



High-performance liquid chromatographic method for the simultaneous determination of 24 fragrance allergens to study scented products[☆]

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Abstract

The European legislation on cosmetic products has recently required the declaration of 26 compounds (24 volatile chemicals and 2 natural extracts) on the label of final products when exceeding a stipulated cut-off level.

In this work a rapid reliable and specific RP-HPLC method coupled with diode array detector (DAD) has been developed for the simultaneous determination and quantification of the 24 volatile chemicals: amyl cinnamal, benzyl alcohol, cinnamyl alcohol, citral, eugenol, hydroxy-citronellal, isoeugenol, amylcinnamyl alcohol, benzyl salicylate, cinnamal, coumarin, geraniol, Lyrall[®] (hydroxy-methylpentylcyclohexene carboxaldehyde), anisyl alcohol, benzyl cinnamate, farnesol, Lilial[®] (2-(4-*tert*-butylbenzyl)propionaldehyde) linalool, benzyl benzoate, citronellol, hexyl cinnamal, limonene, methylheptin carbonate, alpha-isomethyl ionone (3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one). The 24 analytes were appropriately separated over a running time of 40 min, on a C18 column using a simple gradient elution (acetonitrile/water) with flow rate from 0.7 to 1.0 ml/min and UV acquisition at 210, 254 and 280 nm. All calibration curves showed good linearity ($r^2 > 0.99$) within test ranges. The method was successfully applied to the qualitative and quantitative determination of the potential allergens in four commercial scented products, with satisfactory accuracy and precision. The results indicated that this simple and efficient method can be used for quality assessment of complex matrices such as cosmetic scented products.

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Keywords: RP-HPLC; DAD detection; Fragrance allergens; Scented products; Cosmetics

1. Introduction

Fragrance-containing products are part of daily life. The majority of personal-care, household and laundry products on the market contain fragrances. In the cosmetic formulations fragrances are commonly used to give the consumer a feeling of well being and to mask the odour of other chemical ingredients. About 3000 chemical substances and essential oils are commonly used for this scope. Cause of an increasing frequency of allergic contact dermatitis associated with the use of perfumed products, the SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products, today renamed SCCP, Scientific Committee on Consumer Products) identified 26 fra-

grance ingredients, 24 volatile chemicals (Table 1) and two natural extracts (oak moss and tree moss), for which there is need to provide the consumer with information when they are present in cosmetic products and to restrict their use and/or impose certain conditions [1,2].

On the basis of these opinions, Directive 2003/15/EC requires, in the European Union, the declaration of the listed 26 “fragrance allergens” on the label of the final product when present over the stipulated cut-off levels (0.01% for “rinse-off” and 0.001% for “leave-on” cosmetics) [3].

These new health concerns about allergenic reactions have led to an increased interest in the analyses of perfumed products. Qualitative analytical works on the simultaneous identification of the fragrance allergens reported the use of GC-MS [4,5] and, to our knowledge, a HPLC method has never been applied for this scope before.

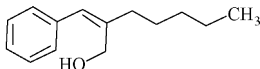
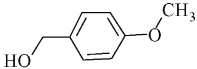
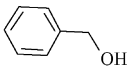
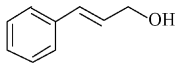
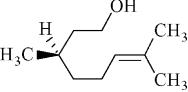
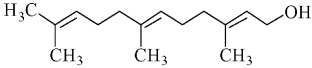
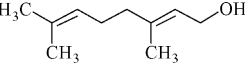
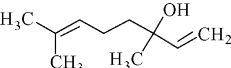
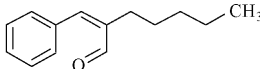
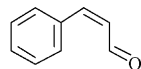
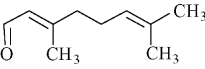
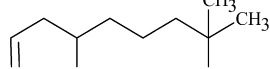
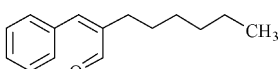
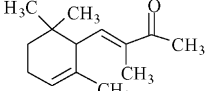
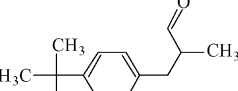
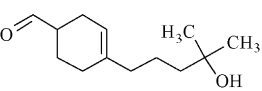
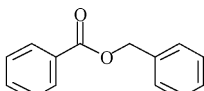
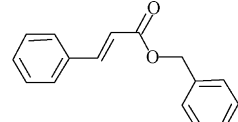
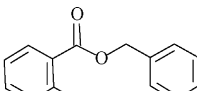
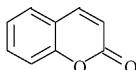
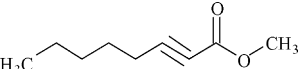
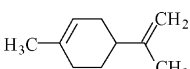
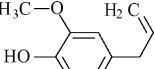
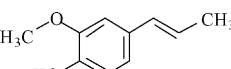
Therefore the aim of this work was to develop a simple, selective and reliable HPLC analytical procedure suitable for

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Table 1
24 selected fragrance allergens

 Amylcinnamyl alcohol (CAS: 101-85-9)	 Anisyl alcohol (CAS: 105-13-5)	 Benzyl alcohol (CAS: 100-51-6)	 Cinnamyl alcohol (CAS: 104-54-1)
 Citronellol (CAS: 106-22-9)	 Farnesol (CAS: 4602-84-0)	 Geraniol (CAS: 106-24-1)	 Linalool (CAS: 78-70-6)
 Amyl cinnamal (CAS: 122-40-7)	 Cinnamal (CAS: 104-55-2)	 Citral (CAS: 5392-40-5)	 Hydroxy-citronellal (CAS: 107-75-5)
 Hexyl cinnamal (CAS: 101-86-0)	 Alpha-isomethyl ionone (3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one) (CAS: 127-51-5)	 Lilial® (2-(4-tert-butylbenzyl) propionaldehyde) (CAS: 80-54-6)	 Lyrall® (hydroxy-methylpentylcyclohexene carboxaldehyde) (CAS: 31906-04-4)
 Benzyl benzoate (CAS: 120-51-4)	 Benzyl cinnamate (CAS: 103-41-3)	 Benzyl salicylate (CAS: 118-58-1)	 Coumarin (CAS: 91-64-5)
 Methylheptin carbonate (CAS: 111-12-6)	 Limonene (CAS: 5989-27-5)	 Eugenol (CAS: 97-53-0)	 Isoeugenol (CAS: 97-54-1)

the simultaneous determination of fragrance allergens potentially present in essential oils and finished scented products. The choice of this chromatographic technique depended on its widespread availability in most laboratories for routine analyses and its efficient application even to very complex matrices [6,7]. The study has been focused on the analysis of the 24 volatile chemicals (excluding the natural extracts) belonging to different classes of compounds with different polarities: alcohols (amylcinnamyl alcohol, anisyl alcohol, benzyl alcohol, cinnamyl alcohol, citronellol, farnesol, geraniol, linalool), carbonyl compounds (amyl cinnamal, cinnamal, citral, hydroxy-citronellal, hexyl cinnamal, alpha-isomethyl ionone, Lilial®, Lyrall®), esters and lactones (benzyl benzoate, benzyl cinnamate, benzyl salicylate, coumarin and methylheptin carbonate), cyclic hydrocarbons (limonene) and phenols (eugenol and isoeugenol).

In this study a reversed-phase liquid chromatographic method with gradient elution and UV detection was developed to simultaneously identify and quantify all the 24 fragrance allergens mentioned. The intrinsic selectivity of the HPLC method was enhanced by the use of a diode array detector which provided

UV spectra for each chromatographic peak, allowed to optimize wavelength of detection, to set spectral references during method development and to confirm peak identity and peak purity. Quantification was carried out by using the internal standard method (*p*-anisaldehyde as the standard).

The method developed was then applied to the detection of the potential allergens in four representative commercially available scented products: a mixture of essential oils (insect repellent for topical use), a “natural” massage oil containing essential oils, an all-purpose moisturizing cream (“leave-on” cosmetic product) and a hair conditioner (“rinse-off” cosmetic product).

2. Experimental

2.1. Reagents and materials

Acetonitrile (MeCN) gradient grade for HPLC analysis was obtained from MERCK (Darmstadt, Germany). Allergen standards were obtained from Accustandard Inc. (New Haven, USA). Internal standard (*p*-anisaldehyde) was supplied by

Aldrich Chimica (Milano, Italy). Deionized water was distilled and filtered by cellulose filters (0.45 μm pore size: Seitz-Filter-Werke, Germany). Millex-GN Millipore (0.2 μm pore size) nylon membrane filters were supplied by Millipore, (Bedford, USA). The commercial scented products were purchased from retail stores.

2.2. Apparatus and chromatographic conditions

Chromatographic experiments were performed with a HPLC system Hewlett-Packard HP1100 (Palo Alto, CA) consisting of a quaternary pump, continuous vacuum degasser, equipped with a Rheodyne 7125 manual sample injector (20 μl injection volume) and a Hewlett-Packard HP UV–vis diode array detector (DAD). A HP ChemStation data system was used for chromatographic acquisition and handling.

Chromatographic separations were achieved by a LiChro-CART Purospher Star RP18-e column (250 mm $\times$ 4.6 mm i.d.) (5 μm) (Merck, Darmstadt, Germany) combined with a Merck LiChroCART 4-4 LiChrospher 100 RP18 (5 μm) guard column.

A gradient elution was carried out with a mobile phase of acetonitrile (MeCN) and water (H_2O). The best chromatographic assays were performed at room temperature at the following conditions:

Time (min)	Flow (ml/min)	MeCN (%)	H_2O (%)
0	0.7	50	50
5	0.7	50	50
15	1.0	60	40
24	1.0	60	40
40	1.0	90	10

The diode array detector was scanned from 190 to 500 nm, and the chromatographic acquisitions were set at three different wavelengths (210, 254 and 280 nm), close to the λ max of all the allergens studied, using the multisignal capability of DAD. Identification was carried out by comparing the retention times (Rt) and the corresponding UV absorbance spectra with those of the single reference standards.

2.3. Stock and working standard solutions

Individual stock solutions of the 24 selected allergens and of the internal standard (Table 2) were prepared by dissolving in acetonitrile each standard (accurately weighed) in volumetric flasks at concentrations of about 10 mg/ml, except for hydroxycitronellal (100 mg/ml). The solutions were kept at 4 $^\circ\text{C}$ and under these conditions they were stable for at least 7 days. Their stability was checked by HPLC verifying if single peak of constant area were present and if purity factors agreed with the value obtained for the solution analyzed just prepared.

Fresh working solutions, prepared daily by appropriate dilutions of the stock ones, were processed individually and in mixture to optimize the operative conditions, to record spectral data and to obtain the calibration curves.

The standard mixture solution of all the tested allergens and internal standard was prepared by diluting a known volume

Table 2

Retention times (Rt) $\pm$ standard deviation (S.D.) related to the mixture of 24 fragrance allergens and internal standard (*p*-anisaldehyde)

Compound	Peak (Fig. 1)	Rt $\pm$ S.D. ^a
Hydroxy-citronellal	1	4.28 $\pm$ 0.02
Anisyl alcohol	2	4.56 $\pm$ 0.02
Benzyl alcohol	3	4.70 $\pm$ 0.07
Cinnamyl alcohol	4	6.43 $\pm$ 0.04
Coumarin	5	6.77 $\pm$ 0.05
Cinnamal	6	9.37 $\pm$ 0.08
Lyrat [®]	7	9.84 $\pm$ 0.07
Eugenol	8	10.44 $\pm$ 0.08
Isoeugenol	9	11.06 $\pm$ 0.09
Geraniol	10	13.29 $\pm$ 0.08
Linalool	11	14.15 $\pm$ 0.08
Citronellol	12	15.75 $\pm$ 0.08
Citral	13	16.47 $\pm$ 0.06
Methylheptin carbonate	14	19.45 $\pm$ 0.08
Amylcinnamyl alcohol	15	21.38 $\pm$ 0.10
Benzyl benzoate	16	22.73 $\pm$ 0.10
Benzyl salicylate	17	26.28 $\pm$ 0.12
Benzyl cinnamate	18	27.46 $\pm$ 0.12
Lilial [®]	19	29.82 $\pm$ 0.08
Farnesol	20	31.26 $\pm$ 0.07
Alpha-isomethyl ionone	21	32.20 $\pm$ 0.08
Amyl cinnamal	22	32.58 $\pm$ 0.06
Hexyl cinnamaldehyde	23	36.90 $\pm$ 0.09
Limonene	24	38.17 $\pm$ 0.02
<i>p</i> -Anisaldehyde	Internal standard	7.62 $\pm$ 0.07

^a Mean of five analytical results.

(from 10 to 50 μl) of each stock solution in volumetric flasks with acetonitrile.

2.4. Calibration curves

Quantitative assays were performed by means of the internal standard procedure. The calibration graphs were constructed from triplicate injections of five solutions at different concentrations for each standard, by plotting the analyte to internal standard peak-area ratios versus the concentration of the compound of interest.

2.5. Commercial scented products

Four representative commercially available products (A–D) were assessed to examine the applicability of the proposed method.

Their composition is reported as labelled. Concerning samples C and D, ingredients are listed according to the INCI (International Nomenclature Cosmetic Ingredients) nomenclature.

- Insect repellent for topical use composed by a mixture of essential oils from *Lavandula angustifolia*, *Cymbopogon nardus*, *Geranium*, *Mentha piperita*.
- “Natural” massage oil containing essential oils from *Rosmarinus officinalis*, *Salvia officinalis*, *Geranium*, *Mentha piperita*, in a hazelnut oil vehicle.

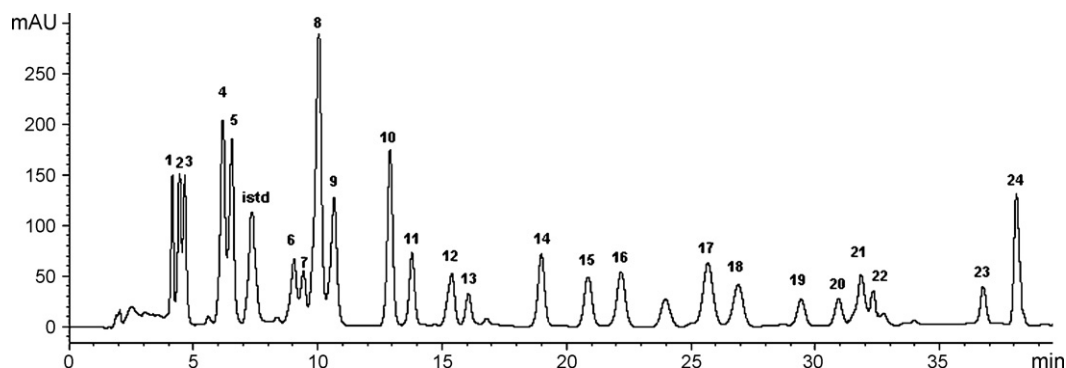


Fig. 1. Typical chromatographic acquisition of the standard mixture of 24 allergens and internal standard at 210 nm. Peak label legend is reported in Table 2.

C. All-purpose moisturizing cream (O/W emulsion) containing the following ingredients: Aqua, paraffinum liquidum, myristyl alcohol, glycerin, butylene glycol, alcohol denat., stearic acid, myristyl myristate, cera microcristallina, glyceryl stearate, hydrogenate coco-glycerides, dimethicone, *Simmondsia chinensis*, tocopheryl acetate, polyglyceryl-2 caprate, sodium carbomer, phenoxyethanol, lanolin alcohol, methyl paraben, butyl paraben, ethyl paraben, isobutyl paraben, propyl paraben, parfum, linalool, citronellol, alpha-isomethyl ionone, butylphenyl methylpropional, limonene, benzyl salicylate.

D. Hair conditioner (O/W emulsion) containing the following ingredients: Aqua, cetearyl alcohol, dimethiconol, cetrimonium chloride, paraffin, glyceryl stearate, parfum, amyl cinnamal, butylphenyl methylpropional, geraniol, hexyl cinnamal, limonene, linalool, citric acid, TEA-dodecylbenzenesulphonate, hydrolyzed keratin, phenoxyethanol, sodium hydroxide.

2.5.1. Sample preparation

2.5.1.1. *Samples A and B.* An accurately weighted amount of each product (about 100 mg for A and 400 mg for B) was added with an appropriate volume of internal standard stock solution (100 μ l), diluted in a 10 ml volumetric flask with acetonitrile and directly injected into the HPLC.

2.5.1.2. *Samples C and D.* An accurately weighted amount of each product (about 1.0 g) was treated with 6 ml of acetonitrile under ultrasonication for 15 min. The suspension was filtered

through a 0.2 μ m nylon membrane, added of an appropriate volume of internal standard stock solution (100 μ l) and diluted to 10 ml with acetonitrile in a volumetric flask. The resulting sample was then subjected to HPLC analysis.

2.5.2. Sample analysis

All commercial samples treated according to the procedures described in Section 2.5.1 were analyzed in triplicate under the best chromatographic conditions. The concentration of each detected allergen was determined from the corresponding calibration curves. R.S.D. of the results was used as a measurement of the reproducibility of the method.

2.6. Validation of the method

The analytical method developed was tested as regards linearity, detection limits, accuracy and precision.

The linearity of the HPLC method was evaluated during preparation of calibration curves, by analyzing in triplicate five concentrations of each fragrance allergen, ranging from 0.1 to 500 μ g/ml for all compounds except for hydroxy-citronellal (from 20 to 5000 μ g/ml). Linear regression analyses were performed by the internal standard method. Linearity for each compound was established by plotting the peak areas ratio (standard to internal standard) versus standard concentrations. Appropriate dilutions of working standard solutions were analyzed to obtain the detection limits (LOD). LODs were evaluated on the basis of a signal-to-noise ratio of 3 ($S/N = 3$).

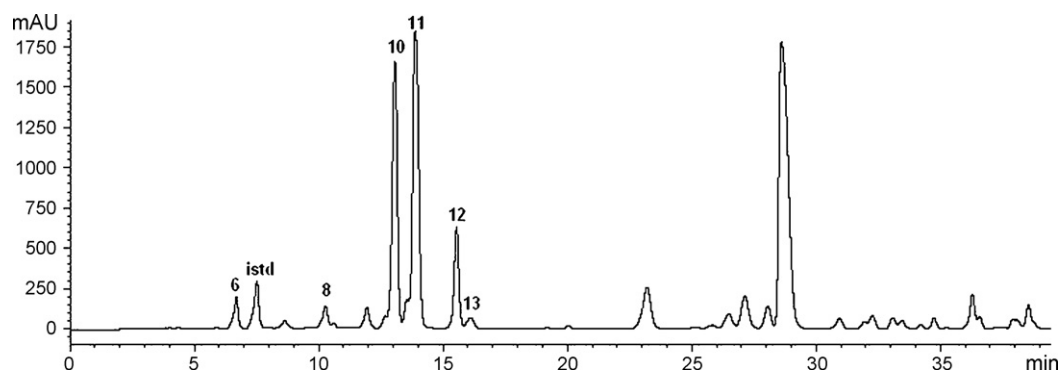


Fig. 2. Typical chromatographic acquisition of sample A (insect repellent) at 210 nm. Peak label legend is reported in Table 3.

To assess accuracy and precision a standard mixture solution in acetonitrile, containing known amounts of all the 24 allergens and internal standard prepared as described in Section 2.3, was processed under the best chromatographic conditions. The solution was analyzed five times within a day to determine the intra-day variability and in triplicate on three different days to value inter-day precision and accuracy.

The accuracy was further assessed by spiking the analytical sample B with known amounts of each of the 24 standards and internal standard before diluting to volume (Section 2.5.1) and the resultant solution was analyzed in triplicate. The total concentration of each analyte was determined from the corresponding calibration curve and accuracy of the measurement was calculated by the following equation:

$$\text{accuracy(\%)} = \left\{ \frac{-(C_{\text{spiked}} - (C_{\text{total}} - C_{\text{original}}))}{C_{\text{spiked}}} \right\} \times 100$$

where C_{total} is the determined total concentration, C_{original} the concentration in the original sample measured in the above described experiment and C_{spiked} is the spiked concentration.

3. Results and discussion

3.1. Chromatography and detection

Reversed-phase chromatographic conditions were found suitable to modulate the retention of all the selected compounds. Due to the different polarity of the analytes a gradient elution (MeCN/H₂O) was adopted; *p*-anisaldehyde was chosen as internal standard (ISTD) because no interference was obtained at the same *R*_t and UV spectra showed absorption bands close to the maximal UV absorbance of all the compounds of interest. Under the chromatographic conditions described above, the 24 analytes were appropriately separated over a running time of 40 min. In Table 2 the retention times (*R*_t) related to all the 24 allergens and ISTD are reported. Values represent the mean of five analytical results $\pm$ S.D. As an example, the typical chromatographic acquisition at 210 nm of the standard mixture containing all the 24 allergens and internal standard is reported in Fig. 1. No critical pair was found all chromatogram long.

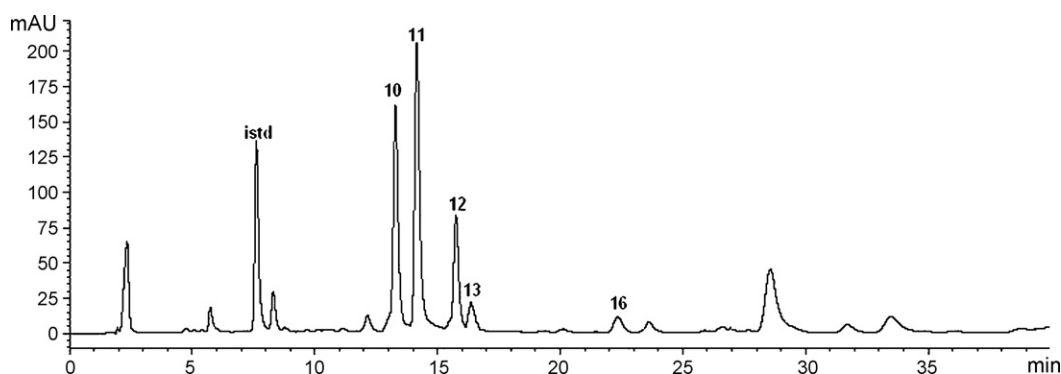


Fig. 3. Typical chromatographic acquisition of sample B (massage oil) at 210 nm. Peak label legend is reported in Table 4.

Table 3

Quantitative assay in commercial scented products: sample A—insect repellent (Fig. 2)

Compound	Peak	Content (%) $\pm$ S.D. ^a	R.S.D. (%)
Coumarin	6	$0.27 \pm 0.14 \times 10^{-2}$	0.52
Eugenol	8	$0.11 \pm 0.21 \times 10^{-2}$	1.91
Geraniol	10	$1.26 \pm 0.14 \times 10^{-2}$	0.11
Linalool	11	$8.80 \pm 2.80 \times 10^{-2}$	0.32
Citronellol	12	$2.45 \pm 1.40 \times 10^{-2}$	0.57
Citral	13	$0.30 \pm 0.10 \times 10^{-2}$	0.33

^a Mean of five analytical results.

Table 4

Quantitative assay in commercial scented products: sample B—massage oil (Fig. 3)

Compound	Peak	Content % ^a $\pm$ S.D. ^a	R.S.D. (%)
Geraniol	10	$0.11 \pm 0.71 \times 10^{-2}$	6.37
Linalool	11	$0.73 \pm 4.20 \times 10^{-2}$	5.75
Citronellol	12	$0.26 \pm 0.70 \times 10^{-2}$	2.69
Citral	13	$1.56 \times 10^{-2} \pm 0.60 \times 10^{-2}$	3.84
Benzyl benzoate	16	$2.10 \times 10^{-3} \pm 1.41 \times 10^{-4}$	6.71

^a Mean of five analytical results.

3.2. Quantitative analysis of commercial products

The study of peak identity and purity showed that any other ingredient present in these samples did not interfere with the attribution and quantification of the potential detected allergens. In sample A (insect repellent for topical use) six potential allergens were detected (coumarin, eugenol, geraniol, linalool, citronellol, citral—peaks 6, 8, 10, 11, 12, 13 respectively) at percentages in a range from 0.27% to 8.80% (Fig. 2, Table 3). In sample B (“natural” massage oil) five potential allergens were found (geraniol, linalool, citronellol, citral, benzyl benzoate—peaks 10, 11, 12, 13, 16, respectively) at percentages in a range from $2.10 \times 10^{-3}\%$ to 0.73% (Fig. 3, Table 4). As regards sample C (“leave on” cosmetic product—all-purpose moisturizing cream) the seven allergens reported on the product label (linalool, citronellol, benzyl salicylate, Lilial®, alpha-isomethyl ionone, limonene—peaks 11, 12, 17, 19, 21, 24 respectively) were all detected at percentages ranging from $9.59 \times 10^{-3}\%$ to $8.50 \times 10^{-2}\%$ (Fig. 4, Table 5).

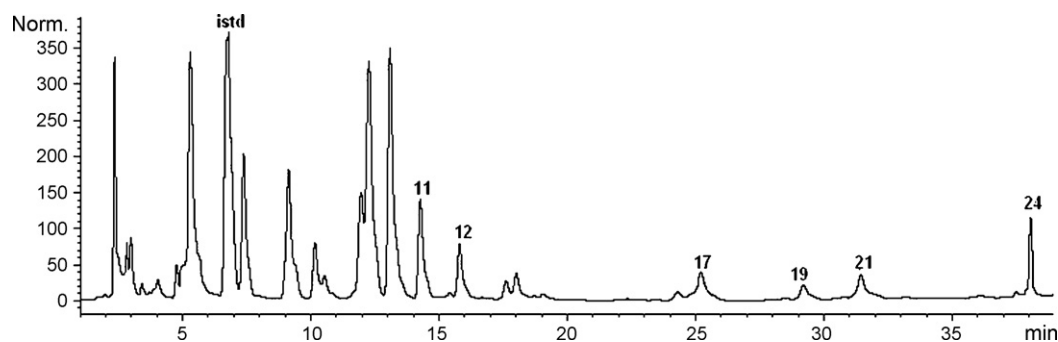


Fig. 4. Typical chromatographic acquisition of sample C (all-purpose moisturizing cream) at 210 nm. Peak label legend is reported in Table 5.

Table 5

Quantitative assay in commercial scented products: sample C—all-purpose moisturizing cream (Fig. 4)

Compound	Peak	Content (%) $\pm$ S.D. ^a	R.S.D. (%)
Linalool	11	$5.55 \times 10^{-2} \pm 1.81 \times 10^{-3}$	3.26
Citronellol	12	$8.50 \times 10^{-2} \pm 0.61 \times 10^{-3}$	0.71
Benzyl salicylate	17	$1.12 \times 10^{-2} \pm 0.12 \times 10^{-3}$	1.03
Lilial®	19	$9.59 \times 10^{-3} \pm 0.32 \times 10^{-3}$	3.33
Alpha-isomethyl ionone	21	$1.99 \times 10^{-2} \pm 0.25 \times 10^{-3}$	1.26
Limonene	24	$3.27 \times 10^{-2} \pm 0.40 \times 10^{-3}$	1.23

^a Mean of five analytical results.

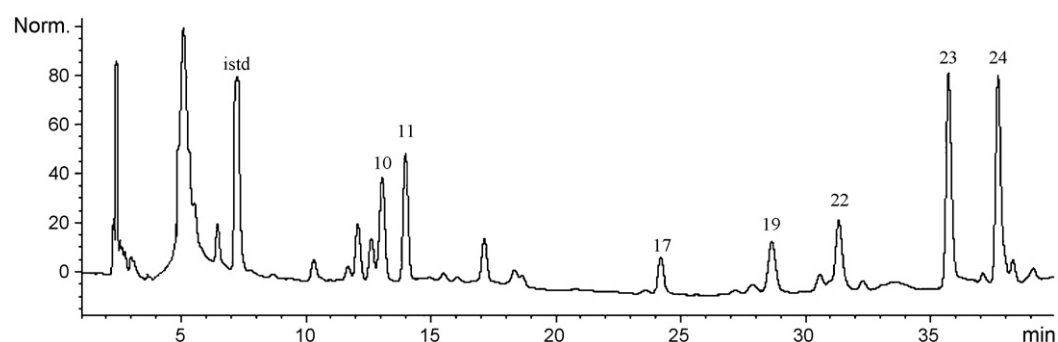


Fig. 5. Typical chromatographic acquisition of sample D (hair conditioner) at 210 nm. Peak label legend is reported in Table 6.

In sample D (“rinse-off” cosmetic product—hair conditioner) all the allergens labelled (geraniol, linalool, benzyl salicylate, Lilial®, amyl cinnamal, hexyl cinnamal, limonene—peaks 10, 11, 19, 22, 23, 24 respectively) were found at percentages in a range from $1.08 \times 10^{-2}\%$ to $3.19 \times 10^{-2}\%$. Moreover benzyl salicylate (not declared) was detected (peak 17, $4.36 \times 10^{-3}\%$) but, according to the cosmetic directive, this amount allows the allergen to be omitted from the label (Fig. 5, Table 6).

3.3. Method validation

3.3.1. Linearity and detection limit

All compounds displayed a good linearity ($r^2 > 0.99$) in a relative wide range concentrations. The results of regression analysis on calibration curves are reported in Table 7.

LODs ranged from 0.01 to 0.74 $\mu\text{g/ml}$ for all compounds except for three synthetic compounds: Lyrall® (1.89 $\mu\text{g/ml}$), Lilial® (1.68 $\mu\text{g/ml}$) and hydroxy-citronellal (10.88 $\mu\text{g/ml}$). All data are reported in Table 7.

3.3.2. Precision and accuracy

In Table 8 the results related to intra-day and inter-day variability obtained from the assay of the standard mixture, are reported. The intra-day precision expressed as R.S.D.% ranged from 0.6% (eugenol) to 3.5% (geraniol) with an accuracy ranging from 90.9% to 104.6%.

Table 6

Quantitative assay in commercial scented products: sample D—hair conditioner (Fig. 5)

Compound	Peak	Content (%) $\pm$ S.D. ^a	R.S.D. (%)
Geraniol	10	$1.08 \times 10^{-2} \pm 0.70 \times 10^{-3}$	6.48
Linalool	11	$3.74 \times 10^{-2} \pm 0.62 \times 10^{-3}$	1.66
Benzyl salicylate	17	$4.36 \times 10^{-3} \pm 0.27 \times 10^{-3}$	6.21
Lilial®	19	$1.08 \times 10^{-2} \pm 0.11 \times 10^{-3}$	1.01
Amyl cinnamal	22	$6.19 \times 10^{-2} \pm 0.76 \times 10^{-3}$	1.22
Hexyl cinnamal	23	$3.56 \times 10^{-2} \pm 0.76 \times 10^{-3}$	2.13
Limonene	24	$5.66 \times 10^{-2} \pm 0.85 \times 10^{-3}$	1.50

^a Mean of five analytical results.

Table 7
Linear regression data and LOD related to the 24 fragrance allergens

Compound	Slope	Intercept	Correlation coefficient (r^2)	Linear range ($\mu\text{g/ml}$)	LOD ^a ($\mu\text{g/ml}$)
Hydroxy-citronellal	0.1626	−0.0077	0.998	20–5000	10.88
Anisyl alcohol	0.8681	−0.5257	0.993	0.5–500	0.13
Benzyl alcohol	0.7708	−0.2192	0.992	0.5–500	0.09
Cinnamyl alcohol	0.7180	−0.3388	0.999	0.5–500	0.05
Coumarin	2.8218	−0.8133	0.999	0.5–500	0.07
Cinnamal	0.4198	−0.1506	0.999	0.5–500	0.06
Lyr ^{al} ®	0.5474	−0.0080	0.996	5.0–500	1.89
Eugenol	1.6236	0.5663	0.996	0.5–500	0.11
Isoeugenol	2.8689	0.0735	0.994	0.5–500	0.10
Geraniol	0.9126	0.0345	0.993	0.5–500	0.21
Linalool	0.4025	0.0006	0.995	1.5–500	0.68
Citronellol	0.2568	−0.0292	0.998	1.5–500	0.71
Citral	3.8057	0.9984	0.999	0.5–500	0.07
Methylheptin carbonate	0.3813	0.0121	0.999	1.0–500	0.37
Amylcinnamyl alcohol	1.3025	−0.1576	0.995	1.0–500	0.22
Benzyl benzoate	1.3924	−0.1811	0.998	1.0–500	0.34
Benzyl salicylate	2.0883	0.0156	0.990	0.5–500	0.17
Benzyl cinnamate	0.6859	1.8555	0.999	0.5–500	0.25
Lilial®	0.3127	−0.0039	0.993	5.0–500	1.68
Farnesol	0.7006	−0.1934	0.999	1.5–500	0.74
Alpha-isomethyl ionone	4.2705	0.0117	0.994	0.5–500	0.12
Amyl cinnamal	0.7778	−0.0081	0.998	0.1–500	0.02
Hexyl cinnamaldehyde	0.7500	−0.0162	0.997	0.1–500	0.03
Limonene	0.6927	−0.1959	0.997	1.5–500	0.48

^a Signal/noise = 3.Table 8
Quantitative analysis of 24 fragrance allergens mixture in acetonitrile

Peak	Compound	Conc. added (mg/ml)	Intra-day precision ^a			Inter-day precision ^b		
			Conc. found (mg/ml)	Accuracy (%)	R.S.D. (%)	Amount found (mg/ml)	Accuracy (%)	R.S.D. (%)
1	Hydroxy-citronellal	0.100	0.0909	90.9	1.7	0.0928	92.8	1.9
2	Anisyl alcohol	0.020	0.0184	92.0	2.5	0.0180	90.0	2.0
3	Benzyl alcohol	0.015	0.0144	96.0	2.1	0.0148	98.6	2.4
4	Cinnamyl alcohol	0.060	0.0610	101.6	1.0	0.0612	102.0	1.0
5	Coumarin	0.030	0.0282	94.0	1.8	0.0295	98.3	3.1
6	Cinnamal	0.015	0.1490	99.3	1.5	0.1478	98.5	1.5
7	Lyr ^{al} ®	0.015	0.0147	98.0	1.2	0.0150	100.0	1.5
8	Eugenol	0.050	0.0489	97.8	0.6	0.0487	97.4	1.1
9	Isoeugenol	0.050	0.0467	94.0	1.7	0.0482	96.4	1.9
10	Geraniol	0.020	0.0190	95.0	3.5	0.0192	96.0	3.0
11	Linalool	0.020	0.0187	93.5	0.9	0.0187	93.5	1.6
12	Citronellol	0.040	0.0418	104.5	0.9	0.0420	105.0	0.9
13	Citral	0.030	0.0314	104.6	2.9	0.0311	103.6	2.7
14	Methylheptin carbonate	0.040	0.0388	97.0	2.0	0.0391	97.7	2.2
15	Amylcinnamyl alcohol	0.015	0.0151	100.7	0.8	0.0149	99.3	1.5
16	Benzyl benzoate	0.015	0.0138	92.0	2.9	0.0142	94.7	3.3
17	Benzyl salicylate	0.020	0.0185	92.5	0.8	0.0193	96.5	1.2
18	Benzyl cinnamate	0.040	0.0386	96.5	1.0	0.0385	96.2	1.3
19	Lilial®	0.030	0.0313	104.3	1.4	0.0316	105.3	1.4
20	Farnesol	0.020	0.0204	102.0	0.8	0.0202	101.0	0.9
21	Alpha-isomethyl ionone	0.015	0.0150	100.0	2.0	0.0155	103.3	1.9
22	Amyl cinnamal	0.020	0.0198	99.0	1.4	0.0188	94.0	1.3
23	Hexyl cinnamaldehyde	0.020	0.0189	94.5	0.8	0.0191	95.5	1.2
24	Limonene	0.040	0.0399	99.7	1.0	0.0375	93.7	1.4

^a Mean of five analytical results.^b Mean of three analytical results.

Table 9

Quantitative results obtained spiking sample B with known amounts of the 24 allergens

Compound	Original amount (%)	Original conc. (mg/ml)	Conc. spiked (mg/ml)	Total conc. (mg/ml)	Conc. found (mg/ml) $\pm$ S.D. ^a	R.S.D.	Accuracy (%)
Hydroxy-citronellal			0.100	0.100	$9.830 \times 10^{-2} \pm 1.45 \times 10^{-3}$	1.48	98.3
Anisyl alcohol			0.020	0.020	$1.820 \times 10^{-2} \pm 5.33 \times 10^{-4}$	2.93	91.0
Benzyl alcohol			0.015	0.015	$1.497 \times 10^{-2} \pm 3.77 \times 10^{-4}$	2.52	99.8
Cinnamyl alcohol			0.060	0.060	$6.006 \times 10^{-2} \pm 4.14 \times 10^{-4}$	0.69	100.1
Coumarin			0.030	0.030	$2.931 \times 10^{-2} \pm 5.50 \times 10^{-3}$	1.88	97.7
Cinnamal			0.015	0.015	$1.495 \times 10^{-2} \pm 2.63 \times 10^{-4}$	1.76	99.7
Lylal [®]			0.015	0.015	$1.492 \times 10^{-2} \pm 2.09 \times 10^{-4}$	1.40	99.5
Eugenol			0.050	0.050	$4.495 \times 10^{-2} \pm 3.64 \times 10^{-4}$	0.81	89.9
Isoeugenol			0.050	0.050	$4.920 \times 10^{-2} \pm 0.95 \times 10^{-3}$	1.90	98.4
Geraniol	0.110	0.044	0.020	0.064	$1.896 \times 10^{-2} \pm 8.72 \times 10^{-4}$	4.60	94.8
Linalool	0.730	0.292	0.020	0.312	$1.908 \times 10^{-2} \pm 1.76 \times 10^{-4}$	0.89	95.4
Citronellol	0.260	0.104	0.040	0.144	$4.184 \times 10^{-2} \pm 4.22 \times 10^{-4}$	1.01	104.6
Citral	1.560×10^{-2}	6.240×10^{-3}	0.030	3.624×10^{-2}	$3.006 \times 10^{-2} \pm 1.06 \times 10^{-3}$	3.54	100.2
Methylheptin carbonate			0.040	0.040	$3.984 \times 10^{-2} \pm 1.07 \times 10^{-3}$	2.70	99.6
Amylcinnamyl alcohol			0.015	0.015	$1.498 \times 10^{-2} \pm 1.44 \times 10^{-4}$	0.96	99.9
Benzyl benzoate	2.100×10^{-3}	0.840×10^{-3}	0.015	1.584×10^{-2}	$1.411 \times 10^{-2} \pm 5.71 \times 10^{-4}$	4.05	94.1
Benzyl salicylate			0.020	0.020	$0.020 \pm 1.70 \times 10^{-4}$	0.85	100.0
Benzyl cinnamate			0.040	0.040	$3.992 \times 10^{-2} \pm 4.43 \times 10^{-4}$	1.11	99.8
Lilial [®]			0.030	0.030	$3.078 \times 10^{-2} \pm 5.11 \times 10^{-4}$	1.66	102.6
Farnesol			0.020	0.020	$2.028 \times 10^{-2} \pm 1.91 \times 10^{-4}$	0.94	101.4
Alpha-isomethyl ionone			0.015	0.015	$1.515 \times 10^{-2} \pm 4.01 \times 10^{-4}$	2.66	101.0
Amyl cinnamal			0.020	0.020	$1.960 \times 10^{-2} \pm 3.10 \times 10^{-4}$	1.58	98.0
Hexyl cinnamaldehyde			0.020	0.020	$1.866 \times 10^{-2} \pm 1.68 \times 10^{-4}$	0.99	93.3
Limonene			0.040	0.040	$3.996 \times 10^{-2} \pm 4.23 \times 10^{-4}$	1.06	99.9

^a Mean of five determinations.

The inter-day precision and accuracy were determined by analyzing in triplicate a standard mixture at the same concentrations on three consecutive days; accuracy ranged from 90.0% and 105.3% with a R.S.D.% from 0.9% to 3.5%.

The overall good precision and accuracy of the analytical method was also confirmed by the study on a spiked real sample (B) (Table 9) with an accuracy ranging from 89.9% to 102.6% and R.S.D. values from 0.85% and 4.60%.

4. Conclusion

The procedure described herein allowed for an efficient simultaneous chromatographic separation and quantitative analysis of 24 fragrance allergens using a conventional HPLC–DAD, widespread available equipment in analytical laboratories.

Even if GC–MS can be considered the chromatographic technique of choice (in the perfumery industry) for the analysis of volatile chemicals such as perfumes or fragrance mixtures, using this simple HPLC procedure it seems possible to efficiently analyze also complex matrices, identifying the potential allergenic fragrances in the presence of other non-volatile ingredients. Thus this method could overcome some problems related to sample preparation, critical point of the analytical procedure, in terms of analyte loss or degradation. Moreover the multisignal capability

of the DAD detector can optimize selectivity in both identification and quantification of the analytes, in the presence of co-eluting peaks.

The results obtained from the analysis of the commercial products seem to indicate that the method developed can be considered a simple, fast, economic and reliable tool useful in routine analyses of complex matrices such as cosmetics.

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