Development of a solid phase dispersion-pressurized liquid extraction method for the analysis of suspected fragrance allergens in leave-on cosmetics

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ABSTRACT

A new method based on solid phase dispersion-pressurized liquid extraction (PLE) followed by gas chromatography-mass spectrometry (GC-MS) has been developed for the determination of 26 suspected fragrance allergens (all the regulated in the EU Cosmetics Directive amenable by GC, as well as pinene and methyleugenol) in cosmetic samples. The effects of the temperature, extraction time and solvent, and dispersing sorbent, affecting the whole proposed procedure, have been evaluated using a multifactor strategy. The optima conditions after the analysis of main and second order effects entailed the extraction at 120 °C for 15 min, using hexane/acetone as solvent, and florisil as dispersing sorbent. The method performance has been studied, showing good linearity ($R \geq 0.996$) as well as good precision (RSD $\leq 10\%$). Detection limits (S/N = 3) ranged from 0.000001 to 0.0002% (w/w), values far below the established restrictions as regard labelling in the European Cosmetics Regulation. Reliability was demonstrated through the quantitative recoveries of all the studied compounds. The absence of matrix effects allowed quantification of the compounds by calibration with standard solutions. The analysis of 10 samples (several moisturizing and anti-wrinkle creams and lotions, hand creams, and sunscreen and after-sun creams), covering very different matrices, showed the presence of suspected allergens in all the analyzed samples; in fact, half of the samples contained an elevated number of them. Although the ubiquity of these compounds was demonstrated, labelling was in all cases in consonance with the European Cosmetics Regulation.

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1. Introduction

The majority of personal care, household and laundry products on the market contain fragrances. Some of the fragrance chemicals have been shown to cause various side effects, like skin sensitivity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. [1–3]. Legislations in force in the three principal markets regarding cosmetic products, i.e., in the European Union [4], the United States (US) [5] and Japan [6], establish that all the ingredients for cosmetics should be included on the label. According to the EU Cosmetics Directive [4], in the case of perfume and aromatic compositions and their raw materials, all together can be referred to under the word "perfume" or "aroma"; nevertheless, its Annex III consists of a list of restricted substances used as ingredients of cosmetic products. Several suspected fragrance allergens are included in this Annex. Two different restrictions are applied to them, i.e., substances that can be included up to a maximum allowed concentration, and substances for which their presence must be indicated in the list of ingredients when their concentrations exceed the 0.001% in leave-on products

and 0.01% in rinse-off products (see in Table 1 the compounds considered in this study and their limitations). The possible negative effects on the health of such substances may drive to at least a decrease of these values. In fact, it has been already observed the inclusion of the term "fragrance free" in several cosmetic products as a positive characteristic.

Hence routine analytical methods are required to ensure that regulations are observed by producers and importers. The variety of matrices in which fragrance compounds have to be analyzed is very broad and includes very complex matrices. In addition, the concentration range of the fragrance compounds in these matrices may fluctuate from low micrograms per gram to milligrams per gram. While liquid samples such as perfumes or perfumed oils, can be directly analyzed usually after simple dilution [7–9], the direct analysis of other cosmetic samples, such as creams and lotions, is quite problematic since the contamination of the chromatographic inlet and column occurs after a few analyses [10], the difficulty of achieving accurate determinations due to the complexity of obtaining homogeneous solutions of the samples, and the coelution of the matrix components.

Therefore, the development of analytical methods for the determination of fragrance allergens in leave-on cosmetics is as challenging as necessary; even though, up to our knowledge, the literature is somewhat scarce in this subject.

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Table 1
Target fragrance allergens, their CAS numbers and purity, chromatographic retention times, as well as their qualification and quantification ions.

Common name	Chemical name	CAS number	Purity	Boiling point (°C)	Retention time (min)	Qualifier and quantifier ions
Pinene	2,6,6-Trimethylbicyclo[3.1.1]-hept-2-ene	80-56-8	≥99%[c]	155	4.75	77,91,93
Limonene[a]	(4R)-1-Methyl-4-(1-methylethenyl)cyclohexene	5989-27-5	97%[d]	176	6.62	67,93,121
Benzyl alcohol[a]	Benzene methanol	100-51-6	99%[e]	205	6.87	77,79,108
Linalool[a]	3,7-Dimethyl-1,6-octadien-3-ol	78-70-6	97%[d]	198	8.05	71,93,121
Methyl-2-octynoate[a]	Methyl heptin carbonate	111-12-6	≥99%[f]	219	9.74	67,95,123
Citronellol[a]	(±)-3,7-Dimethyloct-6-en-1-ol	106-22-9/ 26489-01-0	95%[d]	225	10.06	67,69,81,95
Citral[a]	3,7-Dimethyl-2,6-octadienal	5392-40-5	95%[d]	229	10.20 10.50	67,69,109
Geraniol[a]	3,7-Dimethyl- (2E)-2,6-octadien-1-ol	106-24-1	≥96%[c]	229	10.35	67,69,111,123
Cinnamal[a]	3-Phenyl-2-propenal	104-55-2	≥93%[f]	252	10.52	77,103,131
Anise alcohol[a]	4-Methoxybenzyl alcohol	105-13-5	98%[d]	259	10.66	77,109,121,138
Hydroxycitronellal[a]	7-Hydroxy-3,7-dimethyloctanal	107-75-5	≥95%[f]	241	10.68	59,81,95
Cinnamyl alcohol[a]	3-Phenyl-2-propen-1-ol	104-54-1	98%[e]	250	10.88	91,92,115,134
Eugenol[a]	2-Methoxy-4-(2-propenyl)-phenol	97-53-0	99%[d]	256	11.34	131,149,164
Methyl-eugenol[b]	1,2-Dimethoxy-4-(2-propenyl)-benzene	93-15-2	99%[d]	248	11.71	147,163,178
Isoeugenol[a]	2-Methoxy-4-(1-propenyl)-phenol	97-54-1	98%[d]	267	11.75 12.04	131,149,164
Coumarin[a]	2H-1-Benzopyran-2-one	91-64-5	99%[d]	298	11.98	90,118,146
α-Isomethyl ionone[a]	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	≥85%[c]	266	12.24	107,135,150
Lilial®[a]	2-(4-tert-Butylbenzyl) propionaldehyde	80-54-6	≥95%[c]	279	12.54	131,147,189
Amyl cinnamal[a]	2-Benzylideneheptanal	122-40-7	97%[d]	289	13.21	91,115,203
Lyral®[a]	Hydroxyhexyl-3-cyclohexene carboxaldehyde	31906-04-4	≥97%[c]	319	13.29	77,79,136
Amylcinnamyl alcohol[a]	2-Pentyl-3-phenylprop-2-en-1-ol	101-85-9	≥85%[c]	>200	13.40	91,115,133
Farnesol[a]	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	4602-84-0	95%[d]	283	13.44 13.56	69,81,93
Hexyl cinnamal[a]	2-Benzylideneoctanal	101-86-0	≥95%[f]	308	13.73 13.88	91,115,216
Benzyl benzoate[a]	Phenylmethyl benzoate	120-51-4	98%[e]	324	13.85	91,105,194
Benzyl salicylate[a]	Benzyl-2-hydroxybenzoate	118-58-1	≥99%[c]	320	14.56	65,91,228
Benzyl cinnamate[a]	3-Phenyl-2-propenoic acid phenylmethyl ester	103-41-3	99%[d]	371	16.86	91,131,192,193

[a] According to Regulation (EC) No 1223/2009, the presence of the substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products.

[b] Maximum allowed concentration in fragrance cream: 0.002%, and other leave-on products: 0.0002%.

[c] Purchased from: Fluka Chemie GmbH (Steimheim, Germany).

[d] Sigma–Aldrich Chemie GmbH (Germany).

[e] Chem Service (West Chester, USA).

[f] SAFC Supply Solutions (St. Louis, USA).

Despite the presence of chromophoric groups in the most of these fragrances allows the use of high performance liquid chromatography (HPLC) with ultraviolet detection [11], gas chromatography-mass spectrometry (GC-MS) can be considered the technique of choice for the analysis of this kind of volatile substances [10,12,13].

Pressurized liquid extraction (PLE) has been applied for the analysis of other cosmetic ingredients such as UV filters [14–16], musks [16], preservatives and antimicrobials [15,16] in environmental matrices such as sediments [14] and sewage sludge [15,16]. This technique is fast, increases automation, decreases the amount of organic solvents, and offers the possibility of controlling the selectivity of the extraction by loading different sorbents instead of inert materials into the extraction cell.

The aim of this work is to develop a method based on PLE followed by gas chromatography-mass spectrometry (GC-MS) to simultaneously identify and quantify 26 fragrances in multi-matrix cosmetic samples. To our knowledge, PLE is applied for the first time to the analysis of cosmetics and it is also the first time that it is applied to the analysis of suspected fragrance allergens.

2. Experimental

2.1. Reagents and materials

The 26 studied fragrance allergens, their chemical names and the purity of the standards are summarized in Table 1.

The internal standard PCB-30 (2,4,6-trichlorobiphenyl) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Acetone, ethyl acetate, and n-hexane were provided by Merck (Darmstadt, Germany). Florisil (60–100 mesh) and C18 (70–230 mesh) were achieved from Aldrich (Milwaukee, WI, USA). Before being used, florisil was activated at 130 °C for 12 h and then allowed to cool down in a desiccator. Anhydrous sodium sulphate (99%) was purchased by Panreac (Barcelona, Spain).

Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared in acetone, hexane/acetone (1:1, v/v), and ethyl acetate. All solutions were stored in amber glass vials at −20 °C. All solvents and reagents were of analytical grade.

2.2. Cosmetic samples

Different cosmetics from national and international brands were purchased from local sources. They included moisturizing and anti-wrinkle creams and lotions, hand creams, sunscreen and after-sun creams. Samples were kept in their original containers at room temperature until their analysis.

1 g of the cosmetic sample was weighted exactly into a 10-mL glass vial. When it was necessary, the sample was spiked with 50 μL of the corresponding acetone solution of the target compounds to get the desired final concentration in the cosmetic sample. The sample was then thoroughly mixed with 2 g of drying agent (anhydrous

sodium sulphate, Na_2SO_4) and 2 g of dispersing sorbent (C18 or florisil).

2.3. PLE procedure

Extractions were performed on an ASE 200 system (Dionex, Co., Sunyvale, CA, USA) equipped with a 24-sample carousel, 11-mL stainless steel cells and 40-mL collection vials. Two cellulose filters (Dionex) were placed at each end of the PLE cell. The sample, mixed with the drying agent and the dispersing sorbent, was introduced into the cell, where previously 1 g of clean sand was placed. Finally, the dead volume of the cell was filled up with sand. The cell was tightly closed and placed into the carousel of the ASE system. Extractions were performed by preheating the cell before filling with solvent (preheat method). The extraction pressure was set to 1500 psi, the flush volume was 60% and the purge time was set to 60 s. Hexane/acetone (1:1, v/v) or ethyl acetate were employed as extraction solvents, depending on the experiment. The extraction temperature and extraction time varied during the optimization of the method. After extraction, 20 μL of PCB 30 (100 μg mL^{-1}) were added to the final extract (~15 mL) to correct possible variations of the extract volume. Then, PLE extracts were directly analyzed by GC-MS, without a pre-concentration step, since the detection limits achieved are low enough considering the current cosmetic regulations.

2.4. GC-MS analysis

Analyses were performed on a Varian CP 3900 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) equipped with a 1079 split/splitless injector and an ion trap spectrometer Varian Saturn 2100 (Varian Chromatography Systems). Separation was carried out on a HP5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Injection volume was 2 μL. Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 45 °C (held 2 min) to 100 °C at 8 °C min^{-1}; to 150 °C at 20 °C min^{-1}; to 200 °C at 25 °C min^{-1} (held 5 min); and a final ramp to 280 °C (held 4 min) at 35 °C min^{-1} (total analysis time = 25 min). The splitless mode (held 2 min) was used for injection, after that the split flow was set at 20 mL min^{-1}. The injector temperature was kept at 220 °C. Trap, manifold and transfer-line temperatures were 220 °C, 120 °C and 280 °C, respectively.

The GC-MS system was operated by Saturn GC-MS workstation v5.52 software. In the full scan mode the mass range was varied from 50 to 320 m/z at 0.6 s scan^{-1}, starting at 4 min and ending at 22.5 min. The filament emission current was 15 μA. The analytes were positively identified by comparison of their mass spectra and retention times to those of the standards.

2.5. Statistical analysis

Basic and descriptive statistics, as well as experimental design analysis were performed using Statgraphics-Plus v5.1 (Manugistics, Rockville, MD, USA) as software package. The experimental design was applied in the optimization of the extraction method, to analyze the simultaneous effect of the main parameters affecting PLE.

3. Results and discussion

3.1. Optimization of the dispersive pressurized liquid extraction process

The chromatographic method for the separation of the target allergens was optimized elsewhere [17,18] and it is described in

Table 2
Factors and levels considered in the experimental design.

Factor	Key	Levels		
		Lower (−)	Intermediate	Upper (+)
Temperature (°C)	A	80	100	120
Time (min)	B	5		15
Solvent	C	Hexane/acetone		Ethyl acetate
Dispersing sorbent	D	C18		Florisil

Section 2. Table 1 summarizes the retention times as well as the qualification and quantification ions of the target analytes.

Different parameters affecting the pressurized liquid extraction (PLE) can be optimized in order to achieve fast and efficient extraction. In the usual working range for this technique, pressure generally has a negligible effect on the extraction yield [19], and so, we decided to conduct the experiments at 1500 psi, which is the standard operating pressure in PLE extractions [20]. Flush volume and purge time were set at 60% and 60 s, respectively. The influence of the remaining variables was studied using a multifactor strategy. The studied factors were: extraction temperature (factor A), extraction time (factor B), solvent (factor C) and dispersing sorbent (factor D) (see Table 2). Extraction temperature was studied at three levels from 80 to 120 °C, whereas the other factors were studied at two levels. The second factor considered was the static extraction time that it was assessed at 5 and 15 min. The extraction solvent is one of the most important parameters to optimize in PLE. Two solvents were investigated, hexane/acetone (1:1, v/v), recommended in the 3545 EPA method [21], and ethyl acetate; both solvents with intermediate polarity that should be suitable for the varied range of polarities of the target analytes. The inclusion of an *in situ* clean-up step by adding certain sorbents to the PLE cells favours to the obtaining of clean extracts. In this way, lipids and other co-extractable materials are prevented from coming out to the extract. In addition, these materials can act as dispersing phase, contributing to the consecution of a more efficient extraction. Thus, 2 g of dispersing sorbent (C18 or florisil) were mixed with the sample and packed in the cell.

The study consisted of a $3 \times 2^{3-1}$ mixed level fraction factorial design, involving 12 randomized experiments. Experiments were performed using 1 g of a real moisturizing cream sample containing some of the target analytes (pinene, limonene, linalool, citronellol, geraniol, coumarin, ionone, lilial, hexyl cinnamal, and benzyl salicylate) and fortified with all compounds at 100 μg g^{-1}. Since drying of the sample is essential for an efficient PLE, in all experiments 2 g of anhydrous sodium sulphate were added. Sand was employed to avoid dead volume.

Numerical analysis of the results leads to the ANOVA results shown in Table 3. As it can be seen, temperature (factor A) and time (factor B) were significant for several analytes. In the cases that temperature was significant and the time was also significant. The extraction solvent (factor C) was significant for fewer compounds; and the last factor, the type of dispersing sorbent (factor D), was only significant for two of the most volatile compounds, pinene and limonene. However, the most important factor, which was significant for 25 out of 26 compounds, was a second order factor, the interaction time and extraction solvent (BC). This factor was also the most influential one (see F-values) for most of the analytes. Another interaction effect that must be considered is temperature and extraction solvent (AC), which was significant for 10 compounds. Finally, other interactions were less important and only significant in few cases.

The information included in the ANOVA can be graphically plotted by means of the Pareto charts. In Fig. 1, some examples are showed. In these graphics the length of each bar is proportional to the absolute value of its associated standardized effect. The

Table 3
F-ratios and *p*-values obtained in the analysis of variance.

	Main effects								Interactions													
	A: temperature		B: time		C: solvent		D: sorbent		AA		AB		AC		AD		BC		BD		CD	
	F-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value	*F*-ratio	*p*-value
Pinene	1		37		4		447	+			114		257	+	111		782	+	42		1	
Limonene	281	+	439	+	284	+	640	+			860	+	2414	+	97		4167	+	60		14	
Benzyl alcohol	53		2		420	+	8		48		212	+	721	+			1322	+	87		41	
Linalool	1		1		9		4				8		14		5		87	+			3	
Methyl-2-octynoate	0.1		4		0.1		0.03		3		11		14				64	+	6			
Citronellol	100	+	24	+	8		1		4		60	+	22	+					16		36	+
Citral	12		14		15		1		27		51		83		3		397	+	18			
Geraniol	1		17		558	+	43		268	+	68		497	+	97		1128	+			172	+
Cinnamal	0.2		4		6		0.5				11		16		2		79	+	3			
Anise alcohol	1		17		9		3				24		53		13		300	+	9		14	
Hydroxycitronellal	0.01		5		4		1		2		3		7		1		26	+				
Cinnamyl alcohol	0.02		4		2		0.3		1		2		4		1		20	+				
Eugenol	0.3		7		1		0.5				5		11		3		53	+	2			
Methyleugenol	3		11		0.2		1				8		23	+	5		107	+	2			
Isoeugenol	0.1		28	+	0.4		4		3		14		18				90	+	19	+		
Coumarin	17		41	+	22	+	6		16						2		75	+	21	+	13	
α-Isomethyl ionone	0.4		8		1		0.1				4		16		3		45	+	2			
Lilial®	110		90		0.4		32		10		2		68				888	+	12		3	
Amyl cinnamal	405	+	598	+	6		33				53		688	+	322	+	3138	+	2		57	
Lyral®	49		237	+	29		36				36		50		47		305	+	7		37	
Amylcinnamyl alcohol	187	+	44		319	+	120		64		19		136		37		597	+			97	
Farnesol	24	+	29	+	10		2				15		15		2		42	+	5			
Hexyl cinnamal	17		51	+	0.4		5				10		35	+	13		182	+	1			
Benzyl benzoate	22	+	61	+	5		0.1				9		26	+	3		109	+	6			
Benzyl salicylate	2835	+	6720	+	409	+	76		202	+			6110	+	2694	+	13608	+	295	+	1252	+
Benzyl cinnamate	15		137		17		0.4		39		152		94				399	+	185	+	10	

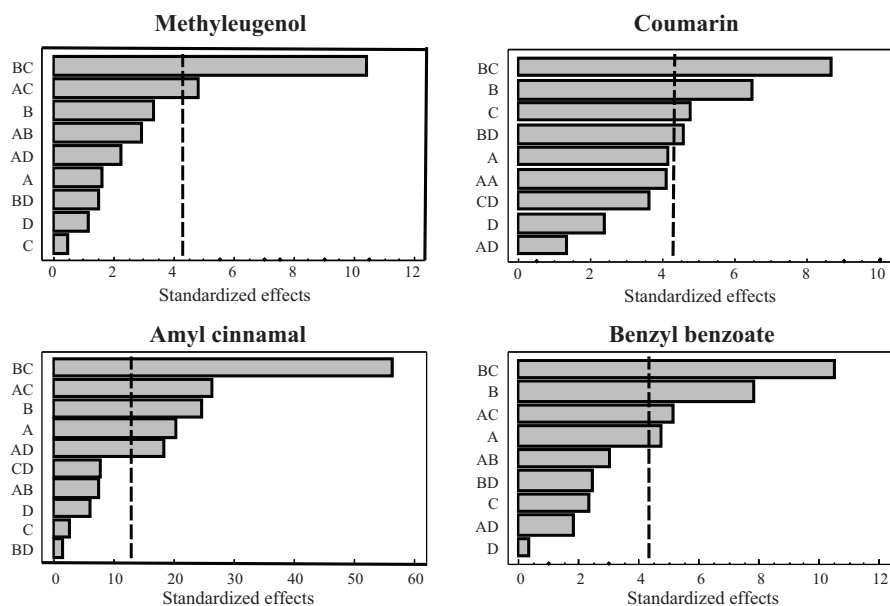+ cell, *p*-value < 0.05; empty cell, *p*-value > 0.05.

Fig. 1. Pareto charts showing the significant factors (95%) for some selected analytes (see factor codes in Table 2).

standardized effect is obtained by dividing the estimated effect of each factor or interaction by the standard error. Vertical line in the graphs represents the statistically significant bound at the 95% confidence level. We can clearly appreciate the notable influence of BC in all cases. Other significant factors were the interaction AC, and the main factors temperature (factor A) and extraction time (factor B).

A very useful graphic option provided by the statistic software is the main effects plot. Fig. 2 shows the main effects diagrams for several representative compounds since the general behaviour was common in most cases. This kind of plots shows the main effects with a line drawn between the low and the high level of the corresponding factors. The length of the line is proportional to the effect magnitude of each factor in the extraction process, and the sign of the slope indicates the level of the factor which produces the highest response. Regarding factors temperature and time, best extractions were generally obtained at the high level of the factors,

which means at 120 °C and 15 min. The solvent was only significant for six compounds, being for some of the analytes more favourable the use of hexane/acetone (see as example benzyl alcohol plot in Fig. 2), and for other compounds ethyl acetate (see coumarin plot). Nevertheless, this factor must be carefully analyzed since it is involved in the most important second order effects: its interaction with the temperature and the extraction time (AC and BC, respectively). Dispersing sorbent was non-significant and, therefore, characterized by a horizontal line, excluding the two most volatile compounds, for which C18 is more suitable than florisil.

As previously commented, the interaction effects must be considered before proposing a general method for the simultaneous extraction of the 26 fragrance allergens, and especially, time–solvent (BC) which was significant for 25 among 26 compounds. The most important second order effects are shown in Fig. 3 for some analytes, as example, since the trends were, in general, the same. Analyzing BC interaction, the most favourable extraction
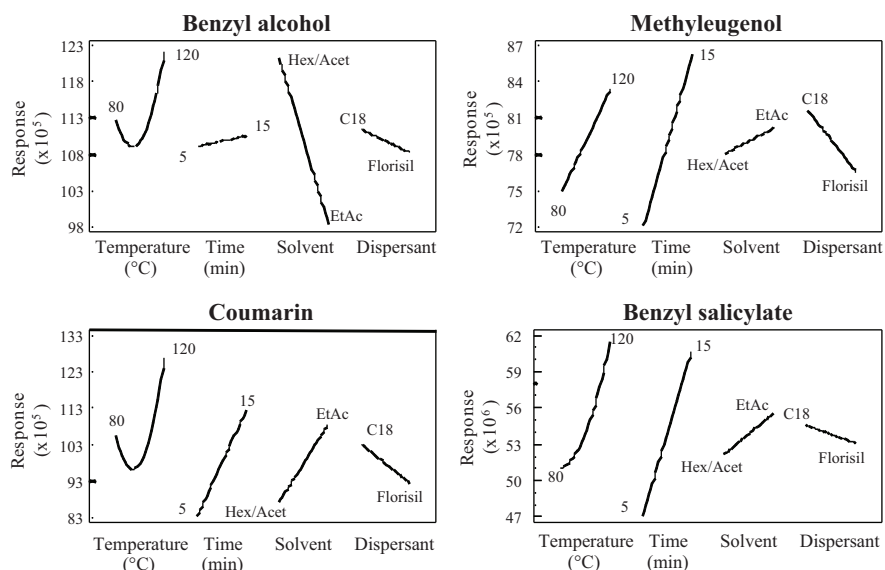

Fig. 2. Main effects plots for some representative fragrance allergens (Hex/Acet: hexane/acetone; EtAc: ethyl acetate).
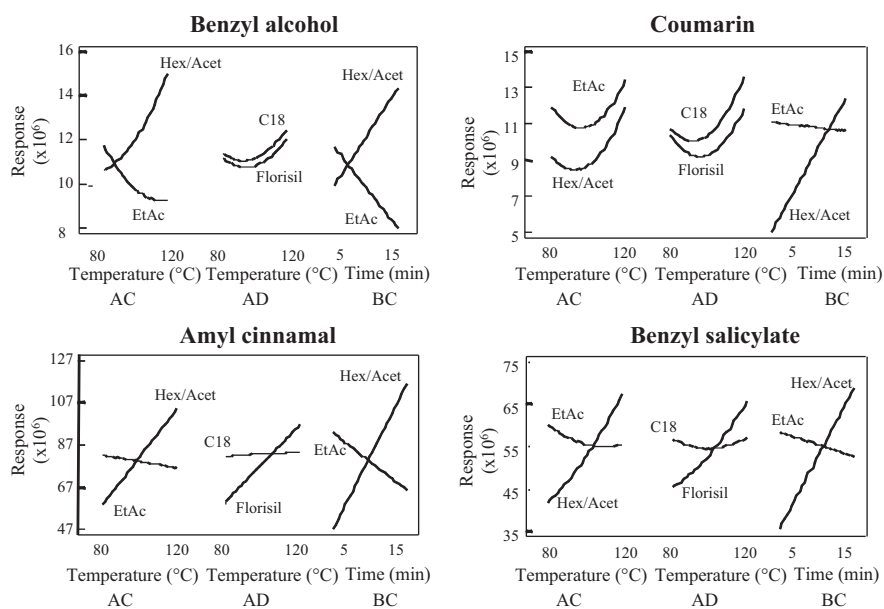
Fig. 3. Interaction effects plots: AC (temperature–solvent); AD (temperature–sorbent); and BC (time–solvent).

conditions implies the extraction with hexane/acetone for 15 min. Even for those compounds, such as coumarin or benzyl salicylate, for which ethyl acetate seemed more favourable after the analysis of only main factors (see Fig. 2), the analysis of second order factors, especially BC, shows the convenience of using hexane/acetone. Regarding AC interaction, the most favourable conditions consist on hexane/acetone extraction at 120 °C. The other interaction effects were not very important with the exception of AD for benzyl salicylate, for which the most favourable conditions are the extraction at 120 °C employing florisil as dispersing phase. In summary, the general conditions selected after the analysis of main and second order effects, involved the extraction at 120 °C for 15 min, using hex-

ane/acetone as solvent, and florisil as dispersing sorbent. Although the use of C18 would be also suitable, florisil was selected since this last dispersant, once it is mixed with the samples, was easier to manipulate as well as the lower prize compared to C18.

3.2. Method validation

Method quality parameters were estimated (Table 4). The instrumental linearity was evaluated at a concentration range between 0.02 and 10 $\mu g\,mL^{-1}$ (including seven concentration levels). Each concentration level was injected in triplicate and the response function was found to be linear with correlation coef-

Table 4
Quality parameters of the method.

Compound	Correlation coefficient (R)	IDL (ng mL^{-1})	Recovery[a] (RSD) (%)		LOD (%, w/w)	LOQ (%, w/w)
			15 $\mu g\,g^{-1}$	75 $\mu g\,g^{-1}$		
Pinene	0.998	4.9	86.1 (4.4)	89.2 (5.0)	0.000052	0.00017
Limonene	0.998	6.0	105 (6.9)	105 (6.1)	0.0000083	0.000028
Benzyl alcohol	0.998	7.5	95.1 (7.4)	95.8 (1.0)	0.000012	0.000040
Linalool	0.999	6.2	98.7 (1.5)	110 (9.7)	0.0000085	0.000028
Methyl-2-octynoate	0.999	5.9	114 (9.2)	86.4 (3.1)	0.000014	0.000046
Citronellol	0.997	7.4	87.4 (9.2)	90.8 (7.2)	0.000043	0.00014
Citral	0.997	25	96.1 (2.5)	92.7 (4.4)	0.000026	0.000086
Geraniol	0.998	11	110 (9.5)	114 (4.2)	0.000021	0.000071
Cinnamal	0.999	6.0	90.4 (2.1)	85.1 (0.3)	0.000018	0.000061
Hydroxycitronellal	0.999	3.8	88.0 (1.4)	98.5 (0.2)	0.000011	0.000021
Anise alcohol	0.998	8.6	111 (0.3)	110 (6.8)	0.000017	0.000055
Cinnamyl alcohol	0.996	17	107 (3.0)	101 (2.2)	0.000021	0.000068
Eugenol	0.999	3.7	91.5 (6.8)	109 (0.6)	0.0000019	0.0000062
Methyleugenol	0.998	0.83	96.0 (4.0)	95.6 (8.0)	0.0000012	0.0000040
Isoeugenol	0.998	5.6	101 (0.8)	99.9 (2.8)	0.0000075	0.000025
Coumarin	0.998	1.5	112 (0.5)	92.0 (6.7)	0.0000036	0.000012
α-Isomethyl ionone	0.998	1.3	87.7 (1.6)	99.0 (4.4)	0.0000032	0.000011
Lilial®	0.999	4.7	97.2 (0.2)	106 (7.2)	0.0000076	0.000025
Amyl cinnamal	0.997	2.6	108 (0.8)	114 (0.5)	0.0000042	0.000014
Lyral®	0.997	5.6	113 (6.9)	91.3 (1.3)	0.000029	0.000097
Amylcinnamyl alcohol	0.998	3.9	91.0 (6.8)	94.0 (6.1)	0.000012	0.000039
Farnesol	0.998	22	85.9 (8.8)	88.3 (4.3)	0.00018	0.00060
Hexyl cinnamal	0.998	2.5	109 (2.6)	112 (1.1)	0.0000063	0.000021
Benzyl benzoate	0.999	1.7	100 (4.0)	85.6 (1.7)	0.0000073	0.000024
Benzyl salicylate	0.998	3.8	n.c.	102 (0.6)	0.0000095	0.000032
Benzyl cinnamate	0.999	6.0	90.4 (5.5)	86.3 (8.9)	0.000012	0.000039

N.c.: not calculated.

[a] $n = 3$.

Table 5
Analysis of real cosmetic samples (MC: moisturizing cream; ML: moisturizing lotion; AW: anti-wrinkle cream; HC: hands cream; SC: sunscreen cream; AS: after-sun cream).

	Concentration (%, w/w)									
	MC1	MC2	MC3	ML	AW1	AW2	HC1	HC2	SC	AS
Pinene	0.00073			0.00030	0.00121					
Limonene[a]	0.02052	0.07904	0.00638	0.01990	0.00050		0.00358	0.00019		
Benzyl alcohol[a]			0.00023	0.00032			0.00433	0.00014		
Linalool[a]	0.06590	0.20321	0.00883	0.01118						
Citronellol[a]	0.00450			0.00101			0.00196			
Citral[a]		0.00114	0.00036	0.00581			0.00192		0.00049	
Geraniol[a]	0.01516						0.00128			
Hydroxycitronellal[a]				0.00216						
Cinnamyl alcohol[a]				0.00074		0.00101				
Eugenol[a]				0.00379	0.00023		0.00027			
Methyleugenol[b]							0.00006			
Isoeugenol[a]					0.00029		0.00012			
Coumarin[a]	0.00211		0.00030	0.00134						
α-Isomethyl ionone[a]	0.00673			0.01511		0.00099	0.00175			
Lilial®[a]	0.19343	0.19835					0.06534			
Lyral®[a]		0.00314								
Farnesol[a]										0.00684
Hexyl cinnamal[a]	0.00369	0.23213		0.02100			0.00859			
Benzyl benzoate[a]		0.01248					0.00486			
Benzyl salicylate[a]	0.12932	0.00019	0.13440							
Total content	0.44210	0.72967	0.15050	0.08265	0.00223	0.00200	0.09406	0.00033	0.00049	0.00684

[a] According to Regulation (EC) No 1223/2009, the presence of the substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products.

[b] Maximum allowed concentration in fragrance cream: 0.002%, and other leave-on products: 0.0002%. Blank cells mean values below LODs.

ficients (R) higher than 0.996. Instrumental limits of detection (IDL) were calculated as the concentration giving a signal-to-noise ratio of three (S/N = 3). Values ranged from 0.83 ng mL^{-1} (methyleugenol) to 25 ng mL^{-1} (citral) (see Table 4).

The other figures of merit were calculated using real cosmetic samples.

Recovery studies were carried out by applying the optimized PLE method to the extraction of cream samples spiked at two different levels, 15 and 75 µg g^{-1}. Previous analyses of this sample showed the presence of some of the target analytes, and these initial concentrations were taken into account to calculate the recoveries. Recoveries were between 85 and 114% (see Table 4). Precision was also evaluated and RSD values were in all cases lower than 10% with an average value of 4.2%.

The limits of detection (LODs) and quantification (LOQs) corresponding to the overall method were calculated as the concentration giving a signal-to-noise ratio of three (S/N = 3) and ten (S/N = 10), respectively. These values are also summarized in Table 4, expressed as percentage (%, w/w) in order to be consequent with the units used in the European Cosmetics Regulation [4]. As it can be seen, the obtained LODs and LOQs are several orders of magnitude lower than the established restrictions (see Table 1); and it is important to emphasize that, if necessary, these limits can be easily reduced (at least one order of magnitude) by concentrating the PLE extract (~15 mL).

3.3. Application to real samples

The method was finally applied to the analysis of several real cosmetic samples including moisturizing creams and lotions, sunscreen and after-sun creams, anti-wrinkle, and hand creams. The PSE extracts were directly analyzed without any further concentration step. In some cases, the extract was properly diluted due to the high concentration of some of the analytes in several samples. Results are shown in Table 5. Fig. 4 shows the extracted ion chromatograms obtained for a moisturizing cream (MC1). Found concentrations ranged from 0.00006% (methyleugenol in sample HC1) to 0.23% (hexyl cinnamal in MC2). Half of the samples contained an elevated number of the studied compounds; in fact,

four of the samples included more than eight fragrance allergens. Three compounds were detected in two samples (limonene and benzyl alcohol in HC2, and citral in SC) labelled as "fragrance free", although the calculated concentrations were below the limits established in the European Cosmetics Regulation [4]. Only six of the target fragrances (methyl-2-octynoate, cinnamal, anise alcohol, amyl cinnamal, amylcinnamyl alcohol, and benzyl cinnamate) were not detected in any sample. Limonene was present in seven out of ten samples, in some cases at quite high concentrations (see concentration values for MC1, MC2, and ML in Table 5). Total fragrance allergen content in the samples almost reached the 1% (0.73%) in some case, with an average value of 0.15%.
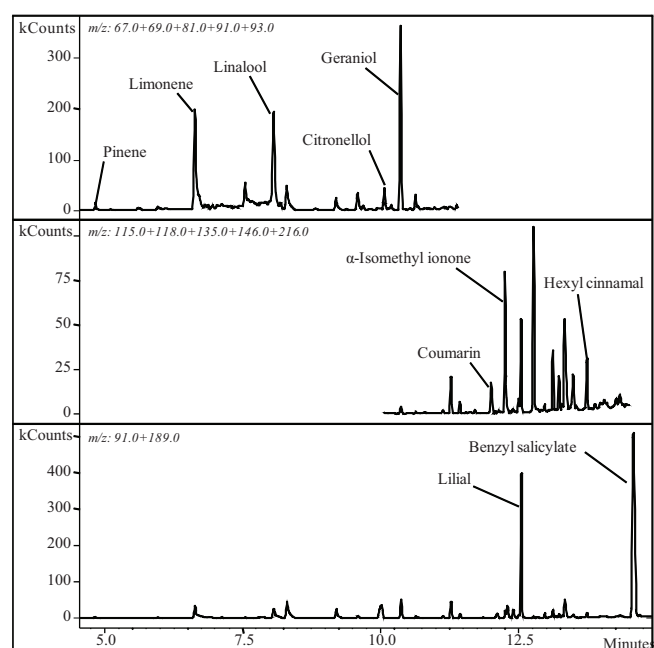

Fig. 4. Extracted ion chromatograms of sample MC1 (see concentrations in Table 5).

As it was commented in Section 1, the presence of these ingredients must be included in the cosmetic label when its concentration exceeds 0.001% (w/w) in ready for use preparation, in the case of leave-on products. The labelling in the samples containing some of these compounds was in consonance with the actual European Cosmetics Regulation.

4. Conclusions

PLE followed by gas chromatography-mass spectrometry (GC-MS) is applied for the simultaneous determination of 26 fragrance allergens in multi-matrix cosmetic samples. This is the first application of PLE to the analysis of cosmetics as well as to the analysis of fragrance allergens. The direct GC-MS analysis without any further step was possible since the obtained extracts were homogeneous and clear, and matrix interferences were not observed in any case. The absence of matrix effect allowed the use of calibration with standard solutions avoiding, in this way, the need of standard addition based quantification procedures. The obtained LODs are far below the established restrictions in Cosmetic Regulations, making this analytical method suitable for routine control. The reliability of the method was demonstrated through a broad range of leave-on cosmetics. The ubiquity of these compounds was demonstrated since they were present in all the analyzed samples and, in most cases, a quite high number of fragrance allergens per sample were detected.

Acknowledgements

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