GC-MS analysis of allergens in plant oils meant to cosmetics

Jacques KALOUSTIAN[1]
Céline MIKAIL[1]
Tarek EL-MOSELHY[2]
Lydia ABOU[1]
Henri PORTUGAL[1]

[1] *Laboratoire de chimie analytique (Faculté de pharmacie) et UMR mixte Aix-Marseille 2 – INSERM 476 – INRA 1260 (Faculté de Médecine), Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France* <Jacques.Kaloustian@pharmacie.univ-mrs.fr>
[2] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Tanta University, Tanta 31527, Egypt*

Abstract: *Cutaneous allergy occurs mainly as a result of the use of domestic products and cosmetics. Some fragrances, present in these products, may contain compounds that are responsible for allergy (allergens). The European Council offered a Directive limiting the level of 26 allergens found in cosmetics. GC-MS technique was used to determine the retention times of 25 allergens, determine detection and quantification limits and make calibration with standard solution of each allergen in concentrations ranging from 10 to 200 mgL^{-1} (21 allergens) and 50 to 200 mgL^{-1} (4 allergens). Quantification was performed by the use of 2 internal standards (tetradecane and hexadecane). Seven oils issued from plants were studied by GC-MS. For all of them, the concentration of potential allergens was lower than their minimum detectable level. The alcoholic solution of extracts issued from different samples of oil did not demonstrate the presence of any quantifiable allergen, even when was concentrated 25 times. GC-MS could be a useful technique in the identification and, if necessary, quantification of allergen in ingredients meant to cosmetics.*

Key words: *GC-MS, allergens, ingredients meant to cosmetics, plant oils, European Council Directive*

Introduction

Allergy falls under three main categories: 1) respiratory such as pollens (trees and herbs) and hair of animals (cats); 2) alimentary such as fruits (strawberry), shellfish, fish, eggs, pharmaceuticals (antibiotics) and 3) cutaneous such as bites of insects (bees and wasps) and industrial products such as domestic products and cosmetics. Cutaneous allergy is accompanied by eczema, hives or oedema of Quincke. The treatment includes the use of antihistamines, corticosteroids and desensitisation as well. The most common reaction to fragrance materials is the allergic contact dermatitis [1].

The increased use of perfumes, essential oils and plant extracts in cosmetics and deodorants, has involved an increased incidence of allergy [2-10]. In addition, domestic products, containing fragrances, have increased eczema of the hands [11-13].

Essential oils are volatile oily substances immiscible with water and usually have a density value lower than one. They have a complex chemical composition (mono- and sesqui- terpenes, oxidised substances and aromatics) [14]. In most cases, the essential oils can be obtained by aqueous distillation, squeezing out from *Citrus* epicarps or dry distillation. Some essential oils can be modified. Redistilled essential oils are deprived of one or several constituents.

Oil and plant extracts may contain ingredients showing, a pharmacological activity inconsistent with a cosmetic use, or potential risk of toxicity to the consumer. The European Council presented a Directive relating to cosmetic products. Twenty six fragrances have been considered as cutaneous contact allergens. The European Union has decided that they should be kept below 100 ppm in products that are designed to be applied to the body and then rinsed off, such as soaps, and must be below 10 ppm in products that are designed to remain on the body throughout the day, such as perfumes. This Directive can be of great value for the consumers capable of reacting to these components and for helping the dermatologists in the diagnosis of cutaneous allergenic reactions [15].

The allergens may be present in natural essential oils or obtained by synthetic ways. Allergenic activity could be due to oxidised derivatives, such as peroxides, as in case of limonene and linalool. On the contrary, phenols present in natural essential oils (lavender), have antioxidant properties and can protect from allergenic reactions [16-24].

Several publications have described the use of Gas Chromatography-Mass Spectrometry (GC-MS) technique in the qualitative and quantitative analysis of many compounds present in essential oils and perfumes [25-32].

The detection and quantification limits for each allergen were determined by GC-MS technique. The regression equations of each allergen standard used in calibration were presented. It also was important to search and determine the allergens in several plant oils.

Article received on 20/06/2006
accepted on 20/12/2006

FONDAMENTAL

doi: 10.1684/ocl.2007.0103

Experimental

Allergens

The allergens and their CAS No are listed in *table 1*. All standards were purer than 95%. To this list methyl-2-octynoate was added. In contrary, the last two components of the list (oak moss and tree moss extracts) were not injected into GC, because they are non volatile.

Sample preparation

Pure essential oils were not tested, because GC studies were described in several references [26-30]. Seven oils issued from plants and produced by S.A. Huiles Bertin (60330 – Lagny-Le-Sec, France) were studied: virgin oil of Sisymbrium, virgin oil of Hazel Nuts, virgin oil of Sweet Almonds, virgin oil of Souchet, virgin oil of Cashew Nuts, virgin oil of Borage, castor oil. 2.5 to 5g of vegetable oils was stirred with 10.0 mL alcohol (96%) or hexane during 15 minutes. Only the alcoholic (or hexanic) solution was injected into GC. Sometimes, this solution was concentrated 10 or 25 times, by heating at 75 °C, for 15 min, under nitrogen sweeping.

Gas chromatography conditions

Tests were done on GC-MS instrument, Varian 3400 - Saturn 4D ion trap. A capillary column (30 m × 0.32 mm ID × 1 μm DB5 film with 5% phenylmethyl silicon). Initial temperature was held for 1 min at 60 °C, then heated at 3 °C min^{-1} until 200 °C. Then the temperature was held at 200 °C for 30 min. Temperatures of the injector and detector were 250 and 285 °C, respectively. The carrier gas used was helium and supplied at 138 kPa head pressure. A 1 μL sample was injected in splitless mode.

Table 1. *List of Allergens from the European Directive 2003/15/CE.*

Skin-sensitising ingredients (trivial name and synonyms)	CAS No
Amylcinnamal or 2-benzylideneheptanal	122-40-7
Benzyl alcohol or α-hydroxytoluene	100-51-6
Cinnamyl alcohol or 3-phenyl-2-propen-1-ol	104-54-1
Citral or neral or geranial or 3,7-dimethyl-2,6-octadien-1-al	5392-40-5
Eugenol or 4-allyl-1-hydroxy-2-methoxybenzene	97-53-0
Hydroxycitronellal or 3,7-dimethyl-7-hydroxyoctanal	107-75-5
Isoeugenol or 4-hydroxy-3-methoxy-1-propenylbenzene	97-54-1
Amylcinnamyl alcohol or 2-pentyl-3-phenylprop-2-ene-1-ol	101-85-9
Benzyl salicylate or benzyl-2-hydroxybenzoate	118-58-1
Cinnamal or 3-phenyl-2-propenal	104-55-2
Coumarin or 2-oxo-1,2-benzopyran	91-64-5
Geraniol or 2-trans-3,7-dimethyl-2,6-octadien-1-ol	106-24-1
Lyral or 4-(4-hydroxy-4methyl-pentyl) cyclohex-3enecarbaldehyde	31906-04-4
Anisyl alcohol or 4-methoxybenzylic alcohol	105-13-5
Benzyl cinnamate or 3-phenyl-2-propenoic acid phenylmethyl ester	103-41-3
Farnesol or 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	4602-84-0
Lilial or 2-(4-tert-butylbenzyl)propionaldehyde	80-54-6
Linalool or 3,7-dimethylocta-1,6-dien-3-ol	78-70-6
Benzyl benzoate or benzyl benzenecarboxylate	120-51-4
Citronellol or 3,7-dimethyl-6-octen-1-ol	106-22-9
Hexylcinnamaldehyde or 2-hexyl-3-phenyl-2-propenal	101-86-0
d-Limonene or (R)-p-mentha-1,8-diene	5989-27-5
Methyl-2-octynoate or methyl heptin carbonate	111-12-6
3-Methyl-α-ionone or 3-methyl-4-(2,6,6-trimethyl-2-cyclohexene-1-yl)-3-butene-2-one or γ-isomethylionone	127-51-5
Evernia prunastri extract or oak moss extract	90028-68-5
Evernia furfuracea extract or tree moss extract	90028-67-4

Detection limit

The concentration of allergen at which the peak was equal or higher than 3 times the background noise was retained (Detection Limit or DL).

Qualitative analysis

Identification was made according to retention times and mass spectra. Three MS libraries were used: NIST, Wiley and TR.

Quantification limit

The concentration of allergen at which the peak was equal or higher than 10 times the background noise was retained (Quantification Limit or QL).

Quantitative analysis

It was based on internal standardisation, using tetradecane and hexadecane as internal standards. Five standard solutions, included in the range of 10 to 200 mg L^{-1} of each allergen, were used in calibration. Intervals of calibration were 50 to 200 mg L^{-1} for benzyl alcohol, anisyl alcohol, cinnamyl alcohol and coumarin.

Table 2. *Retention times (RT) determination of the allergen standards.*

Allergen studied	Mean RT (sec)	SD	RSD (%)
Limonene	890	0.75	0.084
Benzyl alcohol	921	0.71	0.077
Linalool	1098	0.75	0.068
Methyl-2-octynoate	1395	0.98	0.070
Citronellol	1475	0.75	0.049
Citral (1st isomer)	1517	0.75	0.049
Geraniol	1550	0.63	0.041
Citral (2nd isomer)	1602	0.63	0.039
Cinnamal	1640	4.96	0.30
Hydroxycitronellal	1662	1.33	0.080
Anisyl alcohol	1680	7.07	0.42
Methyl-2-nonynoate *	1686	0.89	0.053
Cinnamyl alcohol	1743	6.36	0.36
Eugenol	1857	0.75	0.040
Coumarin	2116	0.71	0.034
Isoeugenol	2130	1.10	0.051
3-Methyl-α-ionone	2183	1.03	0.047
Lilial	2328	1.05	0.045
Farnesol (1st isomer)	2570	1.87	0.073
Amylcinnamal	2624	1.10	0.042
Farnesol (2nd and 3rd isomers)	2634	1.67	0.063
Amylcinnamyl alcohol (1st isomer)	2658	0.75	0.028
Lyral (1st isomer)	2663	1.79	0.067
Lyral (2nd isomer)	2682	1.67	0.062
Farnesol (4th isomer)	2690	1.95	0.072
Amylcinnamyl alcohol (2nd isomer)	2719	0.52	0.019
Hexylcinnamaldehyde	2861	1.10	0.038
Benzyl benzoate	2930	1.05	0.036
Benzyl salicylate	3233	0.52	0.016
Benzyl cinnamate	4335	2.37	0.055

* was not cited in the EC Directive. Standard solutions were prepared in absolute ethanol in a concentration about 1g L^{-1}. SD = standard deviation; RSD = relative standard deviation.

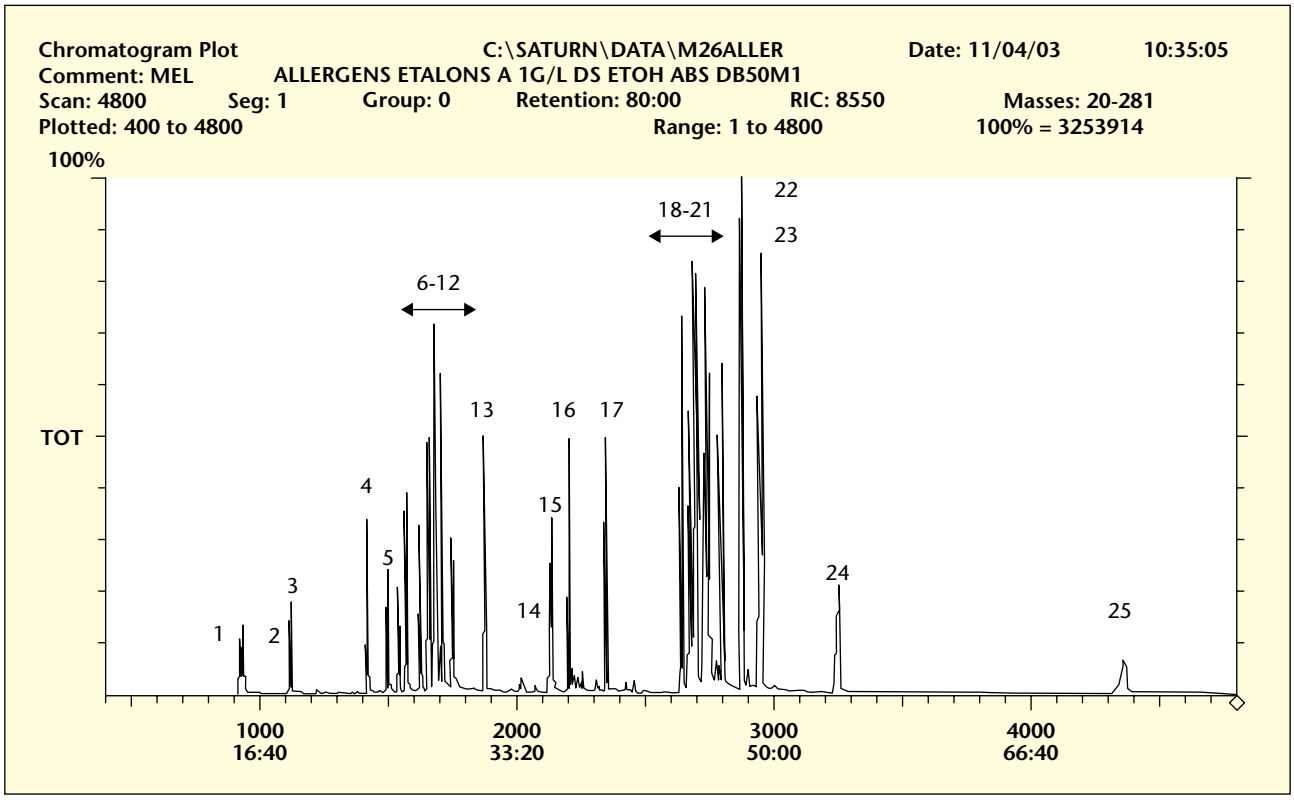

Figure 1. *The chromatogram of the 25 allergen standard mixture.*

Quantification repeatability

Ten tests were done showing a relative standard deviation lower than 2.5% [28].

Results and discussion

Retention times (TR) determination

Ten tests for each standard (about 1 g L^{-1} in absolute alcohol), present in known mixtures, were performed. The mean value of the retention times (RT), standard deviation (SD) and relative standard deviation (RSD), for each allergen standard, are shown in *table 2*. All RSDs were lower than 0.084% except for cinnamal 0.30, anisyl alcohol 0.42 and cinnamyl alcohol 0.36, respectively.

Three allergens (citral, amylcinnamyl alcohol and lyral) consist of two isomers and one allergen (farnesol) consists of four isomers. The % of each isomer was determined by internal normalisation and shown as follows: Citral, 1st peak (*neral*) at 1517 sec (46.3%) and 2nd peak (*geranial*) at 1602 sec (53.7%); amylcinnamyl alcohol, 1st peak at 2658 sec (49.0%) and 2nd peak at 2719 sec (51.0%); lyral 1st peak at 2663 sec (23.9%) and 2nd peak at 2682 sec (76.1%) and farnesol 1st peak at 2570 sec (8.6%), 2nd and 3rd peaks (together) at 2634 sec (43.8%) and 4th peak at 2690 sec (47.6%).

Allergens frequently co-elute with other fragrance plant ingredients, so, mass spectrometry (MS) will be necessary for identification.

Determination of detection and quantification limits of each allergen

First, a mixture of 25 allergen standards (mother solution), each about 1 to 2 g L^{-1} was studied. The chromatogram is shown in *figure 1*.

Then, 3-known mixtures were prepared in alcohol (about 1g L^{-1}) and injected into GC. The composition of the 3-known mixtures is given as follows: *20 allergens* (limonene, linalool, methyl-2-octynoate, citronellol, citral (2 isomers), geraniol, cinnamal, hydroxy-citronellal, methyl-2-nonynoate, cinnamyl alcohol, eugenol, coumarin, 3-methyl-α-ionone, lilial, amylcinnamal, amylcinnamylalcohol (2 isomers), hexylcinnamal, benzyl benzoate, benzyl salicylate and benzyl cinnamate); *5 allergens* (benzyl alcohol, anisyl alcohol, isoeugenol, lyral (2 isomers) and benzyl benzoate) and *2 allergens* (farnesol (4 isomers) and benzyl benzoate). Then several dilutions (1/10 to 1/1000) were made on these mother solutions and injected into GC. The retained DLs and QLs are shown in *table 3*. When two or four isomers were present, only the major peak was considered.

Fundamentals of allergen identification

The following steps were performed:

1) In the chromatogram of unknown plant oil, the peaks with RT close to those of allergen standards was searched.

2) If the peak height was 3 times lower than the background noise, the peak was considered absent.

3) If the height of the peak was 3 times higher than the background noise, the peak was considered present.

4) Identification was done when the theoretical RT given by *TR library* corresponded to the observed RT of the unknown peak, and we compared mass spectra of the unknown peak with those of allergen standards, and after with those standards present in libraries.

5) If a doubt arose about the peak, we re-injected the unknown plant oil enriched with the expected standard. Identification will be confirmed if only one peak appeared without a shoulder.

6) If the height of the peak was 10 times higher than the background noise, quantification would be possible.

Table 3. *Retained detection limit (DL) and quantification limit (QL) for each allergen in mixture.*

Allergen studied	Retained detection limit (mg L^{-1})	Retained quantification limit (mg L^{-1})
Limonene	2.1	11
Benzyl alcohol	40	72
Linalool	2.4	17
Methyl-2-octynoate	2.2	16
Citronellol	2.2	18
Citral (1st or 2nd isomer)	11.5	35
Geraniol	10.1	28
Cinnamal	30	66
Hydroxycitronellal	4.5	25
Anisyl alcohol	50	~100
Methyl-2-nonynoate	4.8	21
Cinnamyl alcohol	43	86
Eugenol	10.9	27
Coumarin	33	~100
Isoeugenol	12.6	30
3-Methyl-α-ionone	2.5	12
Lilial	2.1	12
Amylcinnamal	12.8	26
Amylcinnamyl alcohol (2nd isomer)	2.6	25
Lyral (2nd isomer)	3.9	26
Farnesol (2nd and 3rd isomers)	11.8	32
Hexylcinnamaldehyde	2.2	11
Benzyl benzoate	11.4	24
Benzyl salicylate	11.6	38
Benzyl cinnamate	37	75

Quantification of the allergens present in the samples

The calibration of each allergen was done with the use of two internal standards: tetradecane and hexadecane (*table 4*). The choice of internal standard depends on the presence of interfering peaks. The retention times (sec) of several saturated hydrocarbons were tested and found to be 520, 790, 1390, 1967, 2230 and 2490 for nonane, decane, dodecane, tetradecane, pentadecane and hexadecane, respectively. A linear correlation was observed, $Y = 284.39X - 2037.8$, Y = retention time, X = number of carbons, $R = 0.9998$ and $p < 0.001$. In this paper the choice of tetradecane and hexadecane was based on the best results obtained by analysing different samples. They appeared in retention times that were fairly different from other peaks present in all the samples.

Results of studied samples

Our chromatographic procedure seemed to be slightly longer than that described before [30], but our conditions showed good resolution for about 200 terpenoid compounds determined in general essential oil studies.

From the 25 studied standard allergens, 19 showed a retained DL lower than 13 mg L^{-1} and 5 were between 30 and 50 mg L^{-1}. These variations are well explained by the form of the peaks. A narrow and high peak gave a value lower than 13 mg L^{-1}, in contrary, a broad and low height peak gave a higher value of DL.

GC-MS is considered as a good technique for the determination of volatile substances. Results were obtained with good repeatability.

Table 4. *The calibration data for compounds listed as skin-sensitising ingredients.*

Allergen Studied	Regression Equation*	(R^2)**
Limonene	y = 9.5033 x + 0.0114	0.9958
	y = 8.3883 x + 0.0134	0.9975
Benzyl alcohol	y = 2.9113 x – 0.0243	0.9938
	y = 2.6244 x – 0.0201	0.9922
Linalool	y = 8.6162 x + 0.0143	0.9991
	y = 7.4624 x + 0.0157	0.9979
Methyl-2-octynoate	y = 7.5282 x + 0.0103	0.9986
	y = 6.5170 x + 0.0058	0.9980
Citronellol	y = 8.4145 x + 0.0259	0.9968
	y = 7.2836 x + 0.0273	0.9952
Citral (1st isomer)	y = 5.6307 x + 0.0052	0.9982
	y = 4.8689 x + 0.0025	0.9990
Geraniol	y = 8.6412 x + 0.0002	0.9963
	y = 7.4762 x + 0.0036	0.9929
Citral (2nd isomer)	y = 5.2198 x + 0.0015	0.9985
	y = 4.5067 x + 0.0009	0.9975
Cinnamal	y = 7.0368 x – 0.0617	0.9946
	y = 6.2324 x – 0.0617	0.9935
Hydroxycitronellal	y = 8.9758 x + 0.0618	0.9976
	y = 7.7788 x + 0.0566	0.9953
Anisyl alcohol	y = 2.7664 x – 0.0139	0.9955
	y = 3.9782 x + 0.0063	0.9990
Methyl-2-nonynoate	y = 8.0496 x + 0.0521	0.9925
	y = 7.1888 x + 0.0259	0.9951
Cinnamyl alcohol	y = 3.9441 x + 0.0061	0.9902
	y = 3.7575 x – 0.0027	0.9970
Eugenol	y = 7.9359 x + 0.0262	0.9977
	y = 6.8756 x – 0.0186	0.9939
Coumarin	y = 6.0366 x – 0.0021	0.9990
	y = 5.2234 x – 0.0186	1.0000
Isoeugenol	y = 4.3897 x + 0.0139	0.9955
	y = 3.9782 x + 0.0063	0.9990
3-Methyl-α-ionone	y = 11.267 x + 0.0408	0.9976
	y = 9.7537 x + 0.0411	0.9981
Lilial	y = 11.252 x + 0.0100	0.9997
	y = 9.7475 x + 0.0193	0.9992
Farnesol (1st isomer)	y = 3.4526 x – 0.0008	0.9924
	y = 3.4522 x – 0.0011	0.9969
Amylcinnamal	y = 12.960 x + 0.0574	0.9974
	y = 11.224 x + 0.0571	0.9961
Farnesol	y = 3.3175 x – 0.0032	0.9994
(2nd and 3rd isomers)	y = 3.4368 x – 0.0046	0.9987
Amylcinnamyl alcohol	y = 6.3332 x + 0.0574	0.9949
(1st isomer)	y = 5.4199 x + 0.0512	0.9912
Lyral (1st isomer)	y = 10.494 x + 0.0038	0.9994
	y = 9.2450 x – 0.0008	0.9999
Lyral (2nd isomer)***	y = 7.3230 x – 0.0162	0.9982
	y = 6.3827 x – 0.0158	0.9980
Farnesol (4th isomer)	y = 3.2928 x – 0.0079	0.9930
	y = 3.4702 x – 0.0078	0.9903
Amylcinnamyl alcohol	y = 5.0437 x + 0.0333	0.9992
(2nd isomer)	y = 4.3181 x + 0.0301	0.9982
Hexylcinnamaldehyde	y = 13.896 x + 0.0421	0.9917
	y = 12.026 x + 0.0422	0.9922
Benzyl benzoate	y = 11.739 x – 0.0039	0.9908
	y = 10.158 x + 0.0011	0.9930
Benzyl salicylate	y = 7.0125 x – 0.0578	0.9966
	y = 6.0679 x – 0.0474	0.9960
Benzyl cinnamate	y = 11.739 x – 0.0039	0.9908
	y = 10.158 x + 0.0011	0.9930

(*) 1st data for tetradecane and 2nd for hexadecane when used as internal standards; y = ratio of (allergen peak area/internal standard peak area); x = concentration of allergen (g L^{-1}); (**) R = correlation coefficient; (***) only the 2nd isomer of lyral (the largest peak) must be quantified.

To avoid a possible volatilisation or degradation of allergens, ethanolic solutions were prepared and injected quickly. Solutions were not kept more than one day at 4 °C.
Ethanol is a good solvent for extraction. It dissolves several fractions of vegetable oils, but sometimes hexane will be prefered. For a good extraction, the solvent should be added in excess (2-5 times) more than the volume of the sample. So, to increase the sensitivity, it was necessary to concentrate this alcoholic (or hexanic) extract, 10 to 25 times. But in this case, losses were observed due to volatilisation. In each case, standard solutions were prepared and concentrated in the same conditions as that the studied samples. Allergen loss percentage was then determined. Limonene, benzyl alcohol, linalool, citronellol, citral, cinnamaldehyde, anisyl alcohol and cinnamyl alcohol lose 55% (75%), 15.5% (23%), 10% (23%), 13% (19%), 20% (31%), 20% (20.5%), 26% (33%) and 10% (20.5%) when were concentrate 10 times (25 times), respectively. All the others lost less than 10%. Concentration of 10 and 25 times can improve the determination of DL.

Result expression

Results expression would be done according to the following model:
5.0434 g of Borage oil were stirred with 10.0 mL of alcohol (96%) during 15 min. After centrifugation, the alcoholic volume was 9.0 mL. Then, 10 times concentrated solution was injected into GC. Limonene was considered present (3 times the background noise) and corresponded to the Minimum Detectable Level (MDL), in this concentrated solution (VC = 0.90 mL).
DL * VC = 2.1 * 0.90 * 10^{-3} = 1.89 μg of limonene in the sample.
If we take into account the initial mass of plant oil (5.0434 g) and the loss of 55% of limonene in 10 times concentration, corrected MDL will be:
(1.89 * 100) / 5.0434 * (100 – 55) = 0.83 μg of limonene per 1 g of Borage oil.
All tested oils had potential allergen level lower than DL.
The fragrance mix (FM) is the main screening chemicals used to test for fragrance allergy. FM is composed of 1% of the following 8 chemicals: cinnamal, cinnamyl alcohol, eugenol, isoeugenol, hydroxycitronellal, geraniol, oak moss absolute and amylcinnamaldehyde. Dermatologists have used FM mixture as a standard to test cutaneous allergy. The composition of this mixture should be extended to include other allergens [33-37]. The GC-MS technique, described here, could be applied in allergen determination, in plant oils meant to cosmetics. Evaluation of allergens in cosmetics will be less easier.

Acknowledgements. *F. Turon (S.A. Huiles Bertin) for supplying several samples of plant oils.*

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