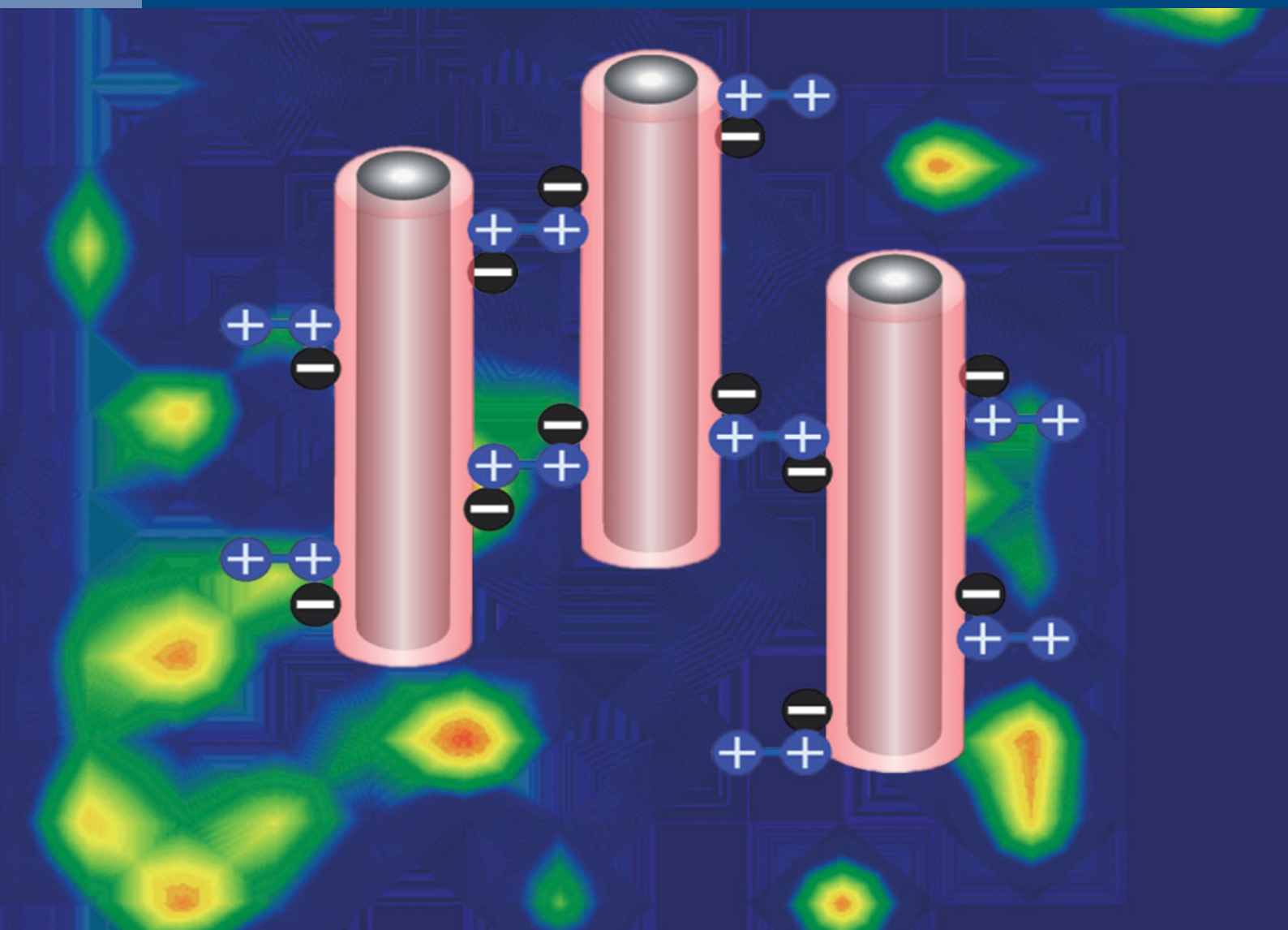


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Research Article

Ultrasound-assisted emulsification microextraction coupled with high-performance liquid chromatography for the simultaneous determination of fragrance allergens in cosmetics and water

A simple, inexpensive, and environmentally friendly method based on ultrasound-assisted emulsification microextraction followed by solidification of floating organic drop and high-performance liquid chromatography coupled to diode array detection was developed for the simultaneous determination of 18 potentially allergenic fragrance substances. Several parameters affecting the microextraction process were investigated in detail by the “one-variable-at-a-time” approach. Optimal conditions were the following: 50 μL of 2-dodecanol as extraction solvent, 10 mL of sample containing 150 g/L of salt, and 5 min of sonication at 35°C. Under the optimized conditions, method showed good linearity in the selected ranges, with squared correlation coefficients ranging from 0.948 to 0.999. Limits of detection ranged from 0.001 to 0.154 $\mu\text{g/mL}$ and enrichment factors from 9 to 237. Precision of the method, expressed as relative standard deviation, was checked at two levels obtaining good results (3.3–14.4%). Recovery studies were made in baby bath water and in eau de cologne showing acceptable accuracy. Finally, the developed method was successfully applied to different commercial cosmetic and water samples. The most commonly found analyte was linalool followed by cinnamal and lilial. Most of the analyzed samples contained at least one of the target compounds.

Keywords: Cosmetics / Fragrance allergens / High-performance liquid chromatography / Ultrasound-assisted microextraction / Water
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1 Introduction

Fragrances are important components of many formulations present in our daily life such as perfumes, cosmetics, personal care items, household, and laundry products. Although the fragrances are generally innocuous, they can sometimes cause skin irritation or allergic reactions. In 1999, the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers identified a list of 24 fragrance related substances with a well-recognized potential to cause allergy [1]. In 2000, two natural moss extracts were also added to the list [2]. Currently, the above-mentioned 24 compounds

and the two natural moss extracts are known in literature as potentially allergenic substances (PAS). In 2003, the European Union published an amendment to the Annex III of its Cosmetic Directive in which requires the declaration of the above-mentioned PAS on product labels if concentration is $>0.001\%$ in leave-on products and 0.01% in rinse-off products [3]. The main route of exposition to these ingredients is the direct application of cosmetics in the skin, but contact with contaminated water must be also considered. Thus, nowadays interest is not being focused only in cosmetic samples but also in water samples.

It is necessary to develop reliable analytical methods for quantification of PAS. Sample preparation is a critical step in analytical methods as it has direct impact on their quality and sensitivity. Recent research trends in sample preparation include miniaturized extraction procedures based on classical extraction techniques. These new techniques are generally simpler, faster, and use fewer amounts of solvents than the traditional ones.

LPME emerged as a solvent-minimized version of the classic LLE in which only several microliters of a water-immiscible solvent (extractant) are put in contact with an aqueous phase (sample) to extract the compounds of interest. From LPME introduction, different approaches have

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Abbreviations: ANOVA, analysis of variance; BBW, baby bath waters; BM, body milk; DAD, diode array detection; DLLME, dispersive liquid–liquid microextraction; EC, eau de cologne; EF, enrichment factor; PAS, potentially allergenic substances; SFOD, solidification of floating organic drop; SPW, swimming pool water; USAEME, ultrasound-assisted emulsification microextraction

been developed, which can be classified into three main categories: single drop microextraction, dispersive liquid–liquid microextraction (DLLME), and hollow-fiber liquid microextraction [4, 5].

DLLME was presented by Rezaee *et al.* [6]. In this technique, dispersion of the extractant is achieved by the addition of a third solvent, miscible with both phases, known as dispersant. As a result, a cloudy solution is formed and the superficial area in contact between the two phases is larger and, thus, extraction is faster. After the extraction, samples are centrifuged to separate the two phases. DLLME has several advantages: simplicity, efficiency, low sample volume, low cost, and high enrichment factor (EF). However, DLLME has also a disadvantage, the necessity of using a disperser solvent. Ultrasound-assisted emulsification microextraction (USAEME) emerged to solve this problem by the use of ultrasound radiation for generating the emulsion and accelerating the mass-transfer process [7].

Although volume of extraction solvents is diminished in LPME, extraction solvents used are still generally toxic. In the last years, there has been a thorough attempt to improve this aspect within the green chemistry framework [8]. Khalili-Zanjani *et al.* proposed a new approach of LPME based on the solidification of floating organic drop (SFOD) [9]. In this method, an extractant with lower density than water, low toxicity, and proper melting point (in the range of 10–30°C) is used. After extraction, organic droplet is solidified in an ice bath and then, it is easily collected with a spatula, melted and finally conducted to analytical determination. Leong *et al.* used this type of solvents in DLLME, developing a new method called DLLME based on SFOD [10]. Finally, USAEME-SFOD was introduced by Kamarei *et al.* [11]. This method possesses all the advantages of both USAEME and SFOD; analyte extraction is very efficient and fast due to the high superficial area between phases, it is an environmentally friendly technique as it only uses low volumes of practically nontoxic solvents and, in addition, it is simple and leads to high EFs.

Analytical methods for the determination of PAS are mainly based on GC–MS [12–18]. This technique has been coupled in several studies to sample pretreatment techniques, including matrix solid-phase dispersion [19] SPME [20], headspace (HS) SPME [21, 22], solid-phase dispersion pressurized liquid extraction [23], DLLME [24] and USAEME [25]. Other determination techniques such as microemulsion electrokinetic chromatography [26], MEKC [27], attenuated total reflectance IR spectroscopy [28], and HPLC [29] have been also used for determination of PAS, but in those cases no pretreatment technique was used.

The aim of the present work was to develop a simple, low cost, and reliable analytical method for simultaneous determination of eighteen PAS using USAEME-SFOD as a preconcentration technique followed by HPLC coupled to diode array detection (DAD). The use of a preconcentration technique, as USAEME-SFOD, could improve the sensitivity of the method and, in consequence, spread out its applications. Also, HPLC–DAD is a very widespread instrumentation

in most routine analysis laboratories; and therefore, this option could be a good alternative to other instrumental techniques. The main parameters influencing extraction and determination of PAS were considered in detail by “one-variable-at-a-time” approach. Then, the optimized procedure was validated and applied to the determination of these fragrance allergens in different water and cosmetic samples.

2 Materials and methods

2.1 Reagents and materials

Anisyl alcohol (98%), benzyl alcohol (99%), cinnamyl alcohol (98%), cinnamal (95%), isoeugenol (98%), geraniol (98%), linalool (97%), citral (96%), methyl-2-octynoate (99%), benzyl benzoate (99%), benzyl salicylate (99%), benzyl cinnamate (99%), lilial (98%), amyl cinnamal (97%), α -isomethyl ionone (85%), hexyl cinnamal (95%), and limonene (96%) were purchased from Sigma–Aldrich (Barcelona, Spain). Amyl-cinnamyl alcohol (45.5%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile (HPLC grade) was obtained from Romil (Cambridge, UK). 2-Dodecanol (99%), 1-undecanol (99%), *n*-hexadecane (99%) and 1-bromohexadecane (97%) were acquired from Sigma–Aldrich (Madrid, Spain). 1-Dodecanol (98%) was supplied by Panreac (Barcelona, Spain). Methanol was obtained from Panreac. Doubly distilled water was used throughout this work.

Individual stock solutions of PAS were prepared in acetonitrile and stored in amber-colored vials at –23°C. Working solutions were prepared weekly by dilution of the stock ones with acetonitrile and also preserved in the freezer and warmed at room temperature before use.

Four eau de toilettes, one eau de cologne (EC), one eau de perfume, and one body milk (BM) were purchased from local shopping center. The swimming pool water (SPW) was obtained from a local swimming pool. The two baby bath waters (BBW) were taken from bathtubs after babies had bathed. All samples were stored in glass bottles.

2.2 Instrumentation and chromatographic conditions

Chromatographic analyses were performed with a LC-20AD system equipped with a SPD-M20A Diode Array Detector (Shimadzu Corporation, Duisburg, Germany) and a manual sample injector (20 μ L injection volume). Data were collected and processed using LC Solution (2.1 version) software. Separations were carried out using XDB-C₁₈ column (250 $\times$ 4.6 mm, 5 μ m) from Agilent (Wilmington, DW, USA) at ambient temperature.

Gradient elution with water and acetonitrile was used for separation of target compounds. The initial mobile phase was held for 6.5 min with 50% acetonitrile, followed by an increase to 60% acetonitrile from 6.5 to 15 min and after that, to reach 80% acetonitrile from 15 to 32 min. The acetonitrile

composition was raised to 90% from 32 to 32.5 min and kept for 1 min. Finally, the acetonitrile composition was decreased for 0.5 min to reach the starting conditions again. These conditions were maintained from 34 to 41 min to obtain the last peaks and homogenize the system. Regarding the flow, it was maintained at 0.5 mL/min for 15 min, from 15 to 16 min was raised to 1 mL/min and, lastly, it was kept until the end. UV-Vis spectra were recorded from 190 to 800 nm.

Extractions were carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX, Papenburg, Germany) with 35 kHz ultrasound frequency. Centrifugation was performed on a Selecta centrifuge (Barcelona, Spain). The cooling bath was a Julabo F26 (Augsburg, Germany).

2.3 USAEME-SFOD procedure

First, 10 mL of a 150 g/L solution of NaCl was placed in a 40 mL glass vial and a mixed solution of fragrance allergens standards was spiked. Then, 50 μ L of 2-dodecanol (extraction solvent) was added to the solution, and it was gently shaken by hand and placed into the ultrasonic bath for sonication at 35 kHz and $35 \pm 1^\circ\text{C}$ during 5 min. As a result, emulsion of extraction solvent in water was formed. Emulsion was then disrupted by centrifugation at 4500 rpm for 15 min and the vial was placed into the thermostatic bath at 3°C for 15 min. The solidified organic drop was then collected with a spatula and transferred to an Eppendorf vial where it melted at room temperature. Once melted, 25 μ L of the organic phase was collected and mixed with 20 μ L of methanol to make it compatible with the HPLC mobile phase. Finally, this mixture was injected in the HPLC and subjected to analysis.

Given the wide range of concentrations in which the compounds appear in samples, convenient dilutions were made when required.

3 Results and discussion

3.1 Peak identification and selection of working wavelengths

Identification of peaks was carried out by comparing the retention times and the UV-Vis spectra of each peak with those of the single reference standards. The PAS sequence and elution time was anisyl alcohol (6.5 min), benzyl alcohol (6.8 min), cinnamyl alcohol (8.8 min), cinnamal (12.8 min), isoeugenol (15.6 min), geraniol (18.0 min), linalool (19.2 min), citral (20.4 and 21.0 min), methyl-2-octynoate (23.4 min), amylcinnamyl alcohol (24.8 min), benzyl benzoate (25.5 min), benzyl salicylate (27.5 min), benzyl cinnamate (27.9 min), lilial (30.3 min), amyl cinnamal (31.9 min), α -isomethyl ionone (32.3 min), hexyl cinnamal (35.2 min), and limonene (38.3 min). Working wavelength for determination of each analyte was selected according to spectra characteristics. A real chromatogram at 200 nm obtained injecting a standard mixture of the allergens is shown in Fig. 1A. This

wavelength, 200 nm, was the working wavelength for the most of the analytes except for isoeugenol (210 nm), methyl-2-octynoate (210 nm), citral (237 nm), α -isomethyl ionone (237 nm), benzyl cinnamate (276 nm), hexyl cinnamal (282 nm) amyl cinnamal, and cinnamal (282 nm).

3.2 Selection of working characteristics

To obtain the best experimental conditions for determination of PAS by USAEME-SFOD along with HPLC–DAD, the influence of different parameters in the obtained signal was tested using the “one-variable-at-a-time” approach. A comparison of USAEME-SFOD with other liquid microextraction techniques was also accomplished. To compare signals, analysis of variance (ANOVA) was used. Concentration of the analytes in the experiments used to fix the experimental conditions was between 0.1 and 2.6 $\mu\text{g/mL}$ depending on the analyte.

3.2.1 Selection of extraction solvent

Selection of an appropriate extraction solvent is important in the optimization process of a microextraction method based on the solidification of organic drop. The extraction solvent should satisfy the following requirements: It should be immiscible with water and have low volatility, low density, and present a melting point near to room temperature. It should also be able to extract the target analytes, and, in the case of chromatography, its peaks should be well separated from those of the analytes [30, 31]. Taking into account these characteristics, 1-undecanol, 1-dodecanol, 2-dodecanol, 1-bromohexadecane, and *n*-hexadecane were chosen as extraction solvents.

First, the compatibility of the extracting solvent with the HPLC mobile phase was checked. 1-Bromohexadecane and *n*-hexadecane were discarded due to their insolubility in some of the compositions of the HPLC mobile phase. Hence, only the alcohols were considered in the following experiments. 1-Dodecanol gave slight problems of solubility, the peak of 1-undecanol overlapped with peaks of amyl cinnamal and α -isomethyl ionone, making their determination not possible, and 2-dodecanol gave higher and better results. Therefore, further experiments were carried out considering 2-dodecanol as the extraction solvent.

3.2.2 Comparison with other liquid microextraction techniques

USAEME was compared with both LPME and DLLME, all of them followed by solidification of the organic drop. In all runs, the following extraction conditions were considered: concentrations of PAS, between 0.1 and 2.6 $\mu\text{g/mL}$; sample volume, 10 mL; extraction solvent, 2-dodecanol; volume of extraction solvent, 50 μ L; extraction temperature 30°C ; and no salt addition. For LPME followed by SFOD, 625 rpm agitation during extraction was used. For DLLME followed by SFOD,

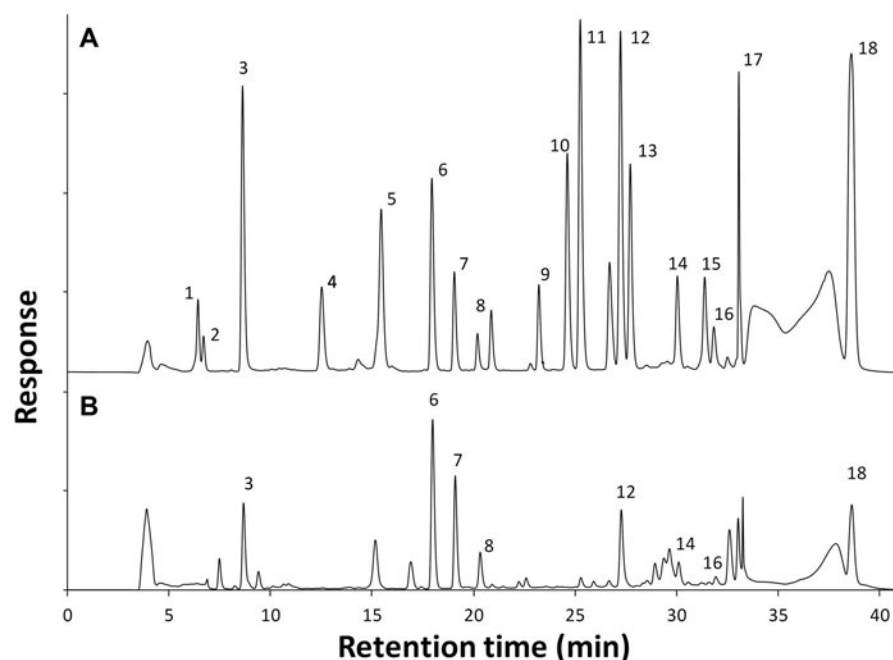


Figure 1. HPLC–DAD chromatograms at 200 nm of (A) the standard mixture of PAS and (B) of BM real sample using the optimized procedure. Assignment of peaks: (1) anisyl alcohol, (2) benzyl alcohol, (3) cinnamyl alcohol, (4) cinnamal, (5) isoeugenol, (6) geraniol, (7) linalool, (8) citral, (9) methyl-2-octynoate, (10) amylcinnamyl alcohol, (11) benzyl benzoate, (12) benzyl salicylate, (13) benzyl cinnamate, (14) lilial, (15) amyl cinnamal, (16) α -isomethyl ionone, (17) hexyl cinnamal, (18) limonene. Chromatogram B is an example of sample chromatograms at 200 nm, for determination of all the analytes using different dilutions.

1 mL of acetonitrile or ethanol was used as dispersants. The solidification conditions (3°C cooling temperature for 15 min) were similar in all cases. For DLLME and USAEME centrifugation was necessary after extraction (10 min at 4500 rpm). Extraction time was 10 min in USAEME and 15 min in LPME and DLLME. As it is shown in Fig. 2A, in general, DLLME offered better extraction recoveries than LPME. Using USAEME, the highest extraction recoveries were obtained for all analytes even when shorter extraction times were used with this method. The application of ultrasonic energy facilitates the emulsification phenomenon and accelerates the mass-transfer process that leads to an increment in the extraction efficiency in a very short time [31].

3.2.3 Selection of centrifugation and cooling conditions

These experimental conditions were fixed according to previous experience in our laboratory [32]. Centrifugation rate and time were the minimum that allowed collecting the cloudy solution into a sedimented drop (4500 rpm, 15 min). Cooling conditions were fixed to obtain a well-formed solidified drop (3°C, 15 min, and 250 rpm of agitation).

3.2.4 Selection of volume of extractant

An increase of the extractant volume leads to a higher volume of the organic phase after centrifugation, resulting in a decrease of the concentration of target compounds due to the dilution [30, 31]. In this work, extractions with volumes of 50, 75, and 100 μ L of 2-dodecanol were carried out. As expected, it was observed that extraction was remarkably better when volume of extractant was lower, as it can be seen in Fig. 2B,

Hence 50 μ L was selected as the lower volume that allowed the collection of the drop after centrifugation.

3.2.5 Selection of the extraction time

Extraction time is a significant variable in LPME methods, as these procedures require a period of time to reach the equilibrium between aqueous and organic phases. Nevertheless, extraction time is not as important in DLLME technique. The reason is that this type of technique is almost time-independent due to the large superficial area between extractant and sample, which allow the target compounds to diffuse quickly into the organic phase [31]. The extraction time was evaluated at 1, 5, and 15 min. ANOVA showed that taken into account the signals of all the analytes at a time, there were no significant differences between signals obtained after different extraction times. These results revealed that owing to the emulsification formed by ultrasound energy, equilibrium was reached in only 5 min for all the analytes. Therefore, 5 min was selected for further experiments.

3.2.6 Selection of the extraction temperature

In general, in LPME, higher temperatures facilitate the diffusion and mass transfer of the analytes from sample solution to the extractant, and time required to reach the equilibrium is decreased. However, at high temperatures, solubility of the organic solvent increases and the possible over-pressurization of the sample vial could make the extraction system unstable; normally, sample solution temperature should not exceed 60°C [31]. In this work, temperature was examined at 35, 45, and 55°C. ANOVA results showed that effect of this parameter was not significant except for anisyl alcohol, where better

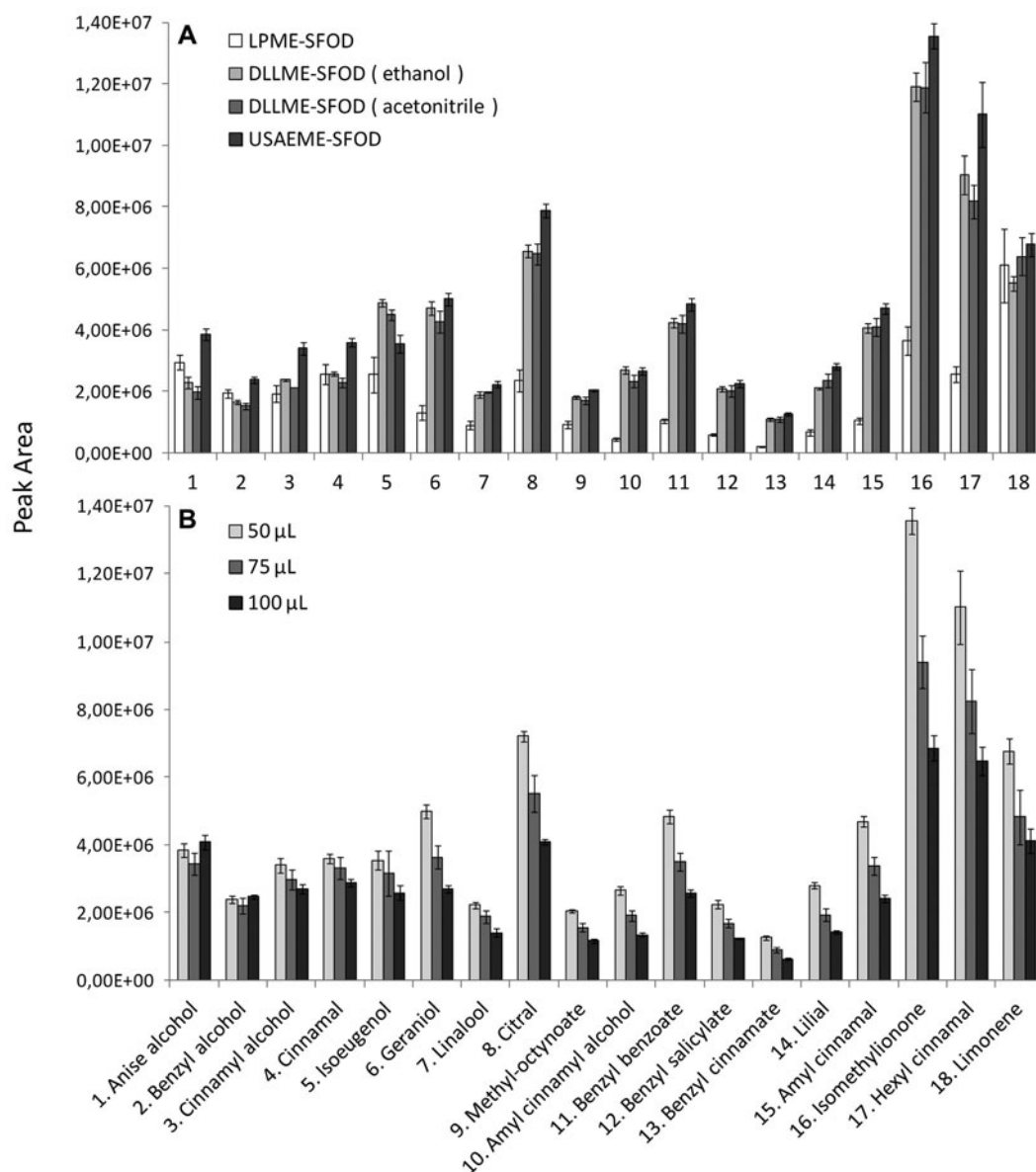


Figure 2. (A) Comparison of three techniques: LPME, DLLME, and USAEME, all of them followed by SFOD. Extraction conditions are expressed in Section 3.2.2. (B) Comparison of obtained signals using different volumes of extraction solvent. Other extraction conditions were the ones explained before.

extraction results were obtained at 35°C. Therefore, 35°C was chosen for further experiments.

3.2.7 Selection of salt addition

Addition of salt into the sample solution may have several effects on microextraction methods. On the one hand, it can improve the extraction efficiency due to the salting-out effect. But, on the other hand, the presence of higher concentrations of salt could change the physical properties of the Nernst diffusion film and thus reduce the diffusion rate of the target compounds into the organic phase [30, 31]. To investigate the influence of ionic strength, experiments at salt concentrations

of 0, 50, 100, and 150 g/L were performed. ANOVA showed that addition of salt was a significant parameter. Looking closely at the results, it was observed that in the most of analytes higher extraction efficiency was reached at 150 g/L of salt. Thus, 150 g/L of salt was chosen.

3.3 Method validation

Analytical figures of merit of the proposed method were obtained under optimal conditions and summarized in Table 1. All analytes showed good linearity in the studied range with squared correlation coefficients (R^2) ranging from 0.948 to

Table 1. Analytical figures of merit of the proposed USAEME-SFOD-HPLC-DAD method for PAS determination

Compound	Linear range ($\mu\text{g/mL}$)	R^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	EF	Intraday precision (RSD, %)		Interday precision (RSD, %)		Recovery (EC) (%)	Recovery (BBW) (%)
						Low ^{a)}	High ^{b)}	Low ^{a)}	High ^{b)}		
1 Anisyl alcohol	0.10–1.09	0.988	0.026	0.079	9	8.7	4.7	8.2	5.1	93	98
2 Benzyl alcohol	0.07–1.00	0.990	0.022	0.065	11	12.3	4.0	11.6	4.2	98	106
3 Cinnamyl alcohol	0.01–0.99	0.993	0.003	0.008	43	6.0	4.1	7.9	4.2	1090	110
4 Cinnamal	0.05–0.96	0.992	0.010	0.031	39	6.6	3.3	7.7	4.0	90	94
5 Isoeugenol	0.11–1.07	0.961	0.020	0.061	118	12.4	10.7	7.6	14.4	138	154
6 Geraniol	0.06–1.14	0.948	0.008	0.023	103	5.3	4.5	8.4	3.9	104	125
7 Linalool	0.01–0.99	0.995	0.002	0.005	117	4.6	3.7	5.7	6.0	105	110
8 Citral	0.04–1.01	0.999	0.006	0.017	117	4.3	3.3	6.3	4.5	104	98
9 Methyl-2-octynoate	0.01–1.08	0.997	0.003	0.008	220	4.3	3.9	5.8	4.9	110	100
10 Amylcinnamyl alcohol	0.05–1.00	0.986	0.009	0.028	190	3.7	4.1	6.9	4.2	121	109
11 Benzyl benzoate	0.05–0.99	0.979	0.007	0.021	197	6.3	4.3	7.6	4.7	128	108
12 Benzyl salicylate	0.01–1.01	0.997	0.002	0.008	226	5.7	4.1	6.0	5.8	109	96
13 Benzylcinnamate	0.01–1.00	0.999	0.001	0.004	194	5.7	3.9	6.2	5.8	103	99
14 Lilial	0.03–0.93	0.986	0.007	0.020	149	3.3	6.0	7.3	5.4	112	136
15 Amyl cinnamal	0.01–1.02	0.998	0.003	0.009	203	4.0	3.6	4.9	4.9	99	99
16 α -Isomethyl ionone	0.01–0.88	0.998	0.001	0.004	237	3.4	3.7	4.5	5.0	108	97
17 Hexyl cinnamal	0.19–0.96	0.963	0.056	0.168	183	4.1	4.4	4.3	4.5	95	90
18 Limonene	0.48–9.64	0.965	0.154	0.463	122	6.1	7.9	4.7	6.8	100	97

a) Low level is around 0.2 $\mu\text{g/mL}$ for anisyl alcohol and isoeugenol, around 0.3 $\mu\text{g/mL}$ for hexyl cinnamal, around 0.7 $\mu\text{g/mL}$ for limonene, and around 0.08 $\mu\text{g/mL}$ for the rest of analytes.

b) High level is around 8 $\mu\text{g/mL}$ for limonene and around 0.8 $\mu\text{g/mL}$ for the rest of analytes.

Table 2. Comparison of the presented work with other methods reported in the literature

	Matrix	Number of PAS	R^2	LOD (ng/mL)	RSD (%)	Recovery (%)	Reference
GC-MS	Perfumes	24	0.996–1.000	0.26–29	0.4–7.7	89–106	13
GC-MS	Fragrances	24	>0.990	0.8–8.8 (mg/Kg)	0.1–5.0		14
GC-MS/MS	Toys	24	0.9850–0.9999	0.02–5.00(mg/Kg)	3.1–13.4	80–107	15
GC $\times$ GC-MS	Fragrances	24	0.9734–0.9996	1–10			17
GC $\times$ GC-FID	Fragrances	24	0.9796–0.9997	4–8	0.51–10.61		17
MSPD-GC-MS	Fragrances	24	0.9970–1.0000	1–12	<10	75–118	19
SPME-GC-MS	Baby bath water	14	0.993–1.000	0.001–0.300	0.4–21	74–124	20
HS-SPME-GC-MS	Water	24	0.9944–0.9998	0.001–1.100	0.6–17	73–136	21
HS-SPME-GC-MS	Wastewater treatment plants	15	0.9851–0.9989	0.01–1.70	4–33	40–100	22
SPD-PLE-GC-MS	Leave on cosmetics	24	0.997–0.999	0.83–25	0.2–9.7	85–114	23
DLLME-GC-MS	Water	21	0.9807–0.9959	0.007–1.000	3–16	29–112	24
USAEME-GC-MS	Water	24	0.9974–1.0000	0.006–1.15	0.2–23	18–116	25
MEEKC	Commercial rinse-off products	18	0.9903–0.9990	–	0.5–6.0	93–107	26
MEKC	Personal care products	8	0.9941–0.9994	1150–11040	2.4–19.2	89–115	27
HPLC	Commercial scented products	24	0.990–0.999	20–10880	0.6–3.5	90–105	29
USAEME-SFOD-HPLC-DAD	Water and cosmetic samples	18	0.948–0.999	1.3–154.3	3.3–14.4	90–145	This work

MEEKC, microemulsion electrokinetic chromatography; MSPD, matrix solid-phase dispersion.

0.999. LODs were calculated based on ten determinations of samples in which the analyte concentration was close to the expected LOD, and then the LODs were calculated as $3 \times \text{SD}$, where SD is the SD of concentration. [33]. The LODs were from 0.001 to 0.154 $\mu\text{g/mL}$. LOQs were calculated as $3 \times \text{LOD}$ and ranged from 0.004 to 0.463 $\mu\text{g/mL}$.

The method was also evaluated considering inter and intraday precision, all of them at two different concentrations for each analyte. Low level was around 0.2 $\mu\text{g/mL}$ for anisyl alcohol and isoeugenol, around 0.3 $\mu\text{g/mL}$ for hexyl cinnamal, around 0.7 $\mu\text{g/mL}$ for limonene, and around 0.08 $\mu\text{g/mL}$ for the other analytes. High level is around

Table 3. Concentration of PAS in real samples

Samples	Compounds ^{a)}																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
EC					98 ± 3 (3.2%)	323 ± 10 (3.0%)	966 ± 38 (3.9%)											
EP		212 ± 11 (5.2%)			5626 ± 344 (6.1%)	2467 ± 371 (15.0%)					32 ± 3 (9.3%)	4 ± 1 (24.0%)		193 ± 10 (5.2%)		592 ± 47 (7.9%)		
ET1						329 ± 5 (1.5%)					172 ± 14 (8.1%)							2291 ± 236 (10.3%)
ET2		16 ± 1 (6.2%)			1607 ± 55 (3.4%)	9094 ± 219 (2.4%)		286 ± 39 (13.6%)										5456 ± 334 (6.1%)
ET3						258 ± 17 (6.5%)								1288 ± 154 (11.9%)				
ET4		1002 ± 71 (7.1%)	2.1 ± 0.1 (4.8%)			2751 ± 7 (0.6%)					292 ± 30 (10.3%)	206 ± 15 (7.3%)		894 ± 31 (3.5%)				
BM			67 ± 5 (7.5%)		253 ± 14 (5.5%)	272 ± 14 (5.1%)		7 ± 1 (14.3%)				54 ± 1 (1.8%)		67 ± 11 (16.8%)		31 ± 1 (3.3%)		219 ± 9 (4.1%)

Mean of triplicates (in mg/kg) ± SD (RSD%). EP, Eau de perfume; ET, Eau de toilette.

a) Compounds are numbered as shown in Table 1.

8 µg/mL for limonene and around 0.8 µg/mL for the other analytes.

For intraday precision, ten measurements were performed in the same day. For interday precision, 12 experiments were carried out on three days over two weeks. Values, expressed as RSD in percentages, ranged between 3.3 and 12.4% for intraday precision and 3.9 and 14.4% for interday precision.

Recovery studies were carried out in one BBW and one EC. EC was performed diluting approximately 1 g of each sample in 50 mL, and in the case of BBW no dilution was necessary. The obtained values were in general satisfactory, ranging from 90 to 110%, except for isoeugenol (137.5%), amylcinnamyl alcohol (121.1%), benzyl benzoate (128.5%), and linal (112.4%) in EC and for isoeugenol (154.0%), geraniol (125.1%), and linal (136.3%) in BBW.

The EF was defined as the ratio between the analyte concentration in the organic phase after the extraction and the initial concentration of analyte within the water sample. EFs ranged from 9 to 237.

The performance of the proposed USAEME-SFOD method was compared with other reported extraction methods for PAS in different matrices that are listed in Table 2. The best LODs were obtained with SPME followed by GC using MS detector. To date, GC with MS has been the most sensitive and used technique. However, other less sensitive techniques for determination of these compounds have emerged as an alternative, HPLC–DAD being one of them. This technique coupled to a preconcentration method, as USAEME-SFOD, could provide similar results to those methods that use GC–MS without a sample pretreatment technique. The RSDs obtained with the proposed procedure were <14.4%. The results of recovery studies taken from consulted reference were made in different and analogous matrices. The comparison of obtained recovery data with results of similar matrices was acceptable.

3.4 Analysis of real samples

The developed method was applied to the determination of PAS in several samples, including two BBW, one SPW, one BM, one EC, one eau de perfume, and four different ETs. Given the wide range of concentrations of fragrance allergens even inside the same sample, different dilutions were applied to each sample to be able to determine all the present analytes. Dilutions of scented products ranged from 0.0029 to 1.0077 g in 50 mL. In case of water no dilution was applied. In SPW and in BBW1 no analyte content was found. In BBW2 presence of anisyl alcohol was observed but this could not be quantified as it was under LOQ. Table 3 shows the results of quantitative analysis of the samples. Data are presented as mean of three independent experiments ± SD (RSD, in %). A chromatogram obtained from BM sample is presented in Fig. 1B showing eight PAS. In this real sample different dilutions were needed for analytes quantification. Linalool was the most commonly found PAS in the samples. Benzyl

alcohol, geraniol, citral, benzyl benzoate, benzyl salicylate, linal, α -isomethyl ionone and limonene were also found in these samples. These reported values are above or below, and are also in accordance with the European Union regulation [34].

4 Concluding remarks

In the present work, a method based on USAEME-SFOD–HPLC–DAD was developed for determination of fragrance allergens. Until now, only one method had been developed for quantification of these compounds using HPLC–DAD and it did not include any sample preparation technique. In this study, sample preparation has been accomplished by USAEME-SFOD, which has been found to be an efficient, environmentally friendly, and nonexpensive technique that allows decreasing LODs of the determination technique.

The developed method has been evaluated in terms of linear range, precision, and accuracy obtaining satisfactory results. Finally, the proposed method has been successfully applied to the analysis of several real samples. In conclusion, the proposed method can be applied for the determination of these fragrance allergens in different water and cosmetic samples.

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