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Research Article

Determination of allergenic hydroperoxides in essential oils using gas chromatography with electron ionization mass spectrometry

Fragrance monoterpenes are widely used commercially due to their pleasant scent. In previous studies, we have shown that air-exposed monoterpenes form hydroperoxides that are strong skin sensitizers. Methods for detection and quantification of the hydroperoxides in essential oils and scented products are thus desirable. Due to thermolability and low UV absorbance, this is a complicated task. We have recently developed a sensitive LC–ESI–MS method, but with limited structural information and separation efficiency for positional isomers and stereoisomers. In the present study, we investigated derivatization with a trimethyl silyl reagent and subsequent GC with electron ionization MS for the determination of monoterpene hydroperoxides. All investigated monoterpene hydroperoxides could be chromatographed as thermostable trimethyl silyl derivatives and yielded the fragment m/z 89 ($[\text{OSi}(\text{CH}_3)_3]^+$) at a higher extent compared to corresponding alcohols. Limonene-2-hydroperoxide and four other hydroperoxide isomers of limonene were separated and detected in sweet orange oil autoxidized for two months. The concentration of limonene-2-hydroperoxide isomers was found to be 19 $\mu\text{g}/\text{mg}$ in total. Also isomers of linalyl acetate hydroperoxide and linalool hydroperoxide were detected in autoxidized petitgrain oil (two months). The presented GC–MS method showed concentrations in the same order as previous LC–MS/MS analysis of the same type of oils.

Keywords: Contact allergy / Essential oils / GC–MS / Monoterpene hydroperoxides / Trimethyl silyl derivatives

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1 Introduction

Fragrances are ubiquitous in everyday consumer products such as household products and cosmetics. This has led to a high frequency of skin (contact) allergy to fragrances, as many of the fragrance compounds are reactive and allergenic or can be activated to such compounds. By estimate, 1–3% of the European population is contact allergic to fragrance ingredients [1–3]. This makes fragrances the most common cause of contact allergy in the population apart from nickel.

Repeated exposure to the specific allergen to which a person has been sensitized, creates a risk of developing chronic dermatitis.

Monoterpenes (synthesized or obtained from plants) are common fragrance compounds due to their pleasant scent. Among the most common terpenes are limonene obtained from citrus oil, linalool with lavender scent, and geraniol exhibiting rose scent. Those are all included in the EU legislation that prescribes labeling of cosmetics containing 24 fragrance compounds known to cause skin sensitization [4]. Also linalyl acetate is very common, another terpene with a lavender scent.

Limonene, linalool, and linalyl acetate are inherently low sensitizing or nonsensitizing, but are activated upon contact with air, forming potent allergenic oxidation products [5]. For geraniol not only activation outside the skin is important, but also the metabolic activation in the skin [6, 7]. The air-induced oxidation (autoxidation) is spontaneous and follows a free radical chain mechanism with initiation, propagation, and termination [5, 8]. The primary oxidation products are hydroperoxides, which with time degrade to a complex mixture of other compounds, such as aldehydes and epoxides. Antioxidants, such as 2,6-di-*t*-butyl-4-methylphenol, may prevent

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Abbreviations: BSTFA/TMCS, *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane; EI, electron ionization; FID, flame ionization detection; GerOOHs, geraniol hydroperoxides; IS, internal standard; Lim-2-OOH, limonene-2-hydroperoxide; LinOAcOOHs, linalyl acetate hydroperoxides; LinOOHs, linalool hydroperoxides; TIC, total ion chromatogram; TMS, trimethyl silyl

the autoxidation for a period of time, but as soon as the antioxidant is consumed, the protection is lost [9]. There is an uncertainty in the use of antioxidants as the protection time varies with, e.g. stability and purity of the actual compound, the storage conditions, and the formulations of the finished product. Interestingly, to achieve the optimal scent of a specific perfume, fragrance mixtures can be matured involving also autoxidation before they are available as consumer products.

The oxidation process of individual monoterpenes has been experimentally investigated [6, 10–12]. In oxidation mixtures from about 10 to 45 weeks of controlled air exposure, a significant content of hydroperoxides was detected. All isolated hydroperoxides were shown to be strong sensitizers in predictive *in vivo* testing [6, 10–12]. Clinical multicenter studies, have shown that oxidized limonene and linalool are frequent causes of contact allergy and around 60% of patients allergic to the oxidized samples reacted when tested with the hydroperoxides [13–17]. Also, more allergic reactions to autoxidized geraniol compared to pure geraniol were found in clinical studies, but the pattern for this compound is more complex compared to limonene and linalool as a metabolic activation also is observed [7, 18, 19].

Due to the sensitizing potency of fragrance hydroperoxides, studies on their occurrence in essential oils or scented consumer products are needed. At present, there is no established method for their determination. Parent fragrance terpenes are usually determined by GC with electron ionization (EI) MS or GC with flame ionization detection (FID). However, such methods are not applicable to the fragrance hydroperoxides as all of them so far tested degrade at the temperatures applied in GC, also by on-column injection. The use of LC with UV detection is limited since fragrance hydroperoxides generally lack useful chromophores. We have in a previous study [20] compared different LC–MS ionization techniques for limonene-2-hydroperoxide (Lim-2-OOH) and linalool hydroperoxides (LinOOHs). The results showed that either ESI or atmospheric pressure chemical ionization is to be preferred over atmospheric pressure photoionization for detection of the monoterpene hydroperoxides [20]. Recently, we have developed an LC–ESI-MS/MS method for quantification of hydroperoxides from limonene, linalool, and linalyl acetate, with an overall method LOD < 0.3 µg/mL [21]. Although the detectability is sufficient for many applications, LC–MS/MS has the drawback of yielding spectra with a minimum of structural information for monoterpene hydroperoxides and, furthermore, the responses vary substantially between different compounds and even between isomers. Without reference compounds, hydroperoxides cannot be identified with this method. As a complementary method to LC–MS/MS for a priori unknown hydroperoxides, a more universal and structurally informative method such as GC–EI-MS could be useful, despite an expected decreased detectability. However, GC–MS analysis requires derivatization of the hydroperoxides in order to increase their thermostability. Due to the variety of applications and matrices involving fragrances, complementary an-

alytical methods are valuable for monoterpene hydroperoxide detection.

In the present study, *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane 99:1 (BSTFA/TMCS) was used for derivatization of the hydroperoxy function. This reagent is well known to produce stable derivatives with excellent chromatographic behavior from compounds such as alcohols, replacing active hydrogens with a trimethyl silyl (TMS) group ($-\text{Si}(\text{CH}_3)_3$). Docherty et al. [22] showed that cumene hydroperoxide could be derivatized by *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) for further GC–MS analysis. However, to our knowledge this is the first time GC–MS analysis is reported for the determination of hydroperoxides in essential oils. The main purpose of the present study was to investigate thermal stability, chromatographic behavior, and spectral properties of TMS derivatives of hydroperoxides. The method was applied on two different air-exposed essential oils known to contain monoterpene hydroperoxides [21].

2 Materials and methods

2.1 Samples and chemicals

Lim-2-OOH **1** (6-hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene, stereoisomers), Lin-7-OOH **2** (7-hydroperoxy-3,7-dimethylocta-1,5-dien-3-ol), Lin-6-OOH **3** (6-hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol), linalyl acetate hydroperoxides LinOAc-7-OOH **4** (7-hydroperoxy-3,7-dimethylocta-1,5-dien-3-yl acetate) and LinOAc-6-OOH **5** (6-Hydroperoxy-3,7-dimethylocta-1,7-dien-3-yl acetate), and geraniol hydroperoxides Ger-7-OOH **6** ((2E,5E)-7-hydroperoxy-3,7-dimethylocta-2,5-dien-1-ol) and Ger-6-OOH **7** ((E)-6-hydroperoxy-3,7-dimethylocta-2,7-dien-1-ol) were synthesized as described in the literature [6, 8, 10, 12]. The structures of the compounds studied are shown in Fig. 1. The hydroperoxides were investigated as mixtures of isomers as in the earlier publication [21], where the quantification and identification are based on the most prominent isomers formed from the autoxidation according to previous studies [6, 8, 10, 12, 23]. The positional isomers of LinOOHs were subjected to preparative HPLC to isolate the two isomers using manual collection. The isolated fractions were concentrated under reduced pressure, and identified by NMR spectroscopy as Lin-7-OOH (**2**) and Lin-6-OOH (**3**). ^1H and ^{13}C data are in agreement with the literature [10], with additional peaks in the ^1H spectrum corresponding to the OOH function at $\delta = 8.5$ ppm for Lin-7-OOH and at $\delta = 8.9$ and 9.0 ppm for Lin-6-OOH. The purity of each hydroperoxide (nonderivatized) was determined to >96% (according to NMR spectroscopy).

The derivatization reagent BSTFA/TMCS (99:1) was purchased from Supelco (Bellefonte, PA, USA). 1,2,3,5-Tetramethylbenzene (purity 90%) obtained from TCI Europe (Zwijndrecht, Belgium) was added as volumetric internal standard (IS) to all standard solutions and samples prior

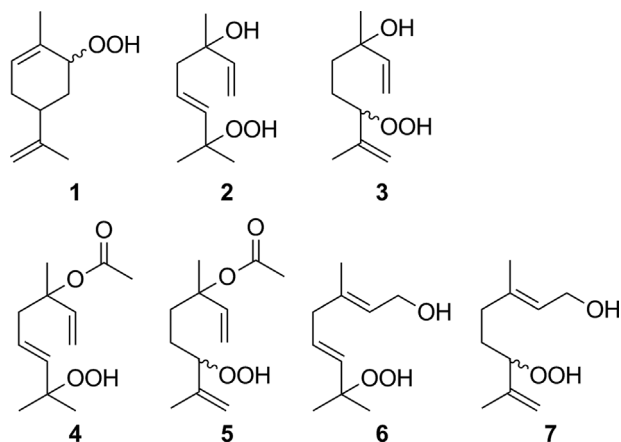


Figure 1. Structures of the hydroperoxides investigated. Lim-2-OOH **1**, Lin-7-OOH **2**, Lin-6-OOH **3**, LinOAc-7-OOH **4**, LinOAc-6-OOH **5**, Ger-7-OOH **6**, Ger-6-OOH **7**. All hydroperoxides were determined as mono-TMS derivatives (at the hydroperoxide function), except for GerOOHs, which formed di-TMS derivatives (derivatized at both alcohol and hydroperoxide function).

to GC–MS analysis. The IS had a purity of 90%, with the remaining 10% corresponding to a positional isomer. This isomer was not identified, but can be observed in the GC–MS chromatogram as a small vicinal peak, marked by an asterisk in Fig. 2. Solvents used were provided from commercial suppliers of Chromasolv quality unless otherwise indicated.

Sweet orange oil (containing limonene) and petitgrain oil (containing linalool, linalyl acetate, and geraniol) were obtained from Kurt Kitzing (Wallerstein, Germany) and used

without further purification. According to ISO standards, the concentration of limonene in sweet orange oil should be 93–96% [24], while the concentration of linalool, linalyl acetate, and geraniol in petitgrain oil should be 15–30%, 40–60%, and 2–4.5% [25], respectively, from GC–FID analyses.

Analysis of the essential oils per se was performed with GC–FID as previously described [21]. The results agreed with the ISO standards as sweet orange oil contained 96% limonene, whereas petitgrain oil contained 16% linalool, 54% linalyl acetate, and 2.6% geraniol.

2.2 Instrumentation and analysis

For GC–MS analyses, a Hewlett-Packard model 6890 GC (Agilent Technologies, Waldbronn, Germany) connected to a Hewlett-Packard model 5973 quadrupole mass selective detector (Agilent Technologies) was used. The GC was equipped with a cool on-column injector, a fused-silica retention gap (SGE, 1 m × 0.53 mm id, deactivated), and a fused-silica column (HP5-MSi, 30 m × 0.25 mm id, 0.25 μm film thickness). The mass selective detector was used in EI mode at 70 eV, scanning and monitoring ions within the m/z range of 50–500 with 3.22 scans/s. Hydroperoxides were localized by extracting the ion trace m/z 89 using the software program (Chemstation, Version D.02.00.275, Agilent Technologies). The GC temperature program started at 90°C for 5 min, followed by a temperature gradient of 10°C/min to 250°C. The final temperature was held for 2 min. The injection volume was 1 μL for samples and standard solutions, respectively.

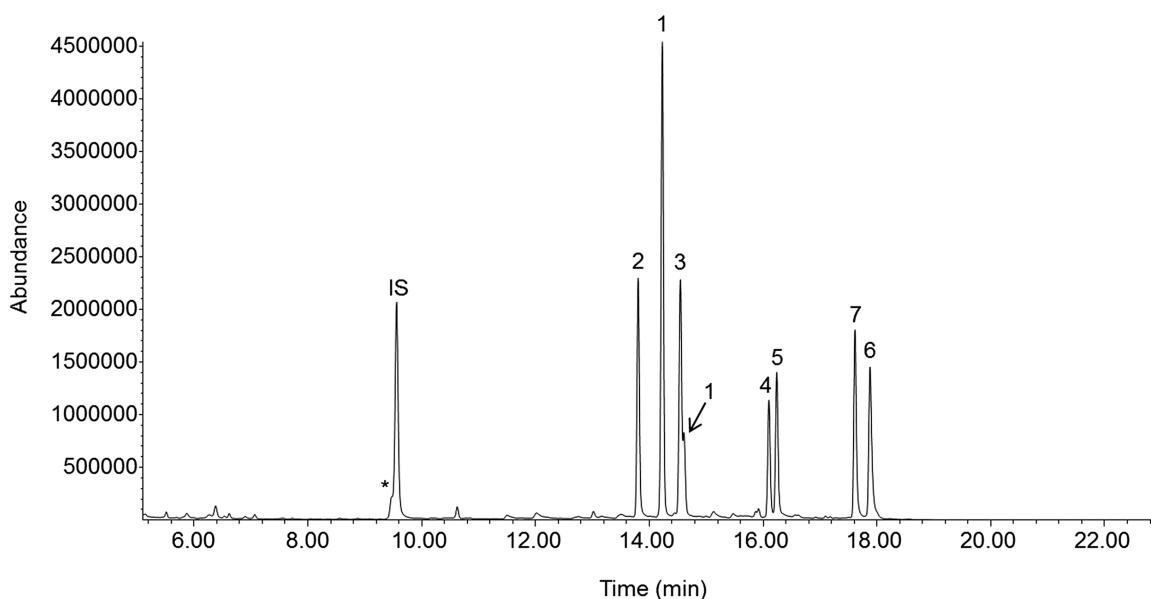


Figure 2. GC–MS chromatogram of TMS derivatives of standard fragrance hydroperoxides. Labeled peaks indicate mono-TMS derivatives of Lim-2-OOH (**1**, 14.2 and 14.6 min), Lin-7-OOH (**2**, 13.8 min), Lin-6-OOH (**3**, 14.5 min), LinOAc-7-OOH (**4**, 16.1 min), and LinOAc-6-OOH (**5**, 16.2 min). Ger-7-OOH (**6**, 17.9 min) and Ger-6-OOH (**7**, 17.6 min) were determined as di-TMS derivatives. The IS (1,2,3,5-tetramethylbenzene, 9.6 min) contained an isomer, corresponding to the peak marked with *. Retention times are given in brackets.

Preparative HPLC was performed with a Gilson HPLC pump (model 305) and Gilson UV/vis detector (model 119) using a ZORBAX RX-SIL column (250 mm × 21.2 mm id, particle size 7 µm, Agilent Technologies). Hexane with 35% *tert*-butyl methyl ether was used as eluent, and the flow rate was 21 mL/min. The UV/Vis detector was operated at a wavelength of 205 nm. ^1H and ^{13}C NMR spectra were recorded on a JEOL Eclipse+ 400 MHz spectrometer (Jeol, Tokyo, Japan) at 400 and 100 MHz, respectively, using CDCl_3 as solvent. Chemical shifts (δ) are reported in ppm relative to CHCl_3 at $\delta = 7.25$ and 77.0 ppm for ^1H and ^{13}C , respectively.

2.3 Preparation of standard solutions and derivatization procedure

Individual stock solutions of each hydroperoxide (nonderivatized) at 3.1 mg/mL were prepared in toluene and stored at -18°C . From these solutions, calibration mixtures were prepared by further dilution in toluene to desired working concentrations prior to derivatization and analysis. From each calibration mixture, an aliquot of 0.8 mL was transferred to test tubes, to which 0.1 mL of IS solution and 0.1 mL of the derivatization reagent BSTFA/TMCS was added. The molar ratio of reagent/hydroperoxide was >100 . The volumetric IS was not derivatized and was used solely to determine the relative responses in the GC–MS analysis. The final individual concentrations of the hydroperoxides prior to derivatization were: 500, 250, 100, 75, 50, 25, and 15 µg/mL, respectively. Each solution contained 57 µg/mL of IS. A blank solution was prepared similarly, except that the reference solution was replaced with 0.8 mL of toluene. The derivatized hydroperoxide solutions were found to be stable for at least one week in the refrigerator after the completion of reaction. Longer periods of time were not tested.

The derivatization yield was tested up to 36 h at room temperature and then up to one week at $+4^\circ\text{C}$ for all investigated compounds, alternatively up to 7 h at 60°C . At room temperature, aliquots were taken every 60 min for 12 h and then after 24 and 36 h. At 60°C , analyses were performed after 30 and 60 min, and then every 60 min up to 7 h.

2.4 Air exposure procedure

Samples of the essential oils, sweet orange oil and petitgrain oil (approximately 70 mL of each sample), were air-exposed in Erlenmeyer flasks (100 mL, Schott Duran, Wertheim/Main, Germany) at room temperature and under a daylight lamp (Philips Master TL-D 90 De Luxe, 18W/950, Eindhoven, The Netherlands) lit for 12 h a day. The necks of the flasks were covered with aluminum foil to prevent contamination. The oxidation mixtures were stirred four times daily for 1 h, as previously described [12]. Minor samples (approximately 1 mL) were taken after two months of autoxidation and stored under argon at -70°C for 12 months and at -20°C for 12 months prior to derivatization and analysis with GC–MS for quantification of the specific hydroperoxides.

2.5 Analysis of essential oils

The content of Lim-2-OOH in air-exposed sweet orange oil (containing limonene) and LinOOHs, LinOAcOOHs, and GerOOHs in air-exposed petitgrain oil (containing linalool, linalyl acetate, and geraniol) was quantified by the standard addition method, spiking each oil prior to derivatization with 100 µL of hydroperoxide reference solutions at six concentration levels. The concentration of each oil was approximately 700 µg/mL after addition of volumetric IS, reference hydroperoxides, and derivatization reagent. Data acquisition was made in a full scan mode. To easily localize the hydroperoxides, the ion trace m/z 89 was then extracted from the acquired data using the computer program (Chemstation, Version D.02.00.275, Agilent Technologies). The same response factor in full scan was assumed for stereoisomers of Lim-2-OOH and also for the positional isomers of LinOOHs, LinOAcOOHs, and GerOOHs, respectively. Equal amounts of IS (20 µg/mL) were added to all essential oil samples and the final added concentrations of each nonderivatized hydroperoxide (sum of isomers) were 25, 20, 15, 10, 5, and 2.5 µg/mL. LOD was determined as three times the SD at zero concentration using the standard addition calibration curve, which was linear within the investigated concentration range. Statistical evaluation was performed using the computer program Winstat (version 3.24, Anders Colmsjö, copyright 1996, Analytical Chemistry, Stockholm University).

3 Results and discussion

TMS derivatives of highly fragile fragrance hydroperoxides were found to be thermostable in GC–MS analysis when using an on-column injector. All investigated compounds were derivatized and the positional isomers could be chromatographically separated, which is an improvement compared to our previously published LC–MS/MS method. The robustness and simplicity using GC was also an improvement.

3.1 Derivatization and chromatography

For all investigated hydroperoxides, derivatization at room temperature was found to be completed after 24 h of reaction. The reaction was considered complete when the GC–MS responses for the TMS derivatives no longer increased or changed and no peaks corresponding to unreacted hydroperoxides were observed in the GC–MS chromatogram. A total ion chromatogram (TIC) from GC–MS of the derivatized fragrance hydroperoxides is shown in Fig. 2. The conversion to TMS derivatives, according to GC–MS of standard and blank solutions, was $>95\%$ for all hydroperoxides ($<5\%$ of the total peak area corresponded to other peaks than the hydroperoxide TMS derivatives). For comparison, a chromatogram of unreacted limonene hydroperoxides is shown in the Supporting Information. The nonderivatized hydroperoxides were almost entirely degraded during the analysis. In the case of Lim

-2-OOH, the major thermal degradation products are carveol and carveone.

The repeatability (using triplicates) showed RSDs at or <4%. The stability of formed derivatives was tested by analyzing samples after storage for one week at +4°C. The mean responses of the stored samples (RSDs < 6%) and those freshly derivatized were not significantly different. To investigate if the entire method, including both derivatization and GC–MS analysis, could be performed within one day, the reactions were also performed at 60°C for 4 h. However, the responses in this case showed a larger variation, probably due to degradation at the higher temperature. Thus, we continued with derivatization at room temperature.

LinOOHs, LinOAcOOHs, and GerOOHs were determined as mixtures of positional isomers (one tertiary and the diastereoisomers of a secondary hydroperoxide (OOH)) in ratios of 5:3 (tertiary OOH/secondary OOH), respectively (Fig. 1), according to NMR spectroscopy. The positional isomers of nonderivatized LinOOHs were separated and isolated by preparative HPLC, which were confirmed by NMR spectroscopy. TMS derivatives of Lin-7-OOH (**2**) and Lin-6-OOH (**3**) were shown to elute at 13.8 and 14.5 min, respectively, on the HP-5 GC column (Fig. 2). The derivative of the secondary hydroperoxide (**3**) showed a more intense peak of m/z 89, as discussed below. Also for derivatized LinOAcOOHs and GerOOHs, one of the corresponding peaks showed a higher response for the m/z 89 ion. Due to a lack of individual reference compounds, we cannot distinguish between the derivatized positional isomers of either LinOAcOOHs nor GerOOHs. However, based on the result from analysis of derivatized Lin-7-OOH (**2**) and Lin-6-OOH (**3**), we propose that the derivatives of LinOAc-7-OOH (**4**) and LinOAc-6-OOH (**5**) eluted at 16.1 and 16.2 min, respectively, and the derivatives of Ger-6-OOH (**7**) and Ger-7-OOH (**6**) eluted at 17.6 and 17.9 min, respectively. GerOOHs became disilylated, i.e. both the hydroxyl group and hydroperoxide group were silylated within the reaction time used, while all other investigated hydroperoxides became monosilylated. The TMS derivatives of the stereoisomers (*cis* and *trans*) of Lim-2-OOH (**1**) were chromatographically separated, but one of them coeluted partly with derivatized Lin-6-OOH (**3**) in a standard mixture of all hydroperoxides.

In general, tertiary alcohols show a slower reaction rate than secondary or primary alcohols, and require longer time to form TMS ethers, which is explained by steric hindrance. Linalool with a tertiary alcohol function generated only minute amounts of the silylated derivative within 24 h, while the primary alcohol geraniol reacted already after 1 h (data not shown). The same tendency was observed for the tertiary hydroperoxides, which showed a slower reaction rate than the secondary analogues. However, tertiary hydroperoxides were found to react much faster than corresponding alcohols and were fully derivatized under the applied experimental conditions. The chromatographic separation of hydroperoxide isomers is an improvement compared with the previously published LC–MS/MS method [21].

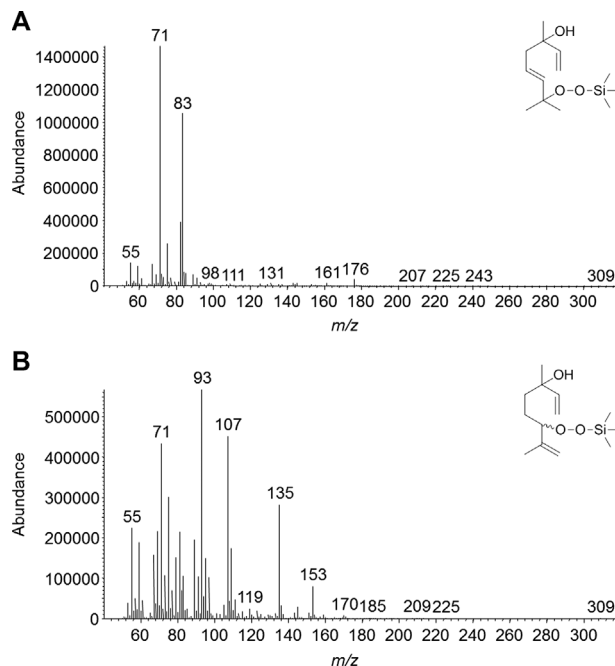


Figure 3. Mass spectrum of the mono-TMS derivative of (A) the tertiary hydroperoxide Lin-7-OOH **2** and (B) the secondary hydroperoxide Lin-6-OOH **3**, showing different fragmentation patterns.

3.2 Mass spectra of TMS derivatives of monoterpene hydroperoxides

The BSTFA/TMCS reagent replaces active hydrogens in, e.g. alcohols with a TMS group, resulting in a mass fragment in EI of m/z 73, corresponding to $[\text{Si}(\text{CH}_3)_3]^+$. This fragment was prominent also for all of the investigated silylated hydroperoxides. Besides m/z 73, a fragment at m/z 89 corresponding to $[\text{OSi}(\text{CH}_3)_3]^+$ was common for all of the TMS peroxides and occurred at a higher intensity than for the corresponding alcohols, although at an intensity of only 1–5% of the TIC. In the chromatograms of essential oils, this fragment could be used to localize the hydroperoxides.

Derivatized secondary hydroperoxides showed a higher intensity of the m/z 89 ion compared to tertiary hydroperoxides. For the latter, the loss of a TMS peroxy radical (–105 Da) is more favored due to formation of a stable tertiary carbocation. TMS derivatives of the monoterpene hydroperoxides exhibited some common fragmentation pathways in EI, such as loss of the TMS peroxy radical (–105 Da) and the m/z 89 fragment. However, the relative intensities of the ions vary significantly. As examples, the spectra of the derivatized LinOOHs are shown in Fig. 3. The Lin-6-OOH (**3**) derivative shows an intense m/z 93, while m/z 71 and 83 are ions common for both isomers. A few fragmentation pathways are proposed in Fig. 4 for the LinOOHs derivatives. Although most of the molecular ions can be expected to be ionized at the silicon (ionization potential 8.1 versus 13.6 eV for oxygen), fragmentation also occurs from molecular ions

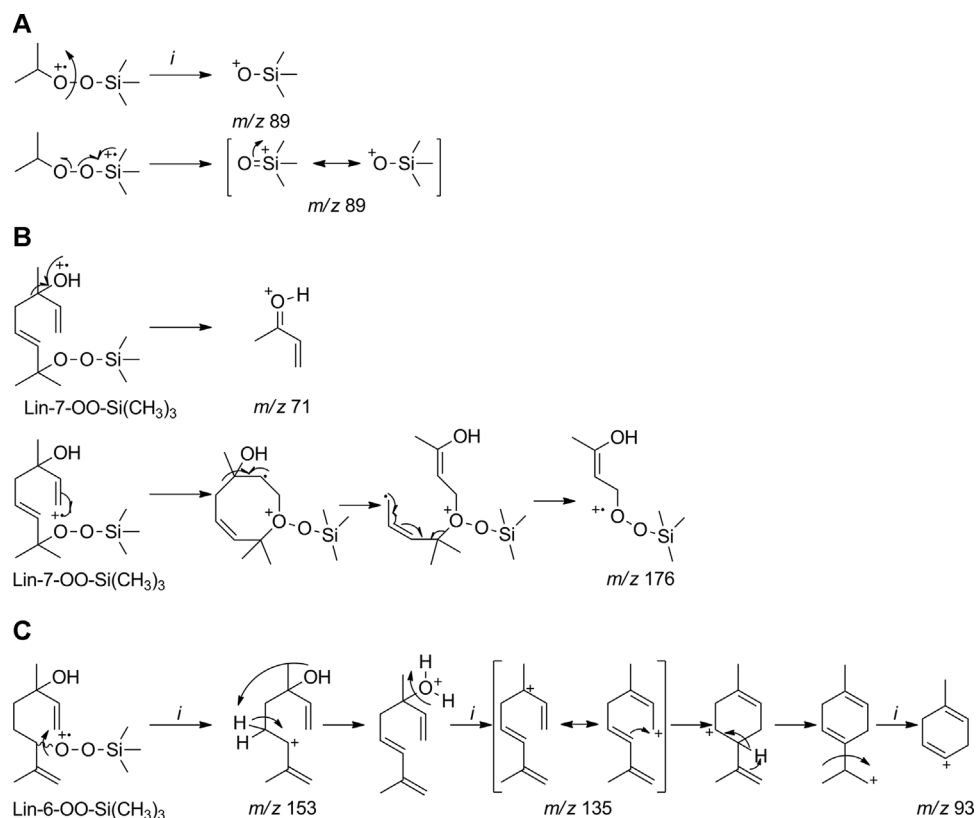


Figure 4. (A) General mechanism for formation of fragment m/z 89 from silyl peroxides and some proposed fragmentation pathways for the mono-TMS derivative of (B) the tertiary hydroperoxide Lin-7-OOH **2**, and (C) the secondary hydroperoxide Lin-6-OOH **3**.

with ionized oxygen. Even if there are similarities between isomers in their GC–EI–MS spectra, the fragmentations in EI yield more specific ions compared with those obtained in LC–MS/MS by collision-induced dissociation. The latter predominantly exhibits water losses from the hydroperoxide ions [21].

3.3 Hydroperoxides in essential oils

Hydroperoxides were identified in both of the air-exposed essential oils investigated, with *cis* and *trans* isomers of Lim-2-OOH (**1**) occurring in sweet orange oil at a concentration of 19 $\mu\text{g}/\text{mg}$ (sum of isomers). In petitgrain oil, LinOAcOOHs (sum of positional isomers) were detected just below the method LOQ (13 $\mu\text{g}/\text{mg}$, $S/N = 10$) and LinOOHs just above the method LOD (3.3 $\mu\text{g}/\text{mg}$, $S/N = 3$). However, GerOOHs could not be detected due to a low initial level of geraniol. The levels of the detected hydroperoxides in the autoxidized essential oils are in the same order as those previously found from LC–MS/MS analyses [21], although there are some discrepancies that most likely can be referred to differences in storage conditions (GerOOHs were not investigated in the previous study). The results from both methods are compared in Table 1. The LOD, as estimated from the standard addition calibration curve, was found to be <3.9 $\mu\text{g}/\text{mL}$ for all investigated hydroperoxides (sum of isomers). Considering that 1 mg of the essential oil was dissolved in 1 mL, this corresponds to a method LOD of <3.9 $\mu\text{g}/\text{mg}$ or 0.4% (w/w). The identi-

fication and quantification is based on the specific isomers most prominent in the oxidation mixtures of the parent compounds according to previous investigations [6, 8, 10, 12, 23]. The determined hydroperoxides are the most stable ones, while the less stable (e.g., primary hydroperoxides) could be formed at lower levels, but could not be identified due to lack of reference compounds. It was beyond the scope of this work to determine all possible isomers, although some of them are tentatively identified.

GC–MS chromatograms from the derivatized essential oils, air-exposed for two months, are shown in Fig. 5A and B. The ion trace of m/z 89 suggests that four additional hydroperoxides are formed in sweet orange oil during autoxidation. This is in accordance with our previous findings [11, 23], where stereoisomers of limonene-1-hydroperoxide at equal amounts and stereoisomers of *p*-mentha-[1(7),8]-diene-2-hydroperoxide at lower amounts were shown to be formed besides Lim-2-OOH isomers in oxidized (*R*)-limonene. Altogether this means that the total concentration of hydroperoxides is considerably >19 $\mu\text{g}/\text{mg}$, the level of Lim-2-OOH solely.

In our earlier work on hydroperoxide determination by LC–MS/MS [21], abundance ratios of fragment ions were used as additional identification points besides retention time and m/z of the fragments. The immanent extensive fragmentation in GC–EI–MS to a large number of low mass ions, on the other hand, makes selective scanning techniques, such as selected-ion monitoring or selected-reaction monitoring, less usable. Instead, entire reference spectra from full-scan

Table 1. Comparison of the concentrations found in this study with levels from the previously published LC–MS/MS method [21]

Sample	Compound	GC–MS ($\mu\text{g}/\text{mg}$)	LC–MS/MS ($\mu\text{g}/\text{mg}$)
Sweet orange oil	Lim-2-OOH 1 (isomers)	19	49
Petitgrain oil	LinOOH 2 and 3	D ^{a)}	3.7
Petitgrain oil	LinOAcOOH 4 and 5	D ^{b)}	5.5
Petitgrain oil	GerOOH 6 and 7	ND	NA

The samples had been stored differently before analysis, which probably explains the differences.

D, detected; ND, not detected; NA, not analyzed.

a) A total of 4.8 $\mu\text{g}/\text{mg}$, below LOQ (11 $\mu\text{g}/\text{mg}$ at S/N = 10).

b) A total of 12 $\mu\text{g}/\text{mg}$, below LOQ (13 $\mu\text{g}/\text{mg}$ at S/N = 10).

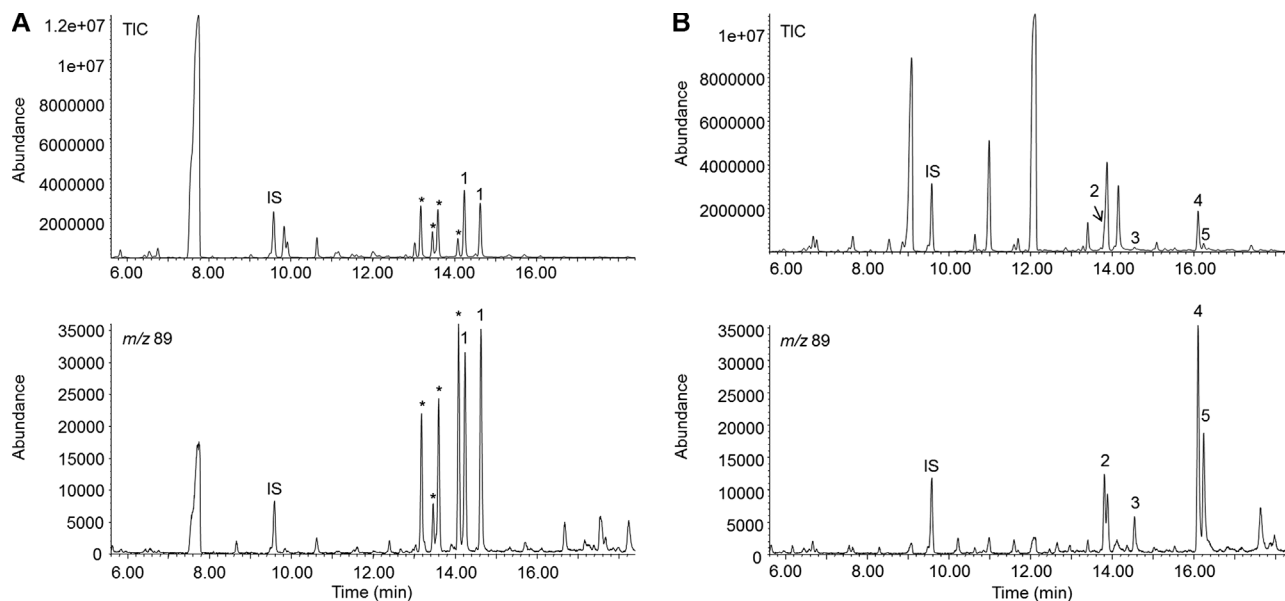


Figure 5. TIC and ion trace of m/z 89 for a derivatized sample of (A) autoxidized sweet orange oil and (B) autoxidized petitgrain oil. Labeled peaks indicate mono-TMS derivatives of Lim-2-OOH **1** in (A), and Lin-7-OOH **2**, Lin-6-OOH **3**, LinOAc-7-OOH **4** and LinOAc-6-OOH **5** in (B). Peaks labeled with * in (A) indicate TMS derivatives of additional limonene hydroperoxide isomers.

acquisition of standard compounds should be used to yield the specificity required for identification.

4 Concluding remarks

The presented method is an additional contribution to the method toolbox for the determination of thermolabile fragrance hydroperoxides. The derivatization method is not novel per se, but as far as we know it has not previously been applied to this important group of compounds. Analytical methods are required to enable an investigation of the exposure to these allergens among the population. However, our methods have so far been limited to essential oils and have to be tested and evaluated also for common fragranced commercial products. For complex products, such as creams and skin lotions, it can be expected that an extensive clean-up from the matrix will be needed. In that case, for an appropriate quantification and to compensate

for losses in the analytical chain, the use of isotope-labeled hydroperoxides would be highly desirable.

Dependent on application or the detection limit needed, either LC–MS or GC–MS is preferred for the separation and detection of monoterpene hydroperoxides. Complementary methods are valuable as such, since there is a wide variety of applications and matrices involving fragrances. GC–MS following derivatization yields a decreased detectability compared to that of our previously published LC/MS method. On the other hand, an advantage with GC–MS is a more universal detection method with less variability in responses and more informative and specific spectra. The higher peak capacity and possibility of separating the different positional isomers of the fragrance hydroperoxides are also beneficial.

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Determination of Allergenic Hydroperoxides in Essential Oils using GC/Electron Ionization-MS

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Supporting information

Figure 1: Chromatogram of underivatized Lim-2-OOH.

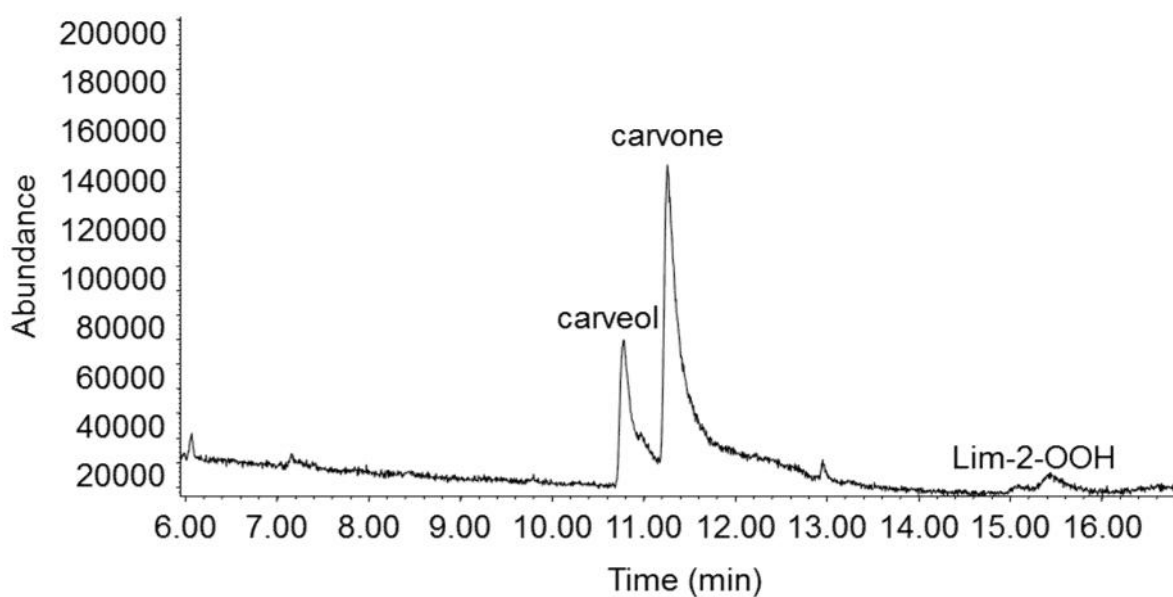


Figure 1. Chromatogram of underivatised limonene-2-hydroperoxide (Lim-2-OOH). Carveol and carvone are thermal degradation products formed during the GC analysis.