

Investigation of the skin sensitizing activity of linalool

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An increasing range of chemicals appears to be capable of causing skin sensitization as a result of their capacity to undergo air oxidation (autoxidation) with the consequent formation of reactive species such as epoxides and hydroperoxides. In this small investigation, the ability of linalool, a common fragrance ingredient, to cause such effects was quantified using the local lymph node assay before and after careful purification by vacuum distillation. The commercially available grade of linalool (97% purity) was shown to be a weak skin sensitizer. Various impurities, including linalool oxide, dihydrolinalool, epoxylinalool, 3-hexenyl butyrate and 3,7-dimethyl-1,7-octadiene-3,6-diol were identified and were completely removed (except for the dihydrolinalool remaining at 1.4%) and the re-purified linalool retested. Neither linalool or dihydrolinalool are protein-reactive compounds. The sensitization potency of the re-purified linalool sample was considerably reduced, but not entirely eliminated, suggesting either that an allergenic impurity could be very quickly reformed by mechanisms of activation or that certain potent undetectable allergens remained. Both possibilities are consistent with what is understood of the chemistry and composition of commercially available linalool.

Key words: autoxidation; impurities; linalool; local lymph node assay; sensitization potency. © Blackwell Munksgaard, 2002. © Blackwell Munksgaard, 2002.

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With the possible exception of certain metals, skin sensitization is caused by chemicals that have the potential to bind covalently to skin proteins (1, 2). However, not all of these materials are directly reactive with skin protein; certain of them require activation either by metabolic enzymes associated with skin, or by air oxidation (autoxidation) [reviewed in (2)]. Examples of compounds that can be metabolically activated include many human contact allergens, such as isoeugenol (3), pentadecyl catechol (4) and cinnamic alcohol (5). Autoxidation has also begun to prove an increasingly important route for the generation of protein reactive species (e.g. 6, 7), and has resulted in suggestions in respect of regulatory assessment of such materials (8). In the present work, we have undertaken a small investigation of the sensitizing capacity of the fragrance chemical linalool (Fig. 1), which has been suggested to be susceptible to autoxidation and also is known to be potential cause of allergic contact dermatitis (ACD) (9, 10).

Materials and Methods

Chemical analysis of linalool

The sample of linalool (97% pure, obtained from Aldrich (Poole, UK; structure in Fig. 1) was analysed by gas chromatography/mass spectrometry (GCMS) to ascertain its purity and identify any impurities that might cause sensitization. The following methods were used: 100 mg of linalool was weighed into a 10-mL volumetric flask and made to volume with methanol. The conditions for the GCMS analysis were as follows:

Gas chromatography conditions Gas chromatograph: Fisons GC8000 series, serial no. 940273. Column: HP-23 (*cis/trans* FAME column) 30 m × 0.25 mm ID, 0.25 m FT. Oven temperature: 140°C isothermal for 10 min. Injector: split at 220°C (300 mL/min split flow). Carrier gas: helium at 15 psi inlet pressure. Injection volume: 0.8 L.

Mass spectrometry conditions Instrument: MD800 serial no. 13215. Mass range: 19–400. Scan

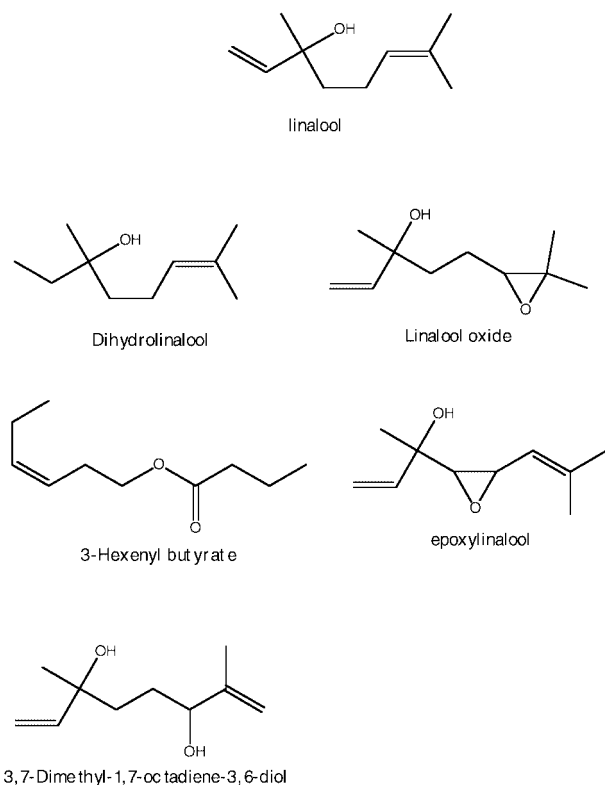


Fig. 1. Chemical structures of compounds found in linalool

time: 0.20 s. Inter scan delay: 0.05 s. Ionization mode: EI+. Source temperature: 220°C. Interface temperature: 220°C. Run time: 10 min.

Purification of linalool

Following the identification of potential sensitizing impurities in linalool, a sample was redistilled in an attempt to eliminate these impurities. A 5-mL sample of linalool was purified by vacuum distillation using an Adiabatic Annular Spinning Band Still (Perkin Elmer Spinning Band Distillation System, Model 131T Microstill). The product of distillation was analysed by GCMS to confirm its purity and identity using the same conditions for the GCMS analysis as described previously.

Local lymph node assay

The protocol used followed the standard methodology described previously (11). Groups of 4 CBA/Ca mice (7–12 weeks of age, Harlan Olac, UK) were treated with 25 μ L of test material, or with an equal volume of the vehicle (4:1 v/v acetone/olive oil) alone on the dorsum of both ears. Treatment was performed once daily for 3 consecutive days. 5 days following the initiation of exposure, all mice were injected via the tail vein with 250 μ L of phosphate-buffered saline (PBS) containing 20 μ Ci of

Table 1. Impurities identified in commercial linalool

Chemical identity of impurity	% by weight
Dihydrolinalool	1.92
Linalool oxide	0.66
3-Hexenyl butyrate	0.18
Epoxy linalool	0.14
3,7-Dimethyl-1,7-octadiene-3,6-diol	0.10

tritiated thymidine (2 Ci/mmol), Amersham International (Amersham, UK). Mice were sacrificed 5 h later and the draining lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells was prepared by mechanical disaggregation. The lymph node cell suspension was washed $2 \times$ in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18 h. Pellets were resuspended in TCA and the incorporation of tritiated thymidine measured by β -scintillation counting. A substance was regarded as a skin sensitizer if at any test concentration the proliferation in treated lymph nodes was 3-fold or greater than that in the concurrent vehicle-treated controls. In addition, the concentration of the chemical required to produce a stimulation index of 3, the EC₃ value, was calculated as described previously (10) to provide a quantitative index of the relative sensitizing potency of chemicals tested.

Results

As supplied, linalool was confirmed to be of 97% purity by GCMS. The impurities (in descending concentration order) in this commercial material were identified as dihydrolinalool, linalool oxide, 3-hexenyl butyrate, epoxy linalool and 3,7-dimethyl-1,7-octadiene-3,6-diol (chemical structures shown in Fig. 1). On a percentage w/w basis, dihydrolinalool accounted for a little over 1/2 of the total impurities (Table 1). Upon redistillation, all impurities were completely removed to below their respective limits of detection except for the dihydrolinalool, which was only reduced to 1.4%, such that the repurified material was in effect 98.6% linalool.

The proliferative response of the lymph node cells are shown in Table 2. Prior to purification, linalool elicited a positive lymphocyte (sensitization) response ((Disintegrations per minute) DPM/node of test/control > 3) at 50.0% and 100%. Linalool was redistilled and then retested at the same concentrations in the same vehicle. On this occasion, a positive sensitization response was obtained at 100% only. Reflecting this difference, the calculated EC₃ value for linalool was 30%, whilst for redistilled linalool it was 55%. This difference is consistent

Table 2. Local lymph node assay results for linalool

Sample	³ HTdR incorporated per lymph node (DPM/node)				
	0%	25%	50%	100%	EC3 value
Original linalool	361	919	1718	2988	30%
Purified linalool	366	771	1043	1777	55%

ent with a substantial weakening in sensitizing potency following purification, although it must be recognized that the accuracy of EC3 predictions at this level is not yet fully appreciated.

Discussion

Many chemicals that have the capacity to cause skin sensitization only express this behaviour as a result of autoxidation following air exposure or possibly via metabolic activation in the skin. This can cause problems in the prospective identification of potential contact allergens on the basis of their chemical structure. Therefore, an enhanced understanding of these aspects is important for the continuing improvement of predictive methodologies such as the expert system, DEREK (12, 13). In addition, the knowledge should permit better diagnosis as well as presenting opportunities to limit the formation of allergen, either by restricting air exposure, monitoring substance shelf lives or permitting considerations relating to allergen substitution.

In the present work, we undertook a modest investigation of the fragrance chemical linalool, whose structure (Fig.1) seemed to us unlikely to be a direct acting hapten. However, evidence had accrued that linalool was in fact a contact allergen (9, 10), suggesting therefore that either impurities, including those potentially formed by autoxidation, and/or metabolic activation might play a key part in sensitization. Testing in the local lymph node assay confirmed that linalool as supplied was an allergen, although its potency as defined by the EC3 value of 30% was low (14). Removal of most of the impurities (increasing linalool purity to 98.6% w/w and decreasing dihydrolinalool impurity to 1.4% w/w in the test sample) resulted in an apparent weakening in potency, with the EC3 value almost doubling to 55%. Neither the linalool nor the dihydrolinalool in the distilled sample are themselves protein-reactive compounds. The results then are consistent with the view that linalool sensitizes largely as a consequence of the formation of reactive species (possibly epoxides) by either autoxidation or metabolic activation or both, given that the identified impurities include compounds such as linalool oxide, epoxy linalool and 3,7-dimethyl-1,7-octadiene-3,6-diol, which

could be formed by such mechanisms of activation; the origin of the 3-hexenyl butyrate is unclear, so it most likely is present as an impurity arising during the commercial manufacture of linalool. No other epoxides such as dihydrolinalool oxide or 1,2-epoxides were detected. It is also possible that the 3,7-dimethyl-1,7-octadiene-3,6-diol could act as an allergenic impurity, but this would need to be reformed in the distilled sample via linalool oxide and be further oxidized at the 6-OH group to form an α,β -unsaturated carbonyl group. The latter could act as a Michael acceptor and bind to protein nucleophiles, though given the activation steps that would be necessary, this seems the most unlikely explanation.

The results emphasize the need to consider susceptibility to compound activation when reviewing the likelihood that a chemical may be a skin sensitizer; such information needs to be built into predictive computer systems used in identifying toxicological hazards. The shelf life and storage of compounds used in testing for sensitization potency also becomes a major issue for compounds that may be activated upon air exposure. As such, limitation of air exposure and the use of antioxidants in association with such chemicals might provide a means by which the risk of ACD could be reduced.

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