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Enantioselective high performance liquid chromatography and supercritical fluid chromatography separation of spirocyclic terpenoid flavor compounds

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ABSTRACT

Chiral spirocyclic terpenoids are abundant natural flavors with significant impact particularly on the food industry. Chromatographic methods for analytical and preparative separation of these compounds are therefore of high interest to natural product chemists in academia and industry. Gas chromatography on chiral stationary phases is currently the standard method for the separation of volatile terpenoids, limiting the scale to analytical quantities. We report herein high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) protocols for the chiral separation of several racemic spirocyclic terpenoids such as the important flavors theaspirane and vitispirane. A screening of mobile phases and 16 commercially available chiral stationary phases (CSPs) largely based on polysaccharides led to identification of protocols for the separation of all terpenoids tested. SFC methods were found to be particularly useful for the separation of these spirocyclic flavors due to the volatility and low polarity of the compounds. The reported chiral HPLC and SFC protocols are scalable alternatives to gas chromatographic separations of volatile terpenoid flavors.

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1. Introduction

Volatile terpenoids are abundant natural flavors of significant industrial relevance particularly for the food industry. An abundant "osmophor" [1] in this heterogeneous class of molecules is the spiroether motif. Many spirocyclic norisoprenoid ethers are thus important flavors present in various plants. Prominent examples are theaspiranes (**1**) and vitispiranes (**2**) as well as some of their oxidized analogs (Fig. 1). Theaspirane has been found in wine [2], grape juice [3], beer [4], various fruits such as raspberry [5], apricot and peach [6], passion fruit [7] and apple [8]. It has also been found in extracts of osmanthus [9], tea [10] and leguminosae [11]. Theaspirone (**3**) was first isolated from black tea and is an important constituent of black tea flavor [12,13].] It has also been identified in oak wood (used for wine maturation) [14], roses [15], and passion fruit [7]. Various syntheses are known [16–20] and theaspirane as well as numerous derivatives have been characterized with respect to their olfactory properties [21]. Vitispirane is an important constituent of wine flavor particularly for wine maturation [22,23], and has been found in roses [24], vanilla [25], liana [26], quince juice [27], and other plants [28]. Vitispirane and close derivatives have been synthesized [20,29,30].

Theaspirane (**1**) and vitispirane (**2**) contain two stereogenic centers allowing four possible stereoisomers each. For vitispirane, theaspirane and theaspirone all four stereoisomers have been isolated to varying extends from different fruits [27]. The stereoisomers of **1** and **2** have been reported to have quite different olfactory properties. For example, (*2R*,*5R*)-**1** has a weak camphoraceous note whereas (*2R*,*5S*)-**1** has an attractive fresh-fruity odor. The odor of (*2S*,*5S*)-**1** has a fresh camphoraceous note and (*2S*,*5R*)-**1** smells almost naphthalene-like [31]. The separation of stereoisomeric spiroethers is therefore important for complete olfactory and structural analysis and eventual applications of these compounds.

In the past, separations of racemic spirocyclic norisoprenoids like theaspirane (**1**) and vitispirane (**2**) have been performed by enantioselective GC [27,31,32]. While these protocols gave good analytical separations on cyclodextrin-based stationary phases, they are not suitable for the separation of larger quantities of the spiroethers. Therefore, our aim was to develop enantioselective HPLC and supercritical fluid chromatography (SFC) methods. To the best of our knowledge neither enantioselective HPLC nor SFC separations have been reported before for this important class of flavors.

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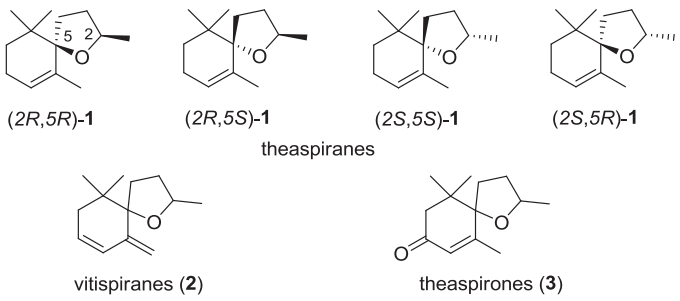

Fig. 1. Selected important spirocyclic C13-norisoprenoid flavor compounds.

Given the scalability of both techniques, these separation protocols would be extremely valuable in an academic and industrial context. They allow the separation of mg-quantities for structural analysis by NMR and biological assays and even larger quantities up to kilogram batches in an industrial context. HPLC on chiral stationary phases (CSPs) may be performed in a reversed phase- or a normal phase mode [33–37]. As CSPs various kinds of selectors have been used and many of them are commercially available [38]. Today Okamoto's, coated or immobilized amylose- and cellulose-based polysaccharide phases on silica together with alkane/alcohol mixtures as mobile phases are most frequently used [35,39–41]. A number of polysaccharide phases is commercially available and a set of four common phases (AD, OD, AS, and OJ termed the "golden four") allows the separation of a broad spectrum of analytes [42]. These phases have also been shown to be effective for enantioselective SFC [43], which has become increasingly attractive for the preparative separation of enantiomers in an industrial context due to efficiency and ecological considerations (although the latter is still a matter of debate) [44–48]. In SFC supercritical CO_2 is utilized as the non-polar part of the mobile phase. The supercritical state is obtained by temperatures above 31 °C and pressures above 74 MPa. Routinely, however, "SFC" separations are performed at 25–35 °C. The term chiral SFC is nevertheless used in most cases to indicate that the major fraction of the mobile phase is compressed CO_2. The advantage of this method is that supercritical CO_2 has properties intermediate between a liquid and a gas. The lower viscosity and thus a higher diffusion rate of the SFC mobile phase allow higher flow rates relative to HPLC. Consequently run times are often significantly shorter compared to HPLC [39,49]. Supercritical carbon dioxide has a polarity approximately equal to hexane [50] and in most cases polar modifiers such as MeOH are added. Due to its low polarity and volatility we assumed that CO_2 is an ideal mobile phase for the separation of non-polar and volatile flavor compounds such as terpenoids.

Herein we describe a screening of HPLC and SFC methods for the enantioselective separation of spirocyclic norisoprenoids involving different commercially available polysaccharide phases.

2. Experimental

2.1. Preparation of analytes

Theaspirane and vitispirane derivatives **1**–**7** (Fig. 2) were obtained as racemic mixtures as described previously [51]. Compound (*2R*,5R**)-**4** was a racemic mixture of two diastereoisomers (overall four stereoisomers). Before chromatography, the compounds were dissolved in MeOH/EtOH 50/50 (v/v) with a concentration of 5 mg/mL. For derivatives (*2R*,5R**)-**4** and (*2R*,5R*,7R**)-**5**, higher concentrations (up to 50 mg/mL) were used because of the low UV-absorption of the compounds. All solutions were stored at +4 °C.

2.2. Chiral stationary phases

The following CSPs were used: CHIRALPAK AD, CHIRALPAK AY, CHIRALPAK AS, CHIRALCEL OD, CHIRALCEL OZ and CHIRALCEL OJ (coated polysaccharide phases supplied by Daicel Corporation, Japan), Lux Amylose-2, Lux Amylose-4 (coated polysaccharide phases, supplied by Phenomenex Inc., USA), CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, CHIRALPAK IF, CHIRALPAK IE, CHIRALPAK ID (immobilized polysaccharide phases supplied by Daicel Corporation, Japan). Chemical structures of these chiral selectors are shown in Table 1. Other chiral columns were the Pirkle- or "brush"-type (R,R and S,S) Whelk-O1 supplied by Regis Technologies Inc. (USA). All columns had a size of 250 × 4.6 mm (i.d.) and a particle size of 5 μm. For SFC screening of theaspirane derivatives (*2R*,5R*,8S**)-**7a** and (*2R*,5R*,8R**)-**7b**, CHIRALPAK IA, IB, IC and ID were used with a size of 150 × 2.1 mm.

As the mobile phases MeOH (Prepsolv), EtOH (Lichrosolv), *i*PrOH (Hipersolv Chromanorm), CH_3CN (Prepsolv) and heptane (Lichrosolv) supplied by Merck KGaA (Germany) were used. For the supercritical fluid chromatography, liquid CO_2 (99.995%) obtained from Linde AG (Germany) was used. As additives trifluoroacetic acid (TFA; 99.5%) from Acros organics (Thermo Fisher Scientific, Belgium) and $HNEt_2$ (≥99.5%) supplied by Sigma-Aldrich Co. LLC (USA) were used.

2.3. Instrumentation and chromatographic conditions

A Waters Alliance system of the 2695 series with 2996 photo diode array detector with column oven was used for HPLC analyses. For SFC, a Waters Acquity UPC2 with Acquity photo diode array detector was used. All systems were connected with a column oven and controlled by Waters Empower software. The flow rates for HPLC were 1 mL/min and for SFC 2.5 mL/min, if not indicated otherwise. The HPLCs were operated in the isocratic mode and the SFC mainly with binary gradients. The solvents for the isocratic mode for HPLC separations were manually prepared. All mixtures were calculated by volumes. When additives were used, their concentration was 0.1% (v/v). The injection volume of the samples and the blanks was between 1 and 10 μL. The photo diode array detector was adjusted to a wavelength of 190–400 nm. The adjusted back pressure for SFC was 2000 psi. All studies were performed at a column temperature of 30 °C. The dead time was determined by the injection of the non-retained marker 1,3,5-tris-*tert*-butylbenzene. The resolution *Rs* of two peaks were calculated by using the following equation:

$$R_s = \frac{(2.35/2)(t_{r(2)} - t_{r(1)})}{W_{50(1)} + W_{50(2)}}$$

where $t_{r(1)}$ and $t_{r(2)}$ are the retention times and $W_{50(1)}$ and $W_{50(2)}$ the corresponding widths at the mid-high of peak 1 and peak 2.

3. Results and discussion

A central motivation was to provide scalable chromatographic methods for the separation of racemic spirocyclic terpenoid flavor compounds. We focused on chiral HPLC as a common laboratory method and on SFC as an attractive option for large scale separations in industry [52–55].

We have studied biocatalytic oxidations of spiroethers such as theaspirane [(*2R*,5R**)-**1**] and vitispirane [(*2R*,5R**)-**2**] by the edible fungus *Pleurotus sapidus* (*PSA*) [56–58]. These conversions with the lyophilisate of *PSA* gave a range of racemic oxidized spiroethers **3**-**7** as shown in Fig. 2 for the oxidation of theaspirane [(*2R*,5R**)-**1**] and vitispirane [(*2R*,5R**)-**2**] [51]. We used this set of racemic

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