

Air oxidation increases skin irritation from fragrance terpenes

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Background: Linalool and limonene are common fragrance terpenes that autoxidize on air exposure. The pure compounds are not allergenic but their oxidation products can cause contact allergy. Little has been investigated regarding the irritancy of oxidized terpenes.

Aim: The aim of this study was to investigate the irritating effect of pure and oxidized *R*-limonene and linalool in concentration series and to study the MNIC (Maximum Non Irritant Concentration) of autoxidized linalool and limonene.

Patients/methods: Patch testing was performed in dermatitis patients and controls with sequentially diluted concentrations of oxidized and non-oxidized linalool, and oxidized and non-oxidized *R*-limonene. Readings were made with visual assessment and using laser Doppler imaging.

Results: The non-oxidized terpenes were non-irritating in all tested concentrations. Both linalool and especially *R*-limonene were more irritating after oxidation compared with the pure compounds. No difference in response was seen between dermatitis patients and controls.

Conclusion: Autoxidation of the fragrance terpenes linalool and *R*-limonene increases irritation. Oxidized linalool is less irritating than oxidized *R*-limonene. In this study, we found no advantages in using laser Doppler technique compared with visual assessment.

Key words: autoxidation; contact allergy; laser Doppler imaging; limonene; linalool; patch testing; skin irritation; terpenes; visual reading. © Blackwell Munksgaard, 2008.

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Fragrance terpenes are ubiquitous in our environment and are widely found in fine fragrances, cosmetics, and hygiene products as well as in household and industrial products. According to several studies (1–4), linalool and limonene are among the most commonly found fragrance ingredients in consumer products presently. Linalool is a naturally occurring terpene, present in large amounts in various plants, e.g. in lavender (5,6). It is also synthetically produced at low costs and widely used as a fragrance chemical due to its

fresh, flowery odour. *R*-Limonene is the main constituent (98%) of peel oil from citrus fruits. In several plants, including essential herb oils, tea tree oil, and turpentine oil, *R*-limonene and its enantiomer and *S*-limonene are found in different ratios and amounts (7). *R*-limonene and *S*-limonene are the mirror images of one another, and exposure of the population to one or the other enantiomeric form depends on the source of the essential oils. *R*-Limonene or a mixture of *R*-limonene and *S*-limonene (dipentene) are also used as solvents and as industrial degreasing agents and cleansers, often in high concentrations. Studies have shown similar oxidation patterns and frequencies of positive patch test reactions to oxidized *R*-limonene and *S*-limonene in consecutively tested dermatitis patients (8). The main source of

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exposure in the population is considered to be the *R*-isomer because the limonene used as an added fragrance ingredient is mainly obtained as a by-product from the citrus juice industry (personal communication with the fragrance industry). Thus, *R*-limonene has been more thoroughly investigated by our group in recent years and was further chosen for research purposes in this study.

Linalool as well as both isomers of limonene have been demonstrated to autoxidize on air exposure at normal room temperature and handling. The pure compounds linalool and limonene seldom cause positive patch test reactions (9–11); however, their oxidation products have been shown to cause contact allergy (8, 9, 12–14). Several oxidation products such as hydroperoxides, aldehydes, ketones, and alcohols were identified in the oxidation mixtures (15–17). For both linalool and limonene, the primary oxidation products, the hydroperoxides, have been identified as the major contact allergens (9, 12–17).

For oxidized linalool (ox. linalool), one multicentre study of 1511 consecutive dermatitis patients has been conducted. This study used ox. linalool, air exposed for 45 weeks, at a test concentration of 2% petrolatum (pet.) and gave 1.3% positive patch test reactions in the patients (9). Oxidized *R*-limonene (Ox. *R*-limonene) has been tested in several multicentre studies. *R*-limonene oxidized 10 weeks was first studied at 2%, 3%, and 5% pet. concentrations (12). The 3% concentration of ox. *R*-limonene was chosen for the studies that followed (8, 12–14) and subsequently caused positive patch test reactions in a frequency of 2–3% in a total of 7400 consecutive dermatitis patients (8, 12–14). The multicentre studies of both ox. linalool and ox. *R*-limonene have all reported a degree of doubtful reactions, in some cases interpreted as irritant reactions.

R-Limonene is known to cause irritancy in high concentrations in humans and animals (18, 19). Irritant contact dermatitis among workers in citrus fruit canning industries is reported in literature (20–22). In humans, non-ox. linalool at a concentration of 20% and non-ox. limonene at 8% are reported as non-irritating (23). As the chemical composition is altered by oxidation, it has previously been shown for other substances that also the irritant potential can be altered (24). However, the difference in irritation between non-oxidized and oxidized forms of terpenes has not been thoroughly investigated.

The patch test concentration used for screening is important as a higher concentration usually increases the possibility of diagnosing contact allergy. However, with high patch test concentra-

tions, adverse events such as irritation or sensitization may occur. It is desirable to find a patch test concentration that detects contact allergy with a low incidence of irritancy or doubtful reactions and a safe level with regard to sensitization. Guidelines on establishing an appropriate patch test concentration with respect to irritancy and active sensitization have been published in a review by Bruze et al. (25).

The aim of the present study was to investigate whether the irritant potential of linalool and limonene was changed due to autoxidation and to study the MNIC (Maximum Non Irritant Concentration) of ox. linalool and ox. *R*-limonene using visual scoring and laser Doppler imaging (LDI) technique.

Materials and Methods

Chemicals

Linalool (3,7-dimethyl-1,6-octadien-3-ol) with a stated purity of 97% was obtained from Sigma Aldrich Chemie (Schnelldorf, Germany). In order to mimic oxidation that happens during handling and storage conditions, a simplified experimental oxidation model was used according to previous experience (17). The oxidation/degradation process was followed using gas chromatography (GC) and high-performance liquid chromatography (HPLC), according to methods previously described (15, 16). After 45 weeks, the oxidation mixture of linalool contained only 30% linalool. At this time, the oxidation mixture contained 19% of linalool hydroperoxides. Details of the chemical analyses of ox. linalool are described elsewhere (15, 16).

R-Limonene (*D*-limonene, *p*-mentha-1,8-diene) with a stated purity of 98% was obtained from Frey & Lau, Henstedt (Ulzburg, Germany). *R*-Limonene was autoxidized in the same way as described for linalool. Samples were taken out on a regular basis and analyzed with GC direct inlet technique and HPLC-ultraviolet to determine the concentrations of *R*-limonene and its major oxidation products (17). After 10 weeks, the oxidation mixture contained 59% *R*-limonene. At this time, the oxidation mixture contained 7% of the most abundant hydroperoxide limonene-2-hydroperoxide [(5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide]. Details of the chemical analyses of ox. *R*-limonene are described elsewhere (17).

Oxidized samples of linalool and *R*-limonene were stored under argon at –20°C, until test preparations were made. Chemical analyses were performed on a regular basis of the stored

non-oxidized and oxidized test material to ensure that the composition had not changed during storage. All test materials were prepared in non-stabilized white pet. (% w/w) (Kebo Lab A/S, Albertslund, Denmark). The testing was performed in two study periods. The oxidized test preparations were kept in -20°C between the study periods, whereas new preparations were made of non-oxidized test preparations before each testing period. The test preparations consisted of non-ox. linalool (5%, 10%, 20%, and 40% pet.), ox. linalool (5%, 10%, and 20% pet.), non-ox. *R*-limonene (2.5%, 5%, 10%, and 20% pet.), and ox. *R*-limonene (2.5%, 5%, 10%, and 20% pet.). The highest concentration of non-ox. linalool was 40% pet., while ox. linalool was tested only up to 20% pet. as we wanted to minimize the risk of active sensitization due to a high content of hydroperoxides in the ox. linalool sample. All test material was stored at -20°C between use during the study periods.

Subjects

Consecutive dermatitis patients ($n = 20$, 4 men and 16 women, mean age 41 years) were asked to participate in the study in addition to their regular patch testing. To investigate if any difference would be obtained between dermatitis patients and persons without dermatitis, nine healthy volunteers (all women, mean age 50 years) were included. Patients with a positive history of fragrance intolerance were not included in the study. Patients numbered 1–9 and all the controls ($n = 9$) were included in the first study period. Patients numbered 10–20 were included in the second study period. Informed consent was obtained.

Exposure and evaluation

Patch test materials of approximately 20 μl were applied in small Finn Chambers[®] (diameter 8 mm, Epitest Ltd Oy, Tuusula, Finland) on Scanpor tape (Norgesplaster A/S, Vennsela, Norway) on the upper right side of the back of the patients. The distribution of the test chambers was randomized but kept constant throughout the study. The chambers were removed by the patient on D2. The visual evaluation and measurement by LDI technique were performed on D3, 24 hr after removal of the patch test material. Initially, readings with both techniques were also performed on D6–D7, but as the reactions were generally weak, there were no visible or measurable changes at the D6–D7 reading. Subsequently, only a regular patch test reading was performed according to International Contact Dermatitis

Research Group guidelines (26) on D6–D7 in the consecutive patients when routine patch test readings were made. The controls were read on D3 only.

The visual evaluation on D3 was made according to the 10-point scale proposed by Basketter et al. (27) (Table 1). Ox. *R*-limonene 20% pet. was tested on eight of the patients and all the controls ($n = 9$), total 17 subjects, but rendered strong reactions, in some cases toxic erythema, and further testing with this concentration was discontinued.

Superficial tissue perfusion

The laser Doppler perfusion imager (Lisca PIM 1.0, Lisca Development AB, Linköping, Sweden) was used to measure superficial skin perfusion. Details of the instrument are given elsewhere (28). To minimize the effect of the variables related to individuals, environment, and technique, the guideline publication for LDI made by the standardization group of the European Society of Contact Dermatitis (ESCD) was followed (29). The test area was uncovered for at least 15 min prior to measurement with LDI. The measurements were performed in a dark room so that the sensitivity of the system and the quality of the images would not be affected by other light sources. No conversation took place during the measurements. The instrument was set to read 19×19 measurement sites for each reaction, which covered a larger area than needed to catch the reaction. The exact location of each test area was marked lightly to show on the associated photo image. A ROI (region of interest) of approximately 100 measurement sites was identified in the LDI image, and the mean perfusion value was in this way calculated from each patch test area.

Table 1. Visual assessment of erythema grading scale used in this study (27)

Grade	Erythematous reaction
0	No reaction
1	Marginal reaction
2	Slight perceptible erythema
3	A greater than slight reaction that is not sufficient to be classified as distinct
4	Distinct erythema
5	A greater than distinct reaction that is insufficient to be classed as well developed
6	Well-developed, possibly spreading erythema
7	A greater reaction that is not sufficient to be classed as strong
8	Strong, deep erythema that may extend beyond the treatment site
9	A more intense reaction than above

Statistical methods

All analyses were carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U*-test and Wilcoxon's signed rank test were used as statistical methods.

To obtain global significance at a level of $P < 0.05$, the significance levels were corrected for multiple comparisons using Bonferroni correction. Based on this correction, a value of $P < 0.0022$ was considered to be statistically significant for each comparison.

The study was performed in two study periods. The first study period was April and May 2005 and the second study period was between January and June 2006. The study was approved by the local ethics committee.

Results

In this study, very discrete reactions were noted in the majority of cases in the lower values of the 0–9 erythema grading scale (27) (Table 1). Altogether, of 440 readings evaluated, 373 were in the range of 0–1. Clinically, an irritant reaction would be noted from 2 to 3 points, and this was also the cut off we chosen in the discussion of the results. No patients or controls were, to the best of our knowledge, sensitized by the study.

Visual readings

The mean values of the results from the visual readings of non-ox. and ox. linalool and *R*-limonene, respectively, on D3 in dermatitis patients and healthy controls, as well as the overall mean value and range for the patients and controls together are shown in Table 2. Two patients reacted with contact allergic reactions to ox. linalool (a ++ reaction to all concentrations tested) and were excluded from the calculations regarding linalool. The irritation from the non-ox. test preparations of both linalool and *R*-limonene was very low, ranging between 0 and 2 points on the 10-point grading scale (Table 1) with a mean value 0–0.22 points. Ox. linalool at 10% and 20% pet. reached irritation scoring of 4 points with a mean value of 1.15 and 1.63, respectively. Ox. *R*-limonene 5% pet. reached 5 points with a mean value of 1.03 points, 10% pet. reached 8 points with a mean value of 2.52 points, while 20% pet. reached the highest score, 9 points with a mean value of 7.29 points, giving erosions in some cases (Table 2). A boxplot of the values is given in Fig. 1.

The visual readings of the dermatitis patients and the controls did not differ statistically

Table 2. The mean values and range of the visual readings of non-ox. and ox. linalool and non-ox. and ox. *R*-limonene, respectively, on D3 in dermatitis patients and controls, as well as the overall mean^a

Concentration (%) (w/w)	Non-ox. linalool ^b				Ox. linalool ^b				Non-ox. <i>R</i> -limonene ^c				Ox. <i>R</i> -limonene ^c			
	5	10	20		5	10	20		2.5	5	10	20	2.5	5	10	20
Mean patients ^d	0.05	0.22	0.11		0	0.66	0.94		1.55	0	0	0.2	0.95	1.2	2.55	7.5
Mean controls ^d	0.11	0.11	0.11		0	0.55	1.55		1.77	0	0	0.11	0.55	0.66	2.44	7.1
Overall mean (range)	0.07 (0–1) ^e	0.22 (0–2) ^e	0.11 (0–1) ^e	0 (0)	0.63 (0–2) ^{e,f}	1.15 (0–4) ^e	1.63 (0–4) ^{e,f}	0.14 (0–1) ^e	0 (0)	0 (0)	0 (0)	0.17 (0–2) ^e	0.83 (0–3) ^{e,g}	1.03 (0–5) ^{e,g}	2.52 (0–8) ^{e,g}	7.29 (2–9) ^{e,g}

Ox. linalool, oxidized linalool; ox. *R*-limonene, oxidized *R*-limonene.

^aThe visual evaluation was made according to a 0- to 9-point scale (27).

^bPatients ($n = 18$) and controls ($n = 9$) as two patients reacted with contact allergic reactions and were excluded from calculations.

^cPatients ($n = 20$) and controls ($n = 9$), except for ox. *R*-limonene 20% where patients ($n = 8$) and controls ($n = 9$).

^dNo statistically significant difference was observed between dermatitis patients and controls.

^eOx. patch test material was statistically significantly more irritating than the non-ox. patch test material at all concentrations ($P \leq 0.002$) when calculating with paired values in individual patients and controls (Wilcoxon's signed rank test).

^fA statistically significant increase in irritation was shown when calculating with paired values in individual patients and controls (Wilcoxon's signed rank test) between ox. linalool 5% and 20% petrolatum (pet.) ($P \leq 0.0005$).

^gA statistically significant increase in irritation was shown when calculating with paired values in individual patients and controls (Wilcoxon's signed rank test) between ox. *R*-limonene 2.5% and 20% pet. ($P \leq 0.0005$) and between ox. *R*-limonene 5% and 10% pet., and between 10% and 20% pet. ($P \leq 0.001$).

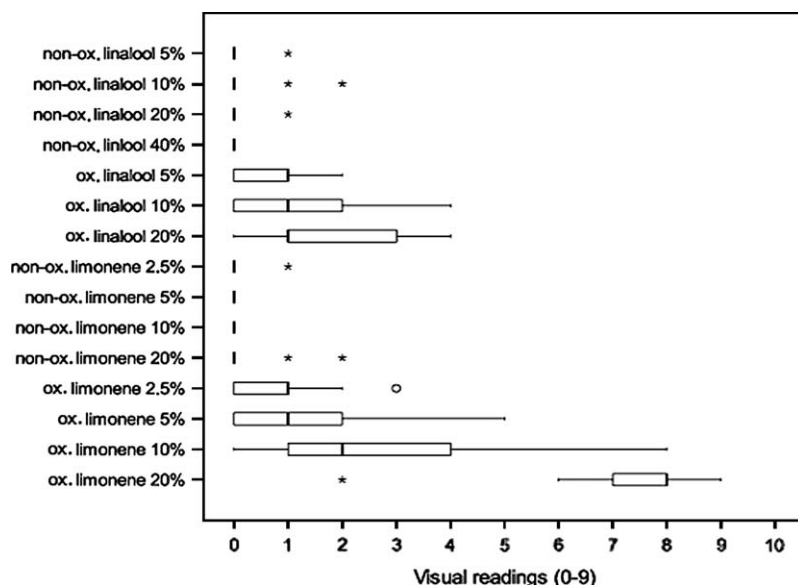


Fig. 1. Boxplots of visual readings (0–9) of non-ox. and ox. linalool and non-ox. and ox. *R*-limonene, according to erythema grading scale shown in Table 1, (27). The box represents the IQ range between first and third quartile containing the middle 50% of the records. The median is indicated by the line across the box. The whiskers show the highest and lowest values that are no greater than 1.5 times the IQ range. Outliers with values between 1.5 and 3 times the IQ range are indicated by circles (○). Outliers with values more than three times the IQ range are indicated by asterisks (*). IQ, interquartile; Ox. linalool, oxidized linalool; Ox. *R*-limonene, oxidized *R*-limonene.

(Mann–Whitney *U*-test). When calculating statistics for the visual readings for the non-ox. and ox. terpenes at similar concentrations, the readings were compared as paired values of the individual patients and controls (Wilcoxon's signed rank test). By visual scoring, the ox. patch test materials were found to be statistically significantly more irritating compared with the non-ox. patch test materials at each given concentration. The difference in the visual readings between non-ox. and ox. linalool at 5% pet. showed a statistical significance of $P = 0.001$, while the difference in the visual readings between non-ox. and ox. linalool at 10% pet. showed a statistical significance of $P \leq 0.002$ and at 20% pet. $P < 0.0005$. The difference in the visual readings between non-ox. and ox. *R*-limonene at 2.5% pet. showed a statistical significance of $P \leq 0.002$, while the difference in the visual readings between non-ox. and ox. *R*-limonene at 5% pet., 10% pet., and 20% pet. all showed a statistical significance of $P < 0.0005$.

The visual readings of rising concentrations of the ox. terpenes were compared as paired values

of the individual patients and controls (Wilcoxon's signed rank test). For ox. linalool, there was a statistically significant ($P < 0.0005$) increase in irritation between the visual readings for the concentrations of 5% and 20% pet. Similarly, for ox. *R*-limonene, a statistically significant increase in irritation was found between the visual readings of 2.5% and 20% pet. ($P < 0.0005$) as well as between 5% and 10% pet. ($P = 0.001$) and between 10% and 20% pet. ($P < 0.0005$) (Table 2).

The visual scores for the 29 subjects were also summed together and are shown in Table 3. The sums of the visual scores (0–9 according to Table 1) of non-ox. linalool from 27 patients and controls for the concentrations 5%, 10%, 20%, and 40% pet., respectively, were 2, 6, 3, and 0. For ox. linalool at the concentrations 5%, 10%, and 20% pet., the corresponding sums were 17, 31, and 44. The sums of the visual scores for non-ox. *R*-limonene from 29 patients and controls for the concentrations 2.5%, 5%, 10%, and 20% pet., respectively, were 4, 0, 0, and 5. For ox. *R*-limonene at the concentrations 2.5%, 5%, and

Table 3. Results of added visual scores from patients, controls, and sum of visual scores [sum of total scoring points 0–9 at D3 readings (27)] to non-ox. and ox. linalool and non-ox. and ox. *R*-limonene at different concentrations^a

Concentration (%) (w/w)	Non-ox. linalool ^b				Ox. linalool ^b			Non-ox. <i>R</i> -limonene ^c				Ox. <i>R</i> -limonene ^c			
	5	10	20	40	5	10	20	2.5	5	10	20	2.5	5	10	20
Sum of visual scores, patients	1	4	2	0	12	17	28	2	0	0	4	19	24	51	60 ^c
Sum of visual scores, controls	1	2	1	0	5	14	16	2	0	0	1	5	6	22	64
Total sum of visual scores	2	6	3	0	17	31	44	4	0	0	5	24	30	73	124 ^c

Ox. linalool, oxidized linalool; ox. *R*-limonene, oxidized *R*-limonene.

^aTwo patients reacted with contact allergic reactions to ox. linalool and were excluded from the calculations regarding linalool.

^bPatients ($n = 18$) and controls ($n = 9$).

^cPatients ($n = 20$) and controls ($n = 9$), except for ox. *R*-limonene 20% where patients ($n = 8$) and controls ($n = 9$).

10% pet., the corresponding sums were 24, 30, and 73, and for the concentration of 20% pet., the sum was 124 based on only 17 persons.

Laser Doppler imaging

The mean values and range for LDI readings for patients, controls, and overall mean on D3 for untreated skin and non-ox. and ox. linalool and *R*-limonene are given in Table 4. A boxplot of the values is given in Fig. 2.

The laser Doppler readings of the dermatitis patients and the controls did not differ statistically (Mann–Whitney *U*-test). When calculating statistics for the LDI readings for the non-ox. and ox. terpenes, the readings were compared as paired values of the individual patients and controls (Wilcoxon's signed rank test). When comparing non-ox. and ox. terpenes at similar concentrations, a statistically significant difference was noted between non-ox. and ox. linalool 20% pet. and between non-ox. and ox. *R*-limonene 20% pet. (both $P < 0.0005$). When comparing the rising concentrations of the ox. terpenes by LDI readings, a significant difference was found between ox. linalool 5% and 20% pet. ($P = 0.002$), ox. *R*-limonene 10% and 20% pet. ($P < 0.0005$), and ox. *R*-limonene 2.5% and 20% pet. ($P < 0.0005$).

Discussion

In our study, we have shown that air oxidation of linalool and *R*-limonene increases skin irritation. The irritation increases as the concentrations of the oxidized test preparations are raised. Ox. *R*-limonene was more irritating than ox. linalool at similar concentrations. For future patch testing, the test concentration of ox. linalool could most probably be raised from the concentration previously used to obtain better detection. There was no statistical difference in skin irritation observed between dermatitis patients and control subjects in this study.

Irritation is important to consider as many cases of occupational contact dermatitis are irritant in nature, mostly regarded to be a result of wet work and surfactants. Our study shows that components other than soaps and detergents in hand cleansers and hygiene products may add to the irritation. Furthermore, the picture is complicated because the sensitizing effect of both linalool and *R*-limonene increases by autoxidation during air exposure.

Studies have shown that the simultaneous exposure to sodium dodecyl sulfate and to nickel, methyldibromo glutaronitrile (MBDGN), or

Table 4. Mean values and range for laser Doppler readings (V) of non-ox. and ox. linalool and non-ox. and ox. *R*-limonene in patients and controls, as well as the overall mean, on D3

	Non-ox. linalool ^a				Ox. linalool ^a				Non-ox. <i>R</i> -limonene ^b				Ox. <i>R</i> -limonene ^b			
	Untreated skin	5%	10%	20%	40%	5%	10%	20%	2.5%	5%	10%	20%	2.5%	5%	10%	20%
Patients ^c	0.61	0.73	0.61	0.61	0.70	0.66	0.7	0.84	0.64	0.68	0.66	0.61	0.75	0.89	0.95	2.61
Controls ^c	0.59	0.72	0.63	0.64	0.65	0.67	0.82	0.79	0.69	0.66	0.71	0.61	0.62	0.71	0.75	2.15
Overall mean	0.60	0.73	0.62	0.62	0.69	0.67	0.74	0.82	0.65	0.67	0.67	0.61	0.71	0.83	0.89	2.37
Range	0.4–1.07	0.38–1.79	0.46–1.15	0.4–1.01 ^d	0.43–1.35	0.4–1.33 ^e	0.38–2.17	0.49–2.6 ^{d,e}	0.36–1.46	0.38–1.65	0.39–1.53	0.44–1.22 ^d	0.47–1.8 ^e	0.44–2.74	0.42–1.72 ^e	0.66–4.76 ^{d,e}

Ox. linalool, oxidized linalool; ox. *R*-limonene, oxidized *R*-limonene.

^aPatients ($n = 18$) and controls ($n = 9$) as two patients reacted with contact allergic reactions to ox. linalool and were excluded from calculations.

^bPatients ($n = 20$) and controls ($n = 9$), except for ox. *R*-limonene 20% where patients ($n = 8$) and controls ($n = 9$).

^cNo statistically significant difference was observed between dermatitis patients and controls.

^dA statistically significant difference was established when calculating with paired values in individual patients and controls (Wilcoxon's signed rank test) between non-ox. and ox. linalool 20% and between non-ox. and ox. *R*-limonene 20% ($P < 0.0005$).

^eA statistically significant difference was established when calculating with paired values in individual patients and controls (Wilcoxon's signed rank test) between ox. linalool 5% and 20%, between ox. *R*-limonene 2.5% and 20%, and between ox. *R*-limonene 10% and 20% ($P < 0.0005$).

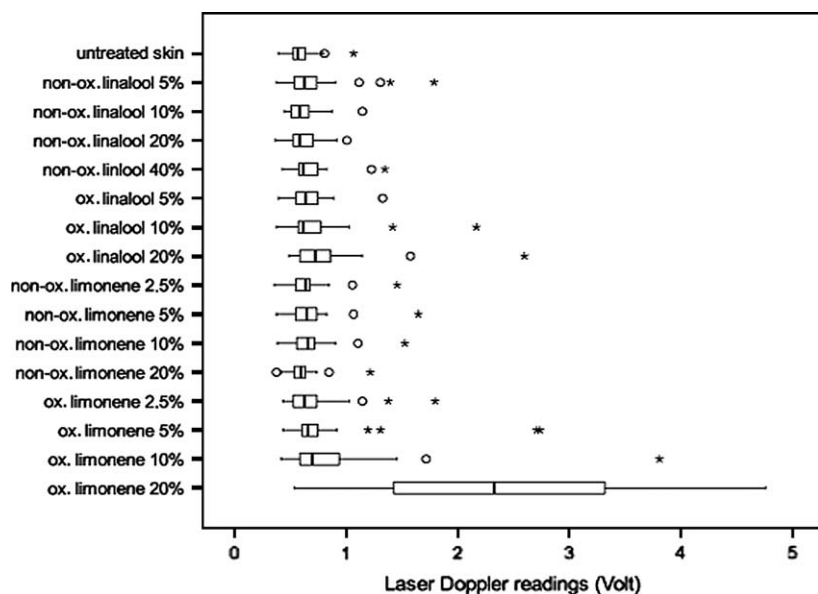


Fig. 2. Boxplots of laser Doppler readings of non-ox. and ox. linalool and non-ox. and ox. *R*-limonene. The box represents the IQ range between first and third quartile containing the middle 50% of the records. The median is indicated by the line across the box. The whiskers show the highest and lowest values that are no greater than 1.5 times the IQ range. Outliers with values between 1.5 and 3 times the IQ range are indicated by circles (O). Outliers with values more than three times the IQ range are indicated by asterisks (*). IQ, interquartile; Ox. linalool, oxidized linalool; Ox. *R*-limonene, oxidized *R*-limonene.

hydroxycitronellal has resulted in an increased response to the allergen in earlier sensitized individuals (30–32). It has also been shown that additive effects of irritants and allergens can increase the risk of sensitization (33). Effects on skin response after exposure to different combinations of allergens and irritants have been extensively reviewed (34). It has been stipulated that sensitization does not come from the antigenic signal alone but from an associated irritancy, inherent in the hapten or by simultaneous exposure, which causes activation of the immune system possibly by upregulation of tumour necrosis factor- α (34 and references therein). For both linalool and *R*-limonene, simultaneous exposure to detergent is very common, i.e. in perfumed hygiene products. Furthermore, occupational exposure to *R*-limonene and *S*-limonene may occur to high concentrations in degreasers, solvents, and hand cleansers, and the combined allergenic and irritant effect of ox. limonene in these products must be considered.

Ox. linalool has, in a multicentre study, rendered a positive patch test frequency of 1.3% (9) when tested at 2% pet. In our study regarding ox. linalool, irritation started to increase at 10% pet., and rose moderately at 20% pet. (Tables 2 and 3, Fig. 1), indicating that the test concentration of ox. linalool could probably be raised to obtain a better detection rate of cases of contact allergy. Ox. *R*-limonene has shown 2–3% frequency of positive patch test reactions when tested at 3% pet. (8, 12–14). Although the present study cannot demonstrate a statistically significant increase in irritation when raising the concentration of ox. *R*-limonene from 2.5% pet. to 5% pet., previous

studies have recorded mounting irritation when ox. *R*-limonene is tested at the higher concentration (12). In the current study, further increase in concentration of ox. *R*-limonene gave considerably more irritation (Tables 2 and 3, Fig. 1). Thus, for practical purposes, we recommend the usage of ox. *R*-limonene in 3% pet. as previously reported.

Visual scoring is commonly regarded as the golden standard when measuring irritation as well as in assessing contact allergic reactions, although quantification of test results by LDI is considered a practically useful method (35, 36). Visual reading of contact allergic reactions as well as of irritation presents problems of individual variation, both on the part of the patient (skin type, sensitive skin) and of the observer. The study by Basketter et al. (27) concerning visual assessment of irritation showed that suitably trained observers can discriminate between relatively subtle grades of erythema in a reliable way. In our study, there was a discrepancy between the methods used, with visual assessment showing clear, statistically significant differences in irritancy between non-ox. and ox. terpenes at similar concentrations, which were not measurable in the blood flow measurements by LDI. Our conclusion is that not much extra information was gained by the LDI measurements, possibly because the time between patch test removal and LDI reading was too long (24 hrs) with the single exposure procedure. In the cases where laser Doppler technique gave statistically significant differences, the irritation was very clear in visual assessment.

In conclusion, we found an increase in irritation for the studied common fragrance terpenes

linalool and *R*-limonene after air oxidation. Ox. linalool was less irritating than ox. *R*-limonene at similar concentrations. Further studies in dermatitis patients, based on ESCD guidelines (25), should be conducted to investigate if a higher patch test concentration of ox. linalool would detect more cases of contact allergy. No difference could be established in the response to the oxidized mixtures between a group of dermatitis patients and a group of healthy controls. Finally, we found no advantages in using laser Doppler technique compared with visual assessment in this study.

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