

Are contact allergens stable in patch test preparations? Investigation of the degradation of *d*-limonene hydroperoxides in petrolatum

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Several of the products formed after oxidation of *d*-limonene exhibit strong contact allergenic properties. Some, e.g., the hydroperoxides, are unstable compounds. In this study, we have examined whether the limonene hydroperoxides are chemically stable in white petrolatum used for patch testing. We found that the stability of the hydroperoxides was strongly dependent on whether or not the petrolatum was stabilized with α -tocopheryl acetate. In the presence of this antioxidant, the hydroperoxides were degraded to a greater extent. The hydroperoxides were shown to be directly reduced to the corresponding alcohols by this agent. On the other hand, the compounds were shown to be stable in non-stabilized petrolatum throughout clinical patch testing for a period of 6 weeks, provided that the preparations were stored in a refrigerator when not used. Thus, it is recommended that vehicles without α -tocopheryl acetate are used when peroxy or hydroperoxy compounds are patch tested or used in sensitization experiments. However, it is important to limit the storage time so that optimal conditions are at hand. A fast method was developed to enable isolation and quantification of the hydroperoxides in white petrolatum. This analytical method may also be applicable to other compositions of patch test preparations.

Key words: hydroperoxides of *d*-limonene; α -tocopheryl acetate; antioxidant; chemical degradation; contact allergens; HPLC; patch test vehicles; white petrolatum; patch testing technique. © Munksgaard, 1999.

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We have previously shown that *d*-limonene itself achieves no significant skin sensitization (1, 2). However, some of the compounds (3) formed upon autoxidation (air oxidation) of *d*-limonene, have been identified as strong contact allergens (2, 4). Among these are 4 main hydroperoxides, which are formed as primary oxidation products (Fig. 1f–i). The hydroperoxides of *d*-limonene are unstable and readily degrade to other secondary oxidation products, such as carvone (Fig. 1j), if further subjected to daylight and air.

At dermatological clinics, patients with a suspected history of allergic contact dermatitis are patch tested with preparations of allergens in suitable vehicles. The most commonly used vehicle is petrolatum. To prevent the petrolatum from oxidation, it is often stabilized (5). A common antioxidant used for this is α -tocopheryl acetate (6).

However, we raised the question whether α -tocopheryl acetate not only protects the petrolatum but also reacts directly with hydroperoxides/peroxides. The aim of this study was therefore to examine the stability of hydroperoxides, when applied in petrolatum with and without α -tocopheryl acetate.

Materials and Methods

Chemicals

d-limonene (Fig. 1a) was purchased from Frey & Lau, Norderstedt, Germany. (R)-(–)carvone ((R)-(–)-*p*-mentha-6,8-dien-2-one, Fig. 1j), (–)-carveol (*p*-mentha-6,8-dien-2-ol, a mixture of *trans* and *cis* isomers, Fig. 1d and 1e), acetophenone and (–)-menthol (Fig. 1k) were all purchased from Aldrich Chemical Company Inc., Milwaukee Wis, USA. The latter compound was used as an internal stan-

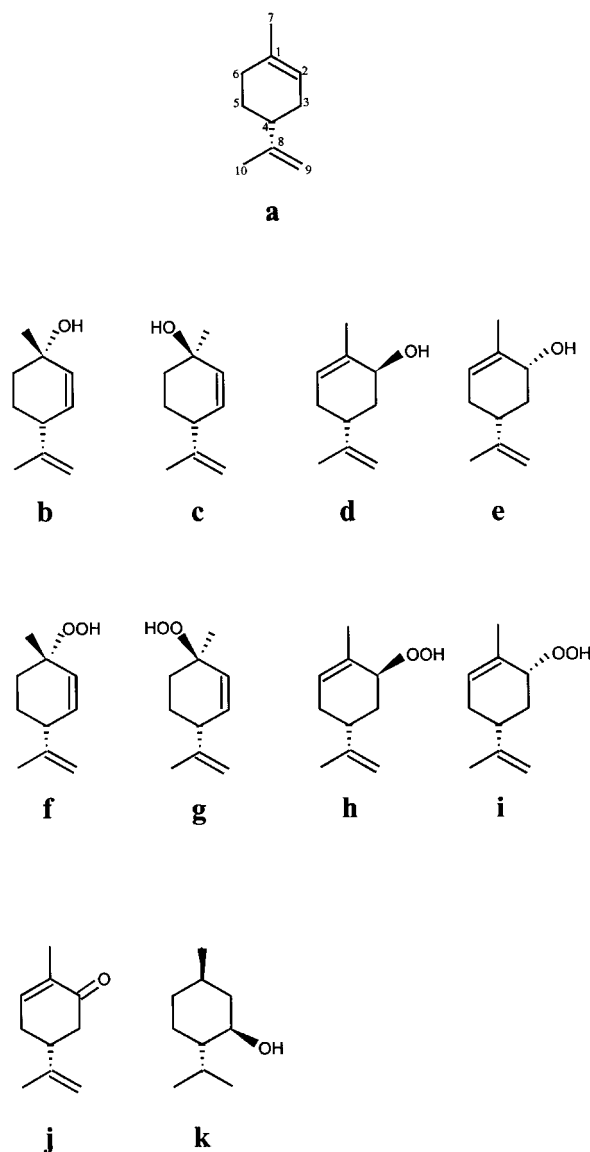


Fig. 1. Chemical structures of the studied limonene alcohols and hydroperoxides **a**=*d*-limonene, **b**=*cis*-*p*-mentha-2,8-dien-1-ol, **c**=*trans*-*p*-mentha-2,8-dien-1-ol, **d**=*trans*-*p*-mentha-6,8-dien-2-ol (*trans*-carveol), **e**=*cis*-*p*-mentha-6,8-dien-2-ol (*cis*-carveol), **f**=*cis*-*p*-mentha-2,8-diene-1-hydroperoxide, **g**=*trans*-*p*-mentha-2,8-diene-1-hydroperoxide, **h**=*trans*-*p*-mentha-6,8-diene-2-hydroperoxide, **i**=*cis*-*p*-mentha-6,8-diene-2-hydroperoxide, **j**=(R)-(-)-carvone, **k**=(-)-menthol (IS).

dard for quantification and was added to the hydroperoxide fraction prior to clean up. Acetophenone was used as a recovery standard and added to the hydroperoxide fraction after isolation with HPLC (High Performance Liquid Chromatography). *Trans* and *cis* isomers of limonene-2-hydroperoxide (*p*-mentha-6,8-diene-2-hydroperoxide, Fig. 1h and 1i) were synthesized and a hydroperoxide fraction was isolated from autoxidized *d*-limonene and kindly provided by one of our col-

leagues at the laboratory, L.P. Shao (4). Hexane of HPLC grade was purchased from Rathburn Ltd. Walkerburn, Scotland. White petrolatum (Snowwhite) stabilized with 10 ppm of α -tocopheryl acetate from Apoteket AB, Sweden, and non-stabilized white petrolatum from Kebo Lab AB, Sweden, were used as vehicles.

Isolation procedure and chemical composition of the hydroperoxide fraction from autoxidized *d*-limonene

Identification of individual compounds was performed by using GC-MS (gas chromatography-mass spectrometry) and reference compounds, as described previously (7, 8). 4 main hydroperoxides were formed upon autoxidation of *d*-limonene, i.e. *cis*-*p*-mentha-2,8-diene-1-hydroperoxide (Fig. 1f), *trans*-*p*-mentha-2,8-diene-1-hydroperoxide (Fig. 1g), *cis*-*p*-mentha-6,8-diene-2-hydroperoxide (Fig. 1i) and *trans*-*p*-mentha-6,8-diene-2-hydroperoxide (Fig. 1h), while only trace amounts of *cis*-*p*-mentha-[1(7),8]-diene-2-hydroperoxide and *trans*-*p*-mentha-[1(7),8]-diene-2-hydroperoxide (not shown in Fig. 1), respectively, were produced. After 3 months, the autoxidized mixture contained approximately equal amounts of hydroperoxides (7%) and corresponding alcohols (5%). The other main constituents were carvone (4%), *cis* and *trans* forms of limonene-1,2-oxide (*p*-menth-8-ene-1,2-oxide, 5%) and *p*-menth-8-ene-1,2-diol (2%). Flash chromatography was used as a 1st clean-up step (4). However, the hydroperoxides and carvone co-eluted in this chromatographic system. By using the same HPLC system as described below, but in preparative scale (column size 20×250 mm, 10 μ m particles in the stationary phase, flow rate of the mobile phase 10 ml/min, 2% of anhydrous ethanol in hexane as mobile phase) more than 90% of the carvone content could be separated from the hydroperoxide fraction. The hydroperoxides started to elute at 13.5 min and carvone eluted at 7.5 min. After clean-up, the enriched hydroperoxide fraction contained 4.3, 2.3 and 5.3% of transcarveol, *cis*-carveol and carvone, respectively. The content of *cis* and *trans* isomers of *p*-mentha-2,8-dien-1-ol was less than 2%.

Handling procedures for the hydroperoxide preparations

Test preparations of the hydroperoxide fraction were used for patch testing consecutive dermatitis patients at the Department of Occupational and Environmental Dermatology, Karolinska Hospital, Stockholm. The preparations were made by addition of about 5 mg/g of the hydroperoxide fraction to non-stabilized and stabilized petrolatum, respectively. At the hospital, the prepara-

tions were used for patch testing 3 days a week and were stored in the refrigerator when not used. Furthermore, samples of the hydroperoxide fraction in non-stabilized and stabilized petrolatum were stored in the refrigerator (6–8°C) at our laboratory, without further handling procedure. Samples were also stored at room temperature (20–22°C) and subjected to daylight. All preparations were stored for totally 6 weeks.

Samples of pure non-stabilized petrolatum with no *d*-limonene hydroperoxides added, stored either in the refrigerator for 6 weeks or at room temperature and in daylight for 2 weeks, were analysed with respect to peroxide content. This was performed by using Merckoquant[®] analytical peroxide test strips, purchased from Merck, Germany. This simple test has previously been shown to work satisfactorily as an indicator of a start of a potential oxidation process (9, 10).

Isolation and quantification of hydroperoxides and corresponding alcohols in the patch test preparations

Every 2 weeks about 40 mg (corresponding to triplicate samples) of each patch test preparation from the hospital and the laboratory were taken out for analysis. The hydroperoxide fraction was isolated from the preparations with HPLC. The HPLC method described is a somewhat modified version of that published in our earlier paper (7). Prior to separation with HPLC, each sample (of about 10 mg) was dissolved in 300 μ l of the HPLC mobile phase and 16.2 μ g (–)-menthol, dissolved in 100 μ l, was added as an internal standard. The whole sample was injected into the HPLC system.

The HPLC system consisted of a Varian 9012 solvent delivery module, a UV detector (Walnut Creek, USA) and an injector (Rheodyne, Cotati, USA) with a loop volume of 500 μ l. An ELDS laboratory data system (Chromatography Data Systems Inc., Sweden) was used for registering, storing and processing the detector signals. A wavelength of 205 nm was used for detection. The HPLC column used was an endcapped CN-HP Novapak cyanopropyl-modified silica column (3.9×300 mm, 5 μ m particles) purchased from Millipore, USA. Hexane, with 0.125% (w/w) of anhydrous ethanol added, was used as mobile phase at a flow rate of 2 ml/min. Addition of ethanol to the mobile phase prevented the hydroperoxides from irreversible adsorption to the stationary phase. The HPLC system was also equipped with a Rheodyne switching valve for reversal of the flow through the HPLC column.

After elution of the petrolatum, the flow was reversed at 2.2 min. A back-flush fraction eluting between 3.5 and 5.5 min was then collected. The vol-

ume of this fraction, containing the hydroperoxides, alcohols, carvone and the internal standard, was reduced to about $\frac{1}{2}$ by evaporation in a gentle stream of nitrogen.

The different hydroperoxides and alcohols in the back-flush fraction were subsequently quantified by using gas chromatography (GC). After evaporation of the HPLC mobile phase and prior to GC analysis, 16.3 μ g of acetophenone was added to each sample as a recovery standard. The Varian 3400 gas chromatograph was equipped with a 30 m×0.25 mm i.d. fused silica capillary column coated with 0.1 μ m DB-5 (J&W Scientific, USA). The injections were made at 35°C using on-column technique. The temperature of the injector was then raised up to 250°C at a rate of 90°C/min. The temperature programming of the GC oven was as follows: 45°C for 0.5 min followed by a temperature increase of 10°C/min up to 250°C. The temperature of the flame ionization detector was held at 310°C. Hydrogen was used as carrier gas, due to better separation efficiency and reduced analysis time compared to both helium and nitrogen. Since the injections were made by using on-column technique, no degradation of the thermally labile hydroperoxides could be detected.

Freshly prepared solutions of the enriched hydroperoxide fraction in hexane were used as reference solutions. Anhydrous ethanol (1% v/v) was added to the standard solutions in order to dissolve the polar hydroperoxides properly. The recoveries were calculated by comparing the GC-analyses of reference solutions with freshly made preparations in non-stabilized petrolatum subjected to the clean-up. Calculation was performed by using a recovery standard, acetophenone, added to both the standard solution and the preparations after clean-up.

Quantification of individual components in the patch test materials was performed by comparison to standard preparations of the compounds in nonstabilized petrolatum, which were subjected to HPLC separation immediately after preparation. Calculation was made by using the internal standard (–)-menthol, added to both standard and patch test preparations prior to clean-up.

Results

Evaluation of the HPLC method

The hydroperoxides and alcohols could quickly be separated from the more non-polar petrolatum and subsequently focused in 1 single peak by simply switching the flow direction immediately after elution of the latter compound. The HPLC chromatogram is shown in Fig. 2. Medium polar compounds, such as the *cis*- and *trans*-isomers of

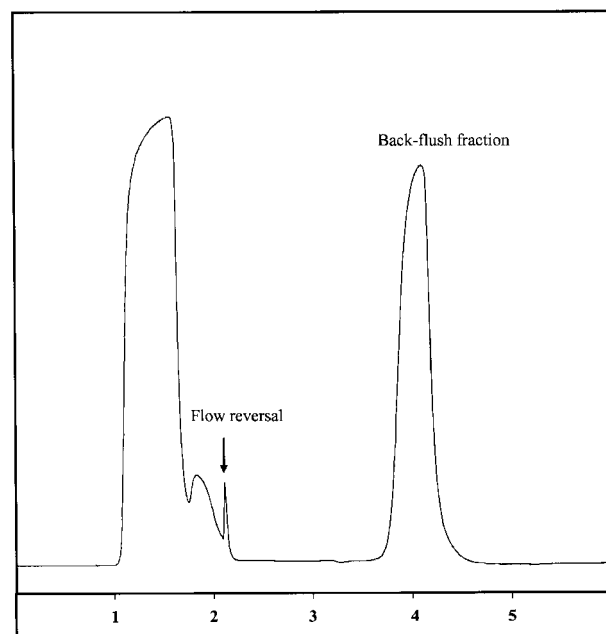


Fig. 2. HPLC-chromatogram showing the backflush separation of hydroperoxides and alcohols from white petrolatum, on a Novapak CN-HP cyanopropylmodified silica HPLC-column (Waters), 3.9×300 mm. The flow was reversed at 2.2 min as marked by the arrow. UV detection at 205 nm. Mobile phase: 0.125% of anhydrous ethanol in hexane, flow 2.0 mL/min, see Materials and Methods section.

p-mentha-8-ene-1,2-oxide, could not fully be separated from petrolatum and consequently not quantified by GC. However, the recoveries of both alcohols and hydroperoxides were above 84%, as shown in Table 1.

Analysis of the d-limonene hydroperoxides in patch test preparations

Table 2 shows the degree of degradation of the 4 main hydroperoxides under different storage conditions. For all samples, the hydroperoxides were

Table 1. Recoveries in % of some of the alcohols and hydroperoxide derivatives of *d*-limonene eluted in the back-flush fraction from the cyanopropylsilica HPLC-column; for HPLC conditions, see Materials and Methods

Compound ¹	Recovery (%)	C.V. ² n=3
<i>trans-p</i> -mentha-6,8-dien-2-ol (trans-carveol)	106	3.6
<i>cis-p</i> -mentha-6,8-dien-2-ol (cis-carveol)	98.6	5.1
<i>cis-p</i> -mentha-2,8-diene-1-hydroperoxide	98.3	4.5
<i>trans-p</i> -mentha-2,8-diene-1-hydroperoxide	102	3.6
<i>trans-p</i> -mentha-6,8-diene-2-hydroperoxide	93.2	5.3
<i>cis-p</i> -mentha-6,8-diene-2-hydroperoxide	84.6	5.5

¹ The compounds are listed in the same order as they elute on a DB-5 GC stationary phase.

² Coefficient of variation (%).

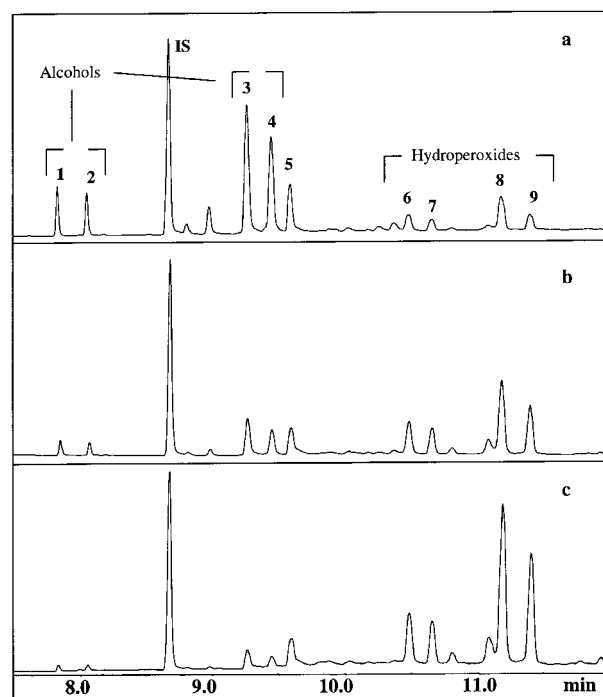


Fig. 3. GC/FID-chromatograms of decomposed hydroperoxides and corresponding alcohols in the hydroperoxide fraction. Injections were made by on-column technique. (a) hydroperoxide fraction after back-flush HPLC separation from white petrolatum stabilised with 10 ppm of α -tocopheryl acetate (b) hydroperoxide fraction after back-flush HPLC separation from non-stabilised white petrolatum and (c) a standard solution of the hydroperoxide fraction in hexane with minor amounts of corresponding alcohols and (R)-(-)-carvone. The hydroperoxide-petrolatum preparations were stored at room temperature and in daylight for 2 weeks before analysis. GC-column: DB-5. For GC conditions, see Materials and Methods. 1=*cis-p*-mentha-2,8-dien-1-ol, 2=*trans-p*-mentha-2,8-dien-1-ol, 3=*trans-p*-mentha-6,8-dien-2-ol (trans-carveol), 4=*cis-p*-mentha-6,8-dien-2-ol (cis-carveol), 5=(R)-(-)-*p*-mentha-6,8-dien-2-one ((R)-(-)-carvone), 6=*cis-p*-mentha-2,8-diene-1-hydroperoxide, 7=*trans-p*-mentha-2,8-diene-1-hydroperoxide, 8=*trans-p*-mentha-6,8-diene-2-hydroperoxide, 9=*cis-p*-mentha-6,8-diene-2-hydroperoxide. For quantification (-)-menthol, eluting at 8.7 min, was used as internal standard (IS).

degraded to a greater extent in petrolatum stabilized with α -tocopheryl acetate than in the corresponding preparations with non-stabilized petrolatum. The 2 most unstable hydroperoxides were the *trans* and *cis* isomers of *p*-mentha-6,8-diene-2-hydroperoxide. In stabilized petrolatum about 57% of the latter compound remained after 6 weeks in the refrigerator, and only 40% was left when used for patch testing during the same period of time. There was little or no significant degradation of the hydroperoxides in non-stabilized petrolatum under similar conditions. No significant difference could be seen between the non-stabilized preparations that had been used for patch testing and those stored in the refrigerator.

Table 2. Degradation of hydroperoxides of *d*-limonene in petrolatum under different conditions; the degree of degradation is presented as percentage of compound left after storage

Compound ¹	Non-stab ² patient ³ 6 weeks (n=6)	Stab ⁴ Patient 6 weeks (n=3)	Non-stab 6–8°C 6 weeks (n=3)	Stab 6–8°C 6 weeks (n=3)	Non-stab 20–22°C day-light 2 weeks (n=3)	Stab 20–22°C day-light 2 weeks (n=3)
cis- <i>p</i> -mentha-2,8-diene-1-hydroperoxide	110 (3) ⁵	78.8 (4)	105 (5)	87.2 (10)	60.4 (6)	43.1 (11)
trans- <i>p</i> -mentha-2,8-diene-1-hydroperoxide	108 (5)	70.2 (3)	102 (8)	92.3 (4)	61.1 (3)	37.5 (11)
trans- <i>p</i> -mentha-6,8-diene-2-hydroperoxide	95.4 (10)	57.8 (6)	89.6 (4)	87.9 (2)	45.2 (14)	41.2 (7)
cis- <i>p</i> -mentha-6,8-diene-2-hydroperoxide	95.8 (6)	40.2 (4)	92.0 (9)	56.7 (5)	34.4 (19)	25.4 (8)

¹ The compounds are listed in the same order as they elute on a DB-5 GC stationary phase.

² Hydroperoxides stored in non-stabilized white petrolatum.

³ Handled during patch testing and stored in refrigerator between the testings.

⁴ Hydroperoxides stored in white petrolatum stabilised with 10 ppm of α -tocopheryl acetate.

⁵ The coefficient of variation values are presented in brackets.

For all samples, degradation proceeded much faster at room temperature than in the refrigerator. The *cis* and *trans* isomers of *p*-mentha-2,8-diene-1-hydroperoxide decomposed significantly faster in the stabilized preparation compared to the non-stabilized. The GC-FID-chromatograms of the hydroperoxide fractions isolated from stabilized and non-stabilized petrolatum are shown in Fig. 3a and Fig. 3b, respectively. In Fig. 3c is shown the chromatogram of a freshly prepared standard solution of the hydroperoxide fraction, containing only minor amounts of the corresponding alcohols. In stabilized petrolatum (Fig. 3a), there was a large increase in the concentrations of these alcohols when the hydroperoxides decomposed. The *cis* and *trans* forms of *p*-mentha-6,8-diene-2-hydroperoxide were reduced to *cis* and *trans*-carveol, respectively, and also *cis*- and *trans*-*p*-mentha-2,8-diene-1-hydroperoxide were reduced to the corresponding alcohols. Only minor increases in concentrations of those compounds were found after decomposition of hydroperoxides in the non-stabilized preparation (Fig. 3b).

The stabilities of samples of pure α -tocopheryl acetate-free petrolatum, stored under similar conditions as the preparations with *d*-limonene hydroperoxides, was also examined (*Materials and Methods*). This was performed in order to be able to assess the suitability of this material to be used as a vehicle substance. No peroxides could be detected in any of the samples and thus it was concluded that no oxidation process had started during the actual time period.

Discussion

In this study, *d*-limonene hydroperoxides were shown to be rapidly reduced to the corresponding alcohols by α -tocopheryl acetate, a stabilizer commonly added to petrolatum. On the other hand,

the hydroperoxides were shown to be stable in non-stabilized petrolatum throughout clinical patch testing for a period of 6 weeks, provided that the preparations were stored in a refrigerator when not used. In the cases when the hydroperoxides were shown to decompose in non-stabilized petrolatum, i.e., at room temperature and in daylight, only a minor increase in concentration of alcohols could be detected by GC-analysis. The latter phenomenon shows that a different degradation process takes place in the absence of α -tocopheryl acetate. This could indicate formation of larger molecules by polymerization, initiated by radicals. However, such compounds could not be detected by means of the analytical methods used in this study. Such polymeric material is, to our knowledge, not possible to separate on a GC-column under normal conditions (7, 11).

The GC analyses were performed on a DB-5 column, despite the fact that not all hydroperoxides that can possibly be formed by oxidation of *d*-limonene can be separated on this type of stationary phase. For total separation of all 6 possible hydroperoxides a more polar stationary phase, such as OV-1701 (8), must be used. On the DB-5, *cis*-*p*-mentha-6,8-diene-2-hydroperoxide and *cis*-*p*-mentha-[1(7)8]-diene-2-hydroperoxide coelute. However, the latter is formed only in trace amounts during autoxidation of *d*-limonene, while about equal amounts of *p*-menth-8-ene-1,2-diol and the former hydroperoxide are formed after 3 months of airo-oxidation. This diol coelutes with *cis*-*p*-mentha-6,8-diene-2-hydroperoxide, one of the major hydroperoxides formed during autooxidation, on the OV-1701 stationary phase (7). Therefore a DB-5 column was chosen for this study.

This HPLC method is a somewhat modified version of that described in our earlier paper (7). One of our aims in the present study was to develop a simplified method, without gradient elution, to en-

able fast clean-up and isolation of mainly *d*-limonene hydroperoxides from patch test preparations. More ethanol was added to the mobile phase compared with the method described earlier, since the retention of alcohols and hydroperoxides on cyanopropyl-modified silica is rather strong. By using this technique, polar oxidation products are extracted from a nonpolar matrix, in this case petrolatum. After elution of petrolatum, both hydroperoxides and alcohols could be isolated in 1 single back-flush fraction with satisfying recoveries. If less polar compounds, such as *cis*- and *trans*-*d*-limonene-1,2-oxide, are to be isolated and separated from petrolatum using the cyanopropyl silica column, a mobile phase with a lower concentration of ethanol has to be used. After some modification of the HPLC system, the method will also be applicable to other types of patch test materials, provided that the allergens to be tested and the vehicle substance differ significantly in polarity. An obvious advantage of having a method for isolation of allergens from patch test preparations is the ability to detect possible decomposition processes during clinical use and also in experimental sensitization studies.

Although the amount of the *d*-limonene hydroperoxides is reduced in the test preparations, the amount is still high enough to give positive reactions (12). Since the test concentration is limited due to irritancy (which is not reduced by the alcohols) an increase in the test concentration of the hydroperoxides relatively to the concentration of alcohols can increase the number of positive patch test reactions. This study only concerns the *d*-limonene hydroperoxides, but it is reasonable to assume that also other types of hydroperoxides will be reduced by α -tocopheryl acetate. For instance, this should be considered for materials like colophony, in which hydroperoxides are major allergens (13, 14). Also regarding fragrances, the role of hydroperoxides and suitable patch test preparations should be taken into consideration. Based on the results found in this study, we conclude that non-stabilized petrolatum is suitable as vehicle for patch testing of hydroperoxides, provided it is handled properly. The test preparations have to be stored in the refrigerator and to be shielded from daylight when not handled for patch testing. It was also shown that non-stabilized petrolatum was stable towards autooxidation, i.e. it did not form any peroxy compounds, under the conditions used in this investigation. On the other hand, we recommend that petrolatum stabilized with α -tocopheryl acetate should not be used for patch testing of hydroperoxides and peroxides, since the stabilizer most likely will make the labile haptens decompose so that false negative-responses could be obtained.

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