

Characterization of skin sensitizers from autoxidized citronellol – impact of the terpene structure on the autoxidation process

Johanna Rudbäck¹, Lina Hagvall^{1,2}, Anna Börje¹, Ulrika Nilsson³ and Ann-Therese Karlberg¹

¹Department of Chemistry and Molecular Biology, Dermatochemistry and Skin Allergy, University of Gothenburg, 412 96 Gothenburg, Sweden,

²Department of Dermatology, Sahlgrenska Academy, University of Gothenburg, 405 30 Gothenburg, Sweden, and ³Department of Analytical Chemistry, Stockholm University, 106 91, Stockholm, Sweden

doi:10.1111/cod.12234

Summary

Background. Citronellol is a frequently used fragrance compound in consumer products. It is present in fragrance mix II, which is used for screening of contact allergy to fragrances. Because of its chemical structure, citronellol could be susceptible to autoxidation.

Objectives. To compare the behaviour of citronellol with that of the structurally similar compounds linalool and geraniol, in terms of ability to autoxidize, the products formed, and the sensitization potencies of these.

Methods. Citronellol was exposed to air, and autoxidation was followed by gas chromatography–mass spectrometry (GC–MS) analysis after derivatization of thermolabile compounds. The sensitizing potencies of the oxidation mixture and its major oxidation compounds were examined with the local lymph node assay.

Results. The concentration of citronellol decreased while the sensitization potency increased in air-exposed samples over time, with hydroperoxides being identified as the major oxidation products and main skin sensitizers.

Conclusions. The present study shows the impact of the absence of the 2,3-double bond in the citronellol structure on the oxidation pathways for formation of oxidation products. The study also shows the usefulness of our new GC–MS method for quantification of the citronellol oxidation products, especially the hydroperoxides. The investigated citronellol hydroperoxides could be important allergens, owing to the high concentrations detected and frequent exposure to citronellol in the population.

Key words: autoxidation; citronellol; contact allergy; FM II; fragrances; GC–MS; prehapten; structure–activity relationships; trimethyl silyl (TMS) derivatives.

Correspondence: Ann-Therese Karlberg, Department of Chemistry and Molecular Biology, Dermatochemistry and Skin Allergy, University of Gothenburg, SE-412 96 Gothenburg, Sweden. Tel: +46-31-786 91 09. E-mail: karlberg@chem.gu.se

Conflicts of interest: The authors have no conflicts of interest to declare. Funding: None.

Accepted for publication 19 February 2014

Citronellol (**1**, 3,7-dimethyl-oct-6-en-1-ol, CAS no. 106-22-9; Fig. 1) is a naturally occurring fragrance terpene found in oils of roses and geranium (1). It is one of the six most frequently used fragrance compounds (2, 3), and was detected in all of the investigated aerosol spray deodorants on the European market in a study from 1998, and in 47% of domestic and occupational products in an investigation from 2001 (4, 5). Citronellol is one of the compounds present in fragrance mix II (FM II) (6) in

the European baseline series. Between 1% and 3% of the general European population is estimated to have contact allergy to fragrance ingredients (7–9). To reduce the risk of skin sensitization and eczema resulting from the use of cosmetic products and protect public health, the EU demands that 24 fragrance compounds and two natural oils that are considered to be the major culprits must be declared on cosmetic products (10). One of the fragrances that must be declared according to this EU legislation is citronellol (10).

In previous studies, we have shown that fragrance terpenes can act as prehaptenes and/or prohaptens (11–15). Prehaptenes are defined as non-sensitizing or low-sensitizing molecules that are transformed into haptens by chemical transformation (abiotic activation) (16). Prohaptens, on the other hand, are defined as non-sensitizing or low-sensitizing molecules that are transformed into haptens by biotic activation (16). As many fragrance terpenes are prone to autoxidation, the formation of allergenic oxidation products can be expected to be rather common under conditions of air exposure. Autoxidation is a free radical chain mechanism with initiation, propagation, and termination (17). Peroxyl radicals are formed after hydrogen abstraction followed by the addition of oxygen. Some selectivity for positions where stable radicals can be formed is observed; that is, allylic hydrogen atoms and hydrogen atoms next to heteroatoms are the most plausible targets (as shown for citronellol in Fig. 1). The fragrance compounds investigated so far with regard to the influence of autoxidation on allergenic potential all have oxidizable allylic positions, and are able to form hydroperoxides as primary oxidation products.

On the basis of our previous experience, we consider it plausible that citronellol could be activated by autoxidation forming allergenic oxidation products. Citronellol lacks the electrophilic moieties necessary for direct covalent bonding, but has oxidizable allylic positions analogous to those in the previously studied fragrance terpenes linalool (3,7-dimethylocta-1,6-dien-3-ol, CAS no. 78-70-6) from lavender scent, and geraniol ((*E*)-3,7-dimethylocta-2,6-dien-1-ol, CAS no. 106-24-1) from rose scent (Fig. 1). Linalool has a very low sensitizing potency itself; however, strong sensitizers are formed because of autoxidation when linalool is in contact with air. The major culprits are the primary oxidation products of linalool, the hydroperoxides, which are present in large amounts in the oxidation mixture (12). When geraniol is activated by autoxidation (13), aldehydes are the major allergens in the autoxidation mixture, and only minor amounts of the tertiary hydroperoxide, which is analogous to the hydroperoxide formed by autoxidation of linalool, can be detected (13).

The aim of the present study was to investigate structural influences on the formation of allergenic compounds upon air exposure by comparing citronellol with the structurally similar linalool and geraniol. Of specific interest was the formation of the secondary and tertiary citronellol hydroperoxides (**2** and **3**; Fig. 1) as a measure of the influence on the oxidation pathways of the absence of the 2,3-double bond in the citronellol structure, which is the difference between it and geraniol. Thus, citronellol was exposed to air, and its sensitizing potency was studied before and after autoxidation, by use of the murine local lymph node assay (LLNA) (18). Specific

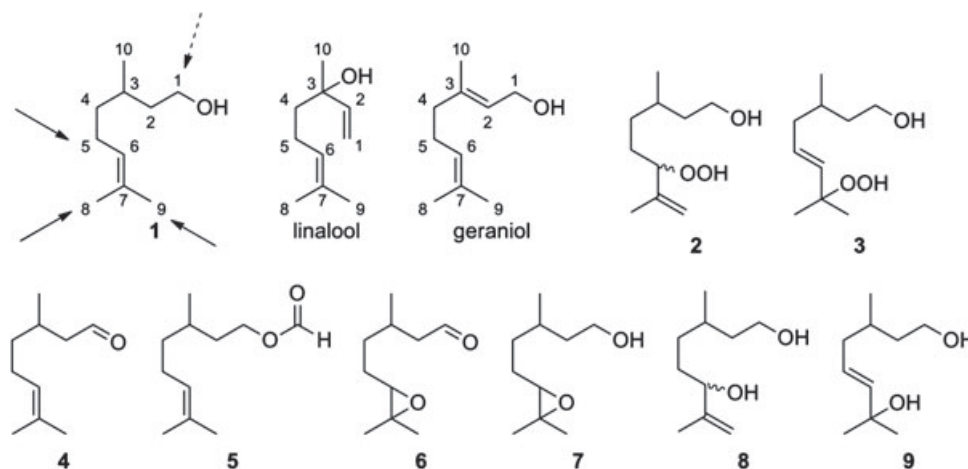


Fig. 1. Structures of citronellol (**1**), linalool, and geraniol, discussed in the text, as well as the oxidation products identified in air-exposed citronellol: isomers of citronellol hydroperoxides (**2** and **3**), citronellal (**4**), citronellyl formate (**5**), epoxycitronellal (**6**), epoxycitronellol (**7**), and isomers of citronellol diols (**8** and **9**). Arrows indicate hydrogen abstraction at allylic positions; dotted arrows indicate hydrogen abstraction next to heteroatoms.

oxidation products were identified, and their sensitizing potencies were investigated. For quantification of the oxidation products in the oxidation mixture, our newly developed analytical method (19) with derivatization prior to gas chromatography (GC)–mass spectrometry (MS) was used for analysis of the oxidation mixture.

Materials and Methods

Chemicals

Citronellol (isomeric mixture) was obtained from Sigma Aldrich (Steinheim, Germany), citronellal (**4**, 3,7-dimethyl-oct-6-en-1-al, isomeric mixture, CAS no. 106-23-0; Fig. 1) was obtained from Alfa Aesar (Karlsruhe, Germany), and citronellyl formate (**5**, 3,7-dimethyl-oct-6-en-1-yl formate, isomeric mixture, CAS no. 105-85-1; Fig. 1) was obtained from Bedoukian Research (Danbury, CT, USA). Citronellol and citronellal were distilled before use to > 98% purity. *N*,*O*-bis (Trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (99:1, CAS no. 25561-30-2) was obtained from Supelco (Bellefonte, PA, USA). The internal standard (IS), 1,2,3,5-tetramethylbenzene (CAS no. 527-53-7), was added volumetrically prior to GC–MS analysis, and obtained from TCI Europe (Zwijndrecht, Belgium). Unless otherwise stated, solvents and reagents were provided by commercial suppliers without further purification.

In studies of the sensitizing potencies, we used [³H]methylthymidine from Perkin-Elmer (Waltham, MA, USA), a scintillation fluid from EcoLume INC Radiochemicals (Solon, OH, USA), acetone, pro analysis, from Merck (Darmstadt, Germany), and olive oil from Apoteket AB (Gothenburg, Sweden). The purity of the synthesized and purchased compounds used in the sensitization experiments was > 98% (GC–MS).

Instrumentation and analysis

Purification of synthesized compounds. Purifications were performed with open-column chromatography on Merck silica gel 60 (230–400 mesh). The reactions and column chromatographic separations were monitored with thin-layer chromatography on silica-plated aluminium sheets (silica gel 60 F₂₅₄). For detection, a visualizing agent consisting of anisaldehyde, sulfuric acid and acetic acid in ethanol was used, followed by heating.

Photo-oxidation. Photo-oxidation was performed with a Rayonet reactor equipped with 16 ultraviolet (UV) lamps (350 nm).

¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy. NMR analyses were performed with a JEOL eclipse+ 400-MHz spectrometer at 400 and 100 MHz, respectively, with CDCl₃ as solvent. Chemical shifts (δ) are reported in ppm relative to CHCl₃ at δ 7.25 and δ 77.0 for ¹H and ¹³C spectra, respectively. To explain the multiplicities, the following abbreviations are used: s = singlet, d = doublet, t = triplet, and m = multiplet.

Preparative high-performance liquid chromatography (HPLC). A Gilson HPLC pump (model 305) was used with a Zorbax RX-SIL column (250 mm × 21.2 mm, 7 μ m; Agilent Technologies, Waldbronn, Germany) and a Gilson UV–visible detector (model 119), operating at a wavelength of 220 nm. *tert*-Butyl methyl ether/hexane (3:2) was used as eluent, and the flow rate was 21.5 ml/min.

Gas chromatography–mass spectrometry. Two GC–MS instruments were used. The development of the method and the quantification of oxidation products were mainly performed on system one, which was a Hewlett-Packard model 6890 gas chromatograph (Agilent Technologies) equipped with an on-column injector, with a fused silica column (HP5-MSi; 30 m × 0.25 mm; film thickness, 0.25 μ m). The GC instrument was connected to a Hewlett-Packard model 5973 quadrupole mass selective detector (Agilent Technologies), which was used in scan mode for monitoring ions within an *m/z* range of 50–500. System two, a Varian 4000 ion trap GC–MS instrument (Varian Inc., Walnut Creek, CA, USA), was mainly used for the quantification of citronellol. The GC instrument (model 3800) was equipped with an autosampler (CP-8400) and an on-column injector (model 1079), with a DB-5 column (30 m × 0.25 mm; film thickness, 0.1 μ m). Electron ionization (EI) was performed at 70 eV in external mode, and the analysis was performed in full-scan mode for monitoring ions of *m/z* 50–500 with three microscans. The emission current was 25 μ A, and the target total ion chromatogram (TIC) was 20 000 counts. The trap temperature, manifold temperature, transfer line temperature and ion source temperature were set to 130, 50, 250 and 180°C, respectively. The injector temperature was initially set to 80°C for 0.5 min, after which the temperature was raised to 250°C at a rate of 150°C/min.

For both systems, the initial setting of the GC oven was 80°C for 0.5 min, and the temperature was then raised to 250°C at a rate of 7°C/min.

β -Scintillation counting. This was performed with a Beckman LS 6000TA instrument.

Air exposure procedure

A distilled sample of citronellol (> 98%, ~70 ml) was air-exposed in an Erlenmeyer flask (100 ml) at room temperature under a daylight lamp (Philips Master TL-D 90 De Luxe, 18 W/950), lit for 12 hr/day. The neck of the flask was covered with aluminium foil to prevent contamination. Citronellol was stirred for 1 hr, four times a day, as previously described (20). Minor samples (~1 ml) were taken on a regular basis, and stored in the freezer under argon at -72°C prior to chemical analysis to determine the concentrations of citronellol and its major oxidation products.

Quantification of citronellol and oxidation products by GC–MS

The degradation of citronellol and the formation of oxidation products were determined for citronellol air-exposed for 0–26 weeks. The oxidation samples were diluted in toluene to 1 or 3 mg/ml. From each diluted oxidation sample, an aliquot of 0.8 ml was transferred to test tubes, to which 0.1 ml of IS solution (0.2 or 0.7 mg/ml) and 0.1 ml of trimethyl silyl (TMS) reagent were added. The mixture was left for 24 hr at room temperature prior to GC–MS (19). The reference compounds of the hydroperoxides (**2** and **3**; Fig. 1) were shown to be fully derivatized within that time period. Quantification was performed in triplicate on the basis of external reference compounds of all compounds studied after extraction of specific ions. Equal amounts of the volumetric IS were added to the air-exposed samples after sampling. The IS was not derivatized, but was used to determine the relative responses in the GC–MS analysis.

Synthesis of reference compounds

6-Hydroperoxy-3,7-dimethyloct-7-ene-1-ol (2) and *(E)-7-hydroperoxy-3,7-dimethyloct-5-ene-1-ol (3)* (citronellol hydroperoxides; Fig. 1). The photo-oxidation reaction was performed as previously described (21). Starting from citronellol (0.794 g, 5.1 mmol) and after a reaction time of 3 hr, the crude product was purified by flash chromatography (ethyl acetate/hexane, 2:3). This resulted in a colourless oil containing a 3:2 mixture of the two diastereoisomers of 6-hydroperoxy-3,7-dimethyloct-7-ene-1-ol (**2**) and the (E)-7-hydroperoxy-3,7-dimethyloct-5-ene-1-ol (**3**) (Fig. 1), in a total yield of 34.5% (0.33 g, 1.8 mmol). For characterization of the hydroperoxides, the compounds were separated with preparative HPLC. Citronellol hydroperoxide **2** (Fig. 1): $^1\text{H-NMR}$ (400 MHz) – δ 0.89 (d, 3H, $J = 6.22$ Hz), 1.06–1.16 (m, 1H), 1.18–1.29 (m, 1H), 1.31–1.48

(m, 2H), 1.51–1.65 (m, 3H), 1.72 (s, 3H), 3.61–3.74 (m, 2H), 4.27 (t, 1H, $J = 6.78$ Hz), 5.0 (m, 2H); $^{13}\text{C-NMR}$ (400 MHz) – δ 17.2/17.3, 19.5/19.6, 28.1/28.2, 29.2/29.4, 32.8/32.9, 39.6/39.7, 61.1, 89.7/90.0, 114.2/114.4, 143.8/144.0. Citronellol hydroperoxide **3** (Fig. 1): $^1\text{H-NMR}$ (400 MHz) – δ 0.89 (d, 3H, $J = 6.59$ Hz), 1.32 (s, 6H), 1.35–1.43 (m, 1H), 1.57–1.75 (m, 2H), 1.92–2.01 (m, 1H), 2.02–2.11 (m, 1H), 3.62–3.74 (m, 2H), 5.52–5.56 (d, 1H, $J = 15.74$ Hz), 5.62–5.72 (m, 1H); $^{13}\text{C-NMR}$ (400 MHz) – δ 19.9, 24.3, 24.4, 29.7, 39.2, 39.9, 61.1, 82.2, 130.2, 135.0.

5-(3,3-dimethyloxiran-2-yl)-3-Methylpentanal (epoxycitronellal, 6; Fig. 1). The synthesis was performed as previously described (20), starting from citronellal. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ data are in agreement with the literature (22).

5-(3,3-dimethyloxiran-2-yl)-3-Methylpentan-1-ol (epoxycitronellol, 7; Fig. 1). The synthesis was performed as previously described (20), starting from citronellol (3.5 g, 22.4 mmol). The crude product was purified with flash chromatography (ethyl acetate/hexane, 2:3), which resulted in a total yield of 86.3% (3.33 g, 19.4 mmol) of a diastereomeric 1:1 mixture. $^1\text{H-NMR}$ (400 MHz) – δ 0.91 (d, 3H, $J = 6.59$ Hz), 1.25 (s, 3H), 1.29 (s, 3H), 1.34–1.44 (m, 2H), 1.46–1.68 (m, 5H), 2.69 (t, 1H, $J = 6.04$ Hz), 3.62–3.74 (m, 2H); $^{13}\text{C-NMR}$ (400 MHz) – δ 18.7/18.8, 19.5/19.7, 24.9, 26.3/26.5, 29.3/29.5, 33.7/33.8, 39.6/39.9, 58.5/58.6, 61.0, 64.7/64.8; EI-MS (70 eV) – m/z (%) 172 (1) (M^+), 85 (37), 71 (44).

3,7-Dimethyloct-7-ene-1,6-diol (8) and (E)-3,7-dimethyloct-5-ene-1,7-diol (9) (citronellol diols; Fig. 1). The synthesis was performed as previously described (13), starting from a mixture of the citronellol hydroperoxides (**2** and **3**; Fig. 1). $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ data are in agreement with the literature (23).

Sensitization experiments

Experimental animals. Female CBA/Ca mice, aged ~8 weeks, were purchased from Scanbur or NOVA SCB Charles River (B&K Sollentuna, Sweden, and Sulzfeld, Germany, respectively). The mice were housed in 'HEPA'-filtered air flow cages with standard laboratory diet and water *ad libitum*. All animal procedures were approved by the local ethics committee in Gothenburg, Sweden.

Sensitizing potencies of oxidation mixtures and oxidation products of citronellol (File S1). The LLNA (18) was used to assess the sensitizing potencies. Mice in six groups of three each were treated by topical application on the dorsum

of both ears of the test compound (25 µl) dissolved in acetone/olive oil (4:1, vol/vol) or of the vehicle alone. All solutions were freshly prepared for every application. Each compound was tested at five different concentrations (with a range of 0.1–80% wt/vol). Treatments were performed daily for three consecutive days (days 0, 1, and 2). Sham-treated control mice received vehicle alone. On day 5, all mice were injected intravenously via the tail vein with [^3H]methylthymidine (2.0 Ci/mmol, 20 µCi) in phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4 (250 µl). After 5 hr, the mice were killed, the draining lymph nodes were excised and pooled for each group, and single-cell suspensions of lymph node cells in PBS were prepared with cell strainers (pore size, 70 µm; Falcon, BD Labware, Franklin Lakes, NJ, USA). Cell suspensions were washed twice with PBS, precipitated with trichloroacetic acid (TCA) (5%), and left in the refrigerator overnight. The samples were then centrifuged, resuspended in TCA (5%) (1 ml), and transferred to scintillation cocktail (10 ml). The [^3H]methylthymidine incorporation into DNA was measured by β -scintillation counting. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), that is, test group/control group ratio. Test materials that, at one or more concentrations, resulted in an SI of > 3 were considered to be positive in the LLNA. EC₃ values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation (24). The sensitizing potencies of the test compounds were classified as follows: $< 0.1\%$, extreme; ≥ 0.1 to $< 1\%$, strong; ≥ 1 to $< 10\%$, moderate; ≥ 10 to $< 100\%$, weak (25).

Results

Air oxidation of citronellol

The content of citronellol decreased over time upon air exposure. Thus, the oxidation mixture contained 84% and 33% citronellol after 10 and 26 weeks, respectively, of air exposure (Fig. 2). The following oxidation products were detected in the oxidation mixture of citronellol: citronellol hydroperoxides, citronellal, citronellyl formate, epoxycitronellal, epoxycitronellol, and citronellol diols. Quantification with GC–MS after 10 and 26 weeks of air exposure gave the results shown in Fig. 3 and Table 1. Isomers of citronellol, citronellol hydroperoxides, epoxycitronellol and citronellol diols were all separated as thermostable TMS derivatives (Fig. 4), whereas citronellal was partly derivatized. Citronellyl formate and epoxycitronellal were only observed non-derivatized. Both derivatized and non-derivatized aldehydes were stable for GC analysis, without

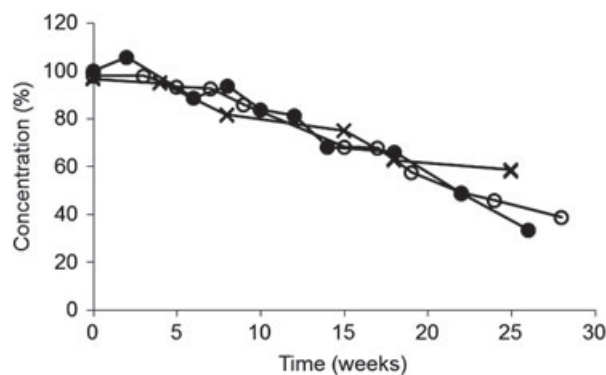


Fig. 2. Degradation of citronellol, linalool and geraniol during air exposure: concentrations of citronellol (●) in air-exposed citronellol, linalool (×) in air-exposed linalool and geraniol (○) in air-exposed geraniol with time. The results for linalool and geraniol are from earlier publications (12, 13), and are included here for comparison with the degradation of citronellol.

any degradation at the high temperatures applied. Non-derivatized compounds are marked with an asterisk in Fig. 4. Upon derivatization, active hydrogens in, for example, alcohols and hydroperoxides are replaced by the derivatization reagent used, in this case a TMS group, forming thermostable derivatives.

Sensitizing potency according to the LLNA

In order to investigate the effect of autoxidation on allergenic activity, the sensitizing potencies were determined for pure citronellol, citronellol air-exposed for 10 weeks, and the synthesized oxidation products citronellol hydroperoxides and epoxycitronellol (Table 1, Fig. 5, and File S1). Pure citronellol was shown to be low-sensitizing or non-sensitizing, as no EC₃ value was obtained at the tested concentrations of 0.6–5.1 M (10–80% wt/vol). In contrast, citronellol air-exposed for 10 weeks [content: pure citronellol 84%, and citronellol hydroperoxides 9.1%] gave an EC₃ value of 0.6 M (9.1% wt/vol). The molarity of the oxidized citronellol mixture was calculated according to the molecular weight of citronellol. A 3:2 mixture of citronellol hydroperoxides was shown to be sensitizing (EC₃ 0.1 M, 2.3% wt/vol). Epoxycitronellol was found to be low-sensitizing or non-sensitizing, as no EC₃ value was obtained at the concentrations tested (0.06–4.7 M, 1–80% wt/vol).

Discussion

Upon air exposure, citronellol was found to follow the oxidation pathways of both linalool and geraniol, although the major oxidation pathway was similar to that of

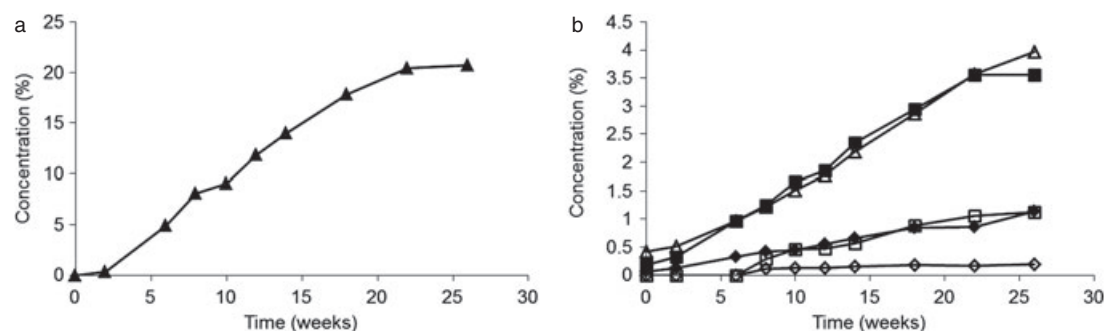


Fig. 3. (a) Concentrations of the primary oxidation products, citronellol hydroperoxides (▲), in air-exposed citronellol over time. (b) Concentrations of the secondary oxidation products, citronellol diols (△), epoxycitronellol (■), citronellal (◆), epoxycitronellal (□), and citronellyl formate (◇), in air-exposed citronellol over time. The quantification of citronellol and the oxidation products was performed with gas chromatography–mass spectrometry after derivatization with bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1). Please note the different scales on the *y*-axes.

Table 1. Citronellol and identified oxidation products: results from quantification after air exposure for 10 and 26 weeks. Sensitization potencies from local lymph node assay investigations of pure citronellol, the identified oxidation products and the oxidation mixture obtained after 10 weeks of air exposure of citronellol are shown

Compound	Amount after 10 weeks of air exposure (%)	Amount after 26 weeks of air exposure (%)	EC3 value M (% wt/vol)
Citronellol	84	33	— ^a
3,7-Dimethyloct-6-en-1-ol			
Citronellol hydroperoxides	9.1	21	0.1 (2.3)
6-Hydroperoxy-3,7-dimethyloct-7-ene-1-ol and (E)-7-hydroperoxy-3,7-dimethyloct-5-ene-1-ol			
Citronellal	0.45	1.1	3.9 (60)
3,7-Dimethylocta-6-enal			
Citronellyl formate	0.13	0.19	NT
3,7-Dimethyloct-6-ene-1-yl formate			
Epoxycitronellal	0.46	1.1	3.4 (57)
5-(3,3-dimethyloxiran-2-yl)-3-Methylpentanal			
Epoxycitronellol	1.7	3.6	— ^a
5-(3,3-dimethyloxiran-2-yl)-3-Methylpentan-1-ol			
Citronellol diols	1.5	4.0	NT
3,7-Dimethyloct-7-ene-1,6-diol and (E)-3,7-dimethyloct-5-ene-1,7-diol			
Oxidized citronellol (10 weeks of air exposure)	—	—	0.6 ^b (9.1)

NT, not tested.

^aTested in concentrations up to 80% in acetone/olive oil (4:1).

^bMolarity was calculated on the basis of the molecular weight of citronellol.

linalool (Fig. 6). The chemical analyses for the autoxidation of citronellol showed that the oxidation rate of citronellol is similar to those of linalool (12) and geraniol (13) (Fig. 2). Oxidation products were formed that increased the sensitizing potency of autoxidized citronellol according to predictive testing with the LLNA. The primary oxidation products were the hydroperoxides, which degrade to secondary oxidation products over time.

We have shown that several terpenes undergo autoxidation, forming allergenic oxidation products,

upon air exposure. Depending on the structure of the terpenes (Fig. 1), they follow different oxidation pathways (12, 13). Hydrogen abstraction by the radical chain mechanism is favoured at allylic positions where stable radicals are formed because of resonance. Citronellol has three allylic positions that are oxidizable (Fig. 1). Therefore, hydrogen abstraction by the radical chain reaction occurs at the 5-position, 8-position, and 9-position. After rearrangement to more stable radicals, isomers of hydroperoxides at the 6-position and 7-position are formed. This follows the same oxidation

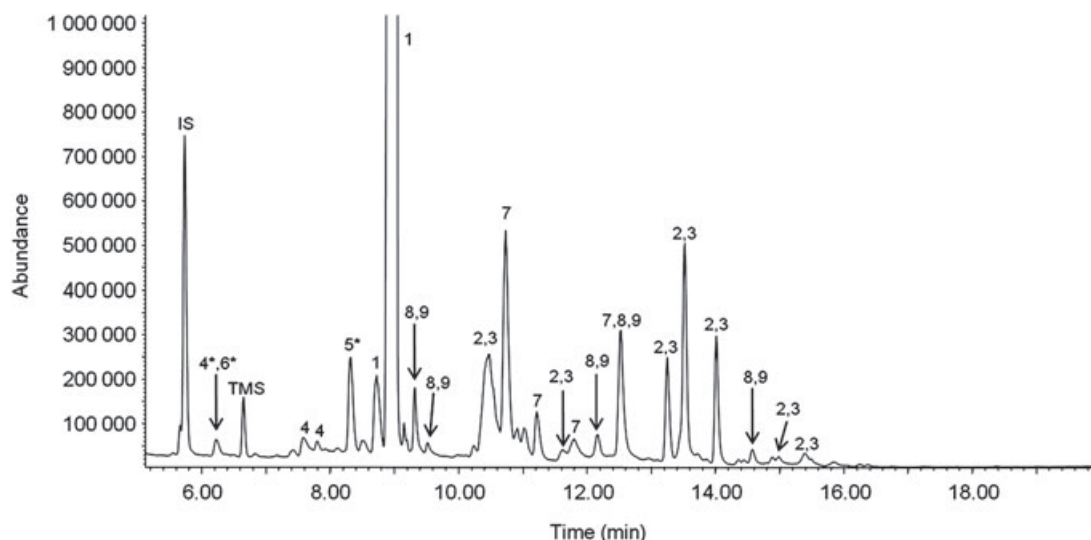


Fig. 4. Gas chromatography–mass spectrometry chromatogram of citronellol (air-exposed for 10 weeks) after derivatization with bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1) for 24 hr. Labelled peaks indicate: isomers of citronellol (**1**), isomers of citronellol hydroperoxides (**2** and **3**), isomers of citronellal (**4**), citronellyl formate (**5**), epoxycitronellal (**6**), isomers of epoxycitronellol (**7**), and isomers of citronellol diols (**8** and **9**). Peaks marked with * indicate compounds not derivatized at the reaction time used. No thermal degradation of non-derivatized or derivatized compounds was observed. The peak labelled TMS originates from the silyl reagent, but the structure is unidentified. TMS, trimethyl silyl.

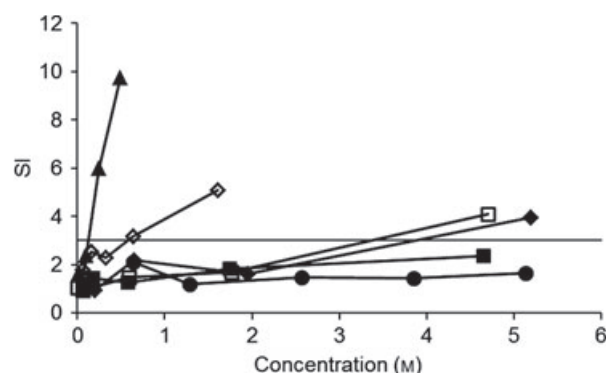


Fig. 5. Dose–response curves for the compounds tested in the local lymph node assay. The molar concentrations of oxidized citronellol were calculated from the molecular weight of citronellol. Citronellol hydroperoxides (▲), 10 weeks air-exposed citronellol (◇), epoxycitronellal (□), citronellal (◆), epoxycitronellol (■) and pure citronellol (●). The results for citronellal and epoxycitronellal are from an earlier publication (22), and are included here for comparison of the possible oxidation products. The horizontal line marks the stimulation index (SI) of 3, the cut-off limit for a compound to be considered a sensitizer according to the method used.

pathway as for linalool, which also has three oxidizable allylic positions located at the same sites of the molecule (Fig. 1); after rearrangement, this results in high amounts of the corresponding hydroperoxides at the 6-position

and 7-position (12). Hydrogen abstraction next to heteroatoms is also favourable. A radical at the 1-position of the citronellol molecule can be formed because of the stabilization of the adjacent hydroxyl group (dashed arrow in Fig. 1). Hydroperoxides formed at the 1-position readily degrade to aldehydes, as hydroxyhydroperoxides are unstable (13). Indirect evidence for the formation of a hydroxyhydroperoxide was found, as citronellal was detected in minor amounts (0.45% at 10 weeks of air exposure) in the oxidation mixture. This is in contrast to geraniol autoxidation, where the corresponding aldehydes are the major oxidation products identified (3.6% at 10 weeks of air exposure) (13). Citronellol is structurally similar to geraniol, with the difference that it lacks a 2,3-double bond (Fig. 1). For geraniol, the peroxy radical formed at the 1-position is stabilized both by the hydroxyl group and by the 2,3-double bond. It is therefore more stable than the corresponding radical at the 1-position of citronellol, which is stabilized only by the hydroxyl group. This gives hydroperoxide formation at the 1-position, resulting in aldehydes as major oxidation products for geraniol. In contrast, only minor amounts of the corresponding aldehyde were detected in the autoxidation of citronellol. This means that the major oxidation route for geraniol differs from that of citronellol (Fig. 6).

In the oxidation mixture of citronellol, we detected epoxycitronellol, whereas for oxidized linalool, two cyclic

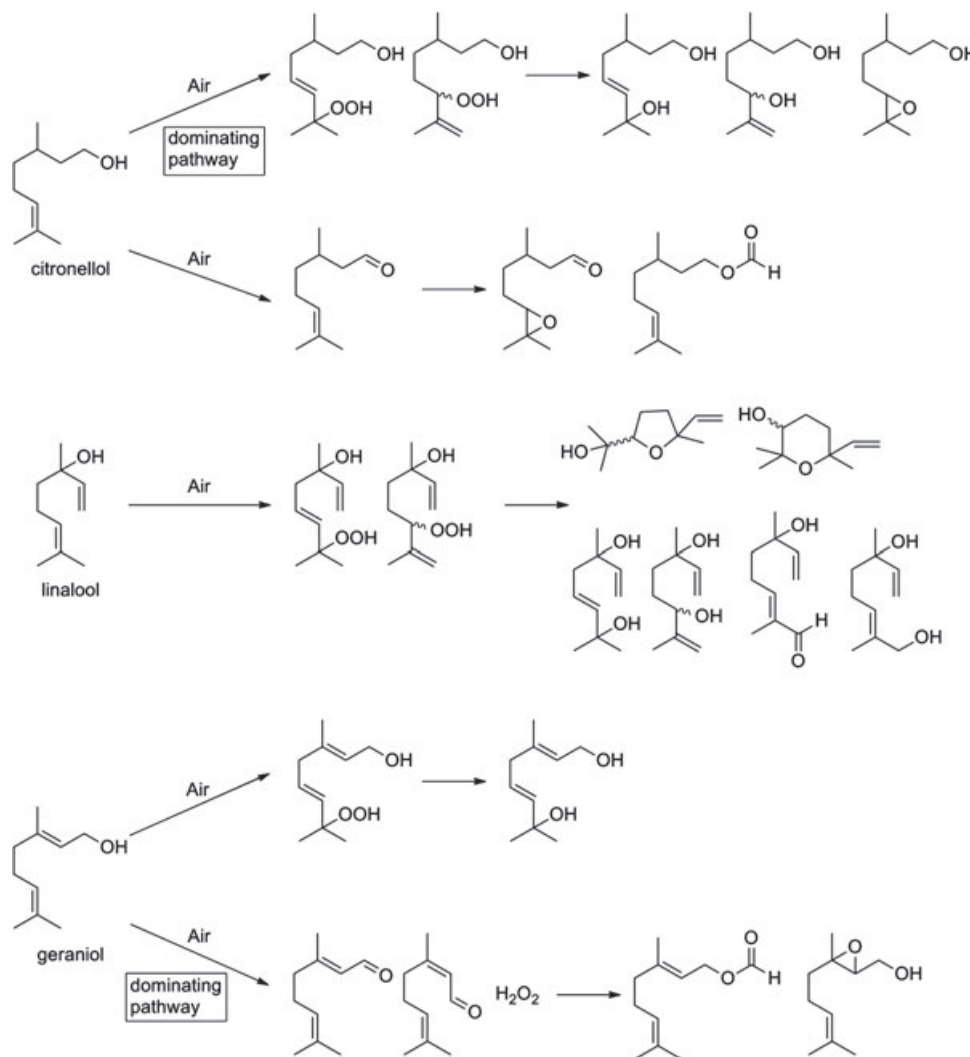


Fig. 6. Oxidation pathways of citronellol, linalool and geraniol based on oxidation products identified in the oxidation mixture after 10 weeks of air exposure. It can be seen that citronellol follows the oxidation pathway of both linalool and geraniol; however, the dominating pathway is similar to that of linalool.

ethers were identified (12) instead of the corresponding epoxide. The formation of these cyclic ethers is explained by the intramolecular attack of the linalool hydroxyl group on the epoxide carbons (positions 6 and 7), forming either the five-membered furan derivative or the six-membered pyran derivative (Fig. 7) (26). For citronellol, an attack of the hydroxyl group would result in either a seven-membered or an eight-membered ring. Formation of seven-membered and eight-membered rings is thermodynamically unfavourable relative to the corresponding five-membered and six-membered rings detected in linalool. As a result, no such products resulting from attack of the hydroxyl group on the epoxide carbons of citronellol were detected (Fig. 7). It should be noted that, for

linalyl acetate epoxide, no ring formation was observed, owing to the acetyl blocking the hydroxyl group (20).

Citronellyl formate is believed to be formed in a similar way as geranyl formate is formed from the autoxidation of geraniol, following a Baeyer–Villiger rearrangement (27). In the radical chain mechanism, a perhydrate can be formed by the formation of a hydroperoxide at the 1-position of citronellol. This perhydrate is similar to the intermediate in the Baeyer–Villiger reaction. For oxidized linalool, the pH was found to decrease over time (12), and the same is supposed to occur for oxidized citronellol. The acidic conditions result in cleavage of the oxygen–oxygen bond, followed by migration and simultaneous transesterification of a citronellol molecule.

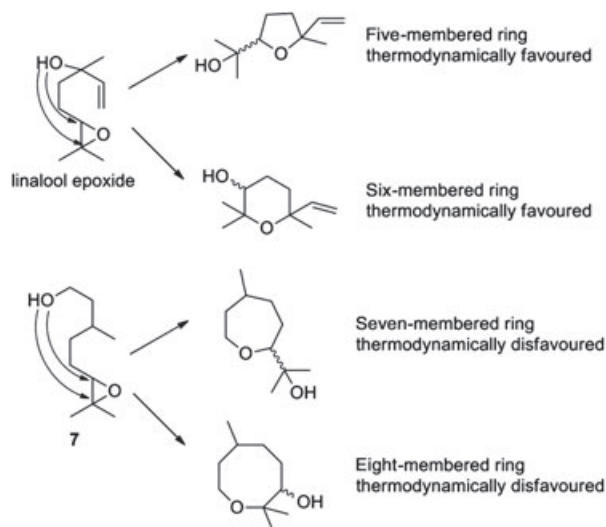


Fig. 7. An intramolecular attack resulting in ring formation is observed only for the linalool epoxide (12), where thermodynamically favoured rings are formed, in contrast to epoxycitronellol (7).

From the present study, it is clear that the sensitizing potency of autoxidized citronellol is mainly attributable to the citronellol hydroperoxides formed (Fig. 5). Epoxycitronellol showed no sensitization potential at concentrations up to 80%. This is in line with previous studies, where linalyl acetate epoxide was also shown to be a non-sensitizer (20). Citronellal and epoxycitronellal have been shown to be low-sensitizing or non-sensitizing in a previous study (EC3 3.9 M, 60% wt/vol, and EC3 3.4 M, 57% wt/vol, respectively) (22). The citronellol diols and citronellyl formate were not tested in the LLNA, as alcohols and formates are known to be low-sensitizing or non-sensitizing (13, 28, 29). The high concentrations of citronellol hydroperoxides in air-exposed citronellol (9.1% after 10 weeks of air exposure, and 21% after 26 weeks of air exposure), indicate slow degradation to secondary oxidation products.

Our results indicate high exposure to the sensitizing hydroperoxides resulting from skin contact with air-exposed citronellol. This could be important from a clinical point of view, because of the frequent exposure to citronellol in the population. We have previously shown that patch testing with oxidized linalool and oxidized geraniol detects additional cases of contact allergy as compared with patch testing with the fragrance markers of the baseline series (30–34). Citronellol is a constituent of FM II, which is used in patch testing for the diagnosis of fragrance contact allergy (6). It is considered to be a weak contact allergen, and allergic reactions to citronellol are

rare in dermatitis patients (6, 35, 36). However, contact allergy to air-exposed citronellol should be investigated analogously to contact allergy to linalool and geraniol, as oxidation of this type of fragrance compound is difficult to prevent when scented consumer products are in contact with air continuously.

In ongoing clinical studies, we have observed that a high percentage of the consecutive dermatitis patients (number tested 400) have positive patch test reactions to oxidized citronellol, indicating that more cases will be detected when patch testing is performed with air-exposed citronellol than when it is performed with FM II only (J Bråred Christensson, 2014).

Besides autoxidation, citronellol may also be metabolically activated, by analogy with geraniol. Possible metabolites of citronellol are, for example, citronellal, epoxycitronellal, and epoxycitronellol, similarly to what has been shown for geraniol (14). These possible metabolites of citronellol were shown to be low-sensitizing or non-sensitizing according to the LLNA (Table 1; Fig. 5), unlike the corresponding metabolites of geraniol (14). Although citronellol and geraniol are structurally similar, the presence of the 2,3-double bond in geraniol has a large impact on the chemical reactivity of the formed products, and hence on the sensitizing potency (22). Metabolic studies of linalool showed the formation of 8-hydroxylinalool, the five-membered furan derivative, and the six-membered pyran derivative (37), compounds that are low-sensitizing or non-sensitizing. On the basis of this, we propose that the allergenic activity of citronellol depends mainly on autoxidation, which is similar to what has been observed for linalool.

The chemical characterization of products is vital in order to investigate the exposure to sensitizers in the population. More specific, chemical analyses should be performed to determine the relevance of the positive patch test reactions to oxidized fragrance compounds that are frequently seen in dermatitis patients (30–34). This especially concerns the hydroperoxides, which, so far, have been identified as the major allergens in most oxidation mixtures. Terpene hydroperoxides are difficult to detect in products, as the chemical structures of terpenes provide only weak chromophores and also low thermostability. We have previously developed a sensitive method using liquid chromatography/electrospray ionization–MS/MS for the quantification of specific hydroperoxides (38). However, in the case of air-exposed citronellol, the separation efficiency of this method was insufficient for accurate determination of all oxidation products. Instead, we used our newly developed GC–MS method (19) for separation of TMS derivatives. Higher peak capacity and better separation of the hydroperoxides were achieved with

the GC–MS method, despite lower sensitivity. We now have two complementary analytical methods available for monoterpene hydroperoxide determination that can be used for different applications and sample complexities.

Conclusions

Citronellol was found to follow the oxidation pathways of both linalool and geraniol, although the main oxidation pathway was similar to that of linalool, with major amounts of highly sensitizing hydroperoxides being formed. Thus, the present study shows the impact of the absence of the 2,3-double bond in the structure of citronellol, as compared with previous studies of geraniol (13), on the oxidation pathways for formation of oxidation products. On the basis of this, the influence of double bonds in conjugation with functional groups should be considered when the autoxidation of additional fragrance terpenes is studied.

Air-exposed citronellol showed stronger sensitizing potency than pure citronellol. The main contributors to the increased sensitizing potency of air-exposed citronellol are considered to be the hydroperoxides. The concentration of the citronellol hydroperoxides increased over time, resulting in high exposure to the sensitizing compounds

after skin contact with air-exposed citronellol. The study shows the usefulness of our new GC–MS method for the identification and quantification of the citronellol oxidation products, especially the hydroperoxides.

Acknowledgements

We are grateful for laboratory work by Maryam Kaveh Ahangaran. J. Rudbäck would like to thank Dr Tamara Delaine for laboratory assistance.

Petri Karhunen, Anders Eliasson, Susanne Exing and Gabriella Wendt are acknowledged for assistance with the LLNA experiments. The work was performed at the Centre for Skin Research Gothenburg (SkinResQU), University of Gothenburg, Sweden.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

File S1. Results from the LLNA with [³H] methylthymidine incorporation (dpm/lymph node) and SI values for pure citronellol, citronellol air-exposed for 10 weeks, citronellol hydroperoxides, and epoxycitronellol.

References

- Lepoittevin J P, Le Coz C. *Dictionary of Contact Allergens*: Berlin, Heidelberg, Springer-Verlag, 2007.
- Buckley D A. Fragrance ingredient labelling in products on sale in the UK. *Br J Dermatol* 2007; **157**: 295–300.
- Belsito D, Bickers D, Bruze M et al. A toxicologic and dermatologic assessment of cyclic and non-cyclic terpene alcohols when used as fragrance ingredients. *Food Chem Toxicol* 2008; **46**: S1–S71.
- Rastogi S C, Johansen J D, Frosch P et al. Deodorants on the European market: quantitative chemical analysis of 21 fragrances. *Contact Dermatitis* 1998; **38**: 29–35.
- Rastogi S C, Heydorn S, Johansen J D, Basketter D A. Fragrance chemicals in domestic and occupational products. *Contact Dermatitis* 2001; **45**: 221–225.
- Frosch P J, Rastogi S C, Pirker C et al. Patch testing with a new fragrance mix – reactivity to the individual constituents and chemical detection in relevant cosmetic products. *Contact Dermatitis* 2005; **52**: 216–225.
- Nielsen N H, Menné T. Allergic contact sensitization in an unselected Danish population. The Glostrup Allergy Study, Denmark. *Acta Derm Venereol* 1992; **72**: 456–460.
- Thyssen J P, Linneberg A, Menné T, Nielsen N H, Johansen J D. The prevalence and morbidity of sensitization to fragrance mix I in the general population. *Br J Dermatol* 2009; **161**: 95–101.
- Meneghini C L, Sertoli A, Nava C, Angelini G, Francalani S, Foti C, Moroni P. Irritant contact dermatitis of the hands in housewives. In: *Irritant Dermatitis: New Clinical and Experimental Aspects (Current Problems in Dermatology)*, Elsner P, Maibach H I (eds): Basel, Karger, 1995: pp. 41–48.
- The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP). *Opinion Concerning Fragrance Allergy in Consumers. A Review of the Problem. Analysis of the Need for Appropriate Consumer Information and Identification of Consumer Allergens*, adopted 8 December 1999, SCCNFP/0017/98 Final: Brussels, SCCNFP, 1999.
- Karlberg A T, Shao L P, Nilsson U, Gäfvert E, Nilsson J L G. Hydroperoxides in oxidized d-limonene identified as potent contact allergens. *Arch Dermatol Res* 1994; **286**: 97–103.
- Sköld M, Börje A, Harambasic E, Karlberg A T. Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization. *Chem Res Toxicol* 2004; **17**: 1697–1705.
- Hagvall L, Bäcktorp C, Svensson S, Nyman G, Börje A, Karlberg A T. Fragrance compound geraniol forms contact allergens on air exposure. Identification and quantification of oxidation products and effect on skin sensitization. *Chem Res Toxicol* 2007; **20**: 807–814.
- Hagvall L, Baron J M, Börje A, Weidolf L, Merk H, Karlberg A T. Cytochrome P450-mediated activation of the fragrance compound geraniol forms potent contact allergens. *Toxicol Appl Pharm* 2008; **233**: 308–313.
- Bergström M A, Luthman K, Nilsson J L G, Karlberg A T. Conjugated dienes as prohapens in contact allergy: in vivo and in vitro studies of structure–activity relationships, sensitizing capacity, and metabolic activation. *Chem Res Toxicol* 2006; **19**: 760–769.
- Gerberick F, Aleksic M, Basketter D et al. Chemical reactivity measurements and the

- predictive identification of skin sensitizers. *Altern Lab Anim* 2008; **36**: 215–242.
- 17 Karlberg A T, Bergström M A, Börje A, Luthman K, Nilsson J L G. Allergic contact dermatitis – formation, structural requirements, and reactivity of skin sensitizers. *Chem Res Toxicol* 2008; **21**: 53–69.
- 18 Gerberick G F, Ryan C A, Dearman R J, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007; **41**: 54–60.
- 19 Rudbäck J, Ramzy A, Karlberg A T, Nilsson U. Determination of allergenic hydroperoxides in essential oils by GC/electron ionization-MS. *J Sep Sci* 2014; doi: 10.1002/jssc.201300843. [Epub ahead of print].
- 20 Sköld M, Hagvall L, Karlberg A T. Autoxidation of linalyl acetate, the main component of lavender oil, creates potent contact allergens. *Contact Dermatitis* 2008; **58**: 9–14.
- 21 Baeckström P, Okecha S, De Silva N, Wijekoon D, Norin T. Photooxidation with simultaneous reduction of hydroperoxides with tetrabutylammonium borohydride. Synthesis of perillenal from myrcene. *Acta Chem Scand B* 1982; **36**: 31–36.
- 22 Delaine T, Hagvall L, Rudbäck J, Luthman K, Karlberg A T. Skin sensitization of epoxyaldehydes – importance of conjugation. *Chem Res Toxicol* 2013; **26**: 674–684.
- 23 Knapp H, Straubinger M, Fornari S, Oka N, Watanabe N, Winterhalter P. (S)-3,7-Dimethyl-5-octene-1,7-diol and related oxygenated monoterpenoids from petals of *Rosa damascena* Mill. *J Agr Food Chem* 1998; **46**: 1966–1970.
- 24 Basketter D A, Lea L J, Dickens A, Briggs D, Pate I, Dearman R J, Kimber I. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *J Appl Toxicol* 1999; **19**: 261–266.
- 25 Kimber I, Basketter D A, Butler M et al. Classification of contact allergens according to potency: proposals. *Food Chem Toxicol* 2003; **41**: 1799–1809.
- 26 Bezaud M, Karlberg A T, Montelius J, Lepoittevin J P. Skin sensitization to linalyl hydroperoxide: support for radical intermediates. *Chem Res Toxicol* 1997; **10**: 987–993.
- 27 Bäcktorp C, Hagvall L, Börje A, Karlberg A T, Norrby P O, Nyman G. Mechanism of air oxidation of the fragrance terpene geraniol. *J Chem Theory Comput* 2008; **4**: 101–106.
- 28 Kern P S, Gerberick G F, Ryan C A, Kimber I, Aptula A, Basketter D A. Local lymph node data for the evaluation of skin sensitization alternatives: a second compilation. *Dermatitis* 2010; **21**: 8–32.
- 29 Bergh M, Shao L P, Magnusson K, Gäfvert E, Nilsson J L G, Karlberg A T. Atmospheric oxidation of poly(oxyethylene) alcohols. Identification of ethoxylated formates as oxidation products and study of their contact allergenic activity. *J Pharm Sci* 1999; **88**: 483–488.
- 30 Hagvall L, Karlberg A T, Christensson J B. Finding the optimal patch test material and test concentration to detect contact allergy to geraniol. *Contact Dermatitis* 2013; **68**: 224–231.
- 31 Bråred Christensson J, Andersen K E, Bruze M et al. Air-oxidized linalool – a frequent cause of fragrance contact allergy. *Contact Dermatitis* 2012; **67**: 247–259.
- 32 Bråred Christensson J, Matura M, Gruvberger B, Bruze M, Karlberg A-T. Linalool – a significant contact sensitizer after air exposure. *Contact Dermatitis* 2010; **62**: 32–41.
- 33 Hagvall L, Karlberg A T, Bråred Christensson J. Contact allergy to air-exposed geraniol: clinical observations and report of 14 cases. *Contact Dermatitis* 2012; **67**: 20–27.
- 34 Matura M, Sköld M, Börje A et al. Selected oxidized fragrance terpenes are common contact allergens. *Contact Dermatitis* 2005; **52**: 320–328.
- 35 Frosch P J, Johansen J D, Menné T et al. Further important sensitizers in patients sensitive to fragrances – I. Reactivity to 14 frequently used chemicals. *Contact Dermatitis* 2002; **47**: 78–85.
- 36 Nardelli A, Carbonez A, Drieghe J, Goossens A. Results of patch testing with fragrance mix 1, fragrance mix 2, and their ingredients, and Myroxylon pereirae and colophonium, over a 21-year period. *Contact Dermatitis* 2013; **68**: 307–313.
- 37 Meesters R J W, Duisken M, Hollender J. Study on the cytochrome P450-mediated oxidative metabolism of the terpene alcohol linalool: indication of biological epoxidation. *Xenobiotica* 2007; **37**: 604–617.
- 38 Rudbäck J, Islam N, Nilsson U, Karlberg A T. A sensitive method for determination of allergenic fragrance terpene hydroperoxides using liquid chromatography coupled with tandem mass spectrometry. *J Sep Sci* 2013; **36**: 1370–1378.

Characterisation of skin sensitisers from autoxidised citronellol - Impact of the terpene structure on the autoxidation process

Johanna Rudbäck¹, Lina Hagvall^{1,2}, Anna Börje¹, Ulrika Nilsson³, and Ann-Therese Karlberg^{1,*}

¹*Department of Chemistry and Molecular Biology, Dermatochemistry and Skin Allergy, University of Gothenburg, SE-412 96 Gothenburg, Sweden*

²*Department of Dermatology, Sahlgrenska Academy, University of Gothenburg, SE-405 30 Gothenburg, Sweden*

³*Department of Analytical Chemistry, Stockholm University, SE-106 91 Stockholm, Sweden*

**Correspondence:* Ann-Therese Karlberg, Department of Chemistry and Molecular Biology, Dermatochemistry and Skin Allergy, University of Gothenburg, SE-412 96 Gothenburg, Sweden, Tel: +46-31-786 91 09, E-mail: karlberg@chem.gu.se

Supporting Information

File S1: Results from the LLNA with [³H]methylthymidine incorporation (dpm/lymph node) and SI values for pure citronellol, citronellol air-exposed for 10 weeks, citronellol hydroperoxides, and epoxycitronellol.

S1. Results from the murine local lymph node assay (LLNA)^a

Compound and test concentration (% wt/vol)	[³ H]Methylthymidine incorporation (dpm/lymph node)	SI	EC3 value M (% wt/vol)	Classification
Citronellol 1			>5.1 (>80)	Weak
Control	1134			
10	2404	2.1		
20	1346	1.2		
40	1665	1.5		
60	1621	1.4		
80	1862	1.6		
10 weeks air-exposed citronellol 1			0.6 ^b (9.1)	Moderate
Control	578			
1	1038	1.8		
2.5	1456	2.5		
5	1319	2.3		
10	1828	3.2		
25	2939	5.1		
Citronellol hydroperoxides 2 and 3 (3: 2 ratio)			0.1 (2.3)	Moderate
Control	243			
0.46	397	1.6		
0.9	244	1.0		
1.8	574	2.4		
4.6	1448	6.0		
9.2	2359	9.7		
Epoxy citronellol 7			>4.7 (>80)	Weak
Control	296			
1	309	1.0		
3	427	1.4		
10	373	1.3		
30	541	1.8		
80	698	2.4		

Sensitising experiments on the citronellol compounds studied.

^aGroups of mice were treated with test substance at five different concentrations, on the dorsum of both ears, for three consecutive days. Sham-treated control mice received vehicle alone. On day 5, all mice were injected intravenously with phosphate-buffered saline (250 µl) containing 20 µCi of [³H]methylthymidine. After 5 h, the mice were sacrificed, the draining lymph nodes were excised and pooled for each group, single-cell suspensions of lymph node cells were prepared, and thymidine incorporation into DNA was measured by β-scintillation counting. The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group, and recorded as stimulation index (SI). The EC3 values (the estimated concentration required to induce an SI of 3) were calculated by using linear interpolation. The sensitising potency was classified according to the following: EC3 < 0.1%, extreme; EC3 ≥ 0.1 - < 1 %, strong; EC3 ≥ 1 - <10%, moderate; EC3 ≥ 10 - < 100%, weak (32).

^bMolarity was calculated on the basis of the molecular weight of citronellol.