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Quantitative Analysis of the 26 Allergens for Cosmetic Labeling in Fragrance Raw Materials and Perfume Oils

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The adoption of the 7th amendment of the European Cosmetic Directive 76/768/EEC requires any cosmetic product containing any of 26 raw materials identified by the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers as likely to cause a contact allergy when present above certain trigger levels to be declared on the package label. Of these 26, 24 are volatile and can be analyzed by GC. This paper describes a method for the quantitative analysis of these volatile raw materials in perfume ingredients as well as complex perfume compositions. The method uses sequential dual-column GC-MS analysis. The full-scan data acquired minimize the false-positive and false-negative identifications that can be observed with alternate methods based on data acquired in the SIM mode. For each sample, allergen levels are determined on both columns sequentially, leading to two numerical results for each allergen. Quantification limits for each allergen in a perfume mixture based on the analysis of a standard are <4 mg/kg. This is well below the level that would trigger label declaration on the consumer good. Calibration curves for all allergens are linear (r > 0.999) and stable for multiple days. Studies on perfumes spiked with multiple allergens at 30, 50, and 70 mg/kg show recoveries close to nominal values.

KEYWORDS: Perfumes; fragrances; allergens; analytical method

INTRODUCTION

In 2003 Directive 2003/15/EC, the 7th amendment of the European Cosmetic Directive 76/768/EEC, was published (*1*). This directive requires that any cosmetic product containing any of 26 raw materials above certain trigger levels must declare these ingredients on the label in descending order of weight. An analytical method for markers in oak moss is reported elsewhere (*7*). Therefore, it was decided not to include tree moss and oak moss in the development of this method. Labeling, using International Nomenclature Cosmetic Ingredient (INCI) names, is required when the level of the individual ingredient exceeds 10 mg/kg in a product intended to remain on the skin or 100 mg/kg in a product to be rinsed off of the skin.

As a result of this directive, it became necessary to develop analytical methods enabling identification and quantification of low levels of these ingredients in the presence of highly complex mixtures, such as fragrances and their raw materials. As with all methods, situations can occur in which the results are negatively influenced by matrix effects. These include coeluting components or closely eluting components. In such situations, false positives, false negatives, or incorrect quantification may result. In response to these issues, we have developed a method in our laboratory that has clear advantages in terms of preventing false positives and false negatives as well as minimizing inaccurate quantification resulting from coelution or other chromatographic disturbances. This paper describes a methodology that we have developed and are now using regularly in our quality control laboratories.

MATERIALS AND METHODS

GC-MS Analysis. GC-MS analysis was carried out on a Shimadzu QP2010 mass spectrometer coupled to a Shimadzu GC-2010 gas chromatograph equipped with a CTC autosampler. The system was equipped with two split/splitless injectors. Each injector was connected to a column of different polarity. Both columns are connected to the MS interface using a dual-hole ferrule. Details of the columns and conditions used are shown in **Table 1**.

Standards Preparation. Each of the 24 ingredients in this study are regularly used fragrance ingredients and are shown in **Table 2**. The purity of each ingredient was verified prior to use. Each was above 95.0% purity as determined by GC-FID area percent measurements. For purposes of this study, the quantities of cis and trans isomers were added together. Exceptions were 3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, which was 89.7% pure, and hydroxymethylpentyl-cyclohexenecarboxaldehyde, which consisted of 72% of the 4-isomer and 27% of the 3-isomer. A special note should be made on *d*-limonene. The EC directive (*1*) refers to *d*-limonene (CAS Registry No. 5989-27-5). The method described in this paper cannot distinguish between *d*-limonene and *l*-limonene. For reasons of consistency reference will be made to *d*-limonene throughout the paper.

Standards were stored as individual chemicals in a freezer (−25 °C) for a maximum of 3 months. Each month, a mixture (mixture A) was prepared from these pure ingredients (~1 g of each ingredient) and stored in a freezer (−25 °C). A calibration stock solution was made

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Table 1. Materials, Equipment, and Settings Used for Allergen Analysis

GC-MS system	Shimadzu QP2010 connected to a Shimadzu GC-2010 equipped with a CTC autosampler
GC column	Varian CpSil 5 CB 50 m × 0.25 mm × 0.25 μm or SGE Solgel1 60 m × 0.25 mm × 0.25 μm (both bonded polydimethylsiloxane)
	Varian CpWax 52 CB 50m × 0.25 mm × 0.20 μm
internal standards	2,3-dichlorotoluene, CAS [32768-54-0]
	1,4-dibromobenzene, CAS [106-37-6]
solvent	acetone, GC grade, CAS [67-64-1]
injection volume	1 μL
injection temperature	250 °C
mode	split, 1:10
column pressure	170 kPa, constant pressure, both columns
carrier gas	helium
temperature program	column 1, 50 °C, 1 min; ramped at 12 °C/min to 250 °C, 11 min; cooled at −40 °C/min to 120 °C, 3 min
	column 2, 120 °C, 3 min, ramped at 4 °C/min to 216 °C, 0 min; ramped at 10 °C/min to 250 °C, 13 min
interface temperature	250 °C
source temperature	200 °C
MS parameters	full scan, *m*/*z* 30−372, scan speed of 2000 amu/s
calibration	eight levels from 2 to 60 mg/kg

Table 2. Fragrance Ingredients and Their CAS Registry Numbers[a]

name	CAS Registry No.
amylcinnamyl alcohol	101-85-9
amyl cinnamal	122-40-7
anisyl alcohol	105-13-5
benzyl alcohol	100-51-6
benzyl benzoate	120-51-4
benzyl cinnamate	103-41-3
benzyl salicylate	118-58-1
cinnamyl alcohol	104-54-1
cinnamal	104-55-2
citral (mixture of neral and geranial)	5392-40-5
citronellol	106-22-9
coumarin	91-64-5
eugenol	97-53-0
farnesol (main isomers, *ZE* and *EE*)	4602-84-0
geraniol	106-24-1
hexyl cinnamic aldehyde	101-86-0
hydroxy-citronellal	107-75-5
isoeugenol	97-54-1
2-(4-*tert*-butylbenzyl) propionaldehyde	80-54-6
d-limonene	5989-27-5
linalool	78-70-6
hydroxy-methylpentyl-cyclohexenecarboxaldehyde	31906-04-4
methyl heptin carbonate	111-12-6
3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)--buten-2-one	127-51-5

[a] CAS Registry No. provided by the author.

Table 3. Quantifier and Qualifier Ions for Allergen Analysis

name	quantifier; qualifier ions (CpSil 5 CB or Solgel 1 or CpWax 52 CB)
amylcinnamyl alcohol	91; 115, 133, 204
amyl cinnamal	145; 115, 129, 202
anisyl alcohol	138; 109, 121, 137
benzyl alcohol	108; 79, 107
benzyl benzoate	105; 91, 212
benzyl cinnamate	131; 91, 192
benzyl salicylate	91; 65, 228
cinnamyl alcohol	134; 92, 105, 115
cinnamal	131; 77, 103, 132
citral	
neral	69; 84, 94, 109
geranial	69; 84, 94, 109
citronellol	81; 95, 123
coumarin	118; 89, 90, 146
eugenol	164; 103, 149
farnesol	
ZE isomer	69; 81, 93
EE isomer	93; 69, 81
geraniol	93; 69, 123
hexylcinnamic aldehyde	145; 117, 129
hydroxycitronellal	59; 71, 81, 96
isoeugenol	164; 103, 149
2-(4-*tert*-butylbenzyl) propionaldehyde	189; 117, 131, 147
d-limonene	68; 67, 93, 121
linalool	71; 80, 93, 121
hydroxy-methylpentyl-cyclohexene-carboxaldehyde	136; 93, 149, 192
methyl heptin carbonate	123; 67, 79, 95
3-methyl-4-(2,6,6-trimethyl-2-cyclo-hexen-1-yl)-3-buten-2-one	150; 107, 135
2,3-dichlorotoluene (IS)	125; 127, 160, 162
1,4-dibromobenzene (IS)	236; 234, 238

weekly from mixture A by diluting 0.1 g of mixture A to 20 g with acetone (∼208 mg/kg each ingredient).

The internal standard solution was prepared bimonthly by diluting 2,3-dichlorotoluene and 1,4-dibromobenzene in toluene (∼1000−1500 mg/L). Each calibration standard was prepared by mixing the appropriate amount (weight) of the stock solution with 100 μL of the internal standard solution. This mixture was then further diluted with acetone to 10 g. This way, calibration standards of approximately 2, 5, 10, 20, 30, 40, 50, and 60 mg/kg of each ingredient were prepared. For quantification, 2,3-dichlorotoluene was used. In those cases when this internal standard coelutes with an unknown containing the same ion used as the quantifier for 2,3-dichlorotoluene, 1,4-dibromobenzene may be used as an alternative.

Sample Preparation. Perfume samples were prepared by adding 100 μL of the internal standard solution to 0.5 g of the neat perfume oil. This mixture was diluted to 10 g with acetone. Raw material samples were prepared by adding 100 μL of the internal standard solution to 0.1 g of the neat raw material. This mixture was diluted to 10 g with acetone. The applied dilution of both perfume and raw material samples in acetone is done to diminish overload in the GC-MS system.

Analysis. Samples were analyzed sequentially on each column with full-scan mass spectral acquisition. The use of full-scan spectra allows for positive identification using a library search. Qualifier and quantifier ions were selected for each target compound as shown in **Table 3**. Response factors are calculated from the quantifier ion signal for the internal standard and target components. For quantification of target components, the above-determined response factor is applied on its quantifier ion signal. Prior to evaluation, all data are subjected to a Savitzky−Golay filter using 11 data points.

RESULTS AND DISCUSSION

Potential Chromatographic and Spectral Issues. A single-column GC run will separate most of the components of a

perfume, but in a substantial number of cases coelution will occur. For correct quantification and identification this does not have to be a problem as long as the GC detector can provide unique information for the components of interest. GC-MS electron impact (EI) generated spectra provide such additional information. However, even with this second dimension, it is often not possible to separate all components of interest. Coeluting components may give rise to mixed spectra.

The analytical method published by the International Fragrance Association (IFRA) (*2*), which is based on GC-MS, uses a combination of retention time and the ratio of selected ions acquired in the SIM mode for positive identification. Quantification is based on a single ion. The method uses multiple SIM windows with mostly three ions and a time width as small as 0.1 min. This method works well with perfume compositions for which no coelution occurs with a component having the same quantifier and qualifier ion of the target component and for which retention times are constant. Sometimes, however, these conditions are not met. For example, a large nonrelated peak that elutes in front of the SIM window for a potential allergen may cause the allergen to shift out of the SIM window, resulting in a false negative. The coelution of an ingredient with ions identical to those used for the identification and quantification of an allergen may also cause problems. First, the coelution may cause the ion ratios of quantifier and qualifier ions to be within set parameters, leading to a false-positive identification. Second, the ion ratio may remain within the parameters set for positive identification, but lead to incorrect quantification. Third, ratios of quantifier and qualifier ions may be outside set limits, leading to false-negative identification.

Multiple alternative approaches for overcoming the above-described situations exist. To prevent false negatives as a result of retention time shift, a wider SIM window with more ions can be used. However, when the equipment is running in SIM, if the component causing the retention time shift does not have any of the ions in the SIM window, the identification relies more heavily on the correct ion ratios because the retention times do not match. A full-scan analysis to verify the retention time shift may be necessary. This approach will not solve the problem of a true coelution of an allergen with a component that contains one of the qualifier or quantifier ions used in the SIM window.

In cases when the ratios of the quantifier and qualifier ions do not match the set ratio criteria, but a peak is found in the SIM window, a full scan can be run to prove the presence or absence of the allergen. This requires an additional analysis and does not solve all coelution problems. It may, however, reveal that one of the ions used in the previous SIM analysis is unique and can be used for quantification instead of qualification. Still, this requires recalibration and reanalysis.

Another way to handle a questionable identification is spiking of the sample with the suspected allergen. This requires a reasonable estimate of the actual allergen level, followed by spiking, and then an additional time-consuming analysis. Other alternatives include GC-MS-TOF (*4*) using mass spectral deconvolution to extract coeluting peaks from complex chromatograms. GC-MS-CI and comprehensive GC have also been reported as alternatives (*5*, *6*).

Advantages of the Current Method. The method developed in our laboratory takes advantage of the benefits of full-scan acquisition and dual-column analysis. The sample is analyzed sequentially on columns of different polarities in full-scan mode. This way, the identical sample is analyzed twice. This setup has a number of advantages for the complex chromatographic situations described above. In the case of coelution, the second column chromatogram is directly available for positive identification. The full-scan spectra allow for positive identification using a library search. Qualifier ions are used to prescreen the chromatogram for target components. Potential retention time shifts have no negative effect because the complete chromatogram is recorded in full scan. Potential coelutions on both columns can easily be overcome by choosing any suitable quantifier ion. Because all spectra are recorded in full scan, no additional acquisition is needed.

Table 4. Quantification Limits of Allergens in a Perfume Oil Determined Using a Standard Mixture of Allergens

name	quantification limit (mg/kg) CpSil 5 CB or Solgel 1	CpWax 52 CB
amylcinnamyl alcohol	2.65	1.77
amyl cinnamal	1.35	2.00
anisyl alcohol	1.42	1.04
benzyl alcohol	0.44	0.32
benzyl benzoate	0.61	0.55
benzyl cinnamate	3.27	2.60
benzyl salicylate	0.82	0.38
cinnamyl alcohol	1.71	1.11
cinnamal	1.11	0.34
citral		
neral	0.17	0.38
geranial	0.25	0.26
citronellol	0.85	1.25
coumarin	1.18	0.51
eugenol	0.56	0.62
farnesol		
ZE isomer	2.03	0.82
EE isomer	1.49	0.96
geraniol	1.93	1.07
hexylcinnamic aldehyde	1.99	0.75
hydroxycitronellal	1.63	0.76
isoeugenol	1.28	0.63
2-(4-*tert*-butylbenzyl) propionaldehyde	0.27	0.23
d-limonene	0.23	0.42
linalool	0.49	0.71
hydroxymethylpentylcyclohexene carboxaldehyde	2.16	1.90
methyl heptin carbonate	0.61	0.35
3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	0.55	0.38

Examination of **Table 3** shows that the most abundant ion is not always chosen as the quantifier. Ions are chosen such that the chances for coelution with isobaric ions are minimized. Dichlorotoluene was used as the primary internal standard for all target components. The calibration curves for all target compounds were found to be linear throughout the calibration range (correlation coefficient $r > 0.999$) and stable for multiple days.

Quantification Limits. Table 4 shows the quantification limit for each target compound in a fragrance oil determined using a sample containing each allergen at a level of ~0.23 mg/kg. It was determined by multiplying the noise of the quantifier ion signal on either side of the target component by 10. Because we used a standard to determine the quantification limit, matrix effects that can occur in raw materials and perfumes were minimized. Such effects can negatively influence the quantification limit. An experiment was done to determine if the calibration of the system would change over time. First, the calibration curves were constructed from the standards. Next, the system was used for other purposes for two full days, analyzing 34 other samples (perfumes and raw materials). After that, calibration standard mixtures of approximately 2 and 6 mg/kg were recorded 10 times. These levels correspond to approximately 40 and 120 mg/kg, respectively, for each allergen in the perfume oil. **Table 5** shows the interval of confidence ($n = 10$, 95%) for each target component for both of these levels expressed as equivalent levels in perfume oils.

Table 5. Interval of Confidence for Allergens Based on 10 Repetitive Analyses of Standards

	confidence intervals ($n = 10$, 95%) (mg/kg) in perfume oil					
	level 1			level 2		
name	nominal	Solgel 1	CpWax	nominal	Solgel 1	CpWax
amylcinnamyl alcohol	31.1	30.3–31.9	31.3–32.4	120	119–124	119–122
amyl cinnamal	33.2	33.4–35.4	32.8–34.0	128	126–134	122–130
anisyl alcohol	32.5	31.1–32.2	32.7–33.4	126	124–127	127–130
benzyl alcohol	32.5	31.7–32.2	32.3–33.1	126	124–126	125–127
benzyl benzoate	32.6	34.8–36.1	33.3–34.0	126	131–135	127–131
benzyl cinnamate	32.7	31.9–33.6	32.2–33.4	126	126–131	130–137
benzyl salicylate	32.6	31.7–32.4	32.0–32.8	126	121–126	123–127
cinnamyl alcohol	32.1	30.5–31.8	31.5–33.1	124	120–124	122–125
cinnamal	32.7	32.0–32.8	32.3–32.8	126	119–127	118–124
citral						
neral	13.6	13.4–13.9	13.5–14.1	53	52–54	52–54
geranial	17.9	17.1–17.7	17.5–18.2	69	68–69	68–69
citronellol	32.4	31.4–32.4	32.2–33.0	125	123–125	125–127
coumarin	32.4	32.3–32.8	32.3–33.1	125	127–129	124–128
eugenol	32.8	32.2–33.1	31.3–32.5	127	127–129	123–127
farnesol						
ZE isomer	15.6	14.2–15.1	15.2–15.8	60	57–59	58–60
EE isomer	15.8	15.4–16.3	15.7–16.3	61	59–61	60–63
geraniol	32.1	31.9–32.8	32.0–33.1	124	121–125	121–125
hexylcinnamic aldehyde	32.5	33.3–34.6	32.8–33.7	126	126–133	124–128
hydroxycitronellal	32.4	31.9–32.6	31.7–32.7	125	125–127	124–127
isoeugenol	32.5	31.5–32.3	31.3–32.0	125	122–128	122–126
2-(4-*tert*-butylbenzyl) propionaldehyde	32.2	33.5–34.1	32.1–32.9	125	126–129	124–127
d-limonene	32.1	32.2–32.6	32.3–33.0	124	123–126	124–126
linalool	31.7	31.4–31.9	31.4–32.0	123	121–123	123–124
hydroxymethylpentylcyclohexene carboxaldehyde	32.6	32.7–34.0	32.5–34.5	126	129–132	124–129
methyl heptin carbonate	32.4	31.5–32.5	32.3–33.1	125	124–125	125–129
3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	29.2	30.0–30.7	28.7–29.3	113	113–117	111–114

Table 6. Quantitative Results for Spiked Perfume Samples

(A) Samples Spiked at 30 mg/kg

	nonspiked sample		30 mg/kg spike level		
component	Solgel (mg/kg)	CPWax (mg/kg)	Solgel (mg/kg)	CPWax (mg/kg)	nominal (mg/kg)
benzyl alcohol			29	34	32
benzyl cinnamate			32	30	31
benzyl salicylate			coelute	35	34
cinnamal			coelute	30	32
citronellol			coelute	30	31
eugenol			coelute	30	32
2-(4-*tert*-butylbenzyl) propionaldehyde			31	30	32
d-limonene	10	9	37	37	30
linalool			30	32	30
hydroxy-methylpentyl-cyclohexene carboxaldehyde			coelute	33	31

(B) Samples Spiked at 50 and 70 mg/kg

	50 mg/kg spike level			70 mg/kg spike level		
component	Solgel (mg/kg)	CPWax (mg/kg)	nominal (mg/kg)	Solgel (mg/kg)	CPWax (mg/kg)	nominal (mg/kg)
benzyl alcohol	50	54	54	74	76	76
benzyl cinnamate	52	53	53	72	69	74
benzyl salicylate	coelute	55	56	coelute	80	79
cinnamal	coelute	51	53	coelute	70	75
citronellol	coelute	53	52	coelute	74	74
eugenol	coelute	50	53	coelute	73	74
2-(4-*tert*-butylbenzyl) propionaldehyde	49	54	53	69	74	74
d-limonene	57	57	50	75	76	71
linalool	53	50	51	72	72	72
hydroxymethylpentylcyclohexene carboxaldehyde	coelute	52	52	coelute	76	73

Full-Scan Quantification Results. To further demonstrate the quantitative performance of the extracted ion full-scan method, a perfume free from the 26 allergens was spiked with 10 of them at nominal levels of 30, 50, and 70 mg/kg. The samples were analyzed using a Solgel 1 column and a CpWax 52CB column as specified above.

Analysis of the nonspiked sample revealed the presence of 9−10 mg/kg of *d*-limonene. Levels of *d*-Limonene in the spiked samples are not corrected for this background level. As the data in **Table 6** show, the average deviation from nominal values is very small. Furthermore, where coelution did occur, no recalibration for a different target ion is needed because quantification from the second column can be used.

The data presented show clearly that a full-scan method for the identification and quantification of the 24 volatile potential allergens identified for cosmetic labeling in perfume oils and perfume ingredients has excellent characteristics. The chance for false positives and false negatives is low, and the quality of

the quantitative data is high. In cases of coelution, reanalyzis is not needed because a second chromatogram is readily available. If this fails to solve the coelution problem, the full-scan data allow for the immediate search of a unique ion. Because the calibration data are also recorded full scan, no rerun of calibration standards is needed. The new calibration can immediately be done on the existing calibration data. The excellent quantitative performance of the method is demonstrated by the quantification limits, interval of confidence data, and recovery results from spiked samples.

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