

Patch testing with oxidized *R*-(+)-limonene and its hydroperoxide fraction

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R-(+)-Limonene is an ubiquitous allergen in our environment. It is one of the most widely used fragrance materials not only in fine fragrances but also most often incorporated in domestic and occupational products. Although the non-oxidized *R*-(+)-limonene itself is not allergenic, it easily forms allergenic products due to autoxidation during handling and storage. 2273 patients at 4 dermatological clinics in Europe were patch tested between 1997 and 1999 in 2 steps. First, the oxidation mixture of *R*-(+)-limonene and 1 selected allergen fraction of the mixture, the limonene hydroperoxides, were tested in 2 different vehicles in consecutive patients. A diverging frequency of positive patch test reactions was observed in the 4 clinics. 3.8% of the consecutive patients tested reacted to oxidized *R*-(+)-limonene in 2 clinics, 6.5% in the 3rd, whereas 0.3% in the 4th clinic. In 2 of the centres, different but significant concomitant positive response rates to other allergens were observed; e.g. to fragrance materials and to colophonium. However, in the total test population, 57% of the limonene-allergic subjects did not react to any of the fragrance allergy markers used in the standard series. In the 2nd step, patients showing positive reactions were retested, also including additional separate allergens of the limonene oxidation mixture (carvone and limonene oxide). 60% of the limonene-allergic patients showed positive results at retesting. The limonene hydroperoxide fraction was proved to be the most important allergen of the oxidation mixture, showing positive reactions in around 60% of the limonene-allergic patients at both test sessions. Testing limonene oxide and carvone separately resulted in very few positive reactions. 3% oxidized *R*-(+)-limonene in non-stabilized petrolatum is most suitable when using only 1 test preparation for diagnosis of contact allergy to oxidized limonene. Our data give clinical support to the European classification of *R*-(+)-limonene, containing oxidation products, as a skin sensitizer.

Key words: allergic contact dermatitis; autoxidation; fragrance allergy; fragrance mix; hydroperoxides; limonene; oxidation products; patch testing. © Blackwell Munksgaard, 2003.

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R-(+)-Limonene is an ubiquitous allergen in our environment. It is a fragrance material not only used in fine fragrances but also most often incorporated in domestic and occupational products (1). It is also used as a solvent, insecticide and a flavouring agent. Although the non-oxidized *R*-(+)-Limonene itself is not allergenic, this was the 1st substance classified with R-43 in Europe as a skin sensitizer, due to the formation of allergenic oxidation/degradation products during handling and storage.

R-(+)-Limonene oxidizes readily in air and forms products with strong allergenic activity. Amongst the oxidation compounds, the major

allergens identified are limonene hydroperoxides, limonene oxide and carvone (Fig. 1). In previous studies, (2) we found that around 1/2 of the subjects reacting to oxidized limonene mixture also show a positive patch test result to limonene hydroperoxides. Furthermore, it was shown that some of the subjects reacted only to the hydroperoxide fraction and not to the mixture. The other 2 allergens identified were not routinely tested on oxidized limonene-allergic subjects.

The appropriate test methods for the diagnosis of subjects allergic to oxidized limonene are not yet agreed on. The present study investigates questions regarding the right patch test vehicle,

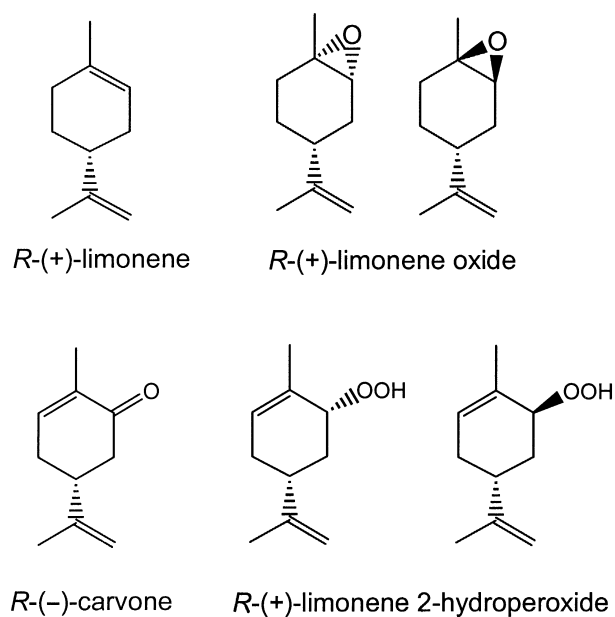


Fig. 1. *R*-(+)-Limonene and identified allergenic oxidation products.

the use of separate allergens/fractions versus oxidation mixture, concomitant reactions and the reproducibility of results.

Subjects and Methods

Chemicals

R-(+)-Limonene (*d*-limonene) (*p*-mentha-1,8-diene) of technical quality was obtained from Frey & Lau (Henstedt, Ulzburg, Germany). According to our gas chromatography analysis, the product consisted of *R*-(+)-limonene 96.0%, α -pinene 0.5%, sabinene 0.2%, myrcene 2.7% and linalool 0.4% (total 99.8%). In order to mimic the oxidation that happens during handling and storage conditions, samples of *R*-(+)-limonene (as purchased) were kept at room temperature on a laboratory counter in open flasks and stirred for 1 h, $\times 4$ a day. After 10 weeks, the oxidation mixture formed, contained only about 40% of *R*-(+)-limonene. This is in accordance with previous studies (3). The mixture was stored under argon at -20° celsius, and preparations for patch testing were made every 2–3 months from the same batch. Analysis of the oxidation mixture and isolation of the limonene hydroperoxide fraction were performed according to previously described methods (2).

R-(-)-Carvone (*l*-carvone) of 98% purity and *R*-(+)-limonene oxide of 97% purity were purchased from Aldrich Chemical Co Inc., (Milwaukee, WI, USA) and used without further purification.

Stabilized petrolatum (USP XXII containing 10 p.p.m. of α -tocopheryl acetate) was purchased from Apoteket AB (Göteborg, Sweden) and non-stabilized pet. from KEBO Laboratory A/S (Albertslund, Denmark).

Subjects and test centres

Consecutive dermatitis patients with suspected allergic contact dermatitis (2273; 1519 female and 754 male) were tested at the participating dermatological clinics of Stockholm, Leuven, Lisbon and Seville during the period 1997–99.

In November 1999, retesting was performed in 30 out of 63 subjects, showing positive reactions at the 1st testing.

Patch tests were applied in small Finn Chambers[®] on Scanpor[®] tape (Epitest, Tuusula, Finland) in Stockholm and Lisbon. Van der Bend[®] chambers (van der Bend, Briele, The Netherlands), mounted on Micropore[®] tape (3M, 3M Health Care, Neuss, Germany), covered by Mefix[®] (Mölnlycke, Hilden, Germany), were used in Leuven. In Seville, Leukotest[®] (Beiersdorf, Hamburg, Germany) was used in the screening part and small Finn Chambers[®] on Scanpor[®] for the retest of the limonene series. Readings were taken twice, on days 2 and 4 (exceptionally on day 3) in Leuven and Seville, and on days 3 and 6/7 in Stockholm and Lisbon and scored according to the ICDRG standard methods (4).

Patch test materials

Standard allergens used for patch testing were obtained in Stockholm from Chemotechnique AB (Malmö, Sweden), in Lisbon and Leuven from Trolab-Hermal (Reinbek, Germany), whereas TRUE Test[™] (Pharmacia A/S, Hillerød, Denmark) was applied in Seville.

During the screening period, 4 different test preparations were used: 3% oxidized *R*-(+)-limonene mixture and 0.5% *R*-(+)-limonene hydroperoxide fraction in stabilized and non-stabilized pet., respectively. In the present paper, the term 'oxidized limonene mixture' is used for naming the patch test material applied, containing all possible allergens formed after oxidation. When we talk about 'oxidized limonene' the term is used generally. The patch test materials were prepared at our laboratory and filled into 5 ml syringes. The syringes were sent to the clinics by express mail and kept in refrigerator between the tests.

In the retest session, the following preparations were used: 3% oxidized *R*-(+)-limonene mixture and 0.5% *R*-(+)-limonene hydroperoxide fraction (from the same batch as used for the

screening tests) in stabilized and non-stabilized pet., 1 and 3% non-oxidized purified *R*-(+)-limonene (prepared by distillation under reduced pressure), 0.2 and 1% *R*-(+)-limonene oxide and 1 and 3% *R*-(-)-carvone in non-stabilized pet. The lower concentrations of limonene oxide and carvone were chosen to correspond approximately to the concentrations of those compounds in the oxidation mixture; the higher concentrations were selected to enhance the detection of contact sensitivity to the compounds, based on previous experience (2). This time, the 4 preparations of oxidized limonene mixture and the hydroperoxides (otherwise identical to the one used in the screening period) were handled in the laboratory 6 weeks before testing in order to mimic conditions in patch test clinics. They were kept in the refrigerator but were left without syringe cap at room temperature for 3 h once every week, for 6 weeks. 1 extra sample of the oxidized *R*-(+)-limonene mixture in non-stabilized pet. was freshly diluted (3%) in the days before the retest session and was additionally tested.

Statistics

For the analysis of frequency of positive test results in the different centres and of concomitant reactions, χ^2 test was used, with the *P*-values given. Student *t*-test and χ^2 test were used for the analysis of drop-out cases at retesting.

Results

In the screening period, the frequency of subjects showing positive patch test reactions to any of the 4 oxidized *R*-(+)-limonene patch test materials in Leuven and Stockholm was similar, 33/877 (3.8%) and 13/331 (3.9%) patients reacting, respectively (Table 1). The number of subjects with positive test reactions in Lisbon 3/850 (0.3%) was significantly ($P < 0.001$) different from that of Leuven and Stockholm. In Seville, 6.5% of the patients showed positive reactions, which is statistically different ($P < 0.01$) from the

results in Leuven. In the total test population, ($n = 2273$) 63 (2.8%) positively reacting subjects were observed. Subjects with doubtful reactions ($n = 52$) were seen at a frequency of 0.1–13.9%, the fewest in Lisbon and the most in Seville.

In a 2nd phase of the study, subjects reacting to any of the limonene patch test materials in the screening period were recruited for retesting. Of the 30 subjects tested, 18 (60%) showed positive test reactions to oxidized *R*-(+)-limonene mixture and/or to the hydroperoxide fraction, whereas 4 subjects (13%) showed doubtful reactions at retesting (Table 2). No reactions were seen to pure *R*-(+)-limonene. 1 patient reacted to *R*-(+)-limonene oxide (positive also to the oxidized limonene mixture) and 1 patient reacted to *R*-(-)-carvone (positive also to the hydroperoxide fraction).

Table 3 gives details on the proportion of reactions to the oxidized limonene mixture and the limonene hydroperoxide fraction at the screening test session. Data obtained at retesting showed similar results. The oxidized limonene mixture gave more positive reactions (84 and 83%, respectively, of all reactions at screening and retesting), compared with the limonene hydroperoxide fraction (59 and 56%, respectively). However, both screening and retest sessions revealed that 16% of the allergic subjects reacted only to the hydroperoxide fraction. The reproducibility of the reactions to the oxidized limonene mixture was 50%, whereas the hydroperoxide fraction showed lower figures (33%) (Table 4).

About 1/2 of the reactions occurred simultaneously to both preparations of the same allergen diluted in stabilized and non-stabilized pet. In the rest of the cases, reactions were found either to the stabilized or to the non-stabilized pet. preparations in statistically insignificant proportion. Analysis of the positive screening patch test results revealed that oxidized limonene mixture in non-stabilized and in stabilized pet. detected 41 and 42 (respectively) out of 63 cases of oxidized limonene allergy (66%). At retesting, oxidized limonene mixture in non-stabilized pet.

Table 1. Results of patch testing with oxidized limonene and its hydroperoxide fraction in 2273 subjects in 4 dermatological clinics

Test centre	Number of patients tested	Subjects with reactions to any limonene preparation ^a	
		Positive (+, ++, +++) (%)	Doubtful (?) (%)
Leuven	877	33 (3.8)	10 (1.1)
Stockholm	331	13 (3.9)	11 (3.3)
Lisbon	850	3 (0.3)	1 (0.1)
Seville	215	14 (6.5)	30 (13.9)

^a3% oxidized *R*-(+)-limonene mixture and 0.5% *R*-(+)-limonene hydroperoxide fraction diluted in stabilized and non-stabilized petrolatum.

Table 2. Positive and doubtful reactions when retesting 30 subjects with oxidized *R*-(+)-limonene and its hydroperoxide fraction

	Number of retested subjects	Positive ^a	Doubtful ^a	Negative ^a
Leuven	11	7	3	1
Stockholm	7	4	1	2
Lisbon	2	2	0	0
Seville	10	5	0	5
Total	30	18 (60%)	4 (13%)	8 (27%)

^aThe numbers indicate the number of subjects with positive, doubtful and negative reactions to any of the 4 patch test materials that also were tested for screening.

Table 3. Simultaneous reactions to oxidized *R*-(+)-limonene 3% petrolatum and its limonene hydroperoxide fraction 0.5% pet. in 2273 consecutive dermatitis patients

	<i>R</i> -(+)-limonene hydroperoxides		
	Positive ^a (%)	Negative	Total
Oxidized <i>R</i> -(+)-limonene mixture			
Positive ^a (%)	27 (43 ^b)	26 (41 ^b)	53 (84 ^b)
Negative	10 (16 ^b)	2210	2220
Total	37 (59 ^b)	2236	2273

^aAll positive responses irrespective of vehicle.

^bThe proportion of reacting subjects among those giving positive responses to any of the oxidized limonene preparations ($n = 63$).

elicited positive reactions in 14 out of the 18 subjects allergic to oxidized limonene (78%).

In the total test population, 57% of the limonene-allergic subjects did not react to any of the fragrance allergy markers in the standard series (i.e. fragrance mix (FM) and balsam of Peru (Myroxylon Pereirae resin). During the screening period, the frequency of positive reactions to FM in the total test population was 10.6% in Stockholm, 13.5% in Leuven, 4.1% in Lisbon and 4.6% in Seville. No significant concomitant reactions to FM were detected amongst subjects allergic to oxidized limonene in Stockholm (2/13 versus 33/138), Lisbon (1/3 versus 34/406) or in

Seville (1/14 versus 9/115). However, in Leuven, 19 out of the 33 limonene-positive subjects reacted to FM, compared with 99 out of 476 subjects negative to oxidized limonene but positive to any of the standard allergens ($P < 0.001$). Significant correlations were observed also to balsam of Peru ($P < 0.001$) and colophonium ($P < 0.001$) (Table 5). Besides FM, other fragrance materials and own perfumes were tested in Leuven and Lisbon when the history indicated a fragrance allergy. In Leuven, 5 subjects with positive reactions to oxidized limonene also reacted to their own perfumes or different essential oils but were negative to FM. In Lisbon, this was the case for 2 out of 3 subjects with a positive reaction to oxidized limonene. In these 2 centres, there was a significant correlation between oxidized limonene allergy and positive patch test reactions to fragrance allergy markers such as FM, balsam of Peru, essential oils and own perfumes (Table 6).

Discussion

Contact allergy to fragrances, besides nickel allergy, is the most common hypersensitivity amongst dermatitis patients. The FM is estimated to detect 50–80% (5) of all cases with fragrance allergy, but new markers have to be identified and introduced for screening in dermatitis patients. The diagnostic importance of positive patch test reactions to other known markers such as balsam of Peru and essential oils is lower and much debated in the literature (5). However, together with other relevant clinical data, their role is still widely accepted as indicators of fragrance allergy. The results of the present study confirm our previous findings that contact allergy to oxidized *R*-(+)-limonene is common amongst subjects with suspected allergic contact dermatitis (2). The highly significant correlation between fragrance allergy and oxidized limonene allergy in Leuven and Lisbon (Table 6) gives clinical support for oxidized limonene being a good fragrance allergy

Table 4. Reproducibility of positive patch test results at retesting (test session II) in 30 subjects with contact allergy to oxidized *R*-(+)-limonene mixture and/or limonene hydroperoxide fraction at the screen testing (test session I)

				Differences in reactions between test session I and II			
		Positive reactions test session II	Positive reactions test sessions I and II	Non-positive test session I–positive test session II		Positive test session I–non-positive test session II	
Positive reactions test session I				Negative test session I	Questionable test session I	Negative test session II	Questionable test session II
Ox. lim. ^a	26	15	13	2	0	7	6
Lim.-OOH ^b	15	10	5	3	2	7	3

^aOx.lim = oxidized *R*-(+)-limonene mixture, independent of the vehicle.

^bLim.-OOH = *R*-(+)-limonene hydroperoxide fraction, independent of the vehicle.

Table 5. Positive reactions to fragrance mix, balsam of Peru and colophonium (tested in the standard series) in allergic subjects^a with positive and negative test results to oxidized limonene

	Seville		Leuven		Stockholm		Lisbon ^b (total population)
	Limonene-positive subjects	Limonene-negative subjects	Limonene-positive subjects	Limonene-negative subjects	Limonene-positive subjects	Limonene-negative subjects	
Fragrance mix	7.1% (1/14)	7.8% (9/115)	57.5% ^c (19/33) <i>P</i> < 0.001	20.8% (99/476)	15.4% (2/13)	23.9% (33/138)	4.11%
Balsam of Peru	14.3% (2/14)	7.8% (9/115)	27.2% ^c (9/33) <i>P</i> < 0.001	8.6% (41/476)	0 (0/13)	15.2% (21/138)	2.8%
Colophonium	14.3% ^c (2/14) <i>P</i> = 0.015	0.8% (1/115)	30.3% ^c (10/33) <i>P</i> < 0.001	8% (38/476)	7.7% (1/13)	13.7% (19/138)	1.4%

^aSubjects with at least 1 allergic reaction at testing.

^bFor Lisbon only the data for the total test population are given because of the few positive results.

^cStatistically significant concomitant reactions in subjects showing positive reactions to oxidized limonene compared with those with negative reactions.

marker. Furthermore, testing with oxidized limonene detects fragrance allergy cases that could not have been diagnosed with the fragrance markers present in the standard series. The role of limonene as an active ingredient of hand cleansers and other industrial products was not investigated in this study. However, occupational hand dermatitis cases of the Stockholm centre might have a different exposure to subjects with fragrance allergy, accounting for the discordance between oxidized limonene allergy and fragrance allergy diagnosed with classical markers. Clinical aspects, such as localization of symptoms and occupation of subjects, suggest that oxidized limonene is a ubiquitous allergen in our chemical environment.

The clinical characteristics and relevance of reactions in the present study are discussed in detail elsewhere (6). However, it should be noted that the frequency of positive reactions to oxidized limonene showed a variation between 0.3 and 6.5% in the 4 dermatological clinics (Table 1). In a study by Frosch et al., (7) the frequency of Lyrall[®] (4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde)-positive reactions showed a variation of 1.2–17% between the test centres. Such variations in sensitization to

fragrance materials are not rare and, besides specific patient referral or selection, they can be explained by local habits in the use of cosmetics. For example, fragrances with citrus notes have a much larger market shares in Spain compared with other countries (unpublished figures from the fragrance industry). The extensive use of citrus peel in the household setting and the use of homemade, citrus-based cosmetics can also explain the high frequency of allergic reactions in Seville. This could also be responsible for the low concordance of positive patch test results to oxidized limonene and other indicators of fragrance sensitivity in this centre. The high number of doubtful reactions to oxidized limonene in Seville may represent cases of low sensitization rather than irritation, particularly in the light of low irritation figures in the other centres (8). All readings were performed according to the ICDRG rules (4). A good correlation in the scoring of skin reactions was guaranteed, because the dermatologists in the study have performed patch test reading together on various occasions.

Many subjects allergic to perfume components react to multiple allergens (unpublished observation of An Goossens), and concomitant sensitization is supposed to be the explanation to this

Table 6. Results of testing subjects allergic to oxidized limonene with fragrance allergy markers (*n* = 63)

Test centre	Leuven	Stockholm	Lisbon	Seville	Total
Number of subjects allergic to fragrance mix	19/33	2/13	1/3	1/14	23/63
Number of subjects allergic to balsam of Peru and +/- essential oils (and not to fragrance mix)	2/33	0/13	1/3	1/14	4/63
Number of subjects allergic only to own fragrances, essential oils or ingredients of the fragrance mix (and not to fragrance mix or balsam of Peru)	5/33	0/13	1/3	1/14	7/63
Total number of subjects allergic to fragrance allergy markers	26/33	2/13	3/3	3/14	34/63 (54%)

observation. A significant number of concomitant reactions were detected in Leuven not only to FM but also to colophonium and balsam of Peru (Table 5). Simultaneous reactions to FM, colophonium and balsam of Peru in dermatitis patients are registered in the literature (9–11). Also, in the previous patch test study on oxidized limonene (2), we noticed a high number of concomitant reactions to FM, balsam of Peru and colophonium in the patients with positive reactions to oxidized limonene compared with those who were negative. The most probable explanation is that this is due to simultaneous sensitization, because we observed no cross-reactivity to oxidized limonene mixture in studies on guinea pigs sensitized to balsam of Peru or colophonium (not published).

All patients showing a positive reaction to any of the oxidized limonene patch test materials at the screening test were asked to participate in a retest session, but only 30 of the total 63 returned. The high dropout rate makes the retest group a special selection. The subjects who returned for retesting had significantly fewer patch test reactions to the allergens present in the standard series at the screening session ($P < 0.01$) and also fewer patch test reactions to oxidized limonene ($P < 0.05$) compared with those not retested. This fact might affect the relatively low rate of positive reactions at retesting (8). Other factors responsible for the discordance might be a difference in penetration or in the reaction threshold of the patient at the 2 test sessions (8). Also a variation in the allergen content of the patch test material might be possible, as the test material used is complex and not yet standardized. However, the overall 60% positive response rate to oxidized limonene at retesting is within the range of reproducibility of patch test results in the standard series when retesting sequentially (12), which shows the weakness of the patch test diagnostic system.

For practical reasons, we would like to find and suggest a single patch test preparation that can be used in clinical practice with maximum diagnostic value to detect contact allergy to oxidized limonene. In both the screening part and at retesting, we used 4 patch test materials (oxidized limonene and its hydroperoxide fraction in stabilized and non-stabilized pet.) for basic testing. At both test sessions, we found that more than 80% of the subjects reacted to oxidized limonene mixture in either of the 2 different vehicles. The remaining cases were detected when testing with the limonene hydroperoxide fraction. Besides hydroperoxides, 2 other allergens found in the oxidized limonene mixture, limonene oxide and carvone

(13), were used for retesting. Positive reactions to these allergens were only rarely observed at retesting and were never observed without positive reactions to the screening substances. Thus, we consider the hydroperoxides to be the most important allergens in the mixture. This is in accordance with our findings regarding colophonium allergy. The major allergen, 15-hydroperoxyabietic acid, detects 60–70% of the cases with known contact allergy to colophonium (14). It should be noted that carvone is found in our environment not only as an oxidation product of limonene but also as a source itself, e.g. in toothpaste (15) and, thus, can cause contact allergy due to exposure separate from that of limonene. Other non-isolated separate allergens in the mixture or synergistic effects of the chemicals might explain the greater number of positive reactions observed in the mixture compared with the hydroperoxide fraction. The clinical relevance of synergistic effects was proved with fragrance substances by dose-response studies by Johansen et al. (16).

The use of 2 different vehicles (stabilized and non-stabilized pet.) increased the rate of positive results. The results at retesting confirmed the results obtained in the screening test. We found that the amount of limonene hydroperoxides in stabilized pet.-containing α -tocopheryl acetate was still enough to cause elicitation. However, there might be inevitable decomposition in the currently investigated patch test material. Positive reactions to oxidized limonene were observed in 4 patients only in the freshly prepared oxidized limonene mixture (1 opposite discordance was also observed). In 3 cases, reactions to the freshly prepared patch test material were stronger than to the same test material that was handled for 6 weeks in the laboratory before patch testing to mimic what happens during storage and usage in the clinic. According to chemical investigations (17), the hydroperoxides are less decomposed in pet. without tocopheryl acetate. 72% of the subjects reacting to any of the patches applied at retesting could be diagnosed by using 3% oxidized *R*-(+)-limonene in non-stabilized pet. We, therefore, conclude that this preparation is most suitable when using only 1 test preparation for diagnosis of contact allergy to oxidized limonene.

The results of this study emphasize not only on the ingredients that were originally applied in the commercial formulations but also on the need for testing compounds that the patients actually may come into contact with. New screeners for the diagnosis of fragrance allergy are searched for, within an EU project on 'Fragrance chemical allergy: a major environmental and consumer

health problem in Europe' (Quality of Life and Management of Living Resources program of the European Commission under the key action Environment and Health). Autoxidation of fragrance terpenes can contribute to fragrance allergy to a great extent; hence, the role of autoxidation products as allergens is investigated in a part of this study.

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