

A sensitive method for determination of allergenic fragrance terpene hydroperoxides using liquid chromatography coupled with tandem mass spectrometry

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Abbreviations: **IS**, internal standard; **LimOOH**, limonene-2-hydroperoxide; **LinOAcOOH**, linalyl acetate hydroperoxides; **LinOOH**, linalool hydroperoxides; **MRM**, multiple-reaction monitoring.

Keywords: Autoxidation, Contact allergy, Fragrance terpenes, Hydroperoxides, Liquid chromatography – tandem mass spectrometry

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Abstract

Different compositions of monoterpenes are utilized for their pleasant scent in cosmetics and perfumes. However, the most commonly used fragrance terpenes easily oxidize upon contact with air, forming strongly skin-sensitizing hydroperoxides. Due to their thermolability and low UV absorbance, detection methods for hydroperoxides are scarce. For the first time, a simple and sensitive method using liquid chromatography/electrospray ionization-tandem mass spectrometry was developed to quantitatively determine hydroperoxides from the common fragrance compounds linalool, linalyl acetate, and limonene. The method was applied to autoxidized petitgrain oil and sweet orange oil. A separation was accomplished using a C₃ column. The method limit of detection for the investigated hydroperoxides in the essential oils was below 0.3 µg/mL, corresponding to 0.3 ppm.

For prevention purposes and according to EU regulations, concentrations in cosmetics exceeding 100 ppm in "rinse-off" and 10 ppm in "stay-on" products of linalool and limonene must be labeled. However, the products may still contain allergens, such as hydroperoxides, formed by oxidative degradation of their parent terpenes. The sensitivity and selectivity of the presented LC/MS/MS method enables detection of hydroperoxides from the fragrance terpenes linalool, linalyl acetate, and limonene. However, for routine measurements, the method requires further validation.

1 Introduction

Fragrances are, after nickel, the most common cause of contact allergy. It is estimated that the frequency of contact allergy to fragrance ingredients in the general population in Europe is 1-3% [1-3]. Contact allergy is a life-long altered specific reactivity in the immune system which can develop following skin contact with a sufficient amount of allergenic compounds. Allergic contact dermatitis develops upon re-exposure to the specific allergen(s) which, in the case of fragrance allergy, is due to contact with every-day products such as cosmetics and household products. Eczema from fragrance compounds typically involves the face, the armpits, and the hands [4, 5]. Patients appear to become sensitized to fragrances primarily from deodorants and perfumes and to a lesser extent from other cosmetic types [6].

In order to reduce the risk of skin sensitization and eczema in the population, the EU legislation lists 26 fragrance compounds for which information should be provided to consumers about their presence in cosmetic products. For products that are designed to stay on the skin for a prolonged time and not to be rinsed off, the limit for labelling is set to 0.001% or 10 ppm (e.g. lotions, deodorants). For those compounds that are rinsed off directly after application, the limit is 0.01% or 100 ppm (e.g. shampoos). The 26 fragrance compounds were introduced into Annex III of the Cosmetics Directive by the 7th amendment (2003/15/EC) on the basis of the opinion of The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) from 1999 [7].

It is important that analytical methods sensitive enough for quantification of the allergenic fragrance ingredients are available to control their presence in complex mixtures. Such methods are in progress or have been developed following the EU legislation [8, 9].

Volatile monoterpenes of natural origin, either synthesized or obtained from plants, are the most commonly used fragrance ingredients due to their pleasant scent. They are also among the allergenic fragrance compounds listed in the legislation. In our research on contact allergy

to terpenes, we have observed that the most commonly used fragrance ingredients, i.e. linalool (synthesized or obtained from essential oils) and limonene (obtained from citrus oils), are inherently non-sensitizing or very weakly allergenic, but are activated upon contact with air, forming highly allergenic oxidation products [10 and refs therein]. Such activation processes are of concern as they increase the risk of skin sensitization. The clinical relevance of the increased risk of contact allergy by air oxidation has been shown in large international multicentre studies on oxidized linalool and limonene [11-15]. A high percentage of eczema patients was diagnosed as allergic to the oxidized fragrance terpenes and a clear connection was seen to allergy to other fragrance ingredients and products [11, 13-15]. Essential oils that are natural mixtures of monoterpenes are used as such in e.g. aroma therapy and as fragrance ingredients in perfumed products in parallel to synthesized compounds. It has been shown that air oxidation (autoxidation) of the fragrance terpenes present in the essential oils occurs in the same way as for the corresponding isolated compounds [16, 17].

Activation by autoxidation can be prevented by appropriate measures at production, storage, and handling. To facilitate prevention, it must be possible to determine the presence of the allergenic oxidation products. Measurements of only the parent compounds can be misleading from an allergenic point of view as they may be degraded to more allergenic oxidation products, such as hydroperoxides which are not detected. Autoxidation is a free radical chain reaction in which hydrogen atom abstraction in combination with addition of oxygen forms peroxy radicals. The reaction shows some selectivity for positions where stable radicals can be formed. So far, all fragrance compounds that have been investigated with regard to the influence of autoxidation on the allergenic potential, including identification of formed oxidation products, have oxidizable allylic positions that are able to form hydroperoxides and/or hydrogen peroxide as primary oxidation products upon air exposure. The hydroperoxides have been identified as major allergens in the oxidation mixtures while

hydrogen peroxide is non-sensitizing [10]. Secondary oxidation products such as aldehydes and epoxides can also be allergenic, thereby further increasing the sensitization potency of the autoxidation mixture [10].

The possibility to detect fragrance hydroperoxides is thus highly desirable due to their strong sensitization potency and extensive occurrence. At present, no established methods are available due to lack of detection methods. In contrast to the parent monoterpenes, which are often determined by GC/MS or GC/FID, the hydroperoxides degrade easily at increased temperatures. Also, LC with UV detection is of limited usability as the terpene hydroperoxides in general lack chromophores. In a previous study, we have shown that either ESI or APCI are the preferred ionization techniques for detection of monoterpene hydroperoxides [18]. In literature, hydroperoxides from squalenes [19, 20] and phospholipids [21, 22] have been identified by APCI or ESI-MS. Recently, Reinnig *et al.* [23] characterized terpenoid hydroperoxy acids after ozonolysis using APCI coupled with ion trap mass spectrometry.

In the present work, we present the development of an LC/ESI-MS method for determination of the major allergenic hydroperoxides that are formed from the commonly used fragrance terpenes linalool, linalyl acetate, and limonene. Two essential oils with known content of relevant parent fragrance terpenes were used to evaluate the method.

2 Materials and methods

2.1 Chemicals

Methanol of LC/MS grade and formic acid of MS grade were obtained from Fluka (Sigma Aldrich, Steinheim, Germany). Isopropanol of LC/MS grade was purchased from Riedel-de Haën (Sigma Aldrich, Seelze, Germany). Water was purified with a Purelab ultra (Genetic) system from Elga Labwater (High Wycombe, UK).

The investigated compounds are shown in Table 1. Linalool and linalyl acetate were purchased from Sigma Aldrich (Steinheim, Germany). Limonene was obtained from Millenium Chemicals, Inc. (Jacksonville, Florida, USA). The monoterpenes were purified by distillation ($\geq 97\%$ according to GC/MS) before use. The hydroperoxides were synthesized and purified according to earlier experience [17, 24, 25]. Identity and purity of the analytes were examined with NMR ($> 96\%$ purity), and the data agreed with published reports. Linalool hydroperoxides (LinOOH, **1**) and linalyl acetate hydroperoxides (LinOAcOOH, **2**) were investigated as mixtures of isomers. The quantification and identification of hydroperoxides are based on the most prominent isomers formed from the autoxidation according to previous studies [17, 24-26]. As shown from the autoxidation of linalool and linalyl acetate [17, 24], the tertiary and secondary hydroperoxides are the most prominent isomers in the oxidation mixtures. The secondary limonene-2-hydroperoxide (LimOOH, **3**, Table 1) is present in the highest amount in the oxidation mixture compared to other isomers of hydroperoxides from limonene [26]. The secondary LimOOH (**3**, Table 1) results in hapten-protein complexes *via* a combination of both radical and classical electrophilic pathways, while the tertiary limonene-1-hydroperoxide only results in hapten-protein complexes *via* radical formation [25]. For linalool and linalyl acetate we have used a mixture as both the tertiary and secondary hydroperoxides have been shown to be important in the oxidation mixtures [17, 24]. The internal standard (IS), [1,1'-biphenyl]-2,2'-diol (2,2'-biphenol), was obtained from Fluka (Buchs, Switzerland). The essential oils, petitgrain oil (containing linalool and linalyl acetate) and sweet orange oil (containing limonene), were obtained from Kurt Kitzing GmbH (Wallerstein, Germany).

2.2 NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on a JEOL Eclipse+ 400 MHz spectrometer at 400 and 100 MHz, respectively, using CDCl_3 as solvent. ^1H and ^{13}C NMR data of LinOOH **1**, and LinOAcOOH **2** (both in a 5:3 ratio of tertiary hydroperoxide:secondary hydroperoxide) and LimOOH **3** agree with literature data [17, 24, 25].

2.3 GC/FID analysis

Analyses of petitgrain oil and sweet orange oil were performed as described according to the ISO standards [27, 28] to check their quality. The GC analyses were carried out using a Hewlett-Packard 6890 gas chromatograph equipped with a FID detector using a polar column (poly(ethylene glycol), J & W Scientific, 30 x 0.25 mm, 0.25 μm film thickness) and nitrogen as carrier gas. Approximately 1 μL of a 10 mg/mL solution in hexane was injected at a split ratio of 1:100. The identification of linalool, linalyl acetate, and limonene was accomplished by comparing the retention time with reference compounds.

According to the ISO standards, the concentration of linalool and linalyl acetate in petitgrain oil should be 15-30% w/w and 40-60% w/w, respectively [27], while the concentration of limonene in sweet orange oil should be 93-96% w/w [28]. Analysis of the essential oils *per se* agreed with the ISO standards as petitgrain oil was shown to contain 16% linalool and 54% linalyl acetate, whereas sweet orange oil contained 96% limonene.

2.4 Liquid chromatography

HPLC analysis was carried out with a system consisting of a binary pump, a temperature-controlled autosampler and a column oven (Agilent 1200 series, Agilent Technologies, Waldbronn, Germany). The separation of compounds was performed using a Zorbax StableBond SB-C₃ (150 x 2.1 mm, particle size 5 μm) narrow-bore analytical column (Agilent Technologies). Autosampler and column oven temperatures were maintained at 4°C and 45°C,

respectively, during analysis. The mobile phase consisted of 30% methanol in water (solvent A) and 10% isopropanol in methanol (solvent B). Both solvents A and B contained 0.1% formic acid. The elution of compounds was achieved at a flow rate of 0.4 mL/min, initiating with 0% B for 9 min, followed by a rapid increase to 28% B over 1 min, then maintaining this condition for 8 min. The gradient program then continued with an increase to 95% B over 2 min, the final composition being held isocratically for 5 min before returning to initial conditions, followed by 10 min column re-equilibration. The column eluent was switched to waste for the initial 3 min.

2.5 Mass spectrometry

Two different LC/MS systems were used. The quantification was mainly performed on system 1, which was an Agilent 6410 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA) equipped with an ESI source. The nebulizer pressure, gas flow rate, and drying gas temperature was 40 psi, 11 L/min, and 300°C, respectively. ESI needle voltage was adjusted to +4.0 kV and both product ion and multiple-reaction monitoring (MRM) detection were applied using nitrogen as collision gas with a dwell time of 200 ms for each transition of hydroperoxide compounds. The hydroperoxide compounds resulted in peak widths of between 0.36 to 0.91 min, corresponding to 12 to 30 data points. System 2 was used for examining the performance of the method including more identification points, i.e. product ion ratios. This system was a TSQ Vantage triple-quadrupole LC/MS from Thermo Scientific. The LC/MS was equipped with an HPLC and an autosampler (both Accela from Thermo Scientific). The settings were as follows: m/z window 0.2, dwell time 200 ms, CE 20 V, Q1 and Q3 0.7 FWHM, S-lens 64 V, Q2 argon pressure 2.0 mTorr.

2.6 Autoxidation of essential oils

Samples (~70 mL) of the essential oils, petitgrain oil and sweet orange oil, were air-exposed in Erlenmeyer flasks, covered with aluminum foils in order to prevent contamination from the environment. The samples were stored at room temperature and stirred for 1 h, 4 times a day for 63 days to mimic handling that can occur during production storage and usage, in accordance with a simple model previously described [17, 29]. The flasks were exposed to light 12 h a day, from a daylight lamp (Philips, 18 W). Small amounts were taken out at a regular basis from the investigated essential oils during the exposure time and stored at -70°C under argon until chemical analysis.

Chemical analyses of the collected aliquots were performed and the content of hydroperoxides was quantified using the developed LC/MS/MS method.

2.7 Standard solution preparation

Stock solutions (1 mg/mL in methanol) of each standard compound were serially diluted to several working concentrations (0.5, 1, 2, 5, 10, 25, 50 µg/mL) in methanol/water (7/3 v/v). An aliquot of each working concentration (100 µL) was spiked with 10 µL of IS (2,2'-biphenol, 22 µg/mL) leading to a final IS concentration of 2.0 µg/mL. Volumes of 5 µL were injected on the LC/MS system.

2.8 Sample preparation

The sample solutions of petitgrain oil and sweet orange oil were prepared at an approximate concentration of 2 mg/mL in methanol/water (7/3 v/v). Aliquots of 110 µL all contained 2.0 µg/mL of IS after spiking, following the same procedure as for the standard working solutions. Volumes of 5 µL were injected on the LC/MS system, for hydroperoxide determination.

3 Results and discussion

An LC/MS/MS method was developed separating allergenic hydroperoxides from the most commonly used fragrance terpenes linalool and limonene and also from linalyl acetate. In the diluted essential oil samples, hydroperoxides from linalool, linalyl acetate, and limonene, were detected at concentrations below 0.3 µg/mL (approximately 0.3 ppm). At a concentration of 0.001% (10 ppm) of the parent compound we can detect the formation of 3% or more of the corresponding hydroperoxides.

3.1 LC separation

The LC separation is shown as extracted ion chromatograms in Fig. 1A using a standard mixture. It is important to attain the best possible chromatographic resolution, due to the complex composition of the essential oils. Moreover, monoterpene hydroperoxides in general show very similar fragmentation patterns in ESI, as in all common MS techniques [18]. A C₃ stationary phase column yielded a much higher chromatographic resolution after optimization of the mobile phase composition, compared to a more lipophilic C₁₈ column (see Figure FS1 in Supporting Information). The combination of methanol and isopropanol as organic modifier resulted in better separation than methanol alone. Different column temperatures were tested for optimal separation efficiency and 45°C was found to be the most appropriate setting (see Figure FS2 in Supporting Information).

3.2 Optimization of LC/MS/MS conditions

LC/ESI-MS/MS product ion spectra of the studied hydroperoxides are shown in Fig. 2. Regardless of the magnitude of fragmentor voltage, LinOOH and LimOOH exhibited $[M+H-H_2O]^+$ (m/z 169.1 and m/z 151.1, respectively) as precursor ion in ESI source, while LinOAcOOH gave rise to the precursor ion $[M+H-CH_3COOH]^+$ (m/z 169.1). As shown, all

three hydroperoxides, but especially LinOOH and LinOAcOOH, exhibited very similar fragmentation pathways because of their structural similarities. Suggestion of a fragmentation pathway for LinOOH is shown in Fig. 3. The parent ions readily undergo further loss of water, rather than hydrogen peroxide. Reinnig *et al.* [23] showed the characteristic neutral loss of hydrogen peroxide for terpenoid hydroperoxy acids in APCI-MS/MS spectra. Most likely, the readily loss of hydrogen peroxide in the work of Reinnig is due to the neighboring carbonyl function.

As shown in Fig. 2, m/z 93 was the most abundant ion for all three hydroperoxides which corresponds to 4-methylcyclohexa-1,4-diene carbocation (see structure in Fig. 3). However, transition to this ion in MRM was not used for quantification, as it gave lower S/N ratio than other less intense product ions due to a higher inherent noise. The most appropriate transitions and settings are shown in Table 2. For each compound one of the product ions was used for quantitation, while 2 others were used as qualifiers. It should be noted that the chosen transition of m/z 151.1 for LimOOH is a secondary transition for LinOOH and LinOAcOOH as seen in Fig. 2.

3.3 Evaluation of the LC/ESI-MS/MS method

Evaluation was performed on spiked essential oils using MRM mode and the settings are presented in Table 2.

The dynamic range of the MS method was tested by setting up calibration curves for standard solutions of LinOOH, LinOAcOOH, and LimOOH. A linear correlation coefficient (r^2), greater than 0.99 in the range of 0.5-50 $\mu\text{g/mL}$, was obtained for all investigated hydroperoxides. Instrumental LOQ (using standard solutions) was 0.50 $\mu\text{g/mL}$ for LinOOH and 0.12 and 0.25 $\mu\text{g/mL}$ for LinOAcOOH and LimOOH, respectively (Supporting Information Table TS1).

The essential oils (petitgrain oil and sweet orange oil) were diluted with methanol/water (7/3 v/v) to approximately 2 mg/mL. Aliquots of the diluted essential oils (from 25 days of autoxidation) were spiked to increase the levels of hydroperoxides by 0.5, 5, and 25 µg/mL before analysis with the developed method. Average accuracy was 89-106% with CVs throughout below 8.5% (Supporting Information Table TS2). The slopes were compared to reference solutions and the results indicate that there was no ion suppression or ion enhancement in the analysis of the essential oils. Method LOQ (using sample solutions of the essential oils) was in the range 0.3-1 µg/mL, corresponding to 0.3-1.0 ppm of the sample volume (Table 3).

3.4 Monitoring formation of hydroperoxides in essential oils

The applicability of the method was tested on the non-spiked essential oils, petitgrain oil (containing linalool and linalyl acetate) and sweet orange oil (containing limonene), subjected to autoxidation. As we have shown previously [10], hydroperoxides were formed from the monoterpenes, with higher concentrations after a longer time of autoxidation. The identification and quantification is based on the specific isomers (Table 1) most prominent in the oxidation mixtures of the parent compounds according to previous investigations [17, 24-26]. Extracted ion chromatograms of non-oxidized and air exposed petitgrain oil and sweet orange oil for 25 days are shown in Fig. 1B – 1E.

Quantifier/qualifier product ion intensity ratios were used for an additional identification point. Those ratios are presented in Table 2. It should be noted that the ion intensity ratios are not constant in LC/MS but are dependent on instrument type, ion source parameters and collision energy setting which must be calibrated against reference compounds. The results of hydroperoxides quantified in the essential oils are shown in Table 4. LimOOH could already be detected at a concentration of 0.05% w/w in fresh sweet orange oil. After 25 days of air

exposure, all three studied monoterpene hydroperoxides were detected (Table 4) in the essential oils. After 63 days of air exposure, petitgrain oil contained 0.37% w/w LinOOH and 0.55% w/w LinOAcOOH, while the hydroperoxide concentration in sweet orange oil was increased resulting in 4.9% w/w LimOOH. For the quantified compounds the ion intensity ratios in standards (5 µg/mL and 50 µg/mL, N=5 of each) were similar to samples (petitgrain oil and sweet orange oil, N=5 of each). The relative standard deviations (RSDs) for the ion ratios were below 6% for LinOOH, below 12% for LinOAcOOH, and below 13% for LimOOH.

Samples of the oils that had been stored in the refrigerator at +4°C protected from daylight for a year before analysis were examined. Of the hydroperoxides investigated, LinOAcOOH was detected at a level of 0.03% w/w in petitgrain oil and LimOOH could be detected at a concentration of 0.35% w/w in sweet orange oil (data not shown). Obviously, storage in the refrigerator did not totally prevent from hydroperoxide formation.

Autoxidation of essential oils will further increase the complexity of the mixture. The hydroperoxides analyzed are the most stable hydroperoxides; less stable (e.g. primary hydroperoxides) can form but cannot be isolated by synthesis, so no reference compounds are available. It should also be noted that the hydroperoxides could degrade to allergenic secondary oxidation products like aldehydes and epoxides.

4 Concluding remarks

Fragrance materials, both defined chemical compounds and natural mixtures of compounds (essential oils), are used in various types of cosmetic products. The fragrance ingredients are mixtures of 20 to over 200 synthetic fragrance chemicals or natural fragrance materials (essential oils).

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According to EU legislation [7], concentrations of linalool or limonene exceeding 0.01% in “rinse-off” products and 0.001% in “stay-on” products must be labeled when present in cosmetics. However, the products may still contain allergens, such as hydroperoxides, formed by oxidative degradation of their parent terpenes. With the sensitivity and selectivity of the presented LC/MS/MS method it is possible to follow the formation of the hydroperoxides. For the hydroperoxides investigated, the lowest detectable concentrations in the diluted essential oil samples were below 0.3 $\mu\text{g/mL}$, corresponding to approximately 0.3 ppm. Assuming an initial level of 0.001% (10 ppm) of a parent terpene in a perfume, it should be possible to detect autoxidation of 3% to a hydroperoxide, not taking into account any clean-up or preconcentration steps. This is the first method for determination of allergenic fragrance hydroperoxides from linalool, linalyl acetate, and limonene in essential oils using LC/MS/MS. However, before it is applied in routine measurements, the method still requires more validation.

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Conflict of interest statement

The authors have declared no conflicts of interest.

Supporting Information

Linearity, instrumental LOQ, and LOD are supplied for the studied terpene hydroperoxides (linalool hydroperoxides, linalyl acetate hydroperoxides, and limonene-2-hydroperoxide) using standard solutions, recovery of the analytes are supplied from the matrices of the essential oils (petitgrain oil and sweet orange oil). Chromatograms from both columns used, C₁₈ and C₃, as well as from different column oven temperatures are provided. Calibration curves of LinOOH are supplied, comparing integration of separate isomer peaks with inclusion of both peaks. The material is available free of charge via the Internet.

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Table 1. Structures of the compounds with molecular weights (Mw) and abbreviations investigated in this study

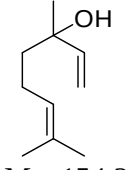
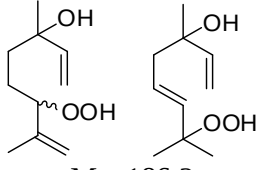
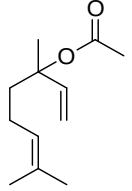
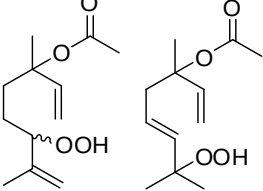
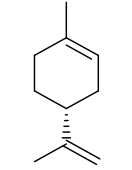
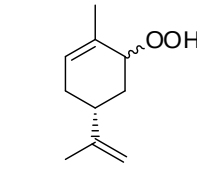
Parent Terpenes		Terpene Hydroperoxides	
Linalool 3,7-Dimethyl-octa-1,6-diene-3-ol	 Mw 154.2	Linalool hydroperoxides (LinOOH, 1) 6-Hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol and 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol	 Mw 186.2
Linalyl acetate 3,7-Dimethyl-octa-1,6-diene-3-yl acetate	 Mw 196.2	Linalyl acetate hydroperoxides (LinOAcOOH, 2) 6-Hydroperoxy-3,7-dimethylocta-1,7-diene-3-yl acetate and 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-yl acetate	 Mw 228.2
Limonene 1-Methyl-4-(prop-1-en-2-yl)cyclohex-1-ene	 Mw 136.2	Limonene-2-hydroperoxide (LimOOH, 3) 6-Hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene	 Mw 168.2

Table 2. Optimal transitions and settings used for the compounds studied. Quantifier and qualifier ions are product ions from the parent ions. The mean ion ratios both in standard (5µg/mL) and in oils, as normalized to the quantifier ion intensity, are within brackets. The first value is for standard, the second for oil.

Compound	Parent ion (<i>m/z</i>)	Quantifier ion (<i>m/z</i>)	Qualifier ion 1 (<i>m/z</i>)	Qualifier ion 2 (<i>m/z</i>)	Dwell time ^d (ms)	Collision energy (V)
LinOOH 1	169.1 ^a	133.1 (1.00)	151.1 (1.59,1.68)	93.1 (6.83,5.75)	200	20
LinOAcOOH 2	169.1 ^b	133.1 (1.00)	151.1 (1.59,1.92)	93.1 (7.71,7.33)	200	20
LimOOH 3	151.1 ^a	107.0 (1.00)	133.2 (0.14,0.16)	123.1 (0.33,0.38)	200	20
2,2'-Biphenol (IS)	187.1 ^c	141.1 (1.00)			200	20

^a [M+H-H₂O]⁺

^b [M+H-CH₃COOH]⁺

^c [M+H]⁺

^d For each transition

Table 3. Method limit of quantification and limit of detection for hydroperoxides in 2 mg/mL oil samples (petitgrain oil and sweet orange oil)

Sample	Compound	LOQ ^a (µg/mL)	LOD ^b (µg/mL)
Petitgrain oil	LinOOH 1	1.0	0.3
Petitgrain oil	LinOAcOOH 2	0.3	0.09
Sweet Orange oil	LimOOH 3	0.6	0.18

^a Limit of quantification defined as S/N = 10

^b Limit of detection defined as S/N = 3

Table 4. Content of the terpene hydroperoxides (LinOOH, LinOAcOOH, and LimOOH) in non-diluted air-exposed essential oils over time analyzed with LC/ESI-MS/MS (% w/w, (%RSD), n = 3)

Time (days)	Petitgrain oil		Sweet orange oil
	LinOOH 1 % (%RSD)	LinOAcOOH 2 % (%RSD)	LimOOH 3 % (%RSD)
0	ND	D	0.05 (2)
3	ND	D	0.06 (1)
7	D	D	0.07 (2)
25	0.07 (2)	0.09 (1)	0.51 (2)
63	0.37 (2)	0.55 (3)	4.90 (1)

D, detected; ND, not detected.

Figure 1. Extracted ion chromatograms of terpene hydroperoxides from LC/ESI-MS/MS analyses. **A.** Reference solution of standard compounds, **B.** Petitgrain oil before subjected to autoxidation, **C.** Petitgrain oil after 25 days of autoxidation, **D.** Sweet orange oil before subjected to autoxidation, **E.** Sweet orange oil after 25 days of autoxidation. Labeled peaks indicate LinOOH (**1**, Rt 5.7 – 6.0 min, transition m/z 169.1 to 133.1), LinOAcOOH (**2**, Rt 13.2 min, transition m/z 169.1 to 133.1), LimOOH (**3**, Rt 13.9 min, transition m/z 151.1 to 107.1), and IS (internal standard, 2,2'-biphenol ([1,1'-biphenyl]-2,2'-diol), Rt 7.4 min, transition m/z 187.1 to 141.1). The peak labeled * in **A** corresponds to geraniol hydroperoxides which was included in the reference solution but were not determined in the essential oil samples.

Rt, retention time.

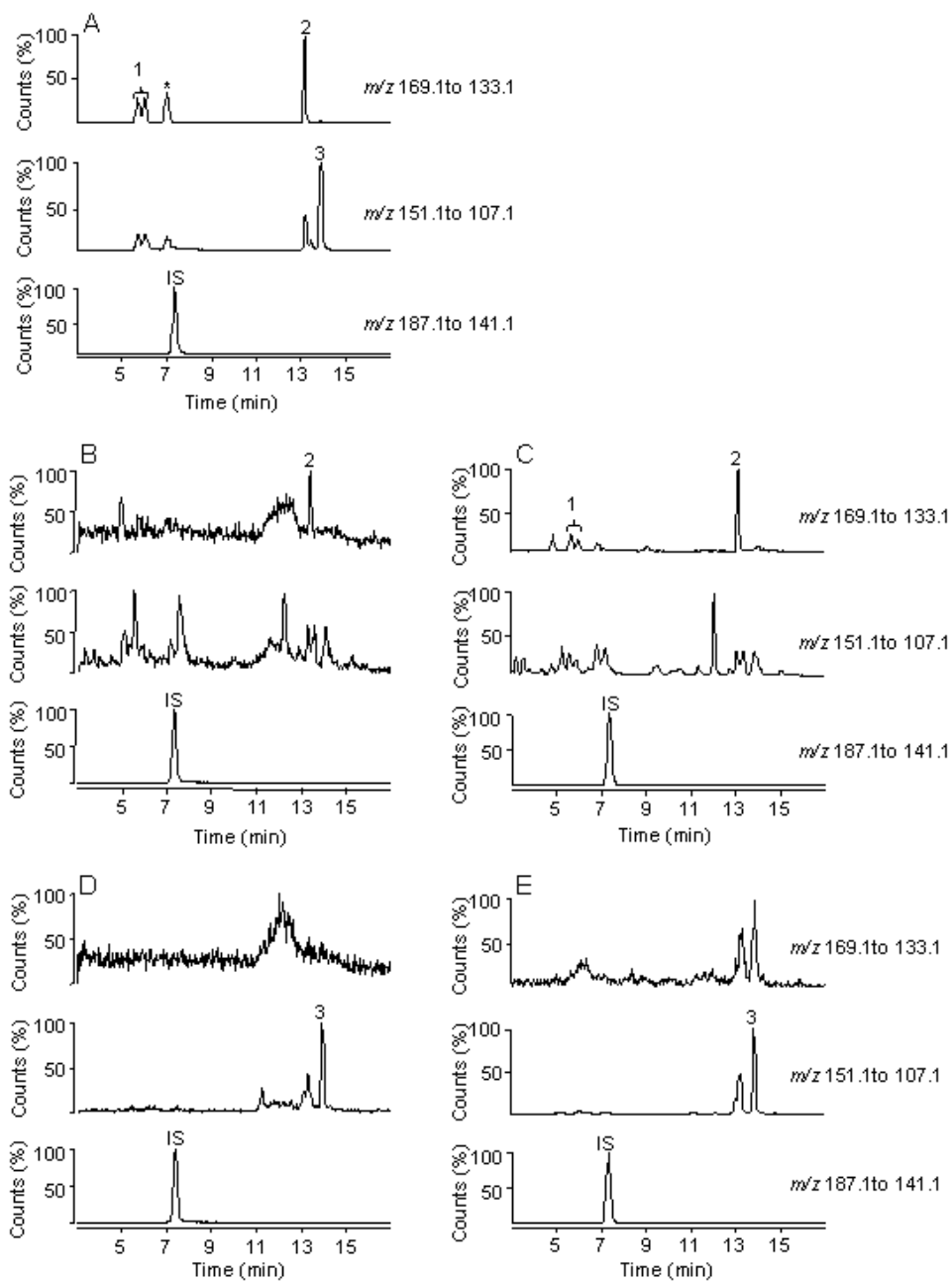


Figure 2. MS/MS product ion spectra of LinOOH (1, A), LinOAcOOH (2, B), and LimOOH (3, C).

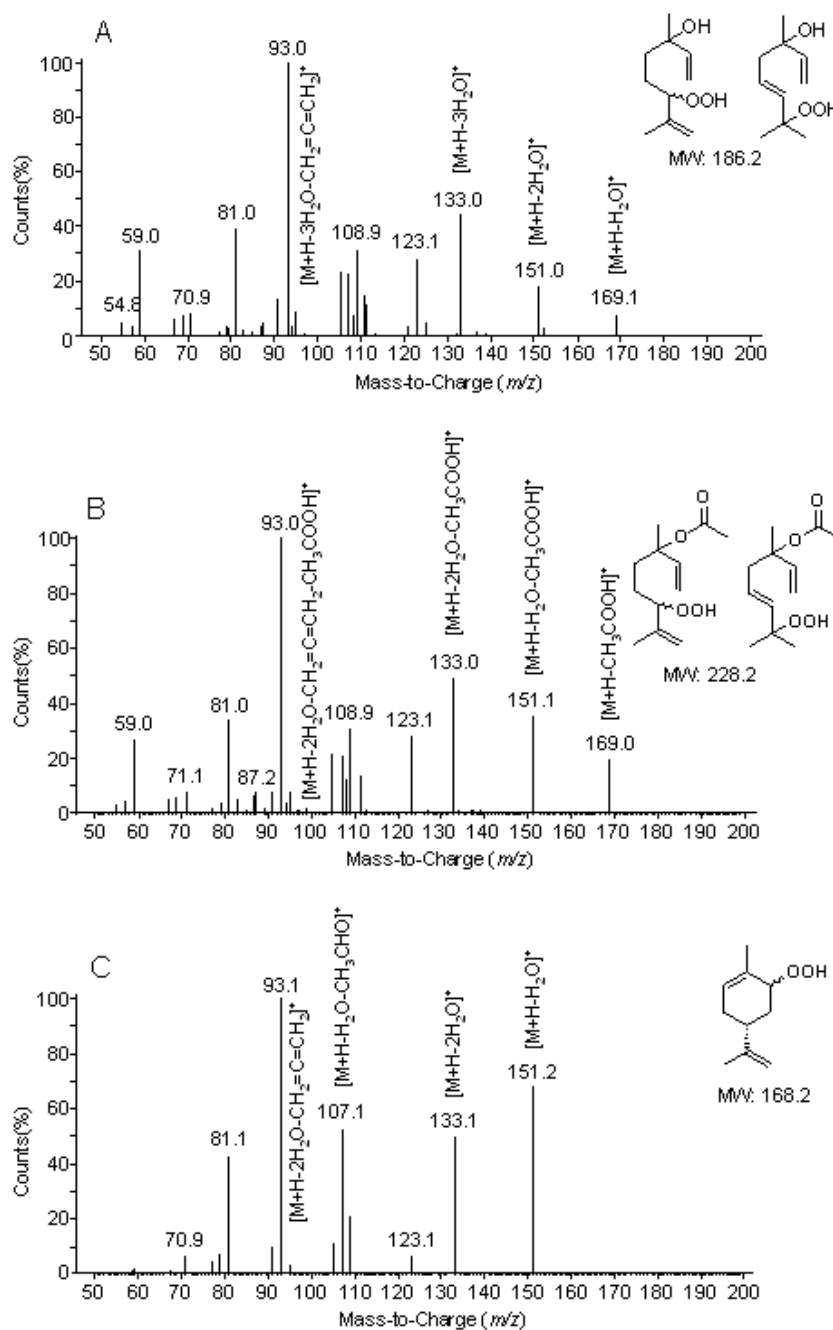
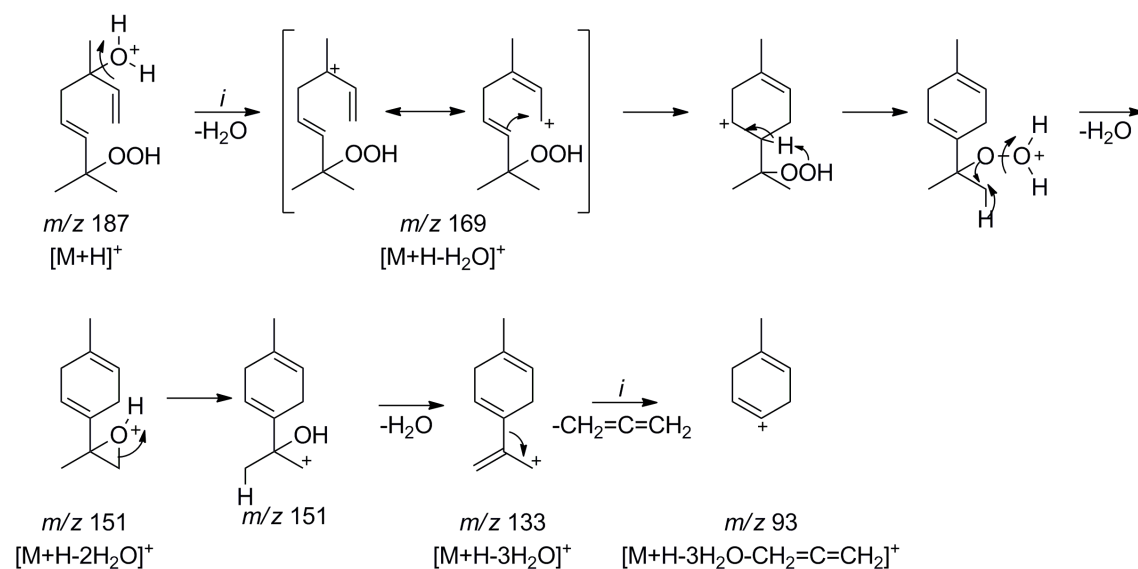


Figure 3. Proposed fragmentation pathway for LinOOH **1** to the base peak m/z 93 (see Fig. 2A).



A sensitive method for determination of allergenic fragrance terpene hydroperoxides using liquid chromatography coupled with tandem mass spectrometry

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Supporting information

Table TS1: Linearity, instrumental LOQ, and LOD of linalool hydroperoxides (LinOOH, **1**), linalyl acetate hydroperoxides (LinOAcOOH, **2**), and limonene-2-hydroperoxide (LimOOH, **3**).

Table TS2: Recovery of the analytes from the essential oil matrices (petitgrain oil and sweet orange oil).

Figure FS1: Total ion chromatograms of terpene hydroperoxides from LC/MS analyses using **A)** C18 column and **B)** C3 column.

Figure FS2: Total ion chromatograms of terpene hydroperoxides from LC/ MS/MS analyses using a column oven temperature of **A)** 35°C, **B)** 40°C, **C)** 45°C, and **D)** 50°C.

Figure FS3. Calibration curves of linalool hydroperoxides (LinOOH, **1**) integrated the two peaks simultaneously and separately.

Table TS1. Linearity, instrumental LOQ, and LOD of LinOOH **1**, LinOAcOOH **2**, and LimOOH **3** (n = 3)

Compound	Range (µg/mL)	Slope ^a	Correlation co-efficient (r ²)	LOQ ^b (µg/mL)	LOD ^c (µg/mL)
LinOOH 1	0.5 – 50	0.183	0.999	0.50	0.16
LinOAcOOH 2	0.5 – 50	0.191	0.998	0.12	0.04
LimOOH 3	0.5 – 50	0.158	0.997	0.25	0.08

^a Slope refers to regression equation $y = mx$

^b Limit of quantification defined as S/N = 10

^c Limit of detection defined as S/N = 3

Table TS2. Recovery of the analytes from essential oils matrices (petitgrain oil and sweet orange oil, n = 3)

Sample	Compound	Conc. spiked ($\mu\text{g/mL}$)	Conc. observed ($\mu\text{g/mL}$)	Mean recovery (%)	CV (%)
Petitgrain oil	LinOOH 1	0.5	0.53 $\pm$ 0.03	105.13	6.16
		5.0	4.43 $\pm$ 0.02	88.63	0.39
		25.0	24.45 $\pm$ 0.40	97.81	1.64
Petitgrain oil	LinOAcOOH 2	0.5	0.52 $\pm$ 0.04	103.73	8.49
		5.0	4.86 $\pm$ 0.13	97.21	2.61
		25.0	24.00 $\pm$ 0.11	96.02	0.47
Orange oil	LimOOH 3	0.5	0.52 $\pm$ 0.01	104.00	2.22
		5.0	4.97 $\pm$ 0.02	99.31	0.31
		25.0	23.60 $\pm$ 0.31	94.39	1.32

Figure FS1. Total ion chromatograms (SCAN, m/z 50-250) of terpene hydroperoxide standards from LC/MS analyses using **A)** ZORBAX SB-C18 Rapid Resolution HT column (50 x 2.1 mm, 1.8 μ m) and **B)** ZORBAX SB-C3 column (150 x 2.1 mm, 5 μ m). Labeled peaks indicate LinOOH (**1**), LinOAcOOH (**2**), and LimOOH (**3**). All isomers detected in SCAN mode could not be detected in MRM mode with the chosen transitions. Only two peaks could be detected for LinOOH and one peak for LinOAcOOH. In this experiment, geraniol hydroperoxides (GerOOH) were additionally included as a standard but were not determined in the essential oil samples.

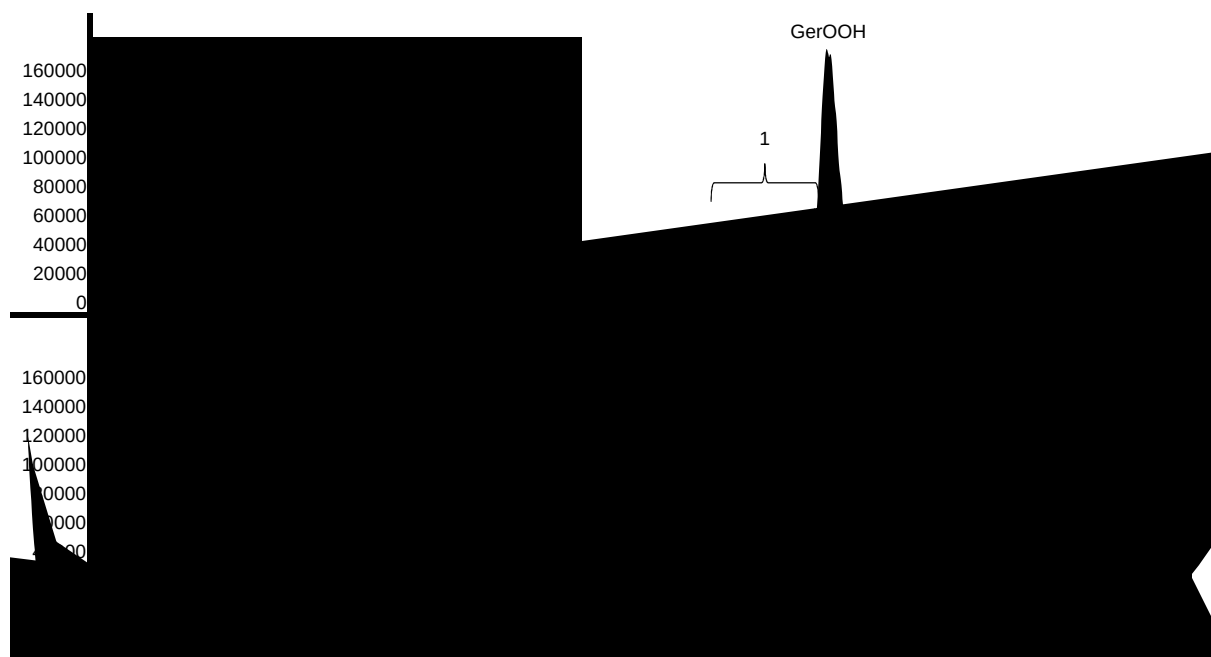


Figure FS2. Total ion chromatograms (MRM) of terpene hydroperoxide standards from LC/MS/MS analyses using a column oven temperature of **A)** 35°C, **B)** 40°C, **C)** 45°C, and **D)** 50°C. Labeled peaks indicate LinOOH (1), LinOAcOOH (2), and LimOOH (3). For A, B, and C the first four minutes went to waste, for D the first three minutes went to waste. Additionally, geraniol hydroperoxides (GerOOH) were included as a standard for this experiment but were not determined in the essential oil samples.

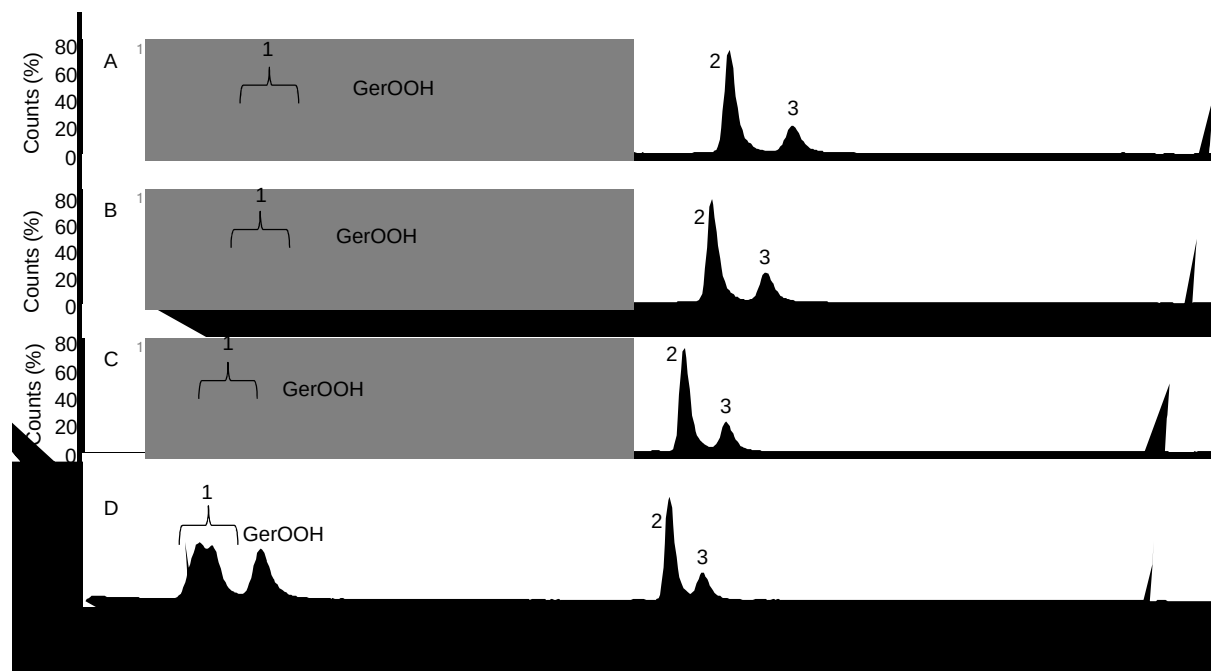


Figure FS3. Calibration curves of linalool hydroperoxides (LinOOH **1**) integrated firstly with two peaks simultaneously and then each peak separately. **A:** Both peaks integrated simultaneously. **B:** First peak in chromatogram (retention time 5.7 min). **C:** Second peak in chromatogram (retention time 6.0 min). All three calibration curves are calculated based on the same total amount of LinOOH **1**.

