

Not only oxidized *R*-(+)- but also *S*-(-)-limonene is a common cause of contact allergy in dermatitis patients in Europe

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Limonene, one of the most often used fragrance terpenes in any kind of scented products, is prone to air-oxidation. The oxidation products formed have a considerable sensitizing potential. In previous patch test studies on consecutively tested dermatitis patients, oxidized *R*-limonene has been proven to be a good and frequent indicator of fragrance-related contact allergy. The current study extends these investigations to 6 European clinics of dermatology, where the oxidation mixture of both enantiomers of limonene (*R* and *S*) have been tested in 2411 dermatitis patients. Altogether, 63 out of 2411 patients tested (2.6%) reacted to 1 or both the oxidized limonene preparations. Only 2.3% reacted to the oxidized *R*-limonene and 2.0% to the oxidized *S*-limonene. In 57% of the cases, simultaneous reactions were observed to both oxidation mixtures. Concomitant reactions to the fragrance mix, colophonium, *Myroxylon pereirae*, and fragrance-related contact allergy were common in patients reacting to 1 or both the oxidized limonene enantiomers. Our study provides clinical evidence for the importance of oxidation products of limonene in contact allergy. It seems advisable to screen consecutive dermatitis patients with oxidized limonene 3% petrolatum, although this patch test material is not yet commercially available.

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Contact allergy to fragrances is an increasing problem with great clinical importance. Fragrances have long been the second most common cause of contact allergy after nickel when patch testing consecutive dermatitis patients. The routinely used diagnostic tool for contact allergy to fragrances, the fragrance mix (FM), contains only 8 common fragrance allergens, while compounded scented products usually contain several hundred fragrance substances. In the last decades, new fragrance allergens have emerged and proved to have an important diagnostic value in fragrance allergy, such as hydroxyisohexyl 3-cyclohexene carboxaldehyde, farnesol, and citral (1–3).

Terpenes are among the most widely used fragrance components not only in fine perfumes but also in occupational and domestic products (4–6).

The fragrance terpenes, limonene and linalool, easily oxidize on exposure to air, turning non-, or low-sensitizing compounds into mixtures with considerable sensitizing capacity (7–9). Some of the oxidation products formed, mainly the hydroperoxides, were shown to be strong allergens (10, 11). Besides the recent reports on contact allergy to oxidized linalool (12), previous investigations on several thousand patients (13, 14) have demonstrated that oxidized *R*-limonene, one of the most frequently used fragrance chemicals, is a common cause of contact allergy in consecutively tested patients.

Peel oil of citrus fruits consists of *R*-limonene. *R*-(+)- and *S*-(-)-limonene (Fig. 1) are found in different ratios and amounts in several plants, including essential herb oils (rosemary, eucalyptus, lavender, caraway, lemongrass, and peppermint),

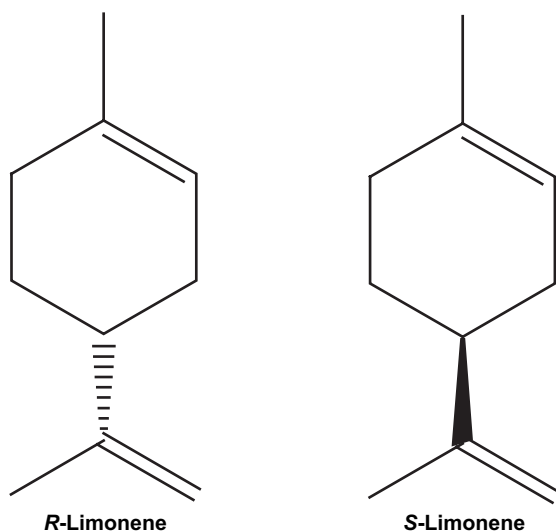


Fig. 1. The structures of the enantiomers *R*- and *S*-limonene.

tea tree oil, and turpentine oil (15). *R*-(+)- and *S*-(-)-limonene are enantiomers, i.e. they are the mirror images of one another (Fig. 1). Exposure of the population to one or the other enantiomeric form depends on the source of the essential oils. Because the limonene used as an added fragrance ingredient is mainly obtained as a by-product from the citrus juice industry, the main source of exposure is considered to be the *R*-isomer (personal communication with the fragrance industry).

The aim of this multicentre study was to investigate the clinical characteristics of patients with positive patch test reactions to oxidized *R*-limonene and compare the results with the results obtained with the simultaneously tested oxidized *S*-limonene, which was tested for the first time.

Materials and Methods

Chemicals

R-(+)-limonene (*d*-limonene) (*p*-mentha-1,8-diene) was used as obtained from Frey & Lau, Henstedt (Ulzburg, Germany). Gas chromatography (GC) analysis showed that 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%). *S*-(-)-Limonene [(*S*)-mentha-1,8-diene] (96%) was purchased from Sigma-Aldrich (Stockholm, Sweden) and purified by flash chromatography. In order to mimic oxidation that happens during handling and storage conditions, samples of *R*-(+)- and *S*-(-)-limonene were kept at room temperature on a laboratory counter in open flasks (covered with aluminium foil to prevent contamination) and stirred for 1 hr, 4 times a day, according to previous experience (8). After

10 weeks, the oxidation mixtures were formed, containing 34% *R*-(+)-limonene and 34% *S*-(-)-limonene. The analyses were performed with GC-direct inlet technique (16). The results obtained were in accordance with the previous studies on the oxidation degradation of *R*-limonene on exposure to air (17). The oxidation mixtures were stored in argon at -20° , and new preparations for patch testing were made every 2–3 months from the same batch at the Department of Dermatochemistry and Skin Allergy, Gothenburg University. The 5-ml syringes were sent to the test centres by airmail and kept refrigerated at the centres between the tests.

Patch test materials

In Malmö, standard allergens used for patch testing were obtained from Chemotechnique AB (Malmö, Sweden), and in Dortmund, Gentofte, Leuven, and London from Trolab-Hermal (Reinbek, Germany). The standard series used in Odense was TRUE Test™ (Mekos A/S, Hillerød, Denmark), supplemented by test substances from Hermal; specifically, the FM 8% petrolatum (pet.) was routinely used in the diagnosis of fragrance allergy.

The following test concentrations of the oxidized terpenes were used: 3.0% (w/w) oxidation mixture of *R*-(+)-limonene and 3.0% (w/w) oxidation mixture of *S*-(-)-limonene, both in nonstabilized white pet. (Kebo Lab A/S, Albertslund, Denmark) according to previous patch test studies (14).

Patch tests were applied in small (8 mm) Finn Chambers® on Scanpor® tape (Epitest, Tuusula, Finland). Readings were taken twice, on days 2 and 4 (exceptionally on D3) in Leuven, London, and Dortmund, and on days 3 and 6/7 in the other centres, and scored according to the International Contact Dermatitis Research Group (ICDRG) standard recommendation (18).

Multicentre study

Consecutive dermatitis patients were tested at the participating dermatology clinics of Copenhagen, Dortmund, Leuven, London, Malmö, and Odense during 2000–2001.

A questionnaire about clinical data (i.e. localization of symptoms, history of atopy, and occupation) was filled out before testing. The patients' history of adverse reactions to fragrances in the past was included and categorized according to the following questions: (i) certain – has reacted with an itching dermatitis to at least 1 fine perfume or aftershave and also to other scented products; (b) probable – has reacted to 1 or more scented products, but no certain product has been

identified as the cause of a clinical reaction; (c) questionable – has reacted to various cosmetics with or without fragrances, i.e. materials other than fragrance constituents may be the cause of reaction; and (d) none – has never reacted to scented materials [modified after Frosch et al. (1)].

Statistical analysis

Chi-square test was used for the analysis of concomitant positive reactions.

The study was approved by the local ethics committees, and all participants were included after informed consent.

Results

Altogether, 63 out of 2411 (2.6%) patients tested in the 6 European centres reacted to 1 or both the oxidized limonene preparations. In those, 49 out of 1812 patients, for whom records on oxidized *R*- and *S*-limonene were kept separately, 41 reacted to the oxidized *R*-limonene (2.3%) and 36 to the oxidized *S*-limonene (2.0%). 13/49 patients reacted only to oxidized *R*-limonene and 8/49 patients only to oxidized *S*-limonene, while 28/49 patients reacted to both oxidation mixtures (Table 1). Questionable reactions were recorded in 83/2411 (3.4%) patients and were reported mainly from two centres, while others observed no such reactions. The two oxidized limonene enantiomers gave rise to approximately equal numbers (71 and 60) of the questionable reactions.

Concomitant reactions to FM, colophonium, and *Myroxylon pereirae* (balsam of Peru) were common in patients reacting to 1 or both the oxidized limonene enantiomers. Of these patients ($n = 63$), 33% showed positive reactions to FM, 32% to colophonium, and 29% to *M. pereirae*. Table 2 shows the respective figures in those

who had a positive reaction either to oxidized *R*- or to *S*-limonene.

Fragrance-related contact allergy was considered in patients who showed positive reactions to own perfumes or perfumed products and/or to known perfume allergens (e.g. FM, *M. pereirae*, ingredients of FM, and essential oils), and/or had a certain or probable history of adverse reactions to fragrances (according to answers in the questionnaire). One or more of the described correlations were detected in 17 out of 36 patients (47%) reacting to oxidized *S*-limonene, and in 20 out of 41 patients (49%) reacting to oxidized *R*-limonene.

In the 63 cases reacting to 1 or both the oxidized enantiomers tested, the hands were affected in 27 patients (43%), the face in 15 patients (24%), and the legs in 14 patients (22%). Axillary dermatitis was observed in 3 cases (5%).

Discussion

The oxidation mixture of *R*-limonene and oxidation mixture of *S*-limonene gave allergic reactions in dermatitis patients in Europe, in a frequency comparable to those of the top 10 substances of the standard series. The frequency of positive reactions to oxidized *R*-limonene (2.3%) corresponds to the results obtained in previous studies (13, 14, 19), where a frequency of 1.8–2.7% was observed in consecutive dermatitis patients tested. Reactions to oxidized *S*-limonene were recorded in the same range of frequency (2%).

Patients with positive patch test reactions to oxidized *R*- and/or *S*-limonene tested had an increased frequency of positive reactions to FM, *M. pereirae*, and colophonium, compared to those who did not show positive reactions. This is in agreement with previous clinical studies (12, 13, 19). Simultaneous reactions to FM, colophonium, and *M. pereirae* in dermatitis patients are frequently

Table 1. Patch test results of testing with oxidized *R*-limonene and oxidized *S*-limonene 3% in consecutive dermatitis patients at 6 dermatological centres

Centre	Number of patients tested	Patient with positive reaction ^a , n (%)	Positive only to oxidized <i>R</i> -limonene ^b	Positive only to oxidized <i>S</i> -limonene ^c	Positive to both oxidation mixtures ^d
London	599	14 (2.4)	nr	nr	nr
Leuven	408	15 (3.7)	6	1	8
Malmö	620	10 (1.6)	2	—	8
Dortmund	253	1 (0.4)	—	—	1
Gentofte	203	5 (2.5)	—	2	3
Odense	328	18 (5.5)	5	5	8
Total	2411	63 (2.6)	13/1812	8/1812	28/1812

nr, not recorded.

^aPositive patch test reaction to 1 or both the oxidation mixtures tested.

^bNumber of positive reactions only to the oxidized *R*-limonene.

^cNumber of positive reactions only to the oxidized *S*-limonene.

^dNumber of positive reactions to both oxidized *R*- and oxidized *S*-limonene.

Table 2. Positive reactions to fragrance mix, *Myroxylon pereirae*, and colophonium (tested in the standard series) in 1812 consecutively tested patients with positive and negative test results to oxidized *R*- and/or oxidized *S*-limonene

	Total test population, <i>n</i> = 1812			
	Positive to oxidized <i>R</i> -limonene (41 patients) (%)	Negative to oxidized <i>R</i> -limonene (1771 patients) (%)	Positive to oxidized <i>S</i> -limonene (36 patients) (%)	Negative to oxidized <i>S</i> -limonene (1776 patients) (%)
Fragrance mix	14/41 (34) ^a	113/1771 (6.4)	11/36 (31) ^a	116/1776 (6.5)
<i>M. pereirae</i>	11/41 (27) ^a	91/1771 (5.1)	12/36 (33) ^a	80/1776 (4.5)
Colophonium	11/41 (27) ^a	62/1771 (3.5)	9/36 (25) ^a	64/1776 (3.6)

^aStatistically significant concomitant reactions in subjects showing positive reactions to oxidized *R*-limonene and/or oxidized *S*-limonene compared to those with negative reactions (*P* < 0.001).

recorded in the literature (20–22), especially in patients with fragrance allergy. A recent study observed a correlation between compositae dermatitis and positive patch test reaction to oxidized terpenes (23). In the present study, fragrance-related contact allergy and/or adverse reaction to fragrances in the patients' history was observed in 47% and 49% of the patients showing positive reactions to oxidized *S*- and *R*-limonene, respectively. In previous studies, a highly significant correlation between fragrance allergy and allergy to oxidized *R*-limonene (13), as well as to oxidized linalool (12), in several test centres gave clinical support for these oxidized terpenes as good fragrance allergy markers. Our current study has shown that the same correlation holds true for oxidized *S*-limonene.

Allergic contact dermatitis from fragrance ingredients may present with localized or generalized symptoms. Facial, hand, and axillary eczema (24), as well as dermatitis on the legs (25), have been frequently reported in fragrance-allergic patients. This is also the case for the patients with contact allergy to the two oxidation mixtures of limonene in this study, as well as in previous studies testing only oxidized *R*-limonene (14) and selected oxidized terpenes (12) in consecutive dermatitis patients.

In the last decade, more than 100 patients with contact allergy to oxidized *R*-(+)-limonene were diagnosed in systematic patch test studies conducted by Karlberg and colleagues (13, 14). Because the formation of allergenic components from limonene was not considered previously, the presence of oxidation products was not investigated in the few earlier articles reporting mostly occupational contact allergy to *R*-limonene (or to dipentene, the mixture of the two limonene enantiomers) (26–28). The same is true for *S*-(-)-limonene in a case report of Doods-Goossens et al. (29), where occupational sensitization to oil of turpentine and peppermint oil with positive patch test reaction to *S*-(-)-limonene was observed. Unpublished data on positive patch

tests to oxidized *S*-(-)-limonene in occupational settings provided evidence for the latter being not only a fragrance related but also an occupational source of contact allergy.

In the present study, 57% of the cases showed simultaneous reactions to oxidized *R*- and *S*-limonene. This observation can be explained by either concomitant sensitization due to their widespread use in fragranced products or by cross-reaction. The first explanation might be plausible because both enantiomers are widely compounded in fragrances. *R*-Limonene is often used as an added ingredient, besides the considerable content in different essential oils, while *S*-limonene most often originates from essential oils (30). The reproducibility of the results of concomitantly applied patch tests of the same allergen has been reported to vary between 56.2% and 95% (31). Therefore, the 57% concordance of positive patch test results obtained with the two oxidized limonene enantiomers could be explained by cross-reactivity as well. The oxidation mixtures of limonene contain several oxidation/degradation products (Fig. 2). Of those with allergenic activity, limonene hydroperoxides, carvone, and limonene oxide have previously been identified, and the hydroperoxides have been demonstrated to be the most important allergens in the oxidation mixture. In the case of cross-reactions, these three major identified allergens have to be taken into account. A recent study showed that hydroperoxides in general do not cross-react (32). In previous experimental sensitization studies on guinea pigs (33), no cross-reaction between *R*-(-)-carvone and *S*-(+)-carvone was observed. Theoretically, *R*- and *S*-limonene can form the same mixture of hydroperoxides during autoxidation. Thus, the simultaneous reactions observed can be due to the presence of the same allergens in the 2 oxidation mixtures.

Our current study provides clinical evidence for the importance of oxidation products of limonene in contact allergy. These results further emphasize the need for testing patients with the compounds

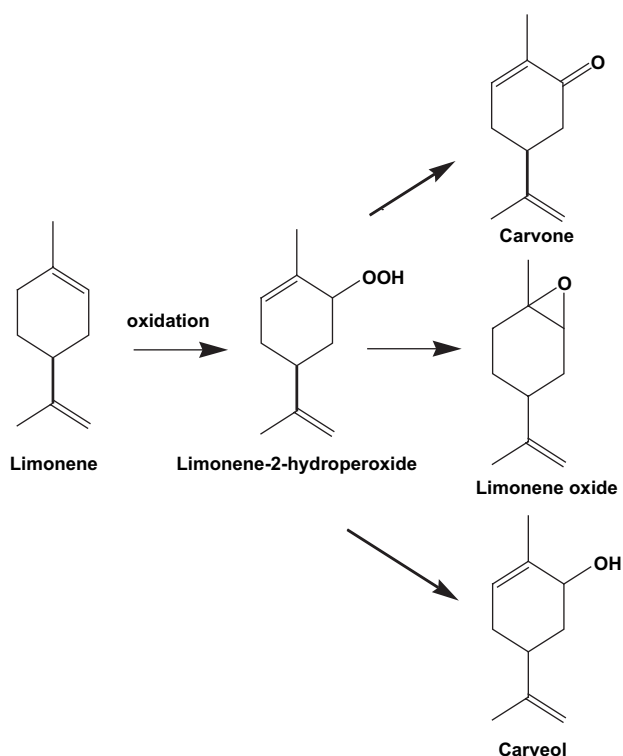


Fig. 2. The structures of the major oxidation products of limonene formed at autooxidation.

that they may actually come in contact with, not only substances originally applied in commercial formulations. It seems advisable to screen consecutive dermatitis patients with oxidized limonene 3% in pet., although such a patch test material is not yet commercially available.

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