad (1)$$

where C_{found} is obtained from the HS-SDME-HPLC-UV calibration method and C_{initial} is the spiked concentration. RR values oscillate from 87.9% for Va to 110% for the pair *m*-Cr + *p*-Cr. The average relative recovery (plus standard deviation) for all analytes was 99.8 ± 5.2%. The limits of detection (LOD) were calculated as three times the standard deviation of residuals divided by the calibration slope [36], and were verified by applying the HS-SDME-HPLC-UV method to aqueous samples spiked at such levels. LODs range from 0.35 μg mL^{-1} for 3-EP to 5.8 μg mL^{-1} for the pair 2,6-DMeP + 3-MeP. Literature detection limits for similar phenols using a HS-procedure are around 0.06 μg mL^{-1} for 3-MeP, 0.07 μg mL^{-1} for 2,6-DMeP, and 0.28 μg mL^{-1} for Va using a HS-SPME-GC–MS [31]. Given the fact that HPLC with UV detection is used in this work, instead of MS detection, the obtained detection limits are quite acceptable.

Table 1
Quality parameters of the HS-SDME-HPLC-UV method.

Analyte	Calibration range[a] ($\mu g\,mL^{-1}$)	(Slope ± SD[b]) × 10^{-4}	R^2	S_{yx}[c] × 10^{-4}	LOD ($\mu g\,mL^{-1}$)	RSD[d] (%)	RR[e] (%)
Va	3–24	0.14 ± 0.01	0.990	0.12	2.4	5.8 (18)	87.9
P	3–18	2.1 ± 0.1	0.996	0.73	1.0	3.8 (10)	98.3
2,6-DMeP + 3-MeP	13–62	0.22 ± 0.01	0.990	0.43	5.8	3.8 (48)	101
m-Cr + *p*-Cr	1–9	3.5 ± 0.1	0.990	1.1	0.92	1.5 (5.0)	110
o-Cr	0.5–4.5	7.4 ± 0.3	0.992	1.0	0.41	7.4 (2.5)	99.9
2-EP	0.5–4.5	40 ± 2	0.991	5.8	0.43	0.7 (2.5)	102
3-EP	0.5–4.5	135 ± 6	0.991	16	0.35	3.0 (2.5)	102
Eu	0.5–4.5	9.3 ± 0.4	0.991	1.4	0.46	5.5 (2.5)	96.6

[a] Working levels in the aqueous sample.
[b] SD: error of the slope for $n = 7$ calibrations levels.
[c] S_{yx}: error of the estimate (standard deviation of the residuals).
[d] RSD: precision for six individual determination (the standard concentration is included in parenthesis, in $\mu g\,mL^{-1}$).
[e] RR: relative recovery for six individual determination spiking deionized waters at the levels indicated in the RSD determination.

3.4. Optimization of the sampling method to determine phenols in biomass smoke

The method developed here to sample phenols in biomass smokes is based in two procedures: the EPA-TO-8 method to sample phenol and cresols in ambient air [20], and the sampling method developed by Conde et al. in a HS-SPME-GC–MS approach [31]. Briefly, both methods are based in the transformation of phenolic gas compounds in aqueous phenolates by using an alkaline medium, in order to increase their solubility in water, and so to trap in water these gaseous analytes from the smoke. The sampling system used (Fig. 1(B)) was optimized to maximize its efficiency, by using an experimental design. Several experimental variables were already fixed attending to previous experiments. The fixed variables were: sampling flow ($1\,mL\,min^{-1}$), and nature and concentration of the sorbent solution to trap the phenolic compounds (NaOH 0.1 N). Sampling conditions were also optimized using pine needles as biomass material, and a burning temperature dictated by its own combustion temperature. The smoking process is traditionally carried out under uncontrolled combustion conditions of the biomass smoking materials. In this sense, the optimization is shifted to minimize the variability of the results due to the own variability of the combustion. Hence, the optimized variables were: amount of biomass to be burned in the sampling system, volume of sorbent solution to trap the phenolic compounds, and sampling time. As a first approximation to obtain a representative response surface of the process, a central design 2^3 + star with two central points was selected, which resulted in 16 randomized experiments. The axial distance was fixed to a level of 1.28719 to fulfill the orthogonality condition. The response used was the normalized peak-area for each analyte. The used levels for the factors were based on previous experiments. The amount of biomass material was varied between 10 and 22 g. Lower amounts did not generate sufficient amount of smoke, whereas higher amounts were excessive for the sample container. Sorbent volumes were varied between 25 and 50 mL. Lower volumes were not tried at first to

Table 2
Intra-days and intermediate precision obtained for the sampling system with pine needles biomass.

Analyte	Average amount found ($mg\,kg^{-1}$)[a]	Intra-days precision (RSD%)	Intermediate precision (RSD%)	$F_{Calculated}$[b]
Va	13.6	13.3 (day 1) 3.75 (day 2) 20.0 (day 3)	10.4	1.241
P	4.91	5.23 (day 1) 5.87 (day 2) 11.6 (day 3)	7.23	0.402
2,6-DMeP + 3-Me-P	200	19.4 (day 1) 3.09 (day 2) 6.29 (day 3)	20.4	8.192
m-Cr + *p*-Cr	1.80	8.14 (day 1) 5.64 (day 2) 11.8 (day 3)	8.33	0.546
o-Cr	0.724	13.3 (day 1) 8.83 (day 2) 10.4 (day 3)	13.0	2.652
2-EP	0.638	8.00 (day 1) 4.13 (day 2) 15.9 (day 3)	12.5	2.963
3-EP	0.468	10.5 (day 1) 14.0 (day 2) 13.5 (day 3)	11.4	0.176
Eu	0.086	2.79 (day 1) 2.06 (day 2) 2.62 (day 3)	2.83	2.056

[a] mg of analyte by kg of pine needles combusted.
[b] $F_{Tabulated} = 5.143$ for $P = 0.05$, $\nu_1 = 2$ and $\nu_2 = 6$ (degrees of freedom).

avoid possible losses of phenols, which could not be converted to phenolates in a low volume of alkaline medium. Higher volumes were not tried to avoid unnecessary dilution of the trapped analytes. Sampling time was varied between 25 and 50 min. The minimum time was selected to imitate the common sampling times used in traditional smoking procedures. Higher times were not used to avoid unnecessary delays in the sampling process. The matrix of the design and the obtained results can be checked in Table S-1 of the Supplementary material.

The obtained results of the design showed a similar behavior for all studied phenols with regards to the three factors studied. The exception was Va, the less volatile analyte of the group. The effects of the factors in the design can be observed in Fig. 4(A) and (B), using 3-EP and Va as representative examples. It was observed that the biomass amount had a positive effect for all analytes (except for Va). On contrary, the sampling time has a negative effect for all analytes (except for Va). The sampling sorbent volume presents an effect, which varies depending on the specific volume considered, being negative at low volumes and positive at higher volumes. The interaction of the effects can be observed in Fig. 4(C) and (D), again using 3-EP and Va as representative examples. It was observed a strong interaction between sampling biomass amount and sorbent volume for all analytes, with the exception of Va. The maximum value is achieved when both factors are in their highest levels. It is worthy to mention the interaction between the sorbent volume and the sampling time, with the maximum response at the highest volume of sorbent, and maximum sampling time for Va and minimum sampling time for the rest of analytes.

In order to improve the optimization, a second central design 2^2 + star, faced centered and with two central points, was carried out using the sorbent volume and the sampling time as factors (given their above mentioned interactions), and fixing the amount of biomass sample. The biomass sample was kept at 20 g, given its positive effect on the extraction, and considering that the utilization of higher amounts was difficult to handle in the sampling system used. The levels of the factors were 5 and 25 min for the sampling time, and 16 and 30 mL for the sorbent volume. This time, lower times were tried to evaluate the possibility of decreasing the overall sampling time, and lower volumes of sorbent were tried to evaluate if phenols were efficiently transformed to phenolates in lower sorbent volumes. The design resulted in 10 random experiments. The matrix of the design and the obtained results can be checked in Table S-2 of the Supplementary material.

Two representative response surfaces (for 3-EP and Va) obtained with this design are shown in Fig. 4(E) and (F). It can be observed that a maximum is not reached under the experimental limits studied. In any case, the maximum efficiency is achieved using the lowest sorbent volume and the maximum sampling time. Furthermore, the extraction efficiency is practically independent on the sorbent volume for the majority of analytes. Given these results, and in order to get the best extraction efficiency for all phenols considered, a sampling time of 25 min and a NaOH sorbent volume of 16 mL were selected as optimum variables.

3.5. Quality parameters of the sampling method

The optimized sampling system to trap phenols from biomass smoke was evaluated in terms of reproducibility and sampling efficiency. The own variability of the non-controlled combustion could lead to non-reproducible results, or to data which do not follow a normal distribution. The reproducibility was studied burning three pine needles samples every day, during three different (and non-consecutive) days, under the above mentioned optimized conditions. The Kolmogorov–Smirnov test was applied to the obtained data, and the results showed that the determined phenols in the smokes followed a normal distribution with a significance level of 0.05. After verifying the normal distribution, the repeatability of the system was evaluated by means of the intra-days precision, and the reproducibility of the system was estimated by calculating the intermediate precision. The results are shown in Table 2. Intra-day precision varied between 2.06% for eugenol and 19.4% for methoxyphenols (as RSD). Intermediate precision values varied between 2.83% for eugenol and 20.4% for the methoxyphenols. The analysis of variance (ANOVA) of one factor indicated that there are no significant differences among the results obtained in different days, for a significance level of $\alpha = 0.05$ of all phenols studied except for the methoxyphenols. It is important to highlight these results, because it must be considered that each burning implies the utilization of a new biomass sample. Samples are homogenized as much as possible before being burned, but evidently slight differences in composition are present.

Table 3
Extraction efficiency and precision of the sampling system.

Analyte	Extraction efficiency[a] (%)	RSD[a] (%)
Va	86.6	14.2
P	85.8	18.2
2,6-DMeP + 3-MeP	106	16.0
m-Cr + *p*-Cr	69.1	12.4
o-Cr	92.5	4.00
2-EP	62.6	0.94
3-EP	63.4	0.12
Eu	58.9	15.9

[a] $n = 4$.

The efficiency of the sampling system was evaluated by volatilizing known amounts of phenols under the optimized procedure, utilizing the scheme shown in Fig. 1(D). The procedure was carried out four times within the same day. The obtained average efficiencies for each analyte are shown in Table 3. They oscillate from 58.9% for Eu to 106% for 2,6-DMeP + 3-MeP, with an average value of 78.1% and an average relative standard deviation of 10.2%. Low sampling efficiencies for some analytes can be related with their relatively high boiling points and vapor pressures or with losses by condensation in the collector tube. Nevertheless, the obtained efficiencies are considered acceptable for this heterogeneous group of analytes with different volatile nature, and sampled under non-controlled combustion conditions.

3.6. Phenolic content in biomass smoke by HS-SDME-HPLC-UV

The optimized method to sample and analysis phenolic compounds in biomass smoke using HS-SDME-HPLC-UV has been applied to four different smoking materials. The biomass materials used for the combustion procedures were rock rose (*C. monpelienisis*), prickly pear (*O. ficus indica*), pine needles (*P. canariensis*), and almonds skin (*P. dulcis*), and they can be observed in Fig. 1(A). These materials have been selected for being the most commonly used in traditional cheese smoking processes in Canary Islands (Spain). The manufacture of these smoked cheeses is regulated by "Protected Denomination of Origin" (P.D.O.), established in some countries and also in Spain, to define and protect high quality traditional products against imitations.

Table 4 shows the average values obtained for the phenolic content in each of the smoking materials. Results have been referred to normal conditions (25 °C and 1 atm) to avoid influences due to changes in the pressure or in the temperature within a sampling. It can be observed that the higher content in phenolic compounds is observed in the smokes of pine needles, with average values of 252 mg kg^{-1} of biomass. The lowest contents correspond to prickly pear, with average values of phenolic compounds of 70 mg kg^{-1} of

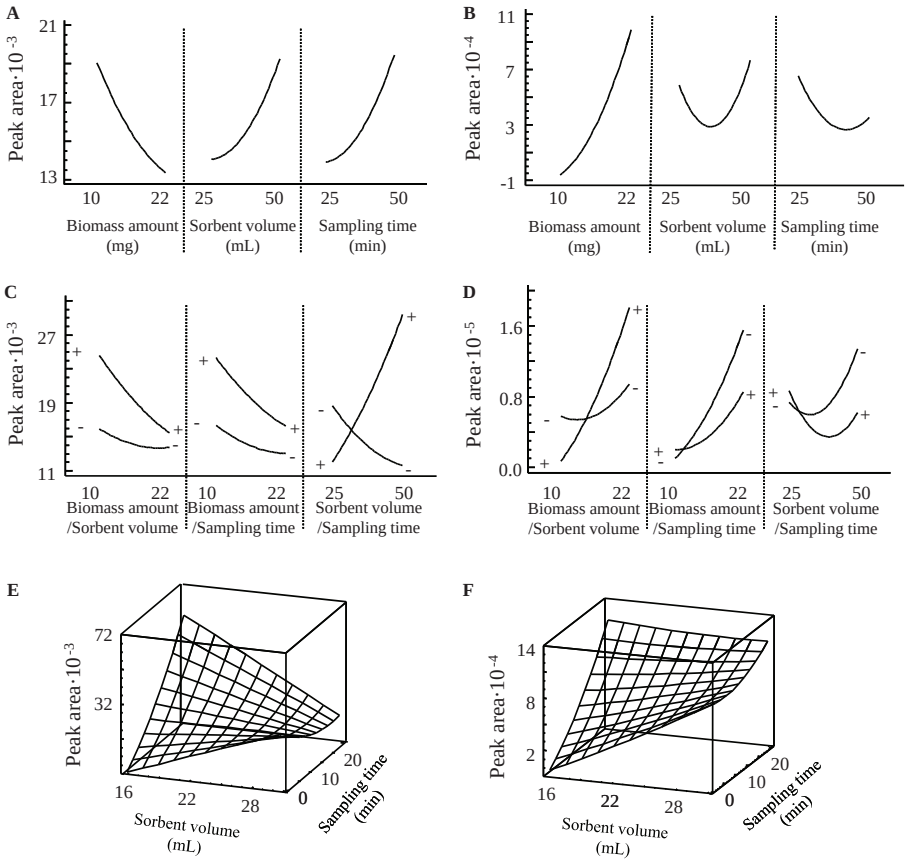

Fig. 4. Plots obtained in the optimization of the sampling procedure using Va (A, C and E) and 3-EP (B, D and F) as representative analytes. The plots are: main effects of the factors in the first design 2^3 + star (A and B), interaction effects of the factors in the first design 2^3 + star (C and D), and response surfaces in the second design 2^2 + star (E and F).

biomass. Average values of 161 and 206 mg kg^{-1} of biomass were obtained for almonds skin and rock rose, respectively.

If the individual concentration of the phenols is considered, it can be observed that the main components of the smokes are Va, P and methoxyphenols (2,6-DMeP and 3-MeP) in all smoking materials studied. The contribution of these phenolic compounds to the overall phenolic content oscillated between 97 and 99% in all samples analyzed for all smoking materials. The contribution of the minority phenolic compounds varied between 0.95 and 3.3%, showing a similar profile (distribution of the relative content) for all smoking materials.

Methoxyphenols (2,6-DMeP and 3-MeP) are the most abundant components in the smokes, and their overall contribution to the smoke composition oscillate between 73 and 92%. These compounds are known by their antioxidant activity [7–11]. However, their contents significantly vary depending on the smoking material. Thus, their presence in the smokes of the biomass materials follows the order: pine needles > rock rose > almonds skin > prickly pear.

The obtained results with methoxyphenols are similar to those obtained for other authors for smokes coming from different vegetal materials. Syringol (2,6-dimethoxyphenol) contents between 397 and 287 mg kg^{-1} have been reported for smokes coming from the combustion of oak and eucalyptus, respectively [37], and between 67.2 and 269 mg kg^{-1} for smokes coming from the combustion of pine and wheat, respectively [38]. 2-Methoxyphenol (guaiacol) contents in biomass smoke have been reported to be 172 mg kg^{-1} (oak), 183 mg kg^{-1} (eucalyptus) and 279 mg kg^{-1} (pine) [37]. The contents of phenol in biomass smoke have been reported to be 82.9 mg kg^{-1} from pine [38], and 145 mg kg^{-1}

Table 4
Phenolic content (mg kg^{-1}) in different biomass smoking materials.

Analyte	Biomass smoking material[a]			
	Pine needles	Almonds skin	Rock rose	Prickly pear
Va	16 ± 2	37 ± 2	14 ± 2	6.3 ± 1.0
P	5.4 ± 0.7	2.2 ± 0.2	0.54 ± 0.04	0.8 ± 0.1
2,6-DMeP + 3-MeP	227 ± 44	118 ± 20	190 ± 32	61 ± 10
m-Cr + *p*-Cr	1.8 ± 0.2	2.4 ± 0.4	0.8 ± 0.1	1.2 ± 0.2
o-Cr	0.84 ± 0.13	0.29 ± 0.05	0.34 ± 0.06	0.06 ± 0.01
2-EP	0.60 ± 0.07	0.38 ± 0.06	0.40 ± 0.04	0.35 ± 0.02
3-EP	0.46 ± 0.03	0.31 ± 0.04	0.33 ± 0.03	0.29 ± 0.01
Eu	0.09 ± 0.01	0.48 ± 0.07	0.030 ± 0.001	0.43 ± 0.06
Total phenolic content	252 ± 44	161 ± 28	206 ± 32	70 ± 10

[a] Average values and standard deviation for seven uncontrolled combustions in the optimized sampling system and HS-SDME-HPLC-UV for each biomass smoking material.

from wheat, 300 mg kg^{-1} from oak, 400 from eucalyptus, and 525 mg kg^{-1} from pine [37]. It can be observed that lower phenol contents are obtained with the biomass smoking materials used in this study. The content of methoxyphenols, on the other hand, is totally comparable with such obtained with literature biomass data.

4. Conclusions

A method to determine phenolic compounds in biomass smoke has been established. The overall method uses a sampling system containing an alkaline solution trap, followed by a HS-SDME-HPLC-UV procedure to determine the phenolates trapped in the alkaline solution. The method can be considered environmental friendly due to the low amounts of organic solvents needed in the extraction step (few microliters). The HS-SDME method presented detection limits ranging from 0.35 to 5.8 μg mL^{-1}, relative standard deviation values ranging from 0.7 to 7.4%, and an average relative recovery of 99.8% with an average standard deviation of 5.2, which reflects the reproducibility and efficiency of the proposed method. The sampling system was also optimized by means of an experimental design, showing to be efficient and reproducible. Sampling efficiencies varied from 58.9 to 106%, and the average precision was 10.2% (as RSD). The precision should be highlighted, attending to the high number of factors involved in the method (sampling step and HS-SDME-HPLC-UV determination), and the own complexity of the smoking materials, which are sampled under non-controlled combustion conditions.

The method was successfully applied to the analysis of the smokes coming from four common biomass smoking materials of the Canary Islands (pine needles, almonds skins, rock rose and prickly pear). The obtained results that methoxyphenols (2,6-DMeP and 3-MeP) are the most abundant components of the smokes, with an overall contribution varying from 73 to 92%, depending on the specific biomass.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.05.046.

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