

Limonene hydroperoxide analogues show specific patch test reactions

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doi:10.1111/cod.12195

Summary

Background. The fragrance terpene *R*-limonene is a very weak sensitizer, but forms allergenic oxidation products upon contact with air. The primary oxidation products of oxidized limonene, the hydroperoxides, have an important impact on the sensitizing potency of the oxidation mixture. One analogue, limonene-1-hydroperoxide, was experimentally shown to be a significantly more potent sensitizer than limonene-2-hydroperoxide in the local lymph node assay with non-pooled lymph nodes.

Objectives. To investigate the pattern of reactivity among consecutive dermatitis patients to two structurally closely related limonene hydroperoxides, limonene-1-hydroperoxide and limonene-2-hydroperoxide.

Methods. Limonene-1-hydroperoxide, limonene-2-hydroperoxide, at 0.5% in petrolatum, and oxidized limonene 3.0% pet. were tested in 763 consecutive dermatitis patients.

Results. Of the tested materials, limonene-1-hydroperoxide gave most reactions, with 2.4% of the patients showing positive patch test reactions. Limonene-2-hydroperoxide and oxidized *R*-limonene gave 1.7% and 1.2% positive patch test reactions, respectively. Concomitant positive patch test reactions to other fragrance markers in the baseline series were frequently noted.

Conclusions. The results are in accordance with the experimental studies, as limonene-1-hydroperoxide gave more positive patch test reactions in the tested patients than limonene-2-hydroperoxide. Furthermore, the results support the specificity of the allergenic activity of the limonene hydroperoxide analogues and the importance of oxidized limonene as a cause of contact allergy.

Key words: allergic contact dermatitis; autooxidation; contact allergy; fragrances; hydroperoxides; limonene; patch testing.

R-Limonene (Fig. 1) is a common fragrance terpene that is widely used in cosmetics and in a variety of domestic and industrial products (1, 2). Pure *R*-limonene

has been shown to be non-allergenic in predictive testing in guinea-pigs (3), and a weak allergen in the murine local lymph node assay (LLNA) (4). Clinical studies have shown that *R*-limonene, tested in non-oxidized form but not specified for purity, seldom causes positive patch test reactions in dermatitis patients (5–7). However, *R*-limonene autooxidizes upon contact with air, forming allergenic oxidation products (3, 8–10). The oxidation mixtures from both enantiomeric forms (*R*-limonene and *S*-limonene) have been shown to be frequent causes of contact allergy in clinical testing (11–14). In earlier studies, oxidized *R*-limonene, tested

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Conflicts of interest: The authors have no conflicts of interest to declare.

Accepted for publication 10 December 2013

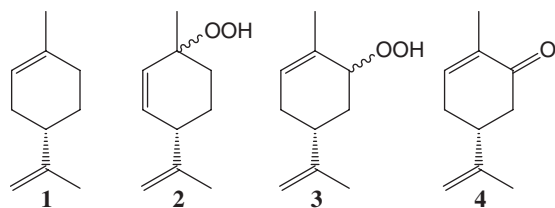


Fig. 1. Structures discussed in this study: *R*-limonene **1**; limonene-1-hydroperoxide [(4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide] **2**; limonene-2-hydroperoxide [(5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide] **3**; carvone **4**.

at a concentration of 3.0% in petrolatum, caused positive patch test reactions at a frequency of 2–3% in consecutive dermatitis patients (11–14). In a recent international multicentre patch test study on consecutive dermatitis patients, oxidized *R*-limonene 3.0% pet., with a stable and controlled concentration of the main haptens, limonene hydroperoxides, at 0.33% pet., gave, overall, 5.2% positive patch test reactions (range 2.3–12.1%) in 2900 patients (15). The variation in frequency was not related to the geographical area. The highest frequencies were seen in the south of Spain, Denmark, and Singapore, and the lowest were seen in Sweden and the north of Spain. In effect, the studies showed that oxidized *R*-limonene is an important sensitizer worldwide.

Upon air exposure of *R*-limonene, different oxidation products are formed, some of which are allergenic. The primary oxidation products, the hydroperoxides (Fig. 1), have been found to be the main allergens in the oxidation mixture (9, 11). They have the potential to form immunogenic hapten–protein complexes in skin via a radical mechanism, and have been identified as skin sensitizers by predictive testing in guinea-pigs (9) and mice (16). Carvone (Fig. 1) is a secondary oxidation product and has been shown to be a skin sensitizer in guinea-pig tests (3, 7) and in the LLNA (17, 18). Carvone is described in the literature as a clinical allergen (19, 20).

In an earlier experimental study (16), we investigated the sensitization potencies of two naturally occurring limonene hydroperoxides, limonene-1-hydroperoxide (Lim-1-OOH) and limonene-2-hydroperoxide (Lim-2-OOH) (Fig. 1), according to the ordinary LLNA protocol used for screening of potential skin sensitizers (21, 22). Both hydroperoxides were found to be potent sensitizers. Lim-1-OOH gave an EC₃ [the estimated concentration required to induce a stimulation index (SI) of 3] value of 0.019 M (0.33% wt/vol), and Lim-2-OOH gave an EC₃ value of 0.049 M (0.83% wt/vol) (16). To determine whether a statistically significant difference in the sensitization potencies could be observed, the hydroperoxides

were further studied in an LLNA with non-pooled lymph nodes (4). Statistical analysis of the SI calculated for each individual mouse showed that Lim-1-OOH was significantly more sensitizing than Lim-2-OOH (4).

In a subsequent small clinical study, 7 patients with known contact allergy to oxidized *R*-limonene were patch tested with the two limonene hydroperoxide analogues. All patients reacted to Lim-1-OOH, and 3 of 7 reacted to Lim-2-OOH (4). The results of both the clinical studies and the experimental studies suggest that the hydroperoxides act as specific haptens, forming specific hapten–protein complexes. This is in accordance with previous findings of no general cross-reactivity among hydroperoxides with various structures (23, 24).

The aim of the present study was to determine whether we could confirm a difference in clinical positive patch test reactions to the structurally closely related limonene hydroperoxides, Lim-1-OOH and Lim-2-OOH, when screening consecutive dermatitis patients. We also wanted to study the pattern of reactions to oxidized *R*-limonene (the oxidation mixture after air exposure of *R*-limonene), Lim-1-OOH, and Lim-2-OOH, and evaluate these three limonene patch test preparations as markers of contact allergy to oxidized limonene.

Materials and Methods

Chemicals

Lim-1-OOH [(4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide] and Lim-2-OOH [(5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide] (Fig. 1) were synthesized as previously described (16). The same batch of autoxidized *R*-limonene as used in the previous small clinical study (4) was used in the present study. *R*-Limonene (limonene) (99%) was bought from Millenium Speciality Chemicals (Jacksonville, FL, USA), and purified by distillation, after which it was air oxidized according to prior experience (3, 4). The content of *R*-limonene in the oxidation mixture was 55%, that of Lim-1-OOH was 1%, and that of Lim-2-OOH was 6%, as determined by high-performance liquid chromatography (HPLC).

Analytical method

HPLC analysis was conducted on a Merck LaChrom 2000 instrument with a diode array detector. An analytical silica column, Purospher® STAR (4.6 × 250 mm, 5-μm particles; Merck, Merck Darmstadt, Germany) was used. The temperature of the column and the sample rack in the autosampler was set to 20°C. The mobile phase consisted of 89% *n*-hexane, 10% *tert*-butyl methyl ether, and 1%

methanol; isocratic elution was performed for 20 min; and the flow rate was 1 ml/min. The separations were monitored at 205 nm. The injection volume was 30 µl. Pure reference compounds were used to make external calibration curves, from which the concentrations of limonene and limonene hydroperoxides in oxidized limonene were determined.

Patch test materials

Patch test materials for the study were prepared at the Dermatochemistry and Skin Allergy unit at the Department of Chemistry and Molecular Biology, University of Gothenburg. The oxidation mixture (oxidized *R*-limonene) and the two synthesized hydroperoxides were stored under argon at -20°C until test preparations were made. Chemical analyses were performed on a regular basis to check the quality. Whenever patch test materials were produced, the composition of the oxidation mixture was checked and found to be intact, that is, in accordance with the analytical result obtained at the start of the study. When patch test materials were made from the isolated hydroperoxides, these were also thoroughly analysed, and, if degradation was seen, they were purified before patch test materials were made.

New patch test preparations were made every 2–3 months in non-stabilized white pet. (Kebo Lab A/S, Albertslund, Denmark). Oxidized *R*-limonene 3.0% (wt/wt) in pet. was placed in 5-ml syringes, and the hydroperoxides (Lim-1-OOH and Lim-2-OOH) 0.5% (wt/wt) in pet. were placed in 1-ml syringes. One syringe (1 ml) of each hydroperoxide preparation was used at a time, and the rest were kept at -20°C until being used. The test preparations for oxidized *R*-limonene and the hydroperoxide syringes in current use were kept refrigerated.

Standard allergens used for patch testing were obtained from Chemotechnique Diagnostics (Vellinge, Sweden). Oxidized linalool used in the current study and in another study was tested concomitantly at 6.0% pet (25). The patch test material for oxidized linalool was prepared at the Dermatochemistry and Skin Allergy unit, University of Gothenburg.

Clinical studies

Consecutive patients undergoing patch testing because of suspected allergic contact dermatitis at the Department of Dermatology, Sahlgrenska University Hospital, Gothenburg, Sweden were screened with oxidized *R*-limonene (3.0% pet.), Lim-1-OOH (0.5% pet.), and Lim-2-OOH (0.5% pet.), in addition to regular patch testing. Patch test

preparations of ~ 20 mg (26) were applied in small Finn Chambers[®] (diameter of 8 mm; inner area of 0.5 cm²; Epitest Ltd Oy, Tuusula, Finland) on Scanpor[®] tape (Norgesplaster A/S, Vennsela, Norway) to the back of the patient, left under occlusion for 48 hr, and then removed by the patient. The dose per unit area was approximately 1.2 mg/cm² of oxidized *R*-limonene 3.0% pet. and 0.2 mg/cm² of the tested hydroperoxide analogues at 0.5% pet. Visual readings were scored according to the International Contact Dermatitis Research Group guidelines (27) on D3–4 and D6–7.

The study was performed between November 2007 and November 2009. In total, 763 patients were tested with the limonene preparations. Owing to a temporary shortage of some of the patch test materials, not all patients were tested with all materials. Those not tested with all three limonene preparations were not included in the analyses. Concomitant positive patch test reactions to other fragrance allergens and/or colophonium and oxidized linalool in the baseline series were compiled for all patients showing positive patch test reactions to any of the studied patch test materials, and also for all tested patients in the test period. When there were positive reactions to any of the test materials, questions regarding exposure to limonene-containing products were asked. A positive patch test reaction was assessed as relevant if the patient used products in which limonene was declared among the contents at the site of eczema, or if the patient had come into contact with products expected to contain limonene (i.e. citrus flavourings, citrus-based degreasers, or citrus-based paint thinners) at the site of eczema.

Statistics

McNemar's exact test was used to compare the numbers of patients reacting to the tested substances, as the same patients were tested with all three materials. Analyses were performed with R version 2.6.0 (The R foundation for Statistical Computing, Vienna, Austria).

Results

The frequencies of positive patch test reactions to oxidized *R*-limonene 3.0% pet., Lim-1-OOH 0.5% pet. and Lim-2-OOH 0.5% pet. among the consecutively tested patients are shown in Table 1. The test results from the same time period for Fragrance Mix I, Fragrance Mix II, *Myroxylon pereirae*, colophonium and oxidized linalool are also shown in Table 1. In all, 25 of 763 patients showed positive patch test reactions to any of the limonene patch test preparations. Of these, only 3 of 25 showed positive patch test reactions to all three test preparations. The

Table 1. Positive patch test reactions to oxidized *R*-limonene, limonene-1-hydroperoxide, limonene-2-hydroperoxide, fragrance markers (Fragrance Mix I, Fragrance Mix II, *Myroxylon pereirae*, and colophonium) and oxidized linalool in the patients patch tested at Sahlgrenska University Hospital, Gothenburg, Sweden between November 2007 and November 2009

Patch test material	Total tested	Positive	%
Oxidized <i>R</i> -limonene, 3.0%	763 ^a	9 ^b	1.2
Limonene-1-hydroperoxide, 0.5%	763 ^a	18 ^b	2.4
Limonene-2-hydroperoxide, 0.5%	763 ^a	13	1.7
Fragrance Mix I	1187	81	6.8
Fragrance Mix II	1178	21	1.8
<i>Myroxylon pereirae</i>	1180	68	5.8
Colophonium	1212	31	2.6
Oxidized linalool, 6.0%	969	38	3.9

^aOnly patients tested with all three limonene preparations were included in the test population. Owing to a periodical shortage of patch test materials, not all patients who were tested during the time period were tested with all three limonene patch test preparations.

^bA statistically significant difference was found between the number of patients reacting to oxidized limonene and the number reacting to limonene-1-hydroperoxide ($p=0.035$). No other statistically significant differences were found.

pattern of concomitant reactions to oxidized limonene and limonene hydroperoxides observed in the patients is shown in Table 2.

Nine of the 25 patients showed positive patch test reactions to oxidized *R*-limonene (Table 2). As stated above, 3 of the 9 patients showing positive patch test reactions to oxidized *R*-limonene also reacted to both Lim-1-OOH and Lim-2-OOH. In total, 6 of 9 reacted to Lim-1-OOH and 4 of 9 reacted to Lim-2-OOH in addition to reacting to oxidized *R*-limonene. Two of 9 did not react to any of the limonene hydroperoxides, and thus only reacted to the oxidation mixture of *R*-limonene.

Eighteen of the 25 patients showed positive patch test reactions to Lim-1-OOH (Table 2). Three of the 18 also reacted to both oxidized *R*-limonene and Lim-2-OOH. In total, 8 of 18 showed positive patch test reactions to Lim-2-OOH in addition to Lim-1-OOH, and 6 showed positive

patch test reactions to oxidized *R*-limonene in addition to Lim-1-OOH. Seven of 18 showed positive patch test reactions only to Lim-1-OOH.

On a similar note, 13 of the 25 patients showed positive patch test reactions to Lim-2-OOH (Table 2). Three of the 13 reacted to both oxidized *R*-limonene and Lim-1-OOH, as stated above. In total, 8 of 13 showed positive patch test reactions to Lim-1-OOH in addition to Lim-2-OOH, and 4 of 13 showed positive patch test reactions to oxidized *R*-limonene in addition to Lim-2-OOH. Four of 13 showed positive patch test reactions only to Lim-2-OOH.

A statistically significant difference was found between the number of patients reacting to oxidized limonene and the number reacting to Lim-1-OOH ($p=0.035$). No other statistically significant differences were found.

The results for individual patients are shown in Table 3. The locations of eczema resulting in the patch testing are shown in Table 3. Overall, the face or head (in 9 of 25 patients) and hands (in 10 of 25 patients) were the most common sites of dermatitis in the patients showing positive patch test reactions to oxidized *R*-limonene or the hydroperoxide analogues. Exposures to limonene-containing domestic or occupational products that were identified at the readings are also shown in Table 3.

Concomitant reactions to other fragrance markers in the baseline series, colophonium and oxidized linalool are shown in Table 4. Seven of 9 patients reacting to oxidized *R*-limonene also showed concomitant reactions to other fragrances and/or colophonium, and 8 of 18 patients reacting to Lim-1-OOH and 8 of 13 patients reacting to Lim-2-OOH showed such concomitant reactions. Concomitant reactions to oxidized linalool were common: 5 of 9, 7 of 18 and 7 of 13 patients reacting to oxidized *R*-limonene, Lim-1-OOH, and Lim-2-OOH, respectively, also showed concomitant reactions to oxidized linalool. Overall, 78% of the patients reacting to oxidized *R*-limonene, 55% of the patients reacting to Lim-1-OOH and 69% of the patients reacting to Lim-2-OOH showed concomitant reactions to other fragrances and/or colophonium and/or oxidized linalool.

Table 2. Pattern of reactions observed in the patients reacting to oxidized *R*-limonene, limonene-1-hydroperoxide and limonene-2-hydroperoxide, showing the concomitant reactions to the other tested limonene patch test materials (i.e. oxidized *R*-limonene, limonene-1-hydroperoxide, and limonene-2-hydroperoxide)

Positive reactions to	Also positive reactions to:			No positive reactions to other limonene markers
	Oxidized <i>R</i> -limonene	Limonene-1-hydroperoxide	Limonene-2-hydroperoxide	
Oxidized <i>R</i> -limonene	9 (total)	6	4	2
Limonene-1-hydroperoxide	6	18 (total)	8	7
Limonene-2-hydroperoxide	4	8	13 (total)	4

Table 3. Clinical data from individual patients showing maximum positive patch test readings to oxidized *R*-limonene, limonene-1-hydroperoxide, and limonene-2-hydroperoxide; the table also shows concomitant positive patch test reactions to fragrance markers and/or colophonium in the baseline series, as well as to oxidized linalool 6.0% pet.

	Age (years)	Sex	Oxidized limonene 3.0%	Limonene-1-hydroperoxide 0.5%	Limonene-2-hydroperoxide 0.5%	Other fragrance markers and oxidized linalool					Location of eczema	Relevant exposure ^a
						Fragrance Mix I	Fragrance Mix II	<i>Myroxylon perellae</i>	Colophonium	Oxidized linalool		
2007	64	F	?	++	++	+	-	+	-	+	Hands	Cleaner
	49	M	-	+	+	-	-	-	-	+	Legs	-
	36	F	-	+	?	-	-	-	-	-	Around mouth	-
	36	M	++	++	++	+	-	+	-	++	Foot	-
2008	33	F	-	+	-	-	-	-	-	-	Axillae	Deodorant
	33	F	-	-	+	-	-	+	-	-	Dry skin, eczema on hands	Shower gel
	81	F	+++	+	+	++	?	+	+	++	Torso	-
	74	F	+	-	-	-	-	+	-	+	Axillae, torso	Positive reaction to deodorant
	66	F	+	+	-	-	-	-	-	-	Hands	Works in cookie factory
	72	F	-	++	+	-	-	-	-	-	Around ears	-
	55	F	?	+	+	?	-	++	++	+	Face	Many perfumed products
	47	F	-	-	+	-	-	-	-	-	Arms, hands	Works with paints
	33	F	+	+	+	++	-	++	++	+	Hands	-
	21	M	?	?	+	++	-	++	-	+	Around mouth	-
2009	49	F	-	+	-	-	-	-	-	-	Face	-
	21	F	-	+	-	-	-	-	-	-	Scalp	-
	14	F	-	+	-	-	-	-	-	-	Face, arms	Shampoo
	54	F	+	+	-	-	-	+	-	-	Face, around mouth	-
	32	M	+	+	?	+	-	?	-	-	Hands, feet, arms	Baker, pizza maker
	32	F	++	-	++	-	-	-	-	-	Genital area	-
	24	F	+	?	-	++	-	-	-	+	Hands	-
	57	F	-	+	+	-	-	++	-	-	Hands	-
	56	F	-	+	?	-	-	-	-	+	Hands, torso	Works in biscuit factory
	14	M	-	+	-	-	-	-	-	-	Face	-
	38	F	-	-	++	-	-	-	-	-	Hands	Sunscreen

^aWhen positive readings for any of the test materials were obtained, questions regarding exposure to limonene-containing products were asked. A positive patch test reaction was assessed as relevant if the patient used products in which limonene was declared among the contents at the site of eczema or if the patient came into contact with products expected to contain limonene (i.e. citrus flavouring, citrus-based degreasers, or citrus-based paint thinners) at the site of eczema.

Table 4. Concomitant reactions to fragrance markers, colophonium and/or oxidized linalool among the patients showing positive patch test reactions to oxidized *R*-limonene, limonene-1-hydroperoxide, and limonene-2-hydroperoxide

Patch test material	Total tested	Positive		Fragrance Mix I		Fragrance Mix II		Myroxylon pereirae		Colophonium		> 1 fragrance marker and/or colophonium		Oxidized linalool		> 1 fragrance marker and/or colophonium and/or oxidized linalool	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Oxidized <i>R</i> -limonene, 3.0%	763	9	1.2	5	45	0	–	5	36	2	2	7	78	5	45	7	78
Limmonene-1-hydroperoxide, 0.5%	763	18	2.4	5	28	0	–	7	39	3	17	8	44	7	39	10	55
Limmonene-2-hydroperoxide, 0.5%	763	13	1.7	5	38	0	–	8	61	3	23	8	61	7	55	9	69
Overall	763	25	3.3	7	28	0	–	10	40	3	12	12	48	10	40	14	56

Discussion

In the present study, we observed a persistent, although not statistically significant, difference in the number of positive patch test reactions to the closely related limonene hydroperoxides Lim-1-OOH and Lim-2-OOH, with more patients reacting to Lim-1-OOH. Despite the lower concentration of Lim-1-OOH found in the oxidation mixture of limonene [quantified at 1%, as compared with 6% of Lim-2-OOH (4)], Lim-1-OOH was found to give more reactions in patch testing of consecutive dermatitis patients in a clinical setting. Thus, the experimental finding that Lim-1-OOH was a stronger sensitizer in the LLNA may be relevant for the clinical prevalence of contact allergy to this analogue. In addition, the present study provides further evidence that the hydroperoxides act as specific haptens, forming specific hapten–protein complexes, as 15 of 23 patients reacting to hydroperoxides reacted to only one of the two tested analogues. This finding is in accordance with earlier studies showing a lack of cross-reactivity and/or the formation of unspecific antigens among hydroperoxides with various structures (23, 24).

We observed in an earlier experimental study that Lim-1-OOH was more sensitizing than Lim-2-OOH in a modified LLNA (4). The closely related hydroperoxides showed a statistically significant difference in sensitization potencies according to the LLNA with non-pooled lymph nodes. Furthermore, Lim-1-OOH detected more cases of contact allergy than did Lim-2-OOH in a small group of limonene-allergic patients (4). The results from the present enlarged clinical study are in accordance with the earlier findings. Again, Lim-1-OOH detected more allergic patients, and thus appears to be the clinically most important allergen. A statistically significant difference was found between the number of patients reacting to oxidized limonene and the number reacting to Lim-1-OOH ($p = 0.035$). No other statistically significant differences were found in this rather limited study population. It should be remembered that the observed simultaneous reactions must be viewed in the light of concomitant exposure, as the hydroperoxides act as specific allergens. For complex mixtures such as oxidized limonene, the composition can vary, depending on, for example, the source of raw material and storage. Thus, individuals can be exposed to various mixtures with unknown contents of different hydroperoxides.

The observation that more patients showed positive patch test reactions to Lim-1-OOH than to Lim-2-OOH, might be attributable to a greater ability of Lim-1-OOH than of Lim-2-OOH to form immunogenic hapten–protein complexes. Radical trapping experiments have been conducted to investigate probable routes for the formation of hapten–protein complexes from

limonene hydroperoxides that might take place in the skin (16). In these investigations, both carbon-centred and oxygen-centred radicals were formed from the limonene hydroperoxides. Lim-2-OOH formed short-lived radical species that produced mainly carvone, which is a moderate sensitizer according to the LLNA (EC3 value of 0.86 M, 13% wt/vol). The short-lived radical species would account for the importance of Lim-2-OOH (EC3 value of 0.049 M, 0.83% wt/vol) as a sensitizer in the oxidation mixture, but the rapid formation of the less sensitizing carvone would diminish the sensitizing effect of Lim-2-OOH. In contrast, Lim-1-OOH (EC3 value of 0.019 M, 0.33% wt/vol) produced large amounts of stable oxygen-centred and carbon-centred radicals. In fact, 18 times more of the carbon-centred radical and ~10 times more of the oxygen-centred radical were trapped from Lim-1-OOH than from Lim-2-OOH (16). The differences in the formation and stability of radicals are closely connected to the structures of the investigated hydroperoxides, and could explain the significant difference in sensitizing potency between the two structural analogues (4).

In previous studies, a hydroperoxide fraction of oxidized limonene containing both Lim-1-OOH and Lim-2-OOH was tested in patients in addition to oxidized limonene (11, 13). In those studies, approximately half of the patients reacting to oxidized limonene did not react to the fraction. In the present study, 7 of 9 patients showing positive patch test reactions to oxidized limonene reacted to one or both limonene hydroperoxide analogues; 6 of these reacted to Lim-1-OOH, and 4 reacted to Lim-2-OOH. In the earlier studies, a fraction was isolated from oxidized *R*-limonene and tested at 0.5% pet., which would correspond to approximately 0.4% of Lim-2-OOH and 0.07% of Lim-1-OOH, if the composition corresponded to the oxidation mixture in the present study. Thus, a similar frequency of reactions to Lim-2-OOH, tested at a comparable concentration, was seen. Lim-1-OOH could not be quantified at the time of the earlier studies, but the concentration of Lim-1-OOH was much lower in the earlier studies than in the present study. The additional cases found with this limonene marker would therefore not have been detected in the earlier study.

In the present study, only 9 of the 25 patients showing positive patch test reactions to any of the test preparations reacted to oxidized *R*-limonene; thus, 64% (16 of 25) of the total number of patients were detected with using the limonene hydroperoxide preparations. This is most probably attributable to the higher concentration of the hapten in the hydroperoxide preparations than that used in testing with the total oxidation mixture. In effect, many of the patients with positive patch test reactions to the main allergens of air-oxidized limonene in this study

would not have been identified with the oxidation mixture of limonene alone. However, there are problems in using hydroperoxide preparations, which may support use of the oxidation mixture. First, the oxidation mixture is expected to represent a steady-state system, whereby, when it is stored and handled according to the instructions, its composition will be maintained, as limonene will continuously oxidize as degradation continues. In the recent multicentre study, careful analyses of remaining patch test material could be performed, owing to a newly developed solid-phase extraction technique (15, 28). These analyses showed that the patch test material was stable while it was used and handled in a clinical setting for 3 months. Owing to the lability of hydroperoxides, it can be expected that degradation will take place more readily in a hydroperoxide preparation, thus leading to loss of efficacy as secondary oxidation products are formed. Much investigative work on stability and analyses needs to be performed before such preparations can be offered. Also, the synthesis of hydroperoxide test material is complicated, and cannot currently be performed on a large scale. Thus, testing with a carefully controlled oxidation mixture may currently be the most efficient way to diagnose contact allergy to oxidized limonene.

In clinical studies on oxidized fragrance terpenes, there is often the question of how the patients have been exposed to the oxidation products causing sensitization. Showing the presence of hydroperoxides and quantification of hydroperoxides in formulated products has been difficult, for technical reasons. Hydroperoxides are labile compounds and will disintegrate if heated, for example for gas chromatographic analyses. However, new methodology has proven to be effective, and limonene hydroperoxides have recently been shown to be present in natural sweet orange oil, both at delivery from the producer, and in increasing amounts after storage and handling (29). We have previously shown that linalool hydroperoxides are formed in lavender oil of both natural and synthetic origin (30). We have also recently reported on a repeated open application test in which low concentrations of oxidized linalool caused eczema in allergic patients, adding evidence for the clinical relevance of the positive patch test reactions (31). Most probably, in our view, oxidation products from fragrance terpenes will be found in a multitude of products in the future where the non-oxidized terpene is declared. The oxidation could take place before the fragrance components are added to the product [as in the sweet orange oil (29)], or at a later time, during storage and handling. Addition of an anti-oxidant will be effective, but only as long as the anti-oxidant is present. When the anti-oxidant is consumed, the oxidation starts immediately,

as has been shown for limonene and BHA (butylated hydroxytoluene) (32). Eventually, further work with analyses and improvements in formulations will increase protection of the consumer.

The clinical relevance of the positive patch test reactions to oxidized *R*-limonene and the limonene hydroperoxides is supported by the high frequency of concomitant reactions to other fragrance markers in the baseline series (Table 4). With 12 of 25 (48%) patients reacting to one or more fragrance markers in the baseline series and/or colophonium, this is in line with previously published studies on such concomitant reactions among fragrance allergens (12, 14, 15, 28). Interestingly, many concomitant reactions to oxidized linalool were recorded among the patients reacting to the limonene markers. If these are included, almost 80% of the patients reacting to oxidized limonene, 55% of the patients reacting to Lim-1-OOH and 70% of the patients reacting to Lim-2-OOH had concomitant reactions to other fragrance allergens. Although both oxidized *R*-limonene and oxidized linalool have hydroperoxides as main allergens, no cross-reactivity, according to the discussion above, would be expected (4, 23, 24). Thus, concomitant sensitizations, owing to the widespread concomitant presence of limonene, linalool and other fragrance chemicals in fragranced products, remain the most plausible explanation for the concomitant positive patch test reactions.

Conclusion

The results are in accordance with the experimental studies, as Lim-1-OOH gave more positive patch test reactions in the tested patients than Lim-2-OOH, although

oxidized *R*-limonene, Lim-1-OOH and Lim-2-OOH all detected additional cases not detected with the other limonene markers. The formation of large amounts of stable oxygen-centred and carbon-centred radicals from Lim-1-OOH may be an explanation for the increased reactivity to this analogue. The results support the specificity of the allergenic activity of the limonene hydroperoxide analogues.

Although, in the present study, Lim-1-OOH and Lim-2-OOH detected the largest number of allergic individuals, there are potential problems with the use of these test materials on a large scale. Oxidized *R*-limonene as a test material has been shown to be stable, and also contains other sensitizers, such as carvone, and the stability of the single hydroperoxides in pet. is of concern. Overall, the test results confirm the importance of air-oxidized limonene and its main allergens as important patch test materials in dermatitis patients. The possibility of using oxidized *R*-limonene and/or single allergens from the oxidation mixture for screening of consecutive dermatitis patients is important, and needs to be further investigated.

Acknowledgements

The skilful statistical assistance of Martin Gillstedt is gratefully acknowledged. Lina Hagvall is gratefully acknowledged for valuable discussions. The work was financially supported by the Swedish Asthma and Allergy Association, Stockholm, Sweden, the Welanders-Finsen Foundation, Stockholm, Sweden, and ALF Västra Götalands Region, Sweden. The work was performed within SkinResQu at the University of Gothenburg, Sweden.

References

- 1 Rastogi S C, Heydorn S, Johansen J D, Basketter D A. Fragrance chemicals in domestic and occupational products. *Contact Dermatitis* 2001; **45**: 221–225.
- 2 Buckley D A. Fragrance ingredient labelling in products on sale in the UK. *Br J Dermatol* 2007; **157**: 295–300.
- 3 Karlberg A-T, Boman A, Melin B. Animal experiments on the allergenicity of d-limonene – the citrus solvent. *Ann Occup Hyg* 1992; **35**: 419–426.
- 4 Bråred Christensson J, Johansson S, Hagvall L, Jonsson C, Borje A, Karlberg A-T. Limonene hydroperoxide analogues differ in allergenic activity. *Contact Dermatitis* 2008; **59**: 344–352.
- 5 Santucci B, Cristaudo A, Cannistraci C, Picardo M. Contact dermatitis to fragrances. *Contact Dermatitis* 1987; **16**: 93–95.
- 6 Schnuch A, Uter W, Geier J, Lessmann H, Frosch P J. Sensitization to 26 fragrances to be labelled according to current European regulation. Results of the IVDK and review of the literature. *Contact Dermatitis* 2007; **57**: 1–10.
- 7 Bruze M, Svedman C, Andersen K E et al. Patch test concentrations (doses in mg/cm²) for the 12 non-mix fragrance substances regulated by European legislation. *Contact Dermatitis* 2012; **66**: 131–136.
- 8 Karlberg A-T, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis* 1992; **26**: 332–340.
- 9 Karlberg A-T, Shao L P, Nilsson U, Gälfvert E, Nilsson J L G. Hydroperoxides in oxidized d-limonene identified as potent contact allergens. *Arch Dermatol Res* 1994; **286**: 97–103.
- 10 Nilsson U, Bergh M, Shao L P, Karlberg A-T. Analysis of contact allergenic compounds in oxidized d-limonene. *Chromatographia* 1996; **42**: 199–205.
- 11 Karlberg A-T, Doms-Goossens A. Contact allergy to oxidized d-limonene among dermatitis patients. *Contact Dermatitis* 1997; **36**: 201–206.
- 12 Matura M, Goossens A, Bordalo O, Garcia-Bravo B, Magnusson K, Wrangsjö K, Karlberg A-T. Oxidized citrus oil (*R*-limonene): a frequent sensitizer in Europe. *J Am Acad Dermatol* 2002; **47**: 709–714.
- 13 Matura M, Goossens A, Bordalo O, Garcia-Brava B, Magnusson K, Wrangsjö

- K, Karlberg A-T. Patch testing with oxidized R-(+)-limonene and its hydroperoxide fraction. *Contact Dermatitis* 2003; **49**: 15–21.
- 14 Matura M, Sköld M, Börje A et al. Not only oxidized R-(+)- but also S-(–)-limonene is a common cause of contact allergy in dermatitis patients in Europe. *Contact Dermatitis* 2006; **55**: 274–279.
 - 15 Bråred Christensson J, Andersen K E, Bruze M et al. An international multicentre study on the allergenic activity of air-oxidized R-limonene. *Contact Dermatitis* 2013; **68**: 214–223.
 - 16 Johansson S, Giménez-Arnau E, Grøtli M, Karlberg A-T, Börje A. Carbon- and oxygen-centered radicals are equally important haptens of allylic hydroperoxides in allergic contact dermatitis. *Chem Res Toxicol* 2008; **21**: 1536–1547.
 - 17 Bergström M A, Luthman K, Nilsson J L G, Karlberg A-T. Conjugated dienes as prohaptens in contact allergy: in vivo and in vitro studies of structure–activity relationships, sensitizing capacity and metabolic activation. *Chem Res Toxicol* 2006; **19**: 760–769.
 - 18 Nilsson A-M, Bergström M A, Luthman K, Nilsson J L G, Karlberg A-T. An alpha,beta-unsaturated oxime identified as a strong contact allergen. Indications of antigen formation via several pathways. *Food Chem Toxicol* 2005; **43**: 1627–1636.
 - 19 Andersen K E. Contact allergy to toothpaste flavors. *Contact Dermatitis* 1978; **4**: 195–198.
 - 20 Paulsen E, Andersen K E, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? *Contact Dermatitis* 1993; **29**: 138–143.
 - 21 Kimber I, Hilton J, Dearman R J et al. An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology* 1995; **20**: 63–73.
 - 22 Gerberick G F, Ryan C A, Dearman R J, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007; **41**: 54–60.
 - 23 Bråred Christensson J, Matura M, Bäcktorp C, Börje A, Nilsson J L, Karlberg A-T. Hydroperoxides form specific antigens in contact allergy. *Contact Dermatitis* 2006; **55**: 230–237.
 - 24 Gäfvert E, Shao L P, Karlberg A T, Nilsson U, Nilsson J L. Contact allergy to resin acid hydroperoxides. Hapten binding via free radicals and epoxides. *Chem Res Toxicol* 1994; **7**: 260–266.
 - 25 Bråred Christensson J, Matura M, Gruvberger B, Bruze M, Karlberg A-T. Linalool – a significant contact sensitizer after air exposure. *Contact Dermatitis* 2010; **62**: 32–41.
 - 26 Bruze M, Isaksson M, Gruvberger B, Frick-Engfeldt M. Recommendation of appropriate amounts of petrolatum preparation to be applied at patch testing. *Contact Dermatitis* 2007; **56**: 281–285.
 - 27 Wahlberg J E, Lindberg M. Patch testing. In: *Contact Dermatitis*, 4th edition, Frosch P, Menné T, Lepoittevin J P (eds): Berlin, Heidelberg, New York, Springer, 2006: pp. 365–390.
 - 28 Bråred Christensson J, Andersen K E, Bruze M et al. Air-oxidized linalool: a frequent cause of fragrance contact allergy. *Contact Dermatitis* 2012; **67**: 247–259.
 - 29 Rudbäck J, Islam N, Nilsson U, Karlberg A-T. A sensitive method for determination of allergenic fragrance terpene hydroperoxides using liquid chromatography coupled with tandem mass spectrometry. *J Sep Sci* 2013; **36**: 1370–1378.
 - 30 Hagvall L, Sköld M, Bråred-Christensson J, Börje A, Karlberg A-T. Lavender oil lacks natural protection against autooxidation, forming strong contact allergens on air exposure. *Contact Dermatitis* 2008; **59**: 143–150.
 - 31 Andersch Björkman Y, Hagvall L, Siwmark C, Niklasson B, Karlberg A-T, Bråred Christensson J. Air oxidized linalool elicits eczema in allergic patients – a repeated open application test study. *Contact Dermatitis* 2013 (Accepted for publication).
 - 32 Karlberg A-T, Magnusson K, Nilsson U. Influence of an anti-oxidant on the formation of allergenic compounds during auto-oxidation of d-limonene. *Ann Occup Hyg* 1994; **38**: 199–207.