

Limonene hydroperoxide analogues differ in allergenic activity

JOHANNA BRÅRED CHRISTENSSON^{1,2}, STAFFAN JOHANSSON¹, LINA HAGVALL¹, CHARLOTTE JONSSON¹,
ANNA BÖRJE¹ AND ANN-THERESE KARLBERG¹

¹Dermatochemistry and Skin Allergy, Department of Chemistry, University of Gothenburg, SE-412 96 Gothenburg, and ²Department of Dermatology, Sahlgrenska University Hospital, SE-413 45 Gothenburg, Sweden

Background: The fragrance terpene *R*-limonene is a very weak sensitizer but forms allergenic oxidation products upon contact with air. Oxidized (ox.) limonene is a frequent cause of contact allergy in clinical testing.

Objectives: This study investigates the sensitizing potencies of ox. and non-ox. limonene and of structurally closely related limonene hydroperoxides. The clinical importance of the difference in sensitizing potency of two hydroperoxides in autoxidized limonene was studied.

Patients/Methods: Ox. and non-ox. limonene were investigated in the murine local lymph node assay (LLNA). Limonene hydroperoxides were investigated using a modified LLNA involving non-pooled lymph nodes and statistical calculations; patch testing of patients with known contact allergy to ox. limonene was performed.

Results: A marked increase in the sensitizing potency of ox. limonene compared with that of pure limonene was observed in the LLNA. One analogue, limonene-1-hydroperoxide, was a significantly more potent sensitizer than the other hydroperoxides and gave more positive test reactions in the allergic patients.

Conclusions: The results support that hydroperoxides have a specific reactivity indicating that oxygen-centred radicals are important in hapten–protein complex formation of hydroperoxides. The primary oxidation products of ox. limonene, the hydroperoxides, have an important impact on the sensitizing capacity of the oxidation mixture.

Key words: allergic contact dermatitis; autoxidation; fragrances; hydroperoxides; limonene; limonene hydroperoxides; local lymph node assay; patch testing; structure activity relationship.
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R-Limonene (Fig. 1) is a common fragrance terpene, widely used in cosmetics and in a variety of domestic and industrial products (1). Pure *R*-limonene (limonene) has been demonstrated to be non-allergenic in predictive testing in guinea-pigs (2). Clinical studies have shown that limonene itself seldom causes positive patch test reactions in dermatitis patients (3, 4). However, limonene autoxidizes upon contact with air forming allergenic oxidation products (2, 5–7), and air-oxidized *R*-limonene (ox. limonene) causes positive patch test reactions in a frequency of 2–3% in consecutive dermatitis patients (8–11).

To act as a hapten, a chemical needs to bind to macromolecules (proteins) in the skin. Some com-

pounds are not active themselves but must be activated either by skin metabolism or outside the body, the latter often by air oxidation (12). The name prehapten has been introduced for those compounds activated outside the body (13, 14), saving the name prohaptens for those compounds that are activated by specific enzymatic systems of the skin. Limonene (in both enantiomeric forms *R*- and *S*-) is an example of a prehapten. On air exposure, different oxidation products are formed, some of which are allergenic. The primary oxidation products are the hydroperoxides. They most probably form immunogenic hapten–protein complexes in the skin through a radical mechanism and have been identified as skin sensitizers

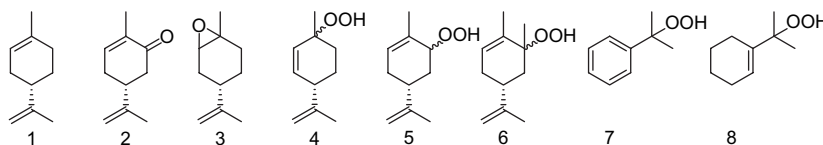


Fig. 1. Structures of limonene and identified oxidation products discussed in this study: *R*-limonene (1), carvone (2), limonene oxide (3), limonene-1-hydroperoxide [(4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide (Lim-1-OOH)] (4), limonene-2-hydroperoxide [(5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (Lim-2-OOH)] (5), limonene-2-methyl-2-hydroperoxide [(5*R*)-5-isopropenyl-1,2-dimethyl-2-cyclohexene-1-hydroperoxide (Lim-2-Me-2-OOH)] (6), cumene hydroperoxide (7), and 1-(1-hydroperoxy-1-methylethyl) cyclohexene (cyclohexene hydroperoxide) (8).

by predictive testing in guinea-pigs (7) and mice (15). Carvone and limonene oxide (Fig. 1) are secondary oxidation products from which hapten–protein complexes can be formed by nucleophilic attacks from electron-rich sites in the proteins. Both compounds are considered as skin sensitizers in guinea-pig tests (2, 5) as well as according to the murine local lymph node assay (LLNA) (16, 17). Carvone is described in literature as a clinical allergen (18, 19).

Hydroperoxides of linalool, geraniol, and linalyl acetate have so far been studied in the LLNA (20–22). They show homogeneous sensitizing potencies in contrast to many other groups of haptens with a structural alert in common where larger differences in the sensitizing potencies are observed (12 and references therein). As hydroperoxides are oxidizing agents, they may oxidize functional groups in the proteins, e.g. the phenolic groups in tyrosine or the sulfur moieties of cysteine and methionine. If so, structurally different hydroperoxides theoretically could form the same oxidized and immunogenic proteins. Hydroperoxides reacting in this manner would be non-specific agents, and it could be anticipated that a similar sensitizing potency would be seen for a wide variety of hydroperoxides. Furthermore, cross-reactions between hydroperoxides with significantly different structures would be observed in animal experiments or at patch testing. We have earlier investigated potential cross-reactivity between hydroperoxides with different structures in guinea-pigs but found that only structurally closely related hydroperoxides showed a pattern of cross-reactivity (23). Cross-reactivity was seen between cumene hydroperoxide (Fig. 1) and its corresponding cyclohexene hydroperoxide (Fig. 1), while no cross-reactivity was observed between limonene-2-hydroperoxide (Lim-2-OOH) (Fig. 1) and cumene hydroperoxide (23). In a recent study (15), the sensitizing capacity of limonene hydroperoxides was investigated according to the ordinary LLNA protocol used for screening (24, 25). The results showed the hydroperoxides to be potent sensitizers. However, a certain difference in sensitizing potency between the two major hy-

droperoxides of ox. limonene, limonene-1-hydroperoxide (Lim-1-OOH) (Fig. 1) and Lim-2-OOH, was observed (15). Because the lymph nodes of the mice in the different groups are pooled together before measurement in the LLNA used for screening, this method does not provide data for a statistical evaluation of a possible difference in the sensitizing potencies observed for two compounds.

The aim of the present study was to investigate if we could demonstrate a statistically significant difference in sensitizing potency between structurally closely related limonene hydroperoxides by using a modified LLNA involving non-pooled lymph nodes and statistical calculations. The compounds studied were Lim-1-OOH [EC3 (see explanation under *Sensitization experiments*) value 0.019 M (0.33% w/v)] and Lim-2-OOH [EC3 value 0.049 M (0.83% w/v)] from ox. limonene and a model compound limonene-2-methyl-2-hydroperoxide (Lim-2-Me-2-OOH) (Fig. 1) [EC3 value 0.071 M (1.29% w/v)], all as mixtures of diastereomers. As the allergenic activity of pure limonene and ox. limonene only have been determined in guinea-pigs, we also wanted to investigate the sensitizing potencies of these using the murine LLNA to be able to visualize the effect of the hydroperoxides on the sensitizing capacity of the ox. limonene. Furthermore, we wanted to investigate if a difference in the sensitizing potency of the two hydroperoxides in ox. limonene would be of any clinical importance.

Materials and Methods

Chemicals

Limonene-1-hydroperoxide [(4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide (Lim-1-OOH)], limonene-2-hydroperoxide [(5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (Lim-2-OOH)], and limonene-2-methyl-2-hydroperoxide [(5*R*)-5-isopropenyl-1,2-dimethyl-2-cyclohexene-1-hydroperoxide (Lim-2-Me-2-OOH)] (Fig. 1) were synthesized as previously described (15). The purity of the synthesized hydroperoxides was

determined to be >96% using nuclear magnetic resonance spectroscopy.

R-Limonene (limonene) (98%) from Frey and Lau (Henstedt-Ulzburg, Germany) was air oxidized according to prior experience (2). A sample of 100 ml was oxidized by exposure to air in an open Erlenmeyer flask in room temperature without direct sunlight. It was gently stirred four times a day 1 hr at a time. After 10 weeks, the content of limonene had decreased to 59%, whereas Lim-2-OOH had accumulated to 7% as determined by high-performance liquid chromatography (HPLC). The ox. limonene was stored in the freezer under argon and used in experimental sensitization tests according to the LLNA and for patch test preparations for testing of consecutive dermatitis patients during the years 2004–2006. A second batch of *R*-limonene (limonene) (99%) was bought from Millenium Speciality Chemicals (Jacksonville, FL, USA) and purified by distillation and tested in the LLNA and also in selected dermatitis patients in 2007. Five hundred millilitres of the distilled limonene was oxidized in a 1000 ml Erlenmeyer flask in accordance with our previous experimental procedure. Samples were taken for analysis every week. This larger sample oxidized more slowly than the sample above, and after 21 weeks of air exposure, the composition corresponded to that of the oxidation mixture previously obtained. The content of limonene had decreased to 55%, whereas the content of Lim-1-OOH had accumulated to 1% and of Lim-2-OOH to 6%. This oxidation mixture was used for clinical testing during 2007.

Quantifications in ox. limonene

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, Darmstadt, Germany) was used. The temperature of the column and the sample rack in the autosampler was set to 20°C. Mobile phase: 89% *n*-hexane, 10% *tert*-butyl methyl ether, and 1% methanol; isocratic elution for 20 min; and flow rate of 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 ox. limonene were determined. The content of Lim-1-OOH in ox. *R*-limonene from Frey and Lau was not calculated because pure Lim-1-OOH was not available as a reference at the time of the analysis.

Sensitization experiments

Sensitization experiments in mice according to the LLNA. The sensitizing potencies of limonene and ox. limonene were investigated using the LLNA (24, 25) according to our previous experience (26). Each compound was tested in three different concentrations, using mice in groups of four (Table 1). All solutions were prepared freshly before application. Results are expressed as mean disintegrations per minute (dpm)/lymph node for each experimental group and as stimulation index (SI), i.e. test group/control group ratio. Test materials, which at one or more concentrations caused a SI greater than 3, were considered positive in the LLNA. EC3 values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation and used to compare the sensitizing capacity of the different test compounds (27). The sensitizing potency of each tested compound was classified according to the following: EC3 < 0.1%, extreme; EC3 ≥ 0.1 to <1%,

Table 1. LLNA responses for limonene and ox. limonene (10 weeks air exposure)^a

Test material	Concentrations (% w/v)	[³ H]thymidine incorporation (dpm/lymph node)	SI	EC3 value		Classification
				%	M	
Limonene				30	2.2	Weak
	25	917	2.2			
	50	2591	6.3			
	100	4328	10			
Control		413				
Ox. limonene				3.0	0.22 ^b	Moderate
	1	689	1.1			
	5	3255	5.0			
	25	11,976	18			
Control		652				

dpm, disintegrations per minute; SI, stimulation index.

^aThe local lymph node experiments were performed as described in *Materials and Methods*. The SIs correspond to the increase in thymidine incorporation of treated groups relative to vehicle-treated controls. EC3 values (the estimated concentration required to induce an SI of 3) were calculated using linear interpolation.

^bCalculated as limonene.

strong; EC3 ≥ 1 to <10%, moderate; EC3 ≥ 10 to <100%, weak (28). All animal procedures were approved by the local ethics committee.

LLNA with non-pooled lymph nodes. Single-cell suspensions of the lymph nodes from individual mice were prepared. The [^3H]thymidine incorporation in each suspension was measured to investigate if a statistically significant difference in the sensitizing capacity could be demonstrated between Lim-1-OOH (1.5% w/v), Lim-2-OOH (1.5%), and Lim-2-Me-2-OOH (1.6%) at equal molar concentrations (0.089 M). The concentration was selected based on results from previous LLNA experiments on these hydroperoxides (15). The test compounds (Lim-1-OOH, Lim-2-OOH, or Lim-2-Me-2-OOH) were dissolved in acetone:olive oil (AOO) (4:1 v/v) and topically applied (25 μl) on the dorsum of both ears to three groups of mice ($n = 8$ per group). A control group of eight mice was treated with the vehicle alone. All solutions were freshly prepared before application. Treatment and measurements were performed according to the ordinary LLNA method, but the single-cell suspensions from the lymph nodes of each mouse were treated separately. Results are expressed as dpm/lymph node for each experimental animal and as SI, i.e. the ratios of

individual test animal/mean of the control group (Table 2).

Statistical analysis. The non-parametric Mann–Whitney *U*-test was used as statistical method. To obtain global significance at a level of $P < 0.05$, the significance levels were corrected for multiple comparisons using Bonferroni correction. For each comparison, a value of $P < 0.004$ was considered statistically significant.

Clinical studies

Patch testing in patients with previous positive reactions to ox. limonene. Individuals with positive patch test reactions (++ or +++) to ox. limonene 3% petrolatum (pet.) were selected from 1985 dermatitis patients patch tested in 2004–2007. A letter was sent to nine individuals who met the above-mentioned criteria. The response rate was eight of nine (89%) individuals of whom seven agreed to further contact. These seven individuals were included in the study. Exclusion criteria were ultraviolet therapy, topical cortisone therapy or sunlight on the back within 4 weeks of testing, and ongoing medication with cytostatic drugs or oral corticosteroids. The mean age was 44 years. The group consisted of one male and six

Table 2. LLNA responses for Lim-1-OOH, Lim-2-OOH, and Lim-2-Me-2-OOH based on data from non-pooled lymph nodes from exposed animals^a

Compounds	Test concentrations		[^3H]thymidine incorporation (dpm/lymph node)	SI	Number of animals
	%	M			
Control			379 ^b	1	8
Lim-1-OOH	1.5	0.089	9257	26	1
	1.5	0.089	6480	18	1
	1.5	0.089	7315	21	1
	1.5	0.089	6172	18	1
	1.5	0.089	5930	17	1
	1.5	0.089	6670	19	1
	1.5	0.089	6528	19	1
	1.5	0.089	4979	14	1
Lim-2-OOH	1.5	0.089	2920	8	1
	1.5	0.089	3129	9	1
	1.5	0.089	3519	10	1
	1.5	0.089	3584	10	1
	1.5	0.089	1683	5	1
	1.5	0.089	1293	4	1
	1.5	0.089	2067	6	1
	1.5	0.089	1402	4	1
Lim-2-Me-2-OOH	1.6	0.089	3365	10	1
	1.6	0.089	2235	6	1
	1.6	0.089	2238	6	1
	1.6	0.089	2795	8	1
	1.6	0.089	2459	7	1
	1.6	0.089	2185	6	1
	1.6	0.089	1830	5	1
	1.6	0.089	2497	7	1

SI, stimulation index.

^aThe local lymph node experiments were performed as described in *Materials and Methods*. The SIs correspond to the increase in thymidine incorporation of treated animals relative to mean dpm/lymph node of vehicle-treated controls.

^bMean dpm/lymph node of 431, 389, 615, 463, 231, 327, 335, and 243.

females. Retesting of four individuals was performed in May 2007, while the remaining three were tested in October 2007 because they had no time for testing before summer. The patch test materials consisted of Lim-1-OOH and Lim-2-OOH in different concentrations in pet., of pure limonene 10%, and ox. limonene 3% in pet. New patch test materials were made within 2 weeks before each testing period. Patch test materials were left under occlusion for 2D. Readings were performed according to the International Contact Dermatitis Research Group recommendation on D3 and D6 (29). The study was approved by the local ethics committee.

Results

Sensitization experiments

Sensitization experiments in mice according to the LLNA. Limonene showed an EC3 value of 30% w/v (2.2 M), while ox. limonene showed an EC3 value of 3% w/v (0.22 M, calculated as limonene) according to the LLNA (Table 1).

LLNA with non-pooled lymph nodes. Results from the modified LLNA experiment are given in Table 2 and Fig. 2. Statistical analyses showed that when using 0.089 M of either hydroperoxide, Lim-1-OOH was more potent to induce lymph node cell proliferation compared with Lim-2-OOH as well as with Lim-2-Me-2-OOH giving a significant difference ($P = 0.0008$) between the groups in both cases (Fig. 2). All three hydroperoxides differed statistically significant from the controls ($P = 0.0008$). No statistical difference was seen between the results obtained for Lim-2-OOH and Lim-2-Me-2-OOH.

Clinical studies

Results from patch testing seven patients with previous positive patch test reactions to ox. limonene are presented in Table 3. A summary of the reactions shows that all patients reacted to Lim-1-OOH, while only three of the seven patients tested reacted to Lim-2-OOH (Table 4). In the patients reacting to both hydroperoxides, reactions were seen to Lim-1-OOH at lower concentrations compared with Lim-2-OOH.

Discussion

For the first time, we could demonstrate a statistically significant difference between the sensitizing potencies of closely related hydroperoxides using an extension of the LLNA. This experimentally demonstrated difference was also found to be

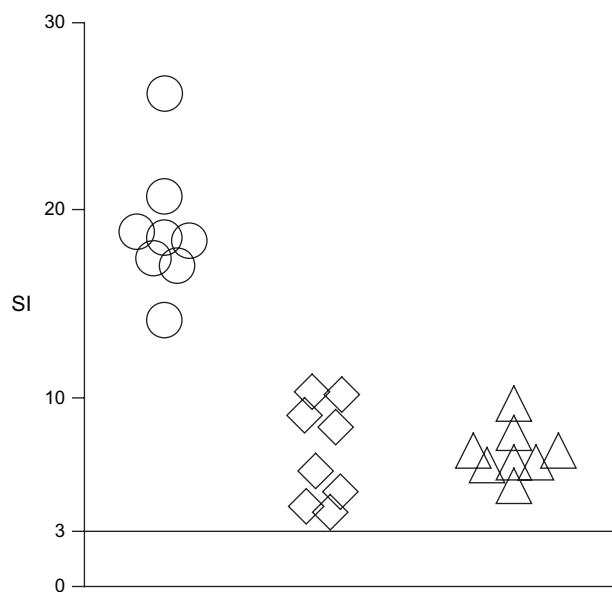


Fig. 2. Results from the modified local lymph node assay experiment using single-cell suspensions of the local lymph nodes from individual mice. Statistical analyses showed that when using 0.089 M of either hydroperoxide, Lim-1-OOH (○) was more potent to induce lymph node cell proliferation compared with Lim-2-OOH (◇) as well as to Lim-2-Me-2-OOH (△) giving a significant difference ($P = 0.0008$) between the groups in both cases. All three hydroperoxides differed statistically significant from the controls ($P = 0.0008$). No statistical difference was seen between the results obtained for Lim-2-OOH and Lim-2-Me-2-OOH. Individual data are given in Table 2. The horizontal line marks a stimulation index (SI) of 3, the cut-off limit for a compound to be considered a sensitizer.

clinically important because the strongest sensitizer in LLNA, Lim-1-OOH, detected more cases of contact allergy compared with Lim-2-OOH. A marked increase in the sensitizing potency of ox. limonene compared with limonene was shown using the LLNA. This result is in accordance with the results obtained in guinea-pig studies (2, 5–7) and patch testing in patients (3, 4, 8–11). In analogy with previously described results from LLNA studies on autooxidation of linalool (20) and geraniol (21), the hydroperoxides were the strongest sensitizers in the oxidation mixture and showed a large impact on the sensitizing capacity of the mixture (Fig. 3).

The two hydroperoxides Lim-1-OOH and Lim-2-OOH present in ox. limonene were investigated together with a synthetic analogue Lim-2-Me-2-OOH. Lim-1-OOH was significantly more sensitizing compared with the other two. This finding that closely related hydroperoxides show a statistically significant difference in sensitizing potency further demonstrates that the hydroperoxides act as specific haptens forming specific hapten–protein complexes. This is in accordance with our previous findings of the lack of non-specific

Table 3. Clinical data from seven retested individuals with previous positive reactions to ox. limonene

Patients	Reading	Limonene % (w/v) in pet.		Ox. limonene	Lim-1-OOH					Lim-2-OOH					Other positive patch test reactions
		10	3		1.0	0.75	0.50	0.25	0.12	1.0	0.75	0.50	0.25	0.12	
May	Female 30 years	D3	+	+	NT	+	NT	+	Neg	Neg	Neg	Neg	Neg	Neg	None
		D6	++	++	NT	NT ^b	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Nickel, chromium
	Female 52 years	D3	+	+	NT	+	NT	Neg	Neg	Neg	Neg	Neg	NT	Neg	
		D6	++	++	NT	+	NT	Neg	Neg	Neg	Neg	Neg	NT	Neg	
	Female 45 years	D3	+	+	NT	+	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Colophonium, lichen acid mix, Myroxylon
		D6	+	+	NT	+	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	pericariae, fragrance mixl
October	Female 71 years	D3	Neg	Neg	NT	+	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Hydroxyisohexyl 3-cyclohexene carboxaldehyde,
		D6	Neg	Neg	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	formaldehyde, formaldehyde releasers
	Female 46 years	D3	Neg	+++	NT	Neg	Neg	Neg	Neg	++	++	++	Neg	Neg	Nickel
		D6	Neg	+++	NT	+	+	Neg	Neg	+	+	+	Neg	Neg	
	Male 23 years	D3	Neg	+++	+	+	+	+	+	+	+	+	+	+	Ox linabool
		D6	Neg	Neg	+	+	+	+	Neg	+	+	+	Neg	Neg	
	Female 41 years	D3	+	++	+	+	++	+	Neg	+	+	++	Neg	Neg	Nickel
		D6	++	++	+	+	++	+	Neg	+	+	++	Neg	Neg	

NT, not tested; nr, not read; pet., petrolatum; Neg, negative.
^aReactions are graded according to International Contact Dermatitis Research Group guidelines (29): +++, vesicles, papules, erythema, and infiltrate; ++, papules, erythema, and infiltrate; +, erythema and infiltrate.
^bThe increased test concentration of Lim-1-OOH was inserted on D3 at the first reading of the patient when a possible positive reaction was found to Lim-1-OOH at 0.25% and was thus read once on D6.

Table 4. Summary of patch testing with limonene, ox. limonene, Lim-1-OOH, and Lim-2-OOH in seven patients with known contact allergy to ox. limonene

	No. of patients tested	Total no. of patients positive
Limonene	7	0
Ox. Limonene	7	6
Lim-1-OOH	7	7
Lim-2-OOH	7	3

cross-reactivity among hydroperoxides with various structures (23). The significant difference obtained in the present study might be due to a higher ability of Lim-1-OOH to form different immunogenic hapten–protein complexes compared with the other two tested compounds (15). It has also been demonstrated (15) that Lim-2-OOH rapidly forms the secondary oxidation product carvone, which is a hapten with moderate-to-weak sensitizing capacity (Fig. 3).

In a recent study (15), radical trapping experiments were conducted to investigate probable routes for formation of hapten–protein complexes from limonene hydroperoxides, which might take place in the skin. In these studies, our investigations showed that both carbon-centred and oxygen-centred radicals were formed when the limonene hydroperoxides were subjected to chemical radical trapping experiments. The amounts of the different radicals identified depended on the structure of the investigated hydroperoxide. Lim-1-OOH generated both carbon-centred and oxygen-centred radicals, indicating the possibility of two parallel routes for formation of hapten–protein complexes. Lim-2-Me-2-OOH gave a carbon-centred radical as the major product.

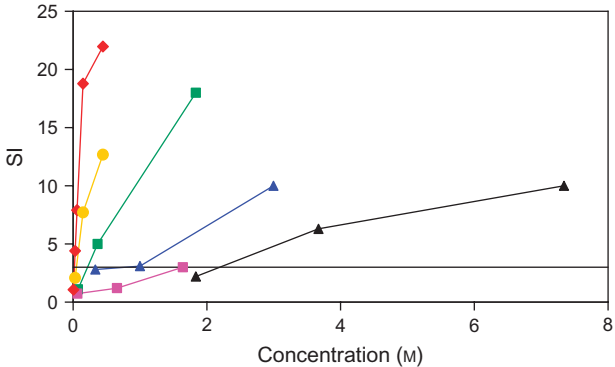


Fig. 3. Dose–response curves for pure and oxidized limonene and identified oxidation products when tested in the local lymph node assay. The concentrations are given in molar. The molar concentrations of oxidized *R*-limonene are calculated using the molecular weight of *R*-limonene. Pure limonene (▲), air-exposed limonene (10 weeks) (■), carvone (▲) (17), limonene oxide (■) (16), Lim-1-OOH (■) (15), and Lim-2-OOH (●) (15). SI, stimulation index.

Although Lim-1-OOH and Lim-2-Me-2-OOH formed almost equal amounts of carbon-centred radicals, Lim-1-OOH is a significantly stronger sensitizer. This indicates that the oxygen-centred radical is of major importance in the formation of immunogenic hapten–protein complexes from hydroperoxides.

When Lim-2-OOH was subjected to chemical radical trapping experiments, only very small amounts of radicals were detected. The sensitizing potency of Lim-2-OOH shows that even small amounts of radicals will be enough to make it an important sensitizer. In the trapping experiments, the main product identified was carvone, which is much less sensitizing (EC3 value 0.86 M, 13% w/v) compared with Lim-2-OOH (17). Interestingly, in an earlier guinea-pig study, no reactions were observed to carvone in animals sensitized to the limonene hydroperoxides (6). This further indicates that the sensitizing capacity of Lim-2-OOH is dependant on the radical formation and that the decomposition to carvone is of minor importance. However, the rapid carvone formation might contribute to the reduced sensitizing capacity of Lim-2-OOH in comparison with that of Lim-1-OOH.

Using the LLNA on individual lymph nodes in the exposed animal groups and choosing one relevant concentration based on an ordinary LLNA for comparison gave the possibility to demonstrate statistically significant differences in the results obtained for the compounds investigated. To the best of our knowledge, this way of extending the LLNA has not been published previously. It provides a tool to discriminate the sensitizing capacity of structurally related compounds with similar EC3 values, which is of importance in structure activity relationship studies as a basis for future non-animal *in silico* methods.

An increase in the sensitizing potency of ox. limonene in comparison with limonene is demonstrated in the ordinary LLNA experiments (Table 1). Unpublished results show the same for the other enantiomeric form, *S*-limonene. When comparing the results from the LLNA performed on the identified oxidation products of limonene to each other and to that of ox. limonene, it is obvious that the hydroperoxides are the strongest sensitizers and that their impact on the sensitizing capacity of the ox. limonene is important (Fig. 3). This is in accordance with corresponding studies of linalool and geraniol (20, 21).

Limonene belongs to the group of fragrance chemicals that must be labelled on cosmetic products when used in concentrations >10 p.p.m. in leave-on products and >100 p.p.m. in rinse-off products, due to their skin sensitizing capacity according to the Cosmetics Directive within the

EU (30). The fragrance chemicals in the directive were included in a recently published multicentre study among dermatitis patients at dermatology clinics to investigate the frequency of contact allergy today (4). As expected, very few reactions (3/2396, 0.1%) to pure limonene were found, and the oxidized form was not tested. This agrees with older studies where pure limonene gave few or no positive patch test reactions (3), whereas the oxidized forms gave 2–3% positive patch test reactions (8–11). In the recent publication (4), the selection of limonene as a common fragrance allergen is questioned. Within the EU regulatory work on the classification and labelling of dangerous substances (led by the European Chemicals Bureau), *R*-limonene and *S*-limonene, as well as the racemic form dipentene, are classified as skin sensitizers (31). The effective basis of this classification is the formation of skin sensitizers resulting from air exposure of limonene. Such a statement needs to be included in the Cosmetic Directive regarding both limonene and other chemicals such as linalool that form allergenic compounds on air exposure, thus acting as prehapten.

In previous studies, the hydroperoxide fraction of ox. limonene containing both Lim-1-OOH and Lim-2-OOH was tested in patients in addition to ox. limonene (8, 10). About half of the patients reacting to ox. limonene reacted to the fraction, indicating that many patients are allergic to other allergens in the oxidation mixture. Additional cases with reactions only to the hydroperoxide fraction were identified as well (8, 10). This can be explained by the higher concentration of the hapten in the hydroperoxide preparation compared with when testing with the total mixture.

The present study indicates that Lim-1-OOH has a greater impact on the allergenic effect of ox. limonene compared with Lim-2-OOH. When retesting the patients with previous patch test reactions to ox. limonene, only three of the seven patients reacted to Lim-2-OOH, while all reacted to Lim-1-OOH. In the patients reacting to both hydroperoxides, reactions were seen to Lim-1-OOH at lower concentrations compared with Lim-2-OOH. It should be noted that one of the patients responded to nothing else but Lim-1-OOH at retesting. It is well known that there is a variation in the response when individuals are tested at different times (32). Our interpretation is that this patient was allergic to Lim-1-OOH in the oxidation mixture and caught by testing with the pure sensitizer at retesting.

It could be advantageous to use Lim-1-OOH for testing of consecutive dermatitis patients because it is desirable to use specific substances for screening of contact allergy. There are,

however, some practical problems using the hydroperoxide in routine testing. Lim-1-OOH is unstable; the test preparation must be handled carefully and be replaced more often than normal test preparations. Furthermore, ox. limonene also contains other sensitizers such as carvone and Lim-2-OOH. The use of ox. limonene and isolated allergens from the oxidation mixture for screening of consecutive dermatitis patients is an important work that needs to be developed further.

Conclusions

One of the analogues of limonene hydroperoxides was a significantly more potent sensitizer than other closely related hydroperoxides in experimental studies. This analogue also gave more positive patch test reactions in dermatitis patients allergic to ox. limonene. The results support our previous findings that hydroperoxides have a specific reactivity. The results also indicate that oxygen-centred radicals seem to play a central role in the formation of hapten–protein complexes of hydroperoxides. The LLNA confirmed the previous guinea-pig studies that autooxidation increased the sensitizing potency of limonene and that the primary oxidation products in the autooxidation mixture, the hydroperoxides, have an important impact on the sensitizing capacity of the ox. limonene.

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- Address:
Johanna Bråred Christensson
Dermatochemistry and Skin Allergy
Department of Chemistry
University of Gothenburg
412 96 Gothenburg
Sweden
Tel: +46 317724726
Fax: +46 317723840
e-mail: johanna.brared-christensson@vgregion.se