Recommendation to test limonene hydroperoxides 0.3% and linalool hydroperoxides 1.0% in the British Baseline patch test series

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What's already known about this topic?

- A significant rate of sensitisation to oxidised limonene and linalool has been demonstrated worldwide
- Distinguishing true positives from irritant reactions may be difficult

What does this study add?

- Higher concentrations of limonene hydroperoxides (0.3%) and linalool hydroperoxides (1.0%) detect more sensitised patients
- Dilutions should be tested in patients with questionable reactions

What's new?

- Limonene hydroperoxides 0.3% and linalool hydroperoxides 1.0% should be added to the British baseline patch test series

ABSTRACT

Background:

There is a significant rate of sensitisation worldwide to the oxidised fragrance terpenes limonene and linalool. Patch testing to oxidised terpenes is not routinely carried out; the ideal patch test concentration is unknown.

Objectives:

To determine the best test concentrations for limonene and linalool hydroperoxides, added to the British baseline patch test series, to optimise detection of true allergy and minimise irritant reactions.

Methods:

During 2013-2014, 4563 consecutive patients in 12 UK centres were tested to hydroperoxides of limonene in petrolatum (pet.) 0.3%, 0.2% and 0.1%, and hydroperoxides of linalool 1.0%, 0.5% and 0.25% pet. Irritant (IR) reactions were recorded separately from doubtful (?+) reactions. Concomitant reactions to other fragrance markers and clinical relevance were documented.

Results:

Limonene hydroperoxide 0.3% gave positive reactions in 241 (5.3%) patients, irritant reactions in 93 (2.0%) and doubtful reactions in 110 (2.4%). Linalool hydroperoxide 1.0% gave positive reactions in 352 (7.7%), irritant reactions in 178 (3.9%), and doubtful reactions in 132 (2.9%). 119 patients with crescendo reactions to 0.3% limonene would have been missed if only tested with 0.1%. 131 patients with crescendo reactions to 1.0% linalool would have been missed if only tested with 0.25%. In almost two-thirds of patients with positive patch tests to limonene and linalool the reaction was clinically relevant. The majority of patients did not react to any fragrance marker in the baseline series.

Conclusions:

We recommend that limonene hydroperoxides be tested at 0.3% and linalool hydroperoxides at 1.0% in the British baseline patch test series.

Background

Limonene and linalool are terpene fragrances of natural origin present in the majority of detergents and cosmetics purchased by the UK consumer.[1] While uncommon fragrance allergens in their natural state, they become potent allergens following air-oxidation.[2] The newly-formed oxidation products include hydroperoxides, largely responsible for sensitisation.

Limonene, the main constituent of citrus peel oil, has a fresh lemon aroma and is found in half of all household detergents and 98% of women's fragrances.[1,3] Linalool has a flowery smell and is present in a variety of essential oils including lavender, jasmine, geranium, ylang-ylang, rosewood and sage. Similarly to limonene, it is found in most fine fragrances.[4] It has been identified as the fragrance terpene to which there is the most frequent exposure.[3,5]

European legislation stipulates that 26 named fragrances, including limonene and linalool, must be indicated in the list of ingredients on cosmetic and detergent products, if their concentration exceeds 0.001% (10ppm) in leave-on products (e.g. a moisturiser) and 0.01% (100ppm) in rinse-off products (e.g. a shampoo). If they are added as part of an essential oil, consumers may not be aware of their presence in certain products.[6] Although essential oils containing limonene and linalool should have them listed on the label if present at threshold levels of 10ppm in the finished product for leave on and 100ppm for rinse off products, aromatherapy products are not covered by the Cosmetics Regulations. (Regulation (EC) No. 1223/2009). A widely-used household cosmetic brand marketed in the UK labelled 'no artificial perfume or colour', contains unlabelled limonene and linalool, inherent constituents of pelargonium graveolens (geranium) oil.

Commercial patch test preparations of oxidised limonene and linalool have in 2012 become available from Chemotechnique Diagnostics (Vellinge, Sweden) (Hydroperoxides of limonene 0.3% in petrolatum (pet.) (H-032A) and Hydroperoxides of linalool 1.0% pet (H-031A)), but are not routinely tested.

A recent national UK audit, published in 2014, demonstrated a significant rate of allergy to limonene hydroperoxides 0.3% and linalool hydroperoxides 1.0%.[2] However, in this audit the numerous doubtful and irritant reactions had not been grouped separately for purposes of analysis, nor had data been gathered on concomitant reactions to other fragrance markers and on clinical relevance.

The ideal concentration for patch testing oxidised terpenes would keep doubtful and irritant reactions to an acceptable level whilst optimising the chances of detecting a true contact allergy, and minimising the risk of active sensitisation. Hence, a further audit, intended to determine the optimal concentration of terpene hydroperoxides for patch testing, was set up. The intentions were: 1.To specifically compare 3 concentrations of each terpene hydroperoxide; 2.To distinguish irritant from doubtful reactions; 3.To assess concomitant reactions to other fragrance markers; and 4.To address the issue of clinical relevance.

Method

During a twelve month period between October 2013 and October 2014, data were collected from 12 UK and Irish dermatology departments (Bath, Birmingham, Cardiff, Cork, Dundee, East Kent, Leeds, Leicester, Newport, Oxford, Sheffield and Swansea). 4563 consecutive patients (including a small number of children and adolescents) were tested to an extended British baseline patch test series including hydroperoxides of limonene at concentrations of 0.3%, 0.2% and 0.1% pet. (equivalent to oxidised limonene 3.0%, 2.0% and 1.0% respectively), and hydroperoxides of linalool 1.0%, 0.5% and 0.25% pet.(equivalent

to oxidised linalool 6.0%, 3.0% and 1.5% respectively), plus other series as clinically indicated. All allergens were produced by Chemotechnique Diagnostics, who confirmed the content of hydroperoxides. The batch to batch variation was: limonene hydroperoxide 0.3%; 0.27-0.33%, linalool hydroperoxide 1.0%; 0.95-1.05%, limonene hydroperoxide 0.2%; 0.18-0.22%, linalool hydroperoxide 0.5%; 0.47-0.53%, limonene hydroperoxide 0.1%; 0.09-0.11%, linalool hydroperoxide 0.25%; 0.24-0.26%.

Allergens were stored and dispensed according to manufacturers' instructions, used within the recommended stability periods (8 months for limonene, 12 months for linalool), and were laid out immediately prior to application by experienced patch test nurses. 8mm Finn Chambers® (Epitest Oy, Tuusula, Finland) on Scanpor® tape (Norgesplaster A/S, Vennsela, Norway) were used in 11 centres. One centre (Swansea) used IQ Ultimate™ chambers. The amount of allergen applied was enough to fill the well of the disk but not extrude when the patch was applied to the patient's back, approximately 20 mg (40 mg/cm2) for Finn chambers and 25 mg (36 mg/cm2) for IQ chambers. Patches were applied for 48 hours.

Readings were carried out on Day 2 (D2) and 4 (D4) (or day 5 (D5) in one centre) by a dermatologist experienced in interpreting patch tests. Allergic patch test reactions were scored according to the guidelines of the International Contact Dermatitis Research Group criteria (Table 1).[7] Irritant (IR) reactions were characterised as well-defined erythema at D2 limited to the exposure area with a lack of infiltrate and with a decrescendo effect between D2 and D4/5, particularly in patients who had irritant reactions to other known irritant patch test preparations, or to the Scanpor tape. Sharp-edged margins and a wrinkled test area surface were other features indicative of irritant reactions. Doubtful (?+) reactions were defined as homogenous macular erythema with minimum to no infiltration, limited to the exposure area, without a decrescendo effect, or appearing only at D4.

Clinical relevance was determined as either relevant or of unknown relevance by the clinician doing the final patch test reading. Examples of relevant reactions were those in patients with a clear current or previous history of fragrance allergy, or dermatitis from a product labeled to contain limonene or linalool, or dermatitis from a botanical oil with limonene or linalool as a constituent, or a positive repeated open application test to such a product. The frequency of positive reactions to each concentration of limonene and linalool hydroperoxide, and the clinical characteristics of patients were recorded. These included age, sex, presence of atopic dermatitis and duration of rash. Reactions to the other fragrance markers in the baseline series (Fragrance mix (FM)1, FM2, *Myroxylon pereirae*, Hydroxyisohexyl 3-cyclohexene carboxaldehyde and colophonium) were recorded.

We compared associations between age, gender, duration and history of atopic dermatitis, and presence of a positive patch test to either limonene 0.3% or linalool 1.0% on day 4/5. We used Chi square test for categorical variables (gender, history of atopy), analysis of variance (ANOVA) for continuous (age) and Kruskal-Wallis for continuous non-normal variables (duration of dermatitis). Due to the repeated nature of the data collected, we used Bhapkar's test of marginal homogeneity to compare reactions to limonene and linalool at each concentration on the same day and between day 2 and day 4. All analyses were undertaken using R version 3.3.2.

Results

A total of 4563 patients were patch tested at 12 UK centres (Table 2). 463 (10.1%, 95% CI 9.3%-11.1%) had positive patch test reactions (1+, 2+, or 3+) to one or both terpene hydroperoxides on D4/5: 111 (2.4%, 95% CI 2.0%-2.9%) were positive to limonene hydroperoxide 0.3% alone and 222 (4.9%, 95% CI 4.3%-5.5%) to linalool hydroperoxide 1.0% alone (Figure 1). 130 of the 463 (2.8%, 95% CI 2.4%-3.4% of the 4563 tested) had a positive reaction (1+, 2+ or 3+) to both limonene and linalool. Hence the total number positive to limonene 0.3% was 241 (5.3%, 95% CI 4.7%-6.0%) and the total positive to linalool 1.0% was 352 (7.7%, 95% CI 7.0%-8.5%). 93 (2.0%, 95% CI 1.7%-2.5%) irritant reactions to limonene hydroperoxide 0.3% were recorded and 178 (3.9%, 95% CI 3.4%-4.5%) to linalool hydroperoxide 1.0% (Table 2). 110 (2.4%, 95% CI 2.0%-2.9%) had doubtful reactions to limonene hydroperoxide 0.3% and 132 (2.9%, 95% CI 2.4%-3.4%) had doubtful reactions to linalool hydroperoxide 1.0%.

The results from the 5 largest centres testing more than 500 patients per year, all of whom used 8mm Finn chambers and a standardised dose, are almost identical to our overall results; 163/3091 (5.3%) of patients were positive to limonene hydroperoxides 0.3% and 245/3091 (7.9%) to linalool hydroperoxides 1.0%.

The mean (+ SD) age of the 241 patients with 1+ to 3+ positive reactions to limonene hydroperoxide 0.3% was 41.3 years (SD 18.1). There were 166 females (81.0%). The median duration of dermatitis was 24 months (IQR 12-60). 81 (33.6%) were atopic. The mean (+SD) age of the 352 patients with 1-3+ positive reactions to linalool hydroperoxide 1.0% was 45.0 years (SD 19.2). There were 226 females (77.4%). The median duration of dermatitis was 33 months (IQR 12-60). 113 (32.1%) were atopic (Table 3).

Table 4 shows the effect of increasing concentration on the number of negative, positive, doubtful and irritant reactions at D2 and D4/5 for limonene and linalool. At D4/5, 0.1% limonene had the lowest rate of positive reactions (1.3%), and 1.0% linalool the highest rate (7.7%). As the concentration of limonene and linalool increased, the rate of both positive and irritant reactions increased (all p-values < 0.001).

Looking for a crescendo effect, indicative of true allergic positive reactions,[8] we compared patch test reactions on day 2 versus day 4 at the different concentrations of limonene and linalool. When testing with limonene 0.3% (Tables 5a-5c; Figure 2), 161 patients had negative patch tests on D2 which became positive on D4, compared with 100 at 0.2%, and 42 at 0.1%. When testing with linalool 1.0% (Tables 5d-5f; Figure 3), 194 patients had negative patch tests on D2 which became positive on D4, compared with 128 at 0.5%, and 63 at 0.25%. We found more positive reactions on day 4 versus day 2 for both limonene and linalool at all concentrations (all P-values < 0.001).

Clinical relevance was recorded in 1017 of the 1047 patients who had IR, ?+, or 1+ to 3+ positive reactions to limonene or linalool. In almost two-thirds of patients with positive patch tests to limonene and linalool the reaction was deemed clinically relevant. Increasing the concentration of limonene and linalool had no effect on clinical relevance (Table 6).

Concomitant reactions were recorded in 824 of the 1047 patients who had an irritant, ?+, or 1+ to 3+ positive reaction to limonene or linalool hydroperoxide. There was no correlation between the concentrations of limonene and linalool and the percentage of patients with concomitant positive reactions to other fragrance markers. In patients with 2+ and 3+ reactions, reactions to other baseline series fragrance markers appeared more frequent than in those with 1+, irritant, doubtful or negative reactions, however the numbers of patients were small in the 2+/3+ group (statistical analysis was not performed). (Tables 7 and 8; Figures 4 and 5). Only 229 of the 824 patients (27.8%) reacted to any other fragrance marker in the baseline series.

Discussion

This large multicentre UK audit confirms the high rate of allergy to 0.3% limonene hydroperoxides (5.3%) and 1% linalool hydroperoxides (7.7%) in consecutively patch tested patients, which we previously reported.[2] A very similar rate of contact sensitization to 0.3% limonene hydroperoxides of 5.1% has recently been reported in Spain.[9] Our results, however, show higher rates of positive patch tests than two recent international multicentre studies of consecutive dermatitis patients from 6-9 test centres, which showed positive reactions to R-limonene hydroperoxides 0.3% in 2.3% of 2411 patients and oxidised linalool 1.0% in 5.3% of 2900 patients respectively.[10,11]

We found a broadly similar rate of questionable (doubtful and irritant) reactions as we had previously. Limonene 0.3% had a rate of irritant reactions of 2.0% and doubtful reactions of 2.4% and linalool 1.0% had a rate of irritant reactions of 3.9% and doubtful reactions of 2.9%. This gives a combined rate of doubtful and irritant reactions to limonene of 4.4% and to linalool of 6.8%, very similar to the overall rate of 7.3% in our previous paper, where irritant and doubtful reactions to limonene and linalool were grouped together.[2] In a recent international multicentre study the combined rate of irritant and doubtful reactions to 0.3% limonene hydroperoxides was 7.9% (irritant 0.9% and doubtful 7.0%).[11] In another study, using 1% linalool hydroperoxides, the combined rate of irritant and doubtful reactions was 6.7%, irritant 0.14-0.3% and doubtful 6.4%).[12]

Differences in rates of irritant and doubtful reactions between studies illustrate the great difficulty in interpretation of patch test results to these chemicals. These differences are also seen within studies, for example Table 2 illustrates the variation between centres in our audit in rates of irritant reactions: from 0-6.8% for limonene and 0-17% for linalool. Similarly, doubtful reactions ranged from 0-17.3% for limonene 0.3% and 0.3-19.2% for linalool 1%. Interpretation of positive patch test reactions requires experienced clinical judgement and is a particular challenge with oxidized terpenes. It should be acknowledged that in this audit patch tests were read on differing days in different centres, with some centres having a D5 reading, which could influence the rate of irritant reactions.

A Repeated Open Application Test (ROAT), is recommended in The European Society of Contact Dermatitis guidelines to be carried out in cases of doubtful reactions.[13] An alternative or additional method is to test serial dilutions of the allergen,[13] as irritant reactions may abruptly disappear at lower concentrations whereas allergic reactions may show a gradually reducing strength of response with reducing concentration. Dilutions of

hydroperoxides of limonene and linalool are now commercially available from Chemotechnique (0.2% hydroperoxides of limonene (H-032B) and 0.5% hydroperoxides of linalool (H-031B).

Brasch et al. showed that crescendo or plateau reaction patterns at Days 1 to 3 were significantly more often observed in relevant allergic reactions.[8] We used a crescendo or plateau pattern to assist in distinguishing positive from doubtful and irritant reactions. As the concentration of limonene increased, the greatest difference in detection rates of patients with negative patch tests at D2 becoming positive at D4/5 (crescendo reactions) was when the concentration of limonene was increased from the lowest concentration, 0.1%, to the highest, 0.3%, resulting in an extra 119 patients with allergy to limonene being detected who would otherwise have been missed. The same was found for the increase in concentration of linalool from 0.25% to 1.0%, when 131 extra patients with linalool allergy were detected. The highest concentrations, 0.3% limonene and 1.0% linalool hydroperoxide respectively, also demonstrated the largest number of cases where a questionable reaction at D2 became a definite positive reaction at D4. This supports the need to test the highest concentrations for both limonene and linalool, as testing at 0.1% or 0.2% limonene and 0.25% or 0.5% linalool only would miss these cases.

The rate of irritant reactions also increased with increasing concentrations. For limonene, the rate of irritant reactions increased from 0.6-2.0% as the concentration increased from 0.1-0.3%. For linalool the rate of irritant reactions increased from 1.1-3.9% as the concentration increased from 0.25-1.0%. It is our opinion that this increase in rate of irritant reactions, while undesirable, is counterbalanced by the increase in detection of sensitisation cases at higher concentrations.

Clinical relevance in patients with positive reactions was present in almost two-thirds of our patients, but there was no correlation between increasing concentration of limonene and linalool hydroperoxide and clinical relevance. A recent international study found that exposure to products containing limonene was assessed as being relevant for the patient's dermatitis in only 36% of all patients with a positive patch test to 0.3% limonene hydroperoxide. However, in the subgroup of patients attending Barcelona and Copenhagen, more than 70% were deemed as relevant, similar to our results.[12]

Recent international multicentre studies showed that only approximately 40% of patients reacting to oxidized linalool and/or R-limonene had concomitant reactions to other markers of fragrance allergy, in keeping with our finding that more than two-thirds of patients sensitised to oxidized terpenes do not have this detected by fragrance screening markers in the baseline patch test series.[10,11] These results are also supported by the finding of Matura et al. that only 29-33% of patients with positive patch tests to oxidized R- or S-limonene also reacted to other fragrance markers,[14] and by other studies.[15-17] A recent paper showed concomitant reactions to another baseline series fragrance marker to occur in 42.1% of patients reacting to 0.3% limonene hydroperoxides[18] and 39.5% reacting to 1.0%. linalool hydroperoxides, fairly similar to our overall results[18].

In our study, there was no clear relationship between the patch test concentration of limonene and linalool and reactions to other fragrance markers. We did, however, find that patients with 2+ and 3+ reactions appeared more likely to have reactions to other fragrance markers than those with 1+ reactions, although the numbers of patients with 2+/3+

reactions were small. This is in keeping with Christensson's finding (testing oxidized linalool 4.0% - 11% pet.) that the strength of the positive patch test reaction correlated with the likelihood of a positive patch test to other fragrance markers, being 28-43% in patients with 1+ reactions and 75-80% in patients with 2-3+ reactions.[19] The same authors have shown that cross-reactivity of hydroperoxide haptens only occurs where the haptens have overall very similar structures.[20] We also found that positivity to other fragrance markers could not be used to clearly distinguish allergic from doubtful reactions. Our results clearly illustrate that standard screening markers for fragrance allergy in the baseline series (such as FM 1 + 2) are not adequate to detect allergy to oxidised terpenes. Patients with fragrance allergy will be missed unless oxidised limonene and linalool are routinely tested.

Atopic dermatitis did not appear to be associated with positive (1+ to 3+) versus irritant or doubtful reactions to limonene or linalool although one might have expected irritant reactions to be more frequent in atopic patients. A previous report found no association between atopy and allergy to FM 1.[21]

It has been demonstrated, through repeated open application testing, that exposure to low concentrations of oxidised linalool causes eczema in allergic patients.[22] This is highly relevant for many consumers previously sensitised to oxidised linalool since they may be at risk of elicitation of dermatitis with the low concentrations present in everyday personal hygiene products. The increasing use of 'natural ingredient based cosmetics' is a further hazard, as these contain essential oils (which may already contain oxidised terpenes when received from a producer,[18] or can subsequently oxidise during storage[23]). Pesonen et al. reported occupational hand dermatitis due to exposure to cosmetic products scented with limonene.[24] The range in occupational groups affected by dermatitis to oxidised limonene is broad; from laboratory technicians to masseurs.[12]

Unless oxidised limonene and linalool are used as a screen for fragrance allergy, patients with clinically relevant sensitisation will be missed. Therefore, we suggest that the baseline British patch test series is extended to include limonene hydroperoxides 0.3% (H-032A) and linalool hydroperoxides 1.0% (H-031A). Patients with doubtful reactions should have a ROAT with suspected products or have dilutions of the hydroperoxides tested (0.2% hydroperoxides of limonene and 0.5% hydroperoxides of linalool).

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Score	Reaction
0	Negative
?+	Doubtful: erythema only
1+	Weak (non-vesicular) positive allergic reaction; erythema, infiltration and possibly papules
2+	Strong (vesicular) positive allergic reaction; erythema, infiltration, papules and vesicles
3+	Extreme positive allergic reaction; bullous reaction
IR	Irritant reaction

Table 1. Scoring of patch tests according to the International Contact Dermatitis Research Group.[7]

Site	Total tested	Limonene 0.3% day 4/5						Linalool 1.0% day 4/5					
		Irritant		?+		1+/2+/3+		Irritant		?+		1+/2+/3+	
		N	%	N	%	N	%	N	%	N	%	N	%
Centres seeing 500 + patients/year													
Leeds	758	0	0.0	5	0.7	26	3.4	0	0.0	5	0.7	34	4.5
Oxford	695	13	1.9	0	0.0	46	7.0	13	1.9	5	0.7	43	6.1
Dundee	557	24	4.3	8	1.4	50	9.4	61	11.0	14	2.5	78	14.3
Newport	550	4	0.7	1	0.2	18	3.0	4	0.7	4	0.7	37	7.0
Sheffield	531	36	6.8	33	6.2	23	4.6	64	12.1	43	8.1	53	10.1
Subtotal	***3091***	***77***	***2.5***	***47***	***1.5***	***163***	***5.3***	***142***	***4.6***	***71***	***2.3***	***245***	***7.9***
Centres seeing <500 patients/year													
Leicester	341	6	1.8	2	0.6	9	2.3	8	2.3	1	0.3	12	3.3
Cardiff	281	0	0.0	1	0.4	14	5.0	0	0.0	1	0.4	21	7.0
Birmingham	250	0	0.0	23	9.2	0	0.0	0	0.0	24	9.6	0	0.0
Swansea	191	0	0.0	11	5.8	32	17	0	0.0	8	4.2	32	17
Bath	165	10	6.1	3	1.8	4	2.2	28	17.0	1	0.6	10	6.0
East Kent	140	0	0.0	5	3.6	11	7.9	0	0.0	6	4.3	26	18.6
Cork	104	0	0.0	18	17.3	8	8.0	0	0.0	20	19.2	6	6.0
Subtotal	***1472***	***16***	***1.1***	***63***	***4.3***	***78***	***5.3***	***36***	***2.4***	***61***	***4.1***	***107***	***7.3***
All	**4563**	**93**	**2.0**	**110**	**2.4**	**241**	**5.3**	**178**	**3.9**	**132**	**2.9**	**352**	**7.8**

Table 2. Total number of patients patch tested at each centre and the number and percentage of irritant, doubtful and positive reactions to hydroperoxides of (a) limonene 0.3% and (b) linalool 1.0% at day 4/5. There are subtotals for the five centres patch testing larger numbers of patients (500+ per year) as well as a subtotal for the remaining seven centres patch testing less than 500 patients per year.

	Irritant	?+	1/2/3+	p value
Limonene 0.3% D4/5				
N	93	110	241	
Mean age (SD)[1]	52.3 (18.6)	43.3 (18.9)	41.3 (18.1)	<0.001
Female gender N (%)[2]	48 (67.6%)	83 (79.8%)	166 (81.0%)	0.06
Median duration, months (IQR)[3]	36 (12-90)	24 (12-84)	24 (12-60)	0.45
Atopic N (%)[2]	33 (35.9%)	29 (33.7%)	81 (33.6%)	0.95
Linalool 1.0% D4/5				
N	178	132	352	
Mean age (SD) [1]	50.6 (17.8)	45.5 (19.6)	45.0 (19.2)	0.03
Female gender N (%)[2]	87 (69.6%)	92 (76.7%)	226 (77.4%)	0.22
Median duration, months (IQR)[3]	24 (12-60)	24 (12-84)	33 (12-60)	0.55
Atopic N (%)[2]	62 (35.6%)	36 (34.3%)	113 (32.1%)	0.83

Table 3. Demographics of 1047 patients with irritant (IR), doubtful (?+) or positive patch test (1 to 3+) reactions to limonene 0.3% and linalool 1.0% on Day 4/5. Patients with irritant reactions were significantly older than those with 1+ or 2+/3+ reactions, for both limonene and linalool.[1]p values from ANOVA, [2]p values from Chi square test, [3]p values from Kruskal-Wallis test.

(a)

	0.1% Limonene				0.2% Limonene				0.3% Limonene			
	Day 2		Day 4/5		Day 2		Day 4/5		Day 2		Day 4/5	
	N	%	N	%	N	%	N	%	N	%	N	%
Negative	4517	99.0	4424	97.0	4449	97.5	4278	93.8	4364	95.6	4119	90.3
Irritant	8	0.2	28	0.6	31	0.7	40	0.9	66	1.4	93	2.0
?+	27	0.6	53	1.2	50	1.1	98	2.1	84	1.8	110	2.4
1+/2+/3+	11	0.2	58	1.3	33	0.7	147	3.2	49	1.1	241	5.3

(b)

	0.25% Linalool				0.5% Linalool				1.0% Linalool			
	Day 2		Day 4/5		Day 2		Day 4/5		Day 2		Day 4/5	
	N	%	N	%	N	%	N	%	N	%	N	%
Negative	4419	96.8	4264	93.4	4290	94.0	4086	89.5	4123	90.4	3901	85.5
Irritant	36	0.8	51	1.1	80	1.8	89	2.0	184	4.0	178	3.9
?+	72	1.6	133	2.9	121	2.7	157	3.4	173	3.8	132	2.9
1+/2+/3+	36	0.8	115	2.5	72	1.6	231	5.1	83	1.8	352	7.7

Table 4. Effect of increasing concentration on the number of negative, irritant, doubtful and positive reactions on day 2 and day 4/5 for (a) limonene and (b) linalool.

	0.1% Limonene, Day 4					
0.1% Limonene, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	4411	25	39	37	5	4517
Row(%)	97.7%	0.6%	0.9%	0.8%	0.1%	
Irritant						
N	0	3	2	3	0	8
Row(%)	0.0%	37.5%	25.0%	37.5%	0.0%	
?+						
N	9	0	11	6	1	27
Row(%)	33.3%	0.0%	40.7%	22.2%	3.7%	
1+						
N	4	0	1	5	1	11
Row(%)	36.4%	0.0%	9.1%	45.5%	9.1%	
2+/3+						
N	0	0	0	0	0	0
Row(%)	0%	0%	0%	0%	0%	
Total	4424	28	53	51	7	4563

Table 5a. 0.1% limonene: Day 2 versus Day 4.

	0.2% Limonene, Day 4					
0.2% Limonene, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	4239	28	82	97	3	4449
Row(%)	95.3%	0.6%	1.8%	2.2%	0.0%	
Irritant						
N	14	11	2	4	0	31
Row(%)	45.2%	35.5%	6.5%	12.9%	0.0%	
?+						
N	20	1	12	16	1	50
Row(%)	40.0%	2.0%	24.0%	32.0%	2.0%	
1+						
N	5	0	2	21	4	32
Row(%)	15.6%	0.0%	6.2%	65.6%	12.5%	
2+/3+						
N	0	0	0	0	1	1
Row(%)	0.0%	0.0%	0.0%	0.0%	100.0%	
Total	4278	40	98	138	9	4563

Table 5b. 0.2% limonene: Day 2 versus Day 4.

	0.3% Limonene, Day 4					
0.3% Limonene, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	4062	66	75	157	4	4364
Row(%)	93.1%	1.5%	5.5%	3.6%	0.1%	
Irritant						
N	31	24	3	8	0	66
Row(%)	47.0%	36.4%	4.5%	12.1%	0.0%	
?+						
N	21	2	27	32	2	84
Row(%)	25.0%	2.4%	32.1%	38.1%	2.4%	
1+						
N	5	1	5	29	4	44
Row(%)	11.4%	2.3%	11.4%	65.9%	9.1%	
2+/3+						
N	0	0	0	0	5	5
Row(%)	0.0%	0.0%	0.0%	0.0%	100.0%	
Total	4119	93	110	226	15	4563

Table 5c. 0.3% limonene: Day 2 versus Day 4.

	0.25% Linalool, Day 4					
0.25% Linalool, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	4228	33	95	61	2	4419
Row(%)	95.7%	0.7%	2.1%	1.4%	0.0%	
Irritant						
N	11	15	4	6	0	36
Row(%)	30.6%	41.7%	11.1%	16.7%	0.0%	
?+						
N	21	2	32	16	1	72
Row(%)	29.2%	2.8%	44.4%	22.2%	1.4%	
1+						
N	4	1	2	26	1	34
Row(%)	11.8%	2.9%	5.9%	76.5%	2.9%	
2+/3+						
N	0	0	0	1	1	2
Row(%)	0.0%	0.0%	0.0%	50.0%	50.0%	
Total	4264	51	133	110	5	4563

Table 5d. 0.25% linalool: Day 2 versus Day 4.

	0.5% Linalool, Day 4					
0.5% Linalool, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	4007	55	100	121	7	4290
Row(%)	93.4%	0.3%	2.3%	2.8%	0.2%	
Irritant						
N	40	25	8	7	0	80
Row(%)	50.0%	31.2%	10.0%	8.8%	0.0%	
?+						
N	33	6	40	41	1	121
Row(%)	27.3%	5.0%	33.1%	33.9%	0.8%	
1+						
N	6	3	8	45	2	64
Row(%)	9.4%	4.7%	12.5%	70.3%	3.1%	
2+/3+						
N	0	0	1	4	3	8
Row(%)	0.0%	0.0%	12.5%	50.0%	37.5%	
Total	4086	89	157	218	13	4563

Table 5e. 0.5% linalool: Day 2 versus Day 4.

	1.0% Linalool, Day 4					
1.0% Linalool, Day 2	**Negative**	**Irritant**	**?+**	**1+**	**2+/3+**	**Total**
Negative						
N	3761	97	71	177	17	4123
Row(%)	91.2%	2.4%	1.7%	4.3%	0.4%	
Irritant						
N	96	63	10	15	0	184
Row(%)	52.2%	34.2%	5.4%	8.2%	0.0%	
?+						
N	41	14	48	65	5	173
Row(%)	23.7%	8.1%	27.7%	37.6%	2.9%	
1+						
N	3	3	3	56	10	75
Row(%)	4.0%	4.0%	4.0%	74.7%	13.3%	
2+/3+						
N	0	1	0	3	4	8
Row(%)	0.0%	12.5%	0.0%	37.5%	50.0%	
Total	3901	178	132	316	36	4563

Table 5f. 1.0% linalool: Day 2 versus Day 4.

	Unknown relevance	Clinically relevant	Total positive (1+, 2+, 3+)
0.1% limonene	19 (33.3%)	38 (66.7%)	57
0.2% limonene	51 (35.4%)	93 (64.6%)	144
0.3% limonene	78 (33.5%)	155 (66.5%)	233
0.25% linalool	47 (41.2%)	67 (58.8%)	114
0.5% linalool	76 (33.2%)	153 (66.8%)	229
1.0 % linalool	124 (36.4%)	217 (63.6%)	341

Table 6. Effect of increasing concentration on clinical relevance for limonene and linalool. Relevance data were recorded in 1017 of the 1047 patients who had irritant, ?+, 1+, or 2+/3+ reactions to hydroperoxides of limonene or linalool.

	Positivity to other fragrance markers		
Limonene, Day 4	**0.1%**	**0.2%**	**0.3%**
Negative			
N	191	156	102
Row(%)	26.9%	26.9%	22.3%
Irritant			
N	4	6	18
Row(%)	20.0%	16.2%	29.0%
?+			
N	15	20	34
Row(%)	31.9%	25.6%	38.2%
1+			
N	16	43	67
Row(%)	37.2%	35.0%	32.7%
2+/3+			
N	3	4	8
Row(%)	75.0%	66.7%	80.0%

Table 7. Day 4 Limonene 0.1%, 0.2%, 0.3%: Proportion of people with positivity to other fragrance markers (recorded in 824 of the 1047 patients; 229 (27.8%) of these had a positive reaction to another fragrance marker).

	Positivity to other fragrance markers		
Linalool, Day 4	**0.25%**	**0.5%**	**1.0%**
Negative			
N	132	96	60
Row(%)	23.0%	22.5%	19.9%
Irritant			
N	8	10	21
Row(%)	18.6%	13.5%	17.4%
?+			
N	42	39	35
Row(%)	38.2%	29.5%	32.1%
1+			
N	45	80	100
Row(%)	47.9%	43.2%	36.6%
2+/3+			
N	2	4	13
Row(%)	66.7%	66.7%	65.0%

Table 8. Day 4 linalool 0.25%, 0.5%, 1.0%: proportion of people with positivity to other fragrance markers (recorded in 824 of the 1047 patients; 229 (27.8%) of these had a positive reaction to another fragrance marker).

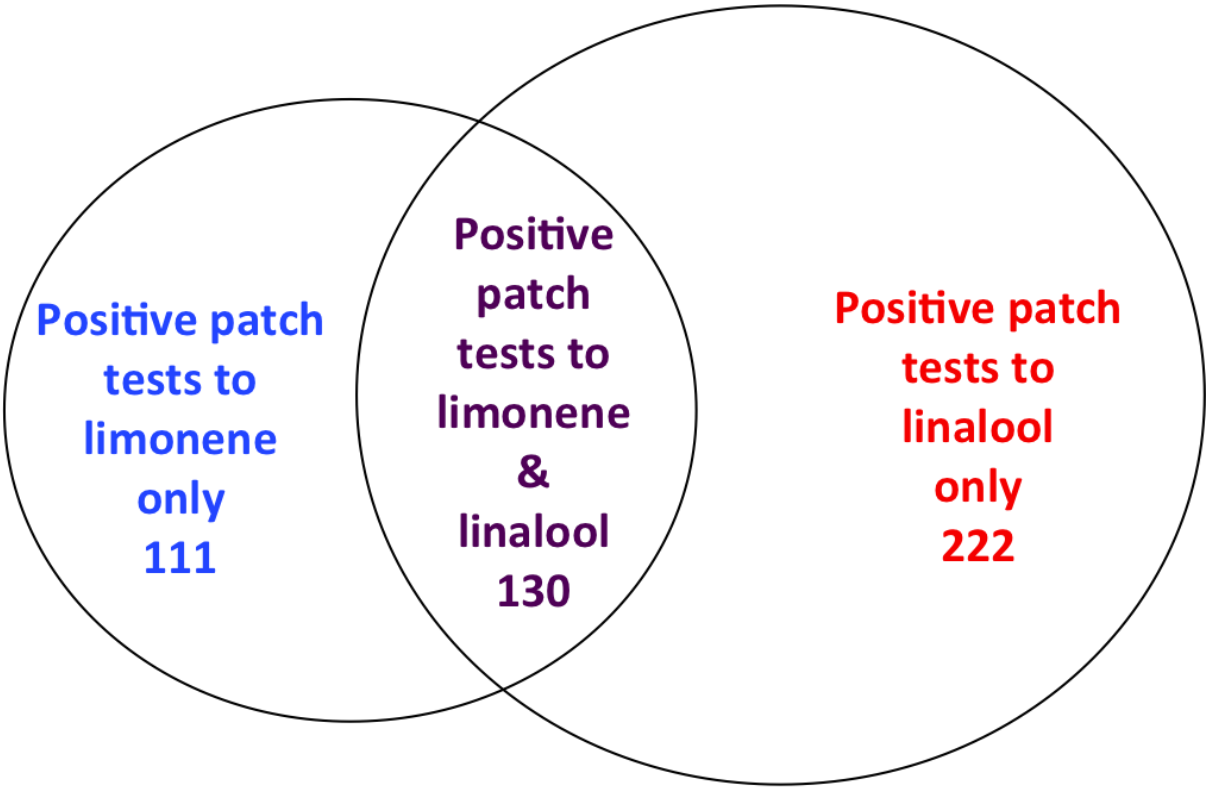

Figure 1. Concomitant positive patch test reactions between oxidised limonene 0.3% and oxidised linalool 1.0%. A total of 463 (10.1%) had positive patch test reactions (1+, 2+, or 3+) to one or both terpene hydroperoxides on D4/5: 111 (2.4%) were positive to limonene hydroperoxide 0.3% alone and 222 (4.9%) to linalool hydroperoxide 1.0% alone. 130 of the 463 had a positive reaction (1+, 2+ or 3+) to both limonene and linalool.

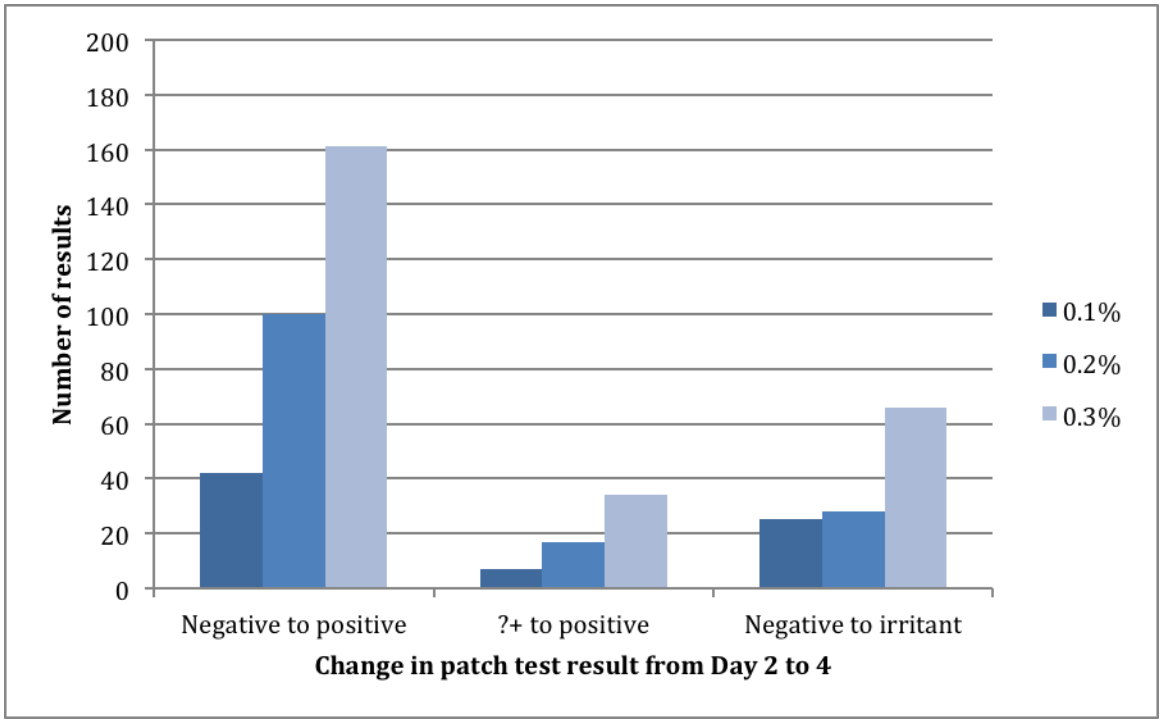

Figure 2. Change in patch test results from negative to positive between Day 2 and Day 4/5 at different concentrations of limonene.

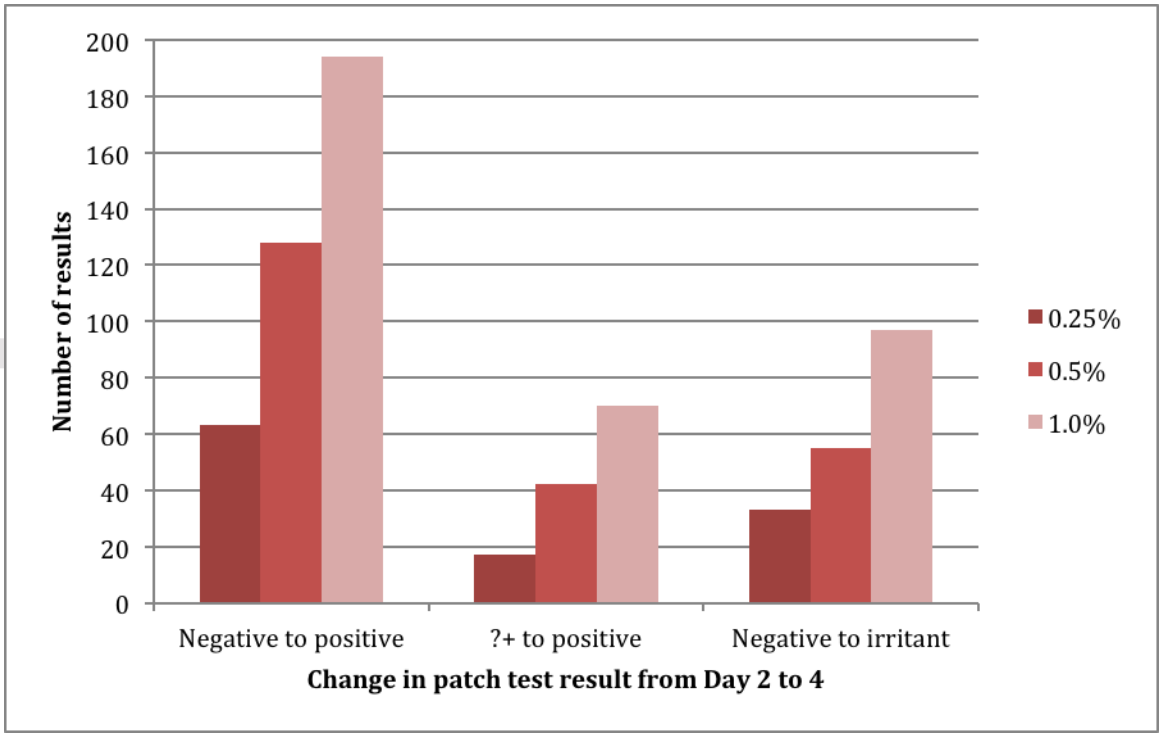

Figure 3. Change in patch test results from negative to positive between Day 2 and Day 4/5 at different concentrations of linalool.

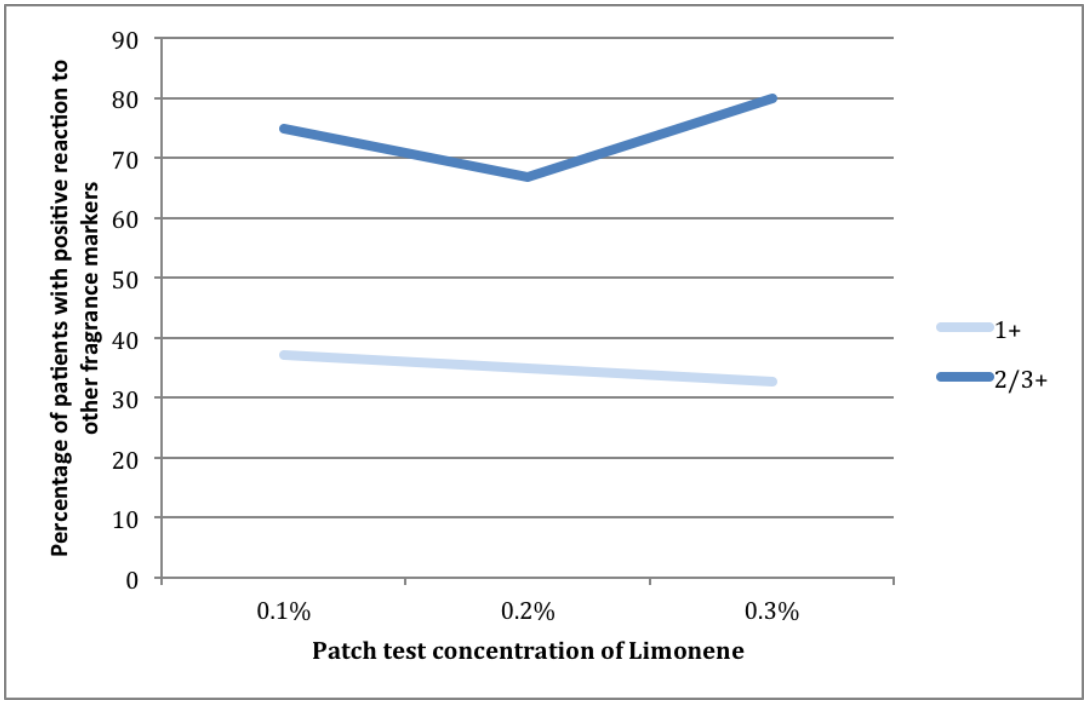

Figure 4. Patch test concentration of limonene and concomitant reactions to other fragrance markers (n=824).

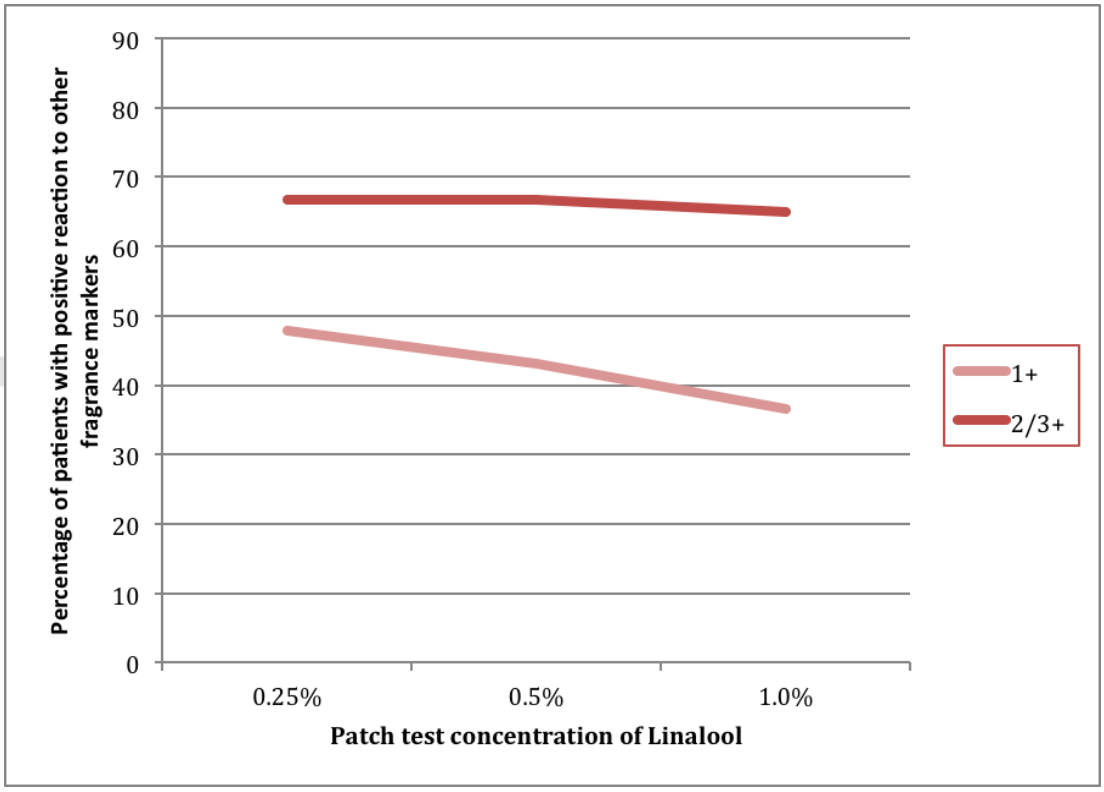

Figure 5. Patch test concentration of linalool and concomitant reactions to other fragrance markers (n=824).