

ORIGINAL ARTICLE

Allergic contact dermatitis caused by hydroperoxides of limonene and dose-response relationship—A repeated open application test (ROAT) study

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Background: Contact allergy to oxidized limonene, with hydroperoxides of limonene (Lim-OOHs) as the main allergens, is common. However, high proportions of weak positive and doubtful patch test reactions have been reported.

Objectives: To determine the clinical relevance, elicitation threshold and dose-response relationship of Lim-OOHs in individuals with a positive or doubtful patch test reaction to standard Lim-OOHs 0.3% pet.

Methods: A multicentre 3-week double-blind vehicle-controlled repeated open application test (ROAT) study with a simulated fine fragrance containing Lim-OOHs at 1260, 420 and 140 ppm, equal to a dose/area per application of Lim-OOHs of 3.0, 0.99 and 0.33 $\mu\text{g}/\text{cm}^2$, was performed.

Results: Among 11 subjects allergic to Lim-OOHs, 11 (100%), 7 (64%), and 3 (27%), respectively, reacted to the applied doses. No reactions were seen in 17 healthy controls exposed to the highest dose. This difference in reactivity was statistically significant ($P < 0.0001$). Among 13 subjects with doubtful patch test reactions to Lim-OOHs, two (15%) had positive ROAT reactions to the highest Lim-OOH dose applied ($P = 0.36$ as compared with controls).

Conclusions: Contact allergy to Lim-OOHs is of clinical relevance in patients with positive patch test reactions. A doubtful patch test reaction to Lim-OOHs 0.3% pet. can be of clinical relevance.

KEYWORDS

allergic contact dermatitis, clinical relevance, contact allergy, dose-response relationship, fragrance substance, limonene hydroperoxides, oxidized limonene, ROAT

1 | INTRODUCTION

Limonene, a cyclic monoterpene, is the major constituent of peel oil from citrus fruit, and is extensively used as a fragrance chemical in scented household and cosmetic products for its fresh citrus odour.^{1,2} Experimental studies have established pure limonene as a prehapten³ that can oxidize upon air exposure (autoxidation) to form sensitizing allergen-specific oxidation products. On the basis of the local lymph

node assay in mice, the EC3 value for oxidized limonene is 10-fold lower than that for pure limonene, indicating a higher sensitizing potency. The main sensitizing haptens formed during autoxidation of limonene are hydroperoxides, including limonene-1-hydroperoxide and limonene-2-hydroperoxide, the former of which has been identified as the strongest sensitizer in oxidized limonene.^{4,5}

Oxidized limonene 3% pet., with a stable and standardized content of the main allergenic hydroperoxides of limonene (Lim-OOHs) of

0.3%, has been commercially available as the patch test preparation "Hydroperoxides of Limonene 0.3% pet." from Chemotechnique Diagnostics (Vellinge, Sweden) since 2012.⁶ High prevalences of contact allergy to Lim-OOHs 0.3% pet. have been reported in consecutive dermatitis patients referred for patch testing, both in an international multicentre study with 5.2% of patients positive overall,⁷ and lately in patch test clinics across Europe with 2.5% to 5.3% positive reactions.^{8–11} In most of these investigations, a high proportion of weak positive as well as doubtful and/or irritant patch test reactions to Lim-OOHs 0.3% pet. have been reported, which has caused some concern about the nature and clinical relevance of positive reactions.^{12,13}

The relevance of a positive or doubtful patch test reaction can be assessed with the repeated open application test (ROAT).¹⁴ The ROAT is a standardized exposure test mimicking daily use of a (cosmetic) product containing a contact allergen of interest.¹⁵ The aim of the ROAT is to investigate whether allergic contact dermatitis can be elicited following twice-daily application for a period of 2 to 4 weeks. In an experimental setting, the ROAT can be used to elicit allergic contact dermatitis, under standardized conditions, in response to specified doses of a contact allergen in order to determine an elicitation threshold and dose-response relationship.¹⁶

In the current study, we wanted to determine the clinical relevance, elicitation threshold and dose-response relationship of Lim-OOHs in individuals with either positive or doubtful patch test reactions to standard Lim-OOHs 0.3% pet. Accordingly, we performed a multicentre 3-week double-blind vehicle-controlled ROAT study with a simulated hydroalcoholic leave-on cosmetic product containing Lim-OOHs at three different concentrations.

2 | MATERIALS AND METHODS

2.1 | Test subjects

The study was conducted in accordance with the Declaration of Helsinki,¹⁷ and was approved by the regional ethical committees in Denmark and Sweden. The study was prospectively registered at www.clinicaltrials.gov (NCT03313232). We identified possible participants among patients who were patch tested with Lim-OOHs 0.3% pet. (oxidized limonene 3% pet., with a stable and standardized content of the main allergenic limonene hydroperoxides of 0.3%), as part of routine patch testing at the Department of Dermatology and Allergy, Gentofte University Hospital (DK) or the Department of Occupational Dermatology, Sahlgrenska University Hospital (SE) between 2012 and 2017. Patients with previous positive patch test reactions (at least one reading) and patients with previous doubtful patch test reactions (at least two readings), aged ≥ 18 years, were invited to participate. Exclusion criteria were active eczema on the upper back or forearms, pregnancy or breastfeeding, recent application of topical immunosuppressant treatment to the upper back or forearms, systemic immunosuppressant treatment, and excessive ultraviolet exposure within 3 weeks of the start of the study. Contact allergy to other allergens was not an exclusion criterion.

Nineteen subjects (DK, 12; SE, 7) with previous positive patch test reactions and 12 subjects (DK, 10; SE, 2) with previous doubtful patch test reactions to Lim-OOHs 0.3% pet. agreed to participate and fulfilled the inclusion criteria. Healthy volunteers with no history of eczema or known contact allergy to Lim-OOHs or other fragrance substances were included as a control group. The exclusion criteria were the same as for the previously patch tested subjects. Eighteen healthy controls (DK, 12; SE, 6), with a similar age and sex distribution as the previously patch tested subjects, were included following advertisement on the website www.forsøgsperson.dk or were recruited among office workers at Sahlgrenska University Hospital. All clinical investigations took place between October 2017 and April 2018.

On the day of inclusion, all participants were subjected to a clinical examination of their skin and filled out a questionnaire on history of eczema and consumer behaviour regarding the use of fragranced products. A diagnosis of atopic dermatitis was made according to the UK Working Party's diagnostic criteria.¹⁸

2.2 | Chemicals and test preparations

Limonene ("pure") and oxidized limonene with a documented content of Lim-OOHs of 12.6% (limonene-1-hydroperoxide and limonene-2-hydroperoxide) from the same batch were purchased from Chemotechnique Diagnostics, and stored under argon or nitrogen at -20°C . The concentrations and doses of oxidized limonene in the test solutions used in the study, and their content of Lim-OOHs, are shown in Table 1. For the ROAT, solutions of oxidized limonene at 1.0%, 0.33%, and 0.11%, respectively, in ethanol/water (80:20) were produced to simulate a fine fragrance as the model for a leave-on cosmetic product. The highest concentration was based on the known concentration of limonene determined by chemical analyses in leave-on cosmetic products intended for non-occlusive use.¹⁹ Prior to initiation of the ROAT, all participants were patch tested with 20 mg of standard Lim-OOHs 0.3% pet., obtained from Chemotechnique Diagnostics, and with a pet. control. In addition, participants with previous positive or doubtful patch test reactions to this test preparation were also patch tested with a dilution series (15 μL on filter discs) of Lim-OOHs in ethanol/water. With respect to oxidized limonene, the highest concentration in the dilution patch test series corresponded in dose per area (1.2 mg/cm^2) to the concentration of oxidized limonene in the standard patch test of Lim-OOHs 0.3% pet. The concentration of Lim-OOHs in the oxidized limonene used for the dilution patch test and ROAT solutions (Lim-OOH content of 12.6%) was higher than that normally found in the standard patch test material of oxidized limonene (Lim-OOH content of $\sim 10\%$). Hence, the calculated concentrations and doses of Lim-OOHs in the test preparations are presented to correctly quantify allergen exposure.

Oxidized limonene diluted to 1% (wt/wt) in ethanol/water (80:20, vol/vol) was investigated for the stability of Lim-OOHs. The formation of Lim-OOHs was investigated in limonene ("pure") diluted to 1% in ethanol/water. Aliquots were taken from each sample and stored in triplicate at either room temperature or at -20°C under argon. After 2 and 3 weeks, the samples were analysed with reversed-phase high-performance liquid chromatography/electrospray ionization-tandem

TABLE 1 Overview of the used concentrations and doses of oxidized limonene, including content of limonene hydroperoxides, and proportions of observed reactions in allergic and doubtful allergic participants

Test solution (vehicle)	Concentration		Dose		Observed reactions, n (%)	
	Concentration of oxidized limonene (%)	Content of limonene hydroperoxides, % (ppm)	Dose per application of oxidized limonene ($\mu\text{g}/\text{cm}^2$)	Content of limonene hydroperoxides per application ($\mu\text{g}/\text{cm}^2$)	Allergic subjects (n = 11)	Doubtful allergic subjects (n = 13)
Confirmatory patch test (pet.) ^a	3	0.3 (3000)	1200	120		
Patch test dilution series (80% ethanol)	4.7	0.59 (5922)	1206	152	11 (100)	13 (100)
	1.6	0.20 (1974)	400	50	10 (91)	8 (62)
	0.52	0.066 (658)	133	17	10 (91)	5 (38)
	0.17	0.022 (219)	44	5.5	6 (55)	1 (7.7)
	0.058	0.0073 (73)	15	1.9	4 (36)	1 (7.7)
	0.019	0.0024 (24)	5.1	0.65	3 (27)	0
	Vehicle	-	-	-	0	0
ROAT solutions (80% ethanol)	1.0	0.13 (1260)	24	3.0	11 ^b (100)	2 (15)
	0.33	0.042 (420)	7.9	0.99	7 (64)	1 (7.7)
	0.11	0.014 (140)	2.6	0.33	3 (27)	1 (7.7)
	Vehicle	-	-	-	0	0

Abbreviation: pet., petrolatum; ROAT, repeated open application test.

^a The confirmatory patch test material was not from the same batch as the oxidized limonene used in the ROAT and patch test dilution series, but from the commercially bought batches used in the participating departments at the time of the study.

^b Including one participant who showed a positive ROAT reaction 12 days after exposure had ended (see Supporting Information Table S1 for additional details).

mass spectrometry. No degradation of Lim-OOHs was observed in the aliquots of oxidized limonene stored at -20°C . A tendency for degradation of Lim-OOHs was observed in oxidized limonene stored at room temperature; however, the decrease over time was not significant and was within the relative SD ($<22\%$) of the analyses. The ratio of limonene-2-hydroperoxide to limonene-1-hydroperoxide in the oxidized limonene was between 6:1 and 5:1 across storage conditions and sampling times. In limonene ("pure"), from which the oxidized limonene was produced, limonene-2-hydroperoxide was detected at a level of 0.28% (wt/wt), corresponding to 0.0028% in the 1% ethanol/water solution, at the time of purchase. This level remained constant, independently of storage conditions and sampling time. Limonene-1-hydroperoxide was below the detection limit at all measurements, so there was no evidence of hydroperoxide formation. For the clinical part of the study, both ROAT solutions and the patch test dilution series were prepared weekly to avoid any changes in Lim-OOH concentrations.

2.3 | Patch testing procedure

A flowchart for the study participants is shown in Figure 1. Prior to performance of the ROAT, patch testing was performed with Finn Chambers (8 mm; SmartPractice, Phoenix, Arizona) applied on the upper back for 2 days with Scanpor tape (Norgesplaster, Vennesla, Norway). The investigators were blinded to placement and reading of the dilution patch test series. Scoring of patch test reactions was performed on day (D) 3 or on D4 and D7.¹⁶ In addition, patch test reactions were scored by use of an extended reading scale: 0 = no reaction; 1 = few (≥ 5) papules with no erythema and no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogeneous infiltration; 4 = erythema with homogeneous infiltration; 5 = erythema, infiltration, and a few

papules; 6 = erythema, infiltration, and papules; 7 = erythema, infiltration, papules, and a few vesicles; and 8 = intensive erythema, infiltration, and vesicles. This was done to detect weaker allergic reactions with regard to defining the minimal eliciting concentration (MEC) for the dilution patch test series, that is: the threshold concentration defined as the lowest dose giving a visible reaction (minimum score of 1 on the extended reading scale), if there was a continuous line of reactions from the highest dose downwards.^{20,21}

On the basis of the results of the confirmatory patch test with Lim-OOHs 0.3% pet., previously patch tested participants were categorized as either allergic (positive patch test reaction: +/++/+++ or doubtful allergic (doubtful patch test reaction: ?+). A total of 11 participants had positive reactions and 13 participants had doubtful reactions to the confirmatory patch test and proceeded to the ROAT (see Supporting Information Table 1 for individual patch test reactions). Previously patch tested participants who had negative confirmatory patch test results with Lim-OOHs 0.3% pet. (n = 7) did not proceed to the ROAT. These included four participants with previous weak positive patch test reactions to Lim-OOHs 0.3% pet., of whom three had at least doubtful patch test reactions to one of the two highest applied concentrations of Lim-OOHs in the dilution patch test series. However, this information was not available to the investigators until after the study had ended, owing to blinding. Among the healthy controls, one participant showed a positive patch test reaction to Lim-OOHs 0.3% pet. and did not proceed to the ROAT.

2.4 | ROAT procedure

In the ROAT, participants were exposed twice daily for up to 21 days to one (healthy controls) or three (allergic and doubtful allergic participants) concentrations of Lim-OOHs in the simulated fine fragrance, as well as a vehicle control. At the start of the ROAT and at weekly evaluations, participants were provided with 500 μL of each test solution

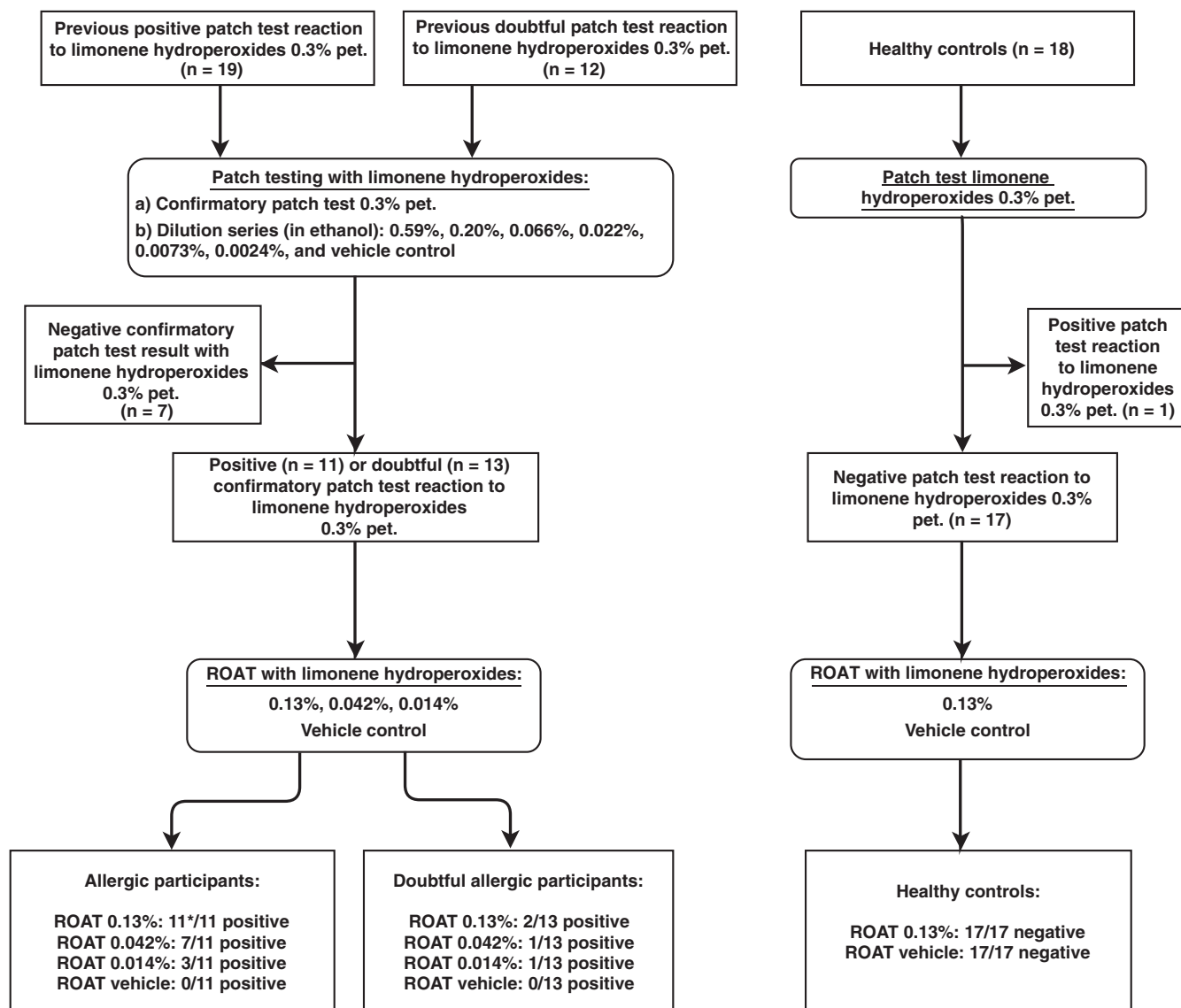


FIGURE 1 Study flowchart for participants with previous positive or doubtful patch test reactions to limonene hydroperoxides 0.3% pet., and healthy controls. *One participant developed a clearly positive reaction on the repeated open application test (ROAT) test area 12 days after exposure had ended in the study (see Supporting Information Table S1 for additional details)

in colour-coded 1.5-mL Eppendorf tubes. Participants were thoroughly instructed on how to apply 25 μ L to each 3 $\times$ 3-cm colour-coded test area on the forearms with a micropipette (DK, LGG-Labware, Meckenheim, Germany; SE, VWR International, Spånga, Sweden). The applied volume of the simulated fine fragrance corresponded to 2.4 mg/cm², which is comparable to the expected daily exposure to hydroalcoholic products on unshaved skin.²² Test solutions were applied in the centre of the test areas, distributed evenly with the side of the pipette tip, and allowed to dry by evaporation. Participants could shower during the ROAT, but were not allowed to use scented products or wash the test areas directly. Placement of test areas on the forearms was randomized for each participant, and both participants and investigators were blinded to the individual contents of the colour-coded test solutions (separate blinding codes for allergic/doubtful allergic participants and healthy controls). Eppendorf tubes were weighed before and after being provided to the participants, to estimate compliance.

Reactions on the ROAT test areas were scored clinically for the development of dermatitis after 7, 14 and 21 days of exposure, or when the participant observed a reaction. Scoring of ROAT reactions involved assessment of the involved area of application, presence and strength of erythema, level of infiltration, and possible presence of vesicles.^{16,23} Application of individual test solutions was continued until a positive ROAT reaction was observed, corresponding to a minimum ROAT score of 5 ($\geq$ 25% involved area, presence of erythema, and signs of infiltration, ie, at least one papule), up to a maximum of 21 days of application. All readings were documented by photography.

2.5 | Statistical analyses

Data management and statistical analyses were performed with SAS ENTERPRISE GUIDE, version 7.1 (SAS Institute, Cary, North Carolina), and two-sided *P* values of < 0.05 were considered to be statistically significant. Graphs were created with GRAPHPAD PRISM version 7 (GraphPad

Software, La Jolla, California). Allergic and doubtful allergic participants were compared with healthy controls by use of the Mann-Whitney *U* test for continuous variables, and Fisher's exact test for categorical variables. Compliance in the ROAT was based on weighing of test solutions, and expressed as the mean percentage of actual use as compared with expected use. In exploratory analyses, the dose-response relationship in Lim-OOH-allergic participants was estimated with threshold logistic regression analyses. The predicted eliciting doses (EDs), including 95% confidence intervals (CIs), that would elicit a patch test reaction or ROAT reaction, respectively, in 10%, 25%, 50%, 75% and 90% of allergic participants were calculated according to Fieller's method. This was performed with probability unit (probit) analyses,²⁴ and fitted four-parameter dose-response curves with constraints on minimum and maximum response frequencies were drawn.

3 | RESULTS

The baseline characteristics for allergic participants, doubtful allergic participants and healthy controls who proceeded to the ROAT are shown in Table 2. The distribution of strength of reaction to the confirmatory patch test with Lim-OOHs 0.3% pet. in the 11 allergic participants was + in three (27.3%), ++ in seven (63.6%), and +++ in one (9.1%). Significantly more allergic participants than healthy controls had current eczema, all with only minor involvement, and significantly fewer allergic participants were exposed to fragranced products in daily life. Doubtful allergic participants were significantly more likely than healthy controls to have a history of atopic dermatitis.

The proportions of observed reactions to the applied patch test and ROAT solutions are summarized in conjunction with the selected exposures in Table 1. All allergic and doubtful allergic participants reacting to the confirmatory patch test also reacted to the highest applied dose of Lim-OOHs in the patch test dilution series. Among allergic participants, three (27%) reacted continuously down to the lowest dose applied (0.65 µg of Lim-OOHs/cm²), corresponding to a MEC in allergic participants of 0.0024%, which is equal to 24 ppm. Among the doubtful allergic participants, one (7.7%) reacted to the second lowest applied patch test dose (1.9 µg of Lim-OOHs/cm²),

corresponding to a MEC of 0.0073% (73 ppm). No reactions were seen to the vehicle control.

In the ROAT, the 17 healthy controls were exposed to the highest dose of 3.0 µg of Lim-OOHs/cm² (1260 ppm), and none reacted. Eleven (100%) allergic participants reacted to the highest dose, after a median of 7 days of exposure, which was significantly different from what was seen in the healthy controls (*P* < 0.0001). This included one allergic participant who showed a positive ROAT reaction to the highest applied dose 12 days after exposure had ended (see additional information in Supporting Information Table 1). Seven (64%) allergic participants reacted in the ROAT to the middle dose of 0.99 µg of Lim-OOHs/cm² (420 ppm) after a median of 14 days of exposure. Three (27%) allergic participants had positive ROAT reactions to the lowest dose of 0.33 µg of Lim-OOHs/cm² (140 ppm) after 14 days (*n* = 2) and 18 days (*n* = 1) of exposure. Among the doubtful allergic participants, two (15%) had positive ROAT reactions to the highest Lim-OOH dose applied, which was not significantly different from what was seen in healthy controls (*P* = 0.36). Of the two doubtful allergic participants, one also reacted to both the middle and lowest doses of Lim-OOHs after 14 days of exposure. Among all participants, no reactions were seen to the vehicle control in the ROAT. Positive ROAT reactions in allergic and doubtful allergic participants are shown in Figures 2 and 3. Compliance in the ROAT, in terms of actual usage of individual test solutions as compared with expected usage, was 116% to 125% in allergic participants, 112% to 116% in doubtful allergic participants, and 119% to 123% in healthy controls. As compared with healthy controls, no significant differences in compliance were observed for allergic or doubtful allergic participants (Supporting Information Table 2).

Fitted dose-response curves based on the frequencies of observed reactions to Lim-OOHs in allergic participants in the patch test and ROAT are shown in Figure 4. The effect of repeated exposure as compared with single patch test exposure is quantified in Table 3, showing the calculated doses (EDs) that will elicit responses in the specified proportions of allergic participants. Following repeated exposure in the ROAT, the calculated ED₁₀ for Lim-OOHs was 0.20 µg of Lim-OOHs/cm² per application (95%CI: 0.016-0.37 µg of Lim-OOHs/cm²), corresponding to an estimated

TABLE 2 Baseline characteristics for participants proceeding to the repeated open application test

	Allergic participants (n = 11)		Doubtful allergic participants (n = 13)		Healthy controls (n = 17)
		As compared with healthy controls, <i>P</i> -value		As compared with healthy controls, <i>P</i> -value	
Age in years, mean (SD)	52 (15)	0.41	40 (17)	0.27	47 (17)
Age range (years)	30-73		20-78		23-73
Female, n (%)	9 (82)	1.0	8 (62)	0.44	13 (76)
History of atopic dermatitis, n (%)	0 (0)	-	6 (46)	0.003	0 (0)
Eczema currently, n (%)	6 (55)	0.007	5 (38)	0.06	1 (5.9)
Facial eczema (n)	1		2		0
Hand eczema (n)	2		3		1
Truncal eczema (n)	1		1		0
Leg eczema (n)	2		1		0
Exposed to fragranced products in daily life, n (%)	2 (18)	0.002	9 (69)	0.67	14 (82)

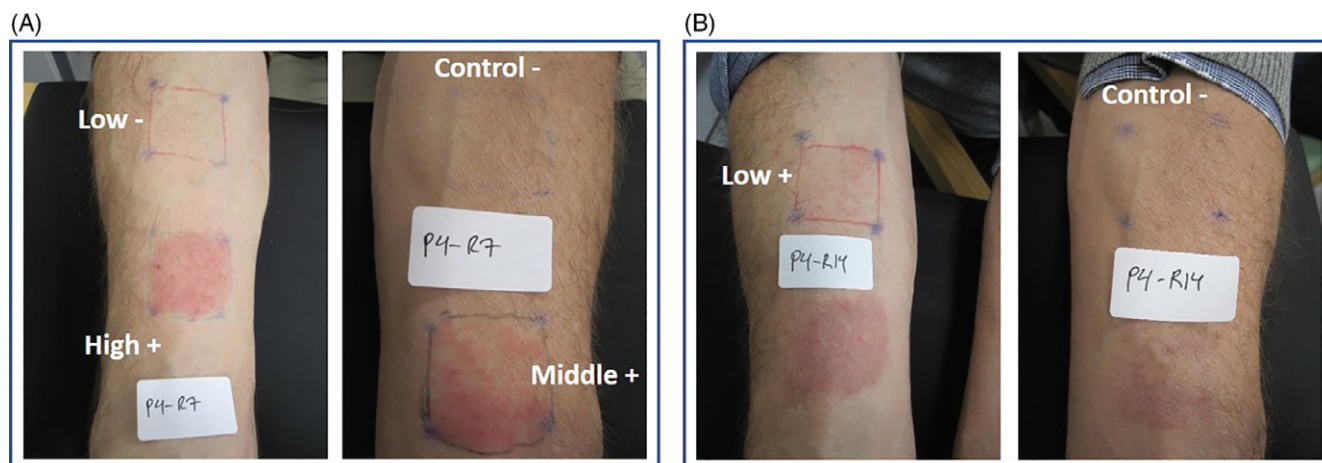


FIGURE 2 Repeated open application test (ROAT) reactions in a participant with a positive (+) confirmatory patch test reaction to limonene hydroperoxides 0.3% pet. (A), On day 7, the participant had positive ROAT reactions to the high dose ($3.0 \mu\text{g}/\text{cm}^2$) and middle dose ($0.99 \mu\text{g}/\text{cm}^2$) of limonene hydroperoxides. (B), On day 14, the subject had a positive ROAT reaction to the low dose ($0.33 \mu\text{g}/\text{cm}^2$) of limonene hydroperoxides. No reaction was seen to the vehicle control (control)

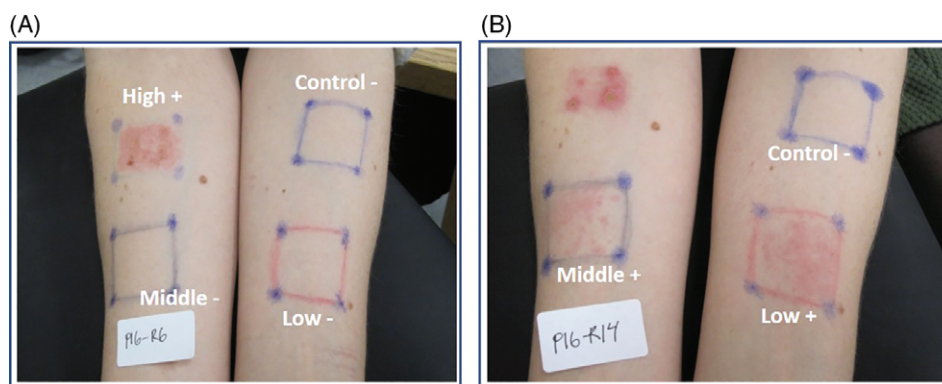


FIGURE 3 Repeated open application test (ROAT) reactions in a participant with a doubtful (?+) confirmatory patch test reaction to limonene hydroperoxides 0.3% pet. (A), On day 6, the participant had a positive ROAT reaction to the high dose ($3.0 \mu\text{g}/\text{cm}^2$) of limonene hydroperoxides. (B), On day 14, the participant had positive ROAT reactions to the middle dose ($0.99 \mu\text{g}/\text{cm}^2$) and low dose ($0.33 \mu\text{g}/\text{cm}^2$) of limonene hydroperoxides. No reaction was seen to the vehicle control (control)

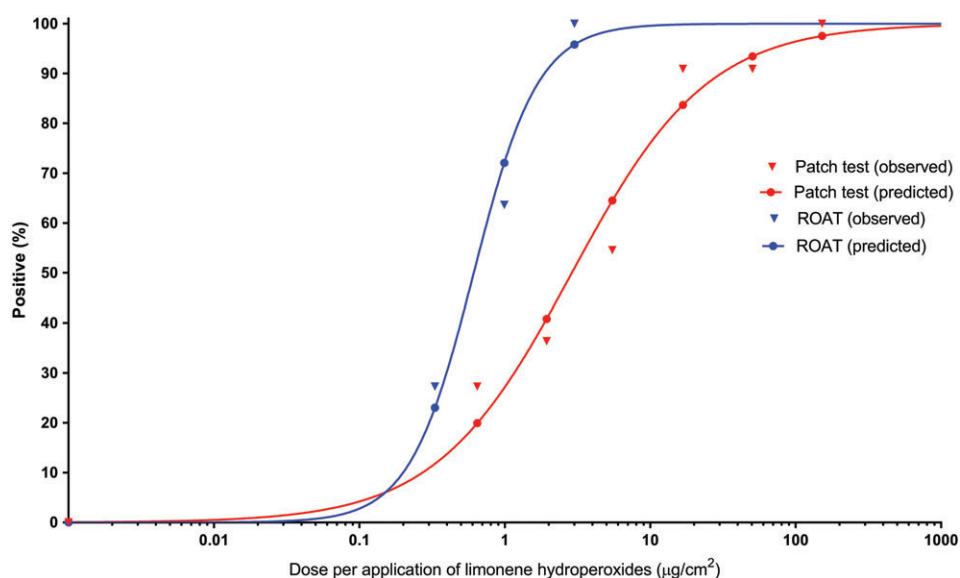


FIGURE 4 Observed and predicted dose-response curves in limonene hydroperoxide-allergic participants following exposure in the patch test dilution series and repeated open application test (ROAT)

TABLE 3 Calculated eliciting doses (EDs) and corresponding 95% confidence intervals (CIs) for specified proportions of limonene hydroperoxide-allergic participants reacting in the patch test and the repeated open application test (ROAT)

	Patch test		ROAT	
	Dose (μg of limonene hydroperoxides / cm^2)	95%CI	Dose (μg of limonene hydroperoxides / cm^2)	95%CI
ED ₁₀	0.27	0.017-0.79	0.20	0.016-0.37
ED ₂₅	0.89	0.15-1.9	0.35	0.076-0.57
ED ₅₀	2.9	1.1-5.9	0.61	0.30-1.0
ED ₇₅	9.4	4.7-31	1.1	0.67-3.3
ED ₉₀	31	13-254	1.9	1.1-15

concentration of Lim-OOHs in the simulated fine fragrance of 85 ppm (95%CI: 6.8-157 ppm). Significantly lower eliciting doses, that is non-overlapping 95% CIs, were estimated for ED₅₀ and ED₇₅ in Lim-OOHs allergic subjects when exposed in the ROAT compared to the single patch test exposure.

4 | DISCUSSION

In recent years, Lim-OOHs present in oxidized limonene have emerged as a very frequent cause of contact allergy in consecutively patch tested dermatitis patients.⁷⁻¹¹ However, high proportions of weak positive and doubtful reactions have caused some concern regarding the clinical relevance of a positive patch test reaction.^{12,13} We studied this question, and found that all patients with currently positive patch test reactions to standard Lim-OOHs 0.3% in pet., and 15% (2/13) of those with currently doubtful patch test reactions at D3/4 or D7, developed allergic contact dermatitis when exposed daily to realistic doses of oxidized limonene. This substantiates the clinical relevance of a positive patch test reaction to standard Lim-OOHs 0.3% pet., and further indicates that even some patients with only doubtful patch test reactions have a weak, but clinically relevant, allergy. No reactions, either allergic or irritant, were observed in the healthy control group.

This ROAT study was conducted in a blinded and randomized fashion, with three concentrations of oxidized limonene with contents of Lim-OOHs of 1260, 420 and 140 ppm, corresponding to doses per area per application of 3.0, 0.99 and 0.33 μg of Lim-OOHs/ cm^2 , respectively. These were formulated in ethanol/water to simulate exposure to a fine fragrance, with 100%, 64% and 27% of patients with positive patch test reactions to standard Lim-OOHs 0.3% pet. developing allergic contact dermatitis at the sites of these exposures. The selected concentrations and doses were chosen to simulate real-life daily exposure to limonene in a hydroalcoholic leave-on cosmetic product; however, quantitative assessment of exposure to Lim-OOHs in commercial fragranced consumer products containing limonene is challenging.²⁵ A recently published study using a method for selective analysis of hydroperoxides in perfumes investigated the content of limonene-2-hydroperoxide in 10 fine fragrances kept and used under normal conditions by consumers for 1 to 5 years after purchase.²⁶ In four of these fine fragrances, limonene-2-hydroperoxide was detected

at concentrations up to 56 ppm, but the levels of the more sensitizing limonene-1-hydroperoxide were not determined. In the current study, we estimated the dose, following repeated exposure in the ROAT, that will elicit allergic contact dermatitis in 10% of sensitized individuals to be 0.20 μg of Lim-OOHs/ cm^2 , corresponding to a concentration of Lim-OOHs in the simulated fine fragrance of 85 ppm. With the known ratio of limonene-2-hydroperoxide to limonene-1-hydroperoxide in the oxidized limonene used for this study, this corresponds to a concentration of limonene-2-hydroperoxide of ~ 70 ppm and a concentration of limonene-1-hydroperoxide of ~ 15 ppm, with the former being comparable to this documented exposure to limonene-2-hydroperoxide in leave-on cosmetic products.

These levels of exposure to Lim-OOHs are in contrast to data published by the fragrance industry, showing that limonene-2-hydroperoxide could be indirectly detected only up to 5 $\mu\text{g/g}$ (ppm) in 14 of 39 aged fine fragrances recalled from consumers.²⁷ On the basis of stability analyses performed in the same study, the authors argued that the formation of Lim-OOHs could be impaired or slowed down when limonene is formulated into a hydroalcoholic solution such as a fine fragrance. Even so, sensitizing Lim-OOHs can already be present from the time of purchase of the limonene added to a cosmetic product. Experimental oxidation studies with the essential oil sweet orange oil (the main compound being limonene) showed that limonene-2-hydroperoxide was present at a concentration of 0.05% (500 ppm) at time of purchase. Following 1 year of storage at 4°C in darkness, the concentration of limonene-2-hydroperoxide had increased significantly to 0.29% (2900 ppm).^{28,29} In a clinical setting, cosmetic products other than fine fragrances, such as deodorants, are more common causes of allergic contact dermatitis in patients with fragrance contact allergy.³⁰ Furthermore, although several possible sources of exposure to oxidized limonene exist, cosmetic products labelled to containing limonene are by far the most common sources of exposure causing allergic contact dermatitis in patients with positive patch test reactions to Lim-OOHs 0.3% pet.³¹ Future research into the formation and stability of Lim-OOHs over time, in cosmetic products with different matrices containing limonene from different sources, would be of interest to further elucidate this complex matter.

The challenges encountered in quantifying the contents of Lim-OOHs in both the added limonene and the finished consumer product are reflected in the current legislation and industry guidelines. In the European Commission's Regulation no. 1223/2009 on cosmetics, the limit reported regarding limonene is "a peroxide value less than 20 mmoles/L", which applies to the added substance and not to the finished cosmetic product.³² The standard for use concentrations of limonene in scented consumer products published by the International Fragrance Association (IFRA) states that: "Such products should have a peroxide value of less than 20 mmoles peroxides per liter, determined according to the FMA method."³³ This method seems to identify all types of peroxide and not just Lim-OOHs, and the accuracy and adequacy of this method are unknown.³⁴ Hence, it is unknown to what extent both the legislation and the IFRA standard limit exposure to Lim-OOHs in cosmetic products containing limonene.

In addition to repeated exposure in the ROAT in the current study, a serial dilution patch test was performed with concentrations of Lim-OOHs from 0.59% to 0.0024% (24 ppm). Among 11 patients

with positive patch test reactions to standard Lim-OOHs 0.3% pet., 27% reacted continuously down to the lowest applied concentration, and hence showed a MEC of 24 ppm. This finding supports the evidence that sensitized individuals can react to even lower concentrations of Lim-OOHs than investigated in the ROAT. However, it is important to remember that the applied dose per area during patch testing is higher than that in the ROAT for a specific concentration of Lim-OOHs. Overall, the dose-response relationship in patients with contact allergy to Lim-OOHs 0.3% pet., following both single patch test exposure and repeated exposure in the ROAT, resembles that of other well-established fragrance contact allergens such as cinnamal,³⁵ isoeugenol,^{36,37} and hydroxyisohexyl-3-cyclohexene carboxaldehyde (Lyrall).^{23,38} The results of the current study are also in accordance with a previous ROAT study on another oxidized fragrance terpene, namely oxidized linalool. Following repeated exposure of allergic subjects, with both a simulated fine fragrance and a simulated cream, the lowest concentration of linalool hydroperoxides that elicited a positive ROAT reaction was 560 ppm.³⁹ As previously established for these fragrance contact allergens, we showed that repeated exposure is important for the elicitation of allergic contact dermatitis when sensitized individuals are exposed to low doses of Lim-OOHs. It would have been desirable had we exposed allergic subjects to even lower concentrations of Lim-OOHs, both in the ROAT and in the dilution patch test, to provide an exposure estimate for a no-effect level with regard to elicitation of allergic contact dermatitis. As this is the first ROAT study to investigate an elicitation threshold for Lim-OOHs, we pragmatically chose the selected dilutions on the basis of experience from previous investigations.

Seven participants with either previous weak positive patch test reactions ($n = 4$, from the Swedish centre) or doubtful patch test reactions ($n = 3$, two from the Danish centre and one from the Swedish centre) to Lim-OOHs 0.3% pet. had negative confirmatory patch tests and did not proceed to the ROAT. It is well known that patch test reactivity can vary over time, and that reactivity can be regained later.^{20,40,41} Importantly, three of the four participants with previous weak positive patch test reactions to Lim-OOHs 0.3% pet. and current negative confirmatory patch test results with Lim-OOHs 0.3% pet. did, in fact, have at least doubtful patch test reactions to one of the two highest applied concentrations of Lim-OOHs in the dilution patch test series. However, as the investigators were blinded to the placement and concentrations of these, eligibility for advancement to the ROAT could not be based on these.

To conclude, patients with positive patch test reactions to standard Lim-OOHs 0.3% pet. develop allergic contact dermatitis when exposed to realistic doses of Lim-OOHs in a simulated leave-on cosmetic product. Furthermore, doubtful reactions to this patch test preparation can be of clinical relevance in some patients. In patients sensitized to Lim-OOHs, the dose-response relationship, following both single patch test exposure and repeated exposure, resembles that of other well-established fragrance contact allergens. Elicitation of allergic contact dermatitis in individuals sensitized to Lim-OOHs was observed with all applied concentrations in the ROAT, with a lowest observed effect level of exposure to Lim-OOHs of 140 ppm.

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary table 1: Individual results (excluding healthy controls) to previous and confirmatory patch testing with limonene hydroperoxides 0.3% pet., as well as individual reactions in the ROAT.

Participant	Age & sex	Atopic dermatitis (UK Working Party criteria)	Previous patch test limonene hydroperoxides 0.3% pet. (test year)	Confirmatory patch test limonene hydroperoxides 0.3% pet.	Positive ROAT, concentration of limonene hydroperoxides (day)
DK-P1	55 F	No	++ (2016)	++	0.13% (21)
DK-P2	73 F	No	++ (2016)	++	0.13% (7), 0.042% (14)
DK-P3	47 M	No	++ (2016)	++	0.13% (7), 0.042% (7)
DK-P4	54 M	No	+ (2012)	+	0.13% (7), 0.042% (7), 0.014% (14)
DK-P5	40 M	No	+ (2014)	?+	No reactions
DK-P6	23 F	Yes	+ (2015)	?+	No reactions
DK-P7	30 F	No	+ (2013)	+	0.13% (7), 0.042% (14), 0.014% (18)
DK-P8	35 F	No	+++ (2014)	++	0.13% (7)
DK-P9	20 F	No	?+ (2017)	?+	No reactions
DK-P10	68 F	No	++ (2015)	+++	0.13% (11), 0.042% (21)
DK-P11	45 F	No	?+ (2015)	?+	No reactions
DK-P12	59 F	No	++ (2015)	++	0.13% (7), 0.042% (21)
DK-P13	61 M	No	?+ (2015)	?+	No reactions
DK-P14	37 M	Yes	?+ (2015)	?+	No reactions
DK-P15	34 F	No	?+ (2016)	Negative	-
DK-P16	40 F	No	+ (2014)	?+	0.13% (6), 0.042% (14), 0.014% (14)
DK-P17	70 F	No	+ (2014)	++	0.13% (7), 0.042% (7), 0.014% (14)
DK-P18	57 F	Yes	?+ (2014)	Negative	-
DK-P19	44 M	Yes	?+ (2014)	?+	No reactions
DK-P20	28 F	Yes	?+ (2014)	?+	No reactions
DK-P21	57 F	Yes	?+ (2012)	?+	No reactions
DK-P22	24 F	Yes	?+ (2013)	?+	0.13% (21)
SE-P1	46 F	No	+ (2017)	+	0.13% (21+12*)
SE-P2	35 F	Yes	+ (2017)	Negative	-
SE-P4	74 F	No	+ (2016)	Negative	-
SE-P5	28 F	No	+ (2017)	?+	No reactions
SE-P6	78 M	No	?+ (2016)	?+	No reactions
SE-P8	46 F	Yes	+ (2015)	Negative	-
SE-P10	28 F	No	?+ (2017)	Negative	-
SE-P11	50 F	No	+ (2016)	Negative	-
SE-P12	38 F	No	+++ (2015)	++	0.13% (7)

***The participant developed a clearly positive reaction on the ROAT test area 12 days after exposure had ended in the study. She had not used any scented products on the test area between finishing the ROAT and developing a reaction.**

Supplementary table 2: Compliance in the ROAT based on weighing of test solutions before and after administration by participants

	Actual use compared to expected use, mean percentage (SD)*				
Test solution	Allergic subjects		Doubtful allergic subjects		Healthy controls
		Compared to healthy controls, p-value		Compared to healthy controls, p-value	
Oxidized limonene 1.0 %	116% (9.9)	0.38	112% (21)	0.23	119% (9.3)
Oxidized limonene 0.33 %	125% (11)	-	116% (21)	-	-
Oxidized limonene 0.11 %	119% (12)	-	112% (20)	-	-
Vehicle	116% (6.0)	0.09	112% (22)	0.11	123% (12)

* Two *allergic subjects* and one *healthy control* did not return one or more test solutions during the study period.