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A two-dimensional non-comprehensive reversed/normal phase high-performance liquid chromatography/tandem mass spectrometry system for determination of limonene and linalool hydroperoxides

Ahmed Ramzi, Hamid Ahmadi, Ioannis Sadiktsis, Ulrika Nilsson*

Department of Environmental Science and Analytical Chemistry, Stockholm University, SE-106 91, Sweden

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ABSTRACT

A two-dimensional non-comprehensive high-performance liquid chromatographic (HPLC) system coupled to electrospray ionization tandem mass spectrometry was developed for the determination of skin allergenic hydroperoxides of limonene and linalool. These compounds are some major components behind skin sensitization and contact (skin) allergy to fragrances.

Fragrance hydroperoxides usually occur in complex compositions, often as constituents of the natural essential oils added to a large number of commercial products. Their similarities to interfering compounds, many with identical elemental composition, make the determination difficult even when using selective detection methods like mass spectrometry. In this work, a first-dimension chromatographic heart-cut isolation of the hydroperoxides on a reversed-phase HPLC system was combined with a second-dimension normal-phase HPLC system for separation of the hydroperoxides. The intersystem transfer was made by trapping the heart-cut fraction on a short graphitized carbon column, exchanging the mobile phase and back-flushing the hydroperoxides into the second dimension.

Each analysis was performed within 60 min without any pretreatment, except dilution, prior to injection. The obtained instrumental limits of detection (LODs) at a signal-to-noise ratio of 3 were lower than 1.2 ng injected on column and method LODs were below 0.3 ppm. An after-shave product was shown to contain the highest concentrations of the measured hydroperoxides, with 445 ± 23 ppm of total linalool hydroperoxides. This level is likely able to elicit skin reactions in already sensitized individuals.

1. Introduction

The increased everyday use of fragranced cosmetics, hygiene and household products in Europe has led to an increased prevalence of contact (skin) allergy, a life-long condition with no cure so far. Fragrances, next to nickel and preservatives, are the most common cause of skin sensitization and it is estimated that between 1–3% of the European population are allergic to one or several fragrance compounds [1–4]. Sensitization implies a life-long change of the immune system specificity and skin allergy is clinically manifested as allergic contact dermatitis (ACD). Once sensitized, ACD rapidly develops upon re-exposure to a sufficient amount of the product containing the allergen.

The most common fragrance compounds are volatile monoterpenoid compounds, such as limonene and linalool. The fragrances are either added to products as synthesized compounds or as constituents of essential oils obtained from plants. It has previously been shown that both linalool and limonene are non-sensitizing or very weakly allergenic *per se*. However, these compounds are easily activated to strong skin sensitizers upon contact with air [5,6]. The major skin allergens are hydroperoxides which are formed as primary oxidation products. This has been shown for oxidized linalool and limonene in large international multicenter studies [7–10], as well as in animal studies [11,12]. So far, the most studied fragrance hydroperoxides are limonene-2-hydroperoxide (Lim-2-OOH), linalool-6-hydroperoxide (Lin-6-OOH), and linalool-7-hydroperoxide (Lin-7-OOH). Due to their strong sensitization potency [11,13–15], the ability to measure these compounds in fragranced products is highly desirable.

Monoterpenoid hydroperoxides, such as the isomers of linalool hydroperoxides (Lin-OOHs) and Lim-2-OOH are challenging to measure, due to low thermal stability and lack of chromophore. Determination of hydroperoxides in autoxidized essential oils has been performed using gas chromatography/mass spectrometry

* Corresponding author.
E-mail address: ulrika.nilsson@aces.su.se (U. Nilsson).

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(GC/MS) with prior derivatization [16]. The problem of thermostability can also be circumvented by use of high-performance liquid chromatography/electrospray ionization - tandem mass spectrometry (HPLC/ESI-MS/MS) [17]. Recently, HPLC/ESI-time-of-flight (ToF)-MS methodology at high resolution (HRMS) was used for measurements of linalool hydroperoxides in some hydroalcholic products (fine fragrances and deodorants) [18]. Indirect measurement of Lim-2-OOH by GC/MS after reduction to more thermostable alcohols has also been presented [19]. Furthermore, a HPLC method to detect hydroperoxides from linalool and limonene in citrus oil using post-column chemiluminescence detection has been published recently [20].

Besides their demanding physiochemical properties, the usually highly complex composition of monoterpenoids both in essential oils and fragranced products make the determination of hydroperoxides even more difficult. The composition can include various parent monoterpenoids together with their oxidation products. Apart from hydroperoxides, this involves secondary oxidation products such as aldehydes, alcohols, diols, epoxides and ketones with different, but generally much lower sensitizing potencies [11,13,21,22]. Both chromatographic and mass spectrometric separation of hydroperoxides from other oxidized compounds are challenging due to similar structures, as well as in many cases identical elemental compositions and similar fragmentation pathways.

In this work, a two-dimensional (2D-) HPLC/ESI-MS/MS system was developed, by coupling normal-phase to reversed-phase HPLC, to increase the selectivity of the chromatographic separation and thereby the detectability of Lin-6-OOH, Lin-7-OOH and Lim-2-OOH. Since the aim was to determine a limited number of target compounds, it was decided to develop a non-comprehensive heart-cut 2D-HPLC system (LC-LC). Comprehensive two-dimensional HPLC, commonly denoted as LCxLC, is a powerful technique to maximize the separation of many components in complex samples. This technique has for instance been used in the separation of peptide digests [23], and its implementations was reviewed in 2008 by Guiochon et al [24]. The drawback of this technique is, however, the more complex instrumentation needed. Different forms of 2D-HPLC, including LC-LC, was recently reviewed by Stoll and Carr [25]

One problem to be solved was how to handle the immiscible solvent. In theory, this could have been handled by evaporation [26] of the solvent, or by using a narrow-bore column in the first dimension running at a very low flow rate [27]. The strategy in the present work was, however, to use a trap column for the intersystem transfer of analytes and for solvent exchange.

The performance of the presented 2D-HPLC/ESI-MS/MS system in terms of linearity, limits of detection (LODs), matrix effects, and applicability for measuring hydroperoxides in authentic perfume samples are presented and discussed.

2. Experimental section

2.1. Solvents and standards

The chemical structures of all discussed compounds are shown in Fig. 1. Linalool (**1**, 97%) and (R)-(+)-limonene (**2**, 97%), were purchased from Sigma Aldrich (St Louis, MO, USA). Individual isomers of hydroperoxides (≥98.0%), linalool-6-hydroperoxide (**3,** Lin-6-OOH), and linalool-7-hydroperoxide (**4,** Lin-7-OOH) and limonene-2-hydroperoxide (**5,** Lim-2-OOH), were synthesized by Greenpharma SAS (Orléans, France) and kept at −80 °C under argon until use. Cumene hydroperoxide (**6**, Cum-OOH, of technical grade 80%), 2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3-ol (**7**, linalool oxide pyranoside), linalool oxide furanoid (**8**, mixture of isomers, ≥97.0%), (+)-limonene oxide (**9**, mixture of cis and trans, 97%), L-carveol (**10**, mixture of cis and trans, ≥95%), L-carvone (**11,** ≥97%), (1S,2S,4R)-(+)-limonene-1,2-diol (**12,** ≥97.0%), citral (**13**, natural mixture of neral and geranial, ≥96%) were all purchased from Sigma Aldrich. Ultrapure water with a resistivity >18 MΩ cm was produced by a MilliQ water purification system from Millipore (Billerica, MA, USA). Toluene, ethyl acetate (EtOAC), and methyl-*tert*-butyl ether (MTBE), all of HPLC grade were purchased from Rathburn Chemicals Ltd (Walkerburn, UK). Methanol (MeOH) of HPLC grade and formic acid (≥99%) were purchased from VWR International (Fontenay-sous-Bois, France). Ethanol of analytical grade (99.5%) was obtained from Solveco AB (Rosersberg, Sweden).

2.2. Preparations of samples and standard solutions

Ten perfume products of different international brands obtained from the Swedish open market (seven eau de toilette, two aftershaves and one eau de cologne) were analyzed. The products had been kept and used by consumers under common conditions for 1–5 years after purchase. Each sample was initially screened for hydroperoxides, and depending on the concentration 3–50 μL of the sample was diluted up to a total volume of 450 μL prior to quantitative analysis. For the Lin-OOH method, ultrapure water was used as a solvent, while MeOH:water 70:30 was used for the Lim-2-OOH method. Finally, 50 μL volumetric internal standard (IS), 100 μg L^{-1} Cum-OOH in ethanol, was added. This IS was used for the determination of both Lim-2-OOH and Lin-OOHs.

Stock solutions of each hydroperoxide standard were prepared in ethanol, bubbled with argon, and then stored at −20 °C until use. Working standard solutions were prepared by dilutions of the stock solutions after addition of IS. For both Lin-6-OOH and Lin-7-OOH dilutions were made with ultrapure water, while MeOH:water 70:30 was used for Lim-2-OOH.

For the analysis with 2D-HPLC/ESI-MS/MS, a 60-μL volume of the final sample or standard solution was injected.

2.3. 2D-HPLC/ESI-MS/MS

A schematic of the 2D-HPLC/ESI-MS/MS system is shown in Fig. 2. The system consisted of a reversed-phase HPLC system, a normal-phase HPLC system, and a porous graphitized carbon (PGC) trap column (Hypercarb, dp = 5 μm, 4.0 mm i.d. × 10 mm, Thermo Scientific, MA, USA), all connected to a divert 6-port valve (Agilent 1200 series, Agilent Technologies, Waldbronn, Germany). Two separate methods were set up, one for linalool hydroperoxides (Lin-OOHs) and one for limonene-2-hydroperoxide (Lim-2-OOH). A detailed description of pump settings, mobile phase compositions, flow rates and switch timings for each of the two methods are given in Supplementary Material and Fig. S1a and S1b, respectively.

2.3.1. First dimension separation

In the first separation dimension, a binary pump P1 (Agilent 1100 series) delivered a mobile phase consisting of ultrapure water and MeOH to a reversed-phase C_8 column (XterraTM MS, d_p 3.5 μm, 3.0 mm i.d. × 50 mm, Waters, Milford, MA, USA). A UV detector (UVD) (Agilent 1100 series with A.06.10 [005] firmware upgrade) was connected to the first dimension C_8 column monitoring the effluent at 200 nm.

2.3.2. Heart-cutting and analyte trapping

Initially, the valve was set in position 1→2, where the mobile phase flow from the C_8 column in the first dimension was diverted to waste. Prior to elution of the hydroperoxide fraction, the valve was switched into position 1→6 and the effluent from the first dimension was diverted into the PGC trap column, after being diluted with a flow of ultrapure water, delivered by a second pump P2 (channel A), through a T-connector (mixing tee 1, void-volume:

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