



Qualitative profiling of mono- and sesquiterpenols in aglycon libraries from *Vitis vinifera* L. Gewürztraminer using multidimensional gas chromatography–mass spectrometry

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Abstract

In grape berries (*Vitis vinifera* L.), sesquiterpenes are mainly accumulated as hydrocarbons in the epicuticular wax layer of grapes, whereas monoterpenes, which are predominantly present as alcohols, are glycosylated and are stored as glycosides in the vacuoles of grape berry cells. In this study, extensive analysis of grape berry hydrolysates by means of comprehensive two-dimensional gas chromatography–time-of-flight–mass spectrometry demonstrated that glycosylated sesquiterpene alcohols show very little structural diversity when compared to the sesquiterpene hydrocarbon fraction in the cuticle and are glycosylated to a rather low extent when compared to monoterpenols. Twenty-four enzymatically released terpenols were found in hydrolysates of the aromatic white wine variety Gewürztraminer (*V. vinifera* subsp. *vinifera*) after previous solid-phase extraction and headspace solid-phase microextraction. The detection of only three sesquiterpene alcohols, namely farnesol, nerolidol and drimenol, shows that most sesquiterpene hydrocarbons do not have a related hydroxylated structure in grapes. Nevertheless, the presence of the acyclic aglycone farnesol and nerolidol may be of importance for the wine aroma, since these structural isomers can be converted into numerous sesquiterpenes by nonenzymatic acid-catalyzed reactions during wine production. Grape-derived glycosidically bound sesquiterpene alcohols, therefore, represent, in addition to free sesquiterpene hydrocarbons, another pool of compounds that may influence the aroma profile of wines.

Keywords Terpenes · Glycosides · Aglycones · Sesquiterpene alcohols · GC×GC · Wine aroma

Introduction

Terpenes play a key role as plant secondary metabolites for the aroma of grapes (*Vitis vinifera* L.) and wines [1]. Both mono- (C_{10}) and sesquiterpenes (C_{15}), which are formed

via the cytosolic mevalonate-dependent metabolic pathway (MVA) and the mevalonate-independent 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate biosynthesis pathway (DOXP/MEP) occurring in plastids [2, 3], are of central importance for the aroma [4, 5]. According to current knowledge, monoterpenes are stored predominantly as sugar bound alcohols in the vacuole whereas sesquiterpenes are accumulated as hydrocarbons in the nonpolar epicuticular wax layer of grape berries [6]. While free and volatile terpenes contribute directly to the aroma, a much larger fraction of glycosidically bound, non-volatile and thus aroma-inactive terpenoids is referred to as “hidden aromatic potential” in ripe grapes [7–9]. In 1974, the first evidence of glycosidically bound terpenes in grape berries was published by Cordonnier and Bayonove [10]. In the following decade, Williams et al. confirmed the presence of monoterpene glycosides by hydrolytic cleavage of the glycosidic bond, identifying both acyclic monoterpene alcohols such as linalool, nerol and geraniol as well as monocyclic terpenols such as α -terpineol as released aglycones

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[11–13]. 6-*O*- α -L-Rhamnopyranosyl- β -D-glucopyranosides, 6-*O*- α -L-arabinofuranosyl- β -D-glucopyranosides, 6-*O*- β -D-apiofuranosyl- β -D-glucopyranosides and β -D-glucopyranosides could be determined as sugar residues of monoterpene glycosides [11, 14]. While monoterpene glycosides in grapes have been extensively investigated in recent years [15–18], glycosidically bound sesquiterpenes in *V. vinifera* are still largely unknown. The first sesquiterpene glycosides in grapes were recently tentatively identified using ultra-high-performance liquid chromatography (UHPLC) quadrupole time-of-flight (qTOF) mass spectrometry (MS), but without further characterization of the aglycones [19]. Since glycosidically bound terpene alcohols are partly released both enzymatically and acid catalytically during winemaking [20, 21], grape-derived sesquiterpene glycosides represent an unexplored aroma potential, in addition to monoterpene glycosides. The potential importance of sesquiterpene alcohols for the wine aroma has recently been demonstrated by microvinification experiments. The yeast-derived, acyclic sesquiterpene alcohols nerolidol and farnesol can be converted into numerous other sesquiterpenes by acid-catalyzed reactions during the vinification process, thus significantly altering the sesquiterpene profile of wines [22]. In the presented study, the terpene alcohols released from grape-derived terpene glycosides were analyzed using the Gewürztraminer variety. The glycosidically bound terpenes from isolated exocarp of grapes were separated and concentrated using solid-phase extraction (SPE) and subsequently hydrolyzed enzymatically. The hydrolysates were analyzed by comprehensive two-dimensional gas chromatography–time-of-flight–mass spectrometry (GC \times GC–TOF–MS) after previous headspace solid-phase microextraction (HS-SPME).

Materials and methods

Chemicals

Milli-Q water, ethanol (purity: $\geq 99.8\%$) and zinc sulfate (purity: 99.9%) were purchased from VWR International (Darmstadt, HE, Germany). The solvents dichloromethane (purity: 99.99%) and methanol (purity: $\geq 99.9\%$) were acquired from Fisher Scientific UK Limited (Loughborough, Leicestershire, UK) and Honeywell (Seelze, NI, Germany). Di-sodium hydrogen phosphate (purity: $\geq 99\%$) and sodium dihydrogen phosphate monohydrate (purity: $\geq 99\%$) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, BY, Germany). Potassium ferrocyanide trihydrate (purity: $\geq 99\%$) was acquired from Acros Organics (Geel, Antwerp Province, Belgium). Citric acid monohydrate (purity: $\geq 99.5\%$) and geraniol (purity: $\geq 90\%$) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe,

BW, Germany) and the enzyme preparation (β -glucosidase, polygalacturonase) from Oenobrand SAS (Montpellier, Occitanie, France). The compounds citronellol (purity: $\geq 95\%$), nerol (purity: 98.7%), carvacrol (purity: 99.4%), thymol (purity: $> 99.9\%$), menthol (purity: 99.3%), dihydrocitronellol (purity: 98.3%) and a C₇–C₃₀ saturated alkane standard solution (certified reference material) were obtained from Sigma-Aldrich (Steinheim, BW, Germany). Nerolidol (*cis* + *trans*, purity: 97.5%), farnesol (mixture of isomers, purity: 98.0%), α -terpineol (purity: 96%) and linalool (purity: 98.5%) were purchased from Alfa Aesar (Ward Hill, MA, USA).

Sample material

The grape berries were sampled on two dates, September 15, 2016 and October 4, 2016 from two aromatic clones (11 Gm and FR 46-106) of Gewürztraminer at the Hochschule Geisenheim University (Geisenheim, HE, Germany; GPS coordinates: 49.98505, 7.94582; altitude: 96 m a.s.l.). The stage of maturation of the grapes was described on the basis of refractometrically determined soluble solids (September 15, 2016: 11 Gm, 19.0°Bx ; FR 46-106, 19.5°Bx and October 4, 2016: 11 Gm, 21.0°Bx ; FR 46-106, 23.0°Bx).

Sample preparation

Tissue extraction

The isolation of the terpene glycosides from the skin of grape berries was carried out based on a previous paper [23]. As described, the skin of the grapes was peeled off and adherent pulp was removed. For the terpene analysis, 20 g (fresh weight) of the grape berry skin was grounded in liquid nitrogen. The material was extracted under nitrogen and exclusion of light in a phosphate buffer (0.1 M; Na₂HPO₄/NaH₂PO₄; pH 7) and 13% (v/v) ethanol for 24 h. The extracts were clarified with Carrez reagents and then centrifuged at $24,000 \times g$, 5°C for 20 min. The supernatant was purified and concentrated by solid-phase extraction.

Solid-phase extraction (SPE)

To separate the free terpenes from the glycosidically bound terpenes a 500 mg Lichrolut EN column from Merck KGaA (Darmstadt, HE, Germany) was conditioned as stated previously [24]. The free terpenes were eluted with dichloromethane and the glycosidically bound terpenes with methanol. The fraction containing the separated glycosidically bound terpenes was concentrated under reduced pressure until dryness.

Hydrolysis

The residue was dissolved in 20 mL citrate-HCl-buffer (0.1 M; pH 4) and 100 mg of the enzyme preparation was added. All samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Sample preparation was performed three times, resulting in three biological replicates.

Analysis of the hydrolysates

The hydrolysates of the grape berries were thawed and homogenized before analysis. A Combi PAL-xt autosampler with a heatable agitator and fiber conditioning station from CTC Analytics (Zwingen, BL, Switzerland) was used. The terpene alcohols were extracted from headspace using a mixed SPME fiber for volatile and semivolatile compounds ($\text{C}_3\text{--C}_{20}$) from Supelco (Bellefonte, PA, USA), based on a method described by Welke et al. [25]. After each analysis, the SPME fiber was baked-out to avoid

sample carryover. In addition, empty vials were measured as control. The injection was carried out into a 7890B gas chromatograph from Agilent Technologies (Bellefonte, PA, USA) equipped with a ZX2 GC \times GC cryogenic modulator from Zoex Corp (Houston, TX, USA). Gas chromatographic separation was performed by combining a highly polar capillary column from Agilent Technologies (Bellefonte, PA, USA) with a medium polar GC column from MEGA s.n.c. (Legnano, MI, Italy), a combination that was used in a previous work for the analysis of sesquiterpene hydrocarbons in grape berry exocarp [26]. The GC \times GC separation was followed by a mass spectrometric analysis using a BenchTOF-Select time-of-flight–mass spectrometer from Markes International Limited (Llantrisant, Wales, UK). The method parameters are listed in Table 1. Available mono- and sesquiterpene standards were diluted in ethanol and the resulting 1 mg/kg solutions were injected with a liquid syringe (1 μL injection volume, split mode).

Table 1 HS-SPME–GC \times GC–TOF–MS conditions used for the analysis of hydrolysates from grape berries (Gewürztraminer cultivar)

| | Conditions |
|--------------------------------|---|
| HS-SPME | |
| HS vial | 10 mL headspace vial (screw cap) |
| Sample volume | 1 mL hydrolysate |
| SPME fiber | DVB/CAR/PDMS ^a ; 50/30 μm ; 2 cm; Stableflex; 24 Ga |
| Incubation conditions | 45 $^{\circ}\text{C}/0\text{ rpm}/10\text{ min}$ |
| Extraction conditions | 45 $^{\circ}\text{C}/0\text{ rpm}/30\text{ min}$ |
| Desorption conditions | 250 $^{\circ}\text{C}/5\text{ min}$ |
| Fiber bake-out conditions | 250 $^{\circ}\text{C}/15\text{ min}$ |
| GC\timesGC | |
| Injector mode | Splitless |
| Injector temperature | 250 $^{\circ}\text{C}$ |
| 1D GC column | DB-WAX Ultra Inert (30 m \times 0.25 mm \times 0.25 μm^b) |
| 2D GC column | MEGA-17 MS FAST (1.7 m \times 0.10 mm \times 0.10 μm^b) |
| Carrier gas | Helium |
| Gas flow | Constant flow, 1.0 mL min ⁻¹ |
| Initial inlet pressure | 24 psi |
| GC oven program | 35 $^{\circ}\text{C}$ (5 min)/5 $^{\circ}\text{C min}^{-1}$ to 120 $^{\circ}\text{C}$ (0 min)/3 $^{\circ}\text{C min}^{-1}$ to 220 $^{\circ}\text{C}$ (5 min) |
| Modulator temperature offset | + 25 $^{\circ}\text{C}$ |
| Modulation period | 5 s |
| Pulse length | 350 ms |
| TOF–MS | |
| MS transfer line temperature | 250 $^{\circ}\text{C}$ |
| Ion source temperature | 250 $^{\circ}\text{C}$ |
| Ionization mode | Electron impact (EI), -70 eV |
| Detector voltage | -2214 V |
| Mass range | 35–250 m/z |
| Scan rate | 100 Hz |

^aDivinylbenzene (DVB)/carboxen (CAR)/polydimethylsiloxane (PDMS)

^bLength \times internal diameter \times film thickness

Data analysis

Data were collected in ProtoToF (version 2.0) as *.LSC files and processed using the software platform ChromSpace (version 1.5.1). Both programs are from Markes International Limited (Llantrisant, Wales, UK). The data files were subjected to a dynamic background compensation (dbc) with a peak width of 100 ms. Data analysis was carried out using filtering methods (extracted-ion chromatogram, parametric filtering settings) and integration algorithms (persistence, deconvolution). Compound identification was performed using mass spectra (MS), retention indices (RI) and authentic standards (STD). Recorded mass spectra were compared with library spectra from the National Institute of Standards and Technology (NIST) database of the mass spectral search program (NIST, Gaithersburg, MD, USA; version 2.2). The similarity of the mass spectra was indicated by match factors (MF) and reverse match factors (RMF). After liquid injection of a saturated *n*-alkane standard solution (C₇–C₃₀), the retention indices (RI_{exp}) were calculated using the method of van Den Dool and Kratz for temperature-programmed gas chromatography [27]. The mono- and sesquiterpene alcohols were subsequently identified by comparing the experimentally determined retention indices with literature values (RI_{lit}). The sesquiterpene alcohols (*E*-nerolidol and farnesol as well as the monoterpene alcohols linalool, menthol, dihydrocitronellol, α -terpineol, β -citronellol, nerol, geraniol, thymol and carvacrol were additionally identified by commercially available standard compounds. To determine the ratio of sesquiterpene aglycones to monoterpene aglycones, the mean values of the peak areas of all terpenols were summed and the percentage of each analyte calculated.

Results and discussion

The gas chromatographic separation of terpene aglycones from grapes (*V. vinifera* L.) requires prior cleavage of the glycosidic bond by means of enzymes or acids. By combining suitable extraction methods (SPE–HS–SPME) with comprehensive two-dimensional gas chromatography–time-of-flight–mass spectrometry (GC×GC–TOF–MS), a total of 24 terpene aglycones were found in hydrolysates of grape berry exocarp. Figure 1 shows the two-dimensional separation of the released terpene alcohols.

Twenty-one monoterpene alcohols were identified in the hydrolysates of grape berries of the terpene-rich white wine variety Gewürztraminer (*V. vinifera* subsp. *vinifera*, clones 11 Gm and FR 46-106). Among these, the structural diversity of the *p*-menthane-related monoterpenols is remarkable. The detection of menthol is particularly interesting because its precursor piperitone contributes to the positive mint aroma of red Bordeaux wines, as described by Picard et al. [28]. The large number of monoterpenols found in the hydrolysates is in marked contrast to the few free monoterpene alcohols found in the volatile profile of ripe grape berries (Gewürztraminer cultivar) identified by May [29]. The GC×GC–TOF–MS measurements thus confirm the assumption that monoterpenols are predominantly glycosidically bound in grapes [6].

Although more than 80% of the peak area of all terpene aglycones detected can be attributed to only six monoterpenols, nerol, α -terpineol, linalool, geraniol, β -citronellol and dihydrocarveol, the first detection of grape-derived sesquiterpene aglycones in the Gewürztraminer variety, especially (*E*-nerolidol and farnesol [30, 31], is noteworthy. While previous studies showed that free mono- and sesquiterpene hydrocarbons as well as monoterpene alcohols mainly characterize the wine aroma, the detection of grape-derived

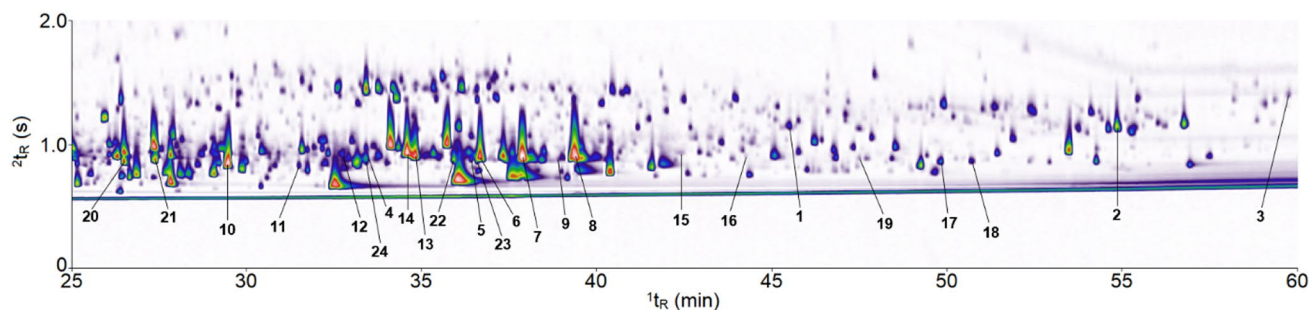
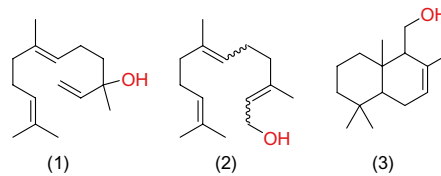


Fig. 1 GC×GC–TOF–MS total ion chromatogram (TIC) of a hydrolysate from grape berries of the white wine variety Gewürztraminer (*Vitis vinifera* subsp. *vinifera*). The x axis represents the primary column retention time (1t_R) and the y axis corresponds to the retention time on the secondary column (2t_R). The peak intensity, which is proportional to analyte concentration, is represented by color-coding

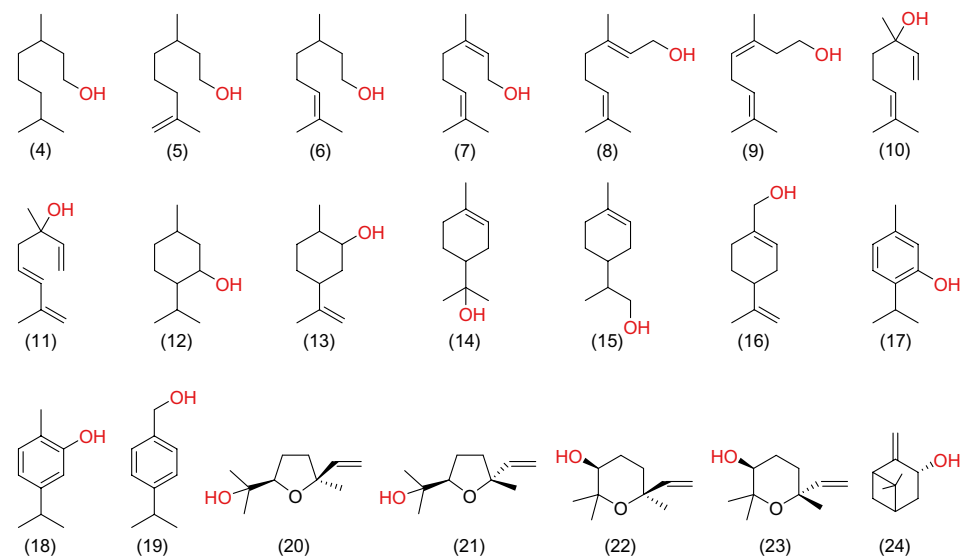
from low (violet) to high (red) on a white background. Note that a logarithmic color gradient has been selected, as numerous trace volatile compounds would otherwise not be visible in a single contour plot due to some very dominant peaks. Peak numbers of mono- and sesquiterpene aglycones released by enzymatic hydrolysis refer to those of Fig. 2 and Table 2

Fig. 2 Structural formulas of the enzymatically released terpene alcohols from grape-derived terpene glycosides, grouped by sesquiterpene and monoterpene aglycones. 1–2 acyclic sesquiterpene alcohols, 3 bicyclic sesquiterpene alcohol, 4–11 acyclic monoterpene alcohols, 12–23 monocyclic monoterpene alcohols, 24 bicyclic monoterpene alcohol

Sesquiterpene aglycones



Monoterpene aglycones



sesquiterpene aglycones reveals an undiscovered pool of potential aroma components in the Gewürztraminer variety.

It is known that wine yeasts are able to form the sesquiterpene alcohols farnesol and nerolidol [32]. Recently, deuterium-labeling experiments showed that yeast-derived farnesol and nerolidol significantly alter the terpene profile of wines during the fermentation process [22]. The sesquiterpene alcohols act as precursors for the formation of numerous other sesquiterpenes. Due to the slightly acidic pH during vinification, many sesquiterpenes can already be formed by acid catalysis from nerolidol and farnesol without the presence of corresponding sesquiterpene cyclases [33].

In addition to the acyclic sesquiterpene alcohols nerolidol and farnesol, the first tentative and indirect detection of glycosidically bound drimenol in grape berry exocarp was obtained by GC×GC–TOF–MS analysis. The bicyclic drimenol has a drimane skeleton that has not been found in *V. vinifera* L. so far. The detection of drimenol is particularly interesting due to the antifungal activity against *Botrytis cinerea* [34], a pathogen that affects numerous plants including grapes [35]. At this point, it should be mentioned

that drimenol can also be formed by rearrangement from farnesol [36].

The fact that only three sesquiterpene alcohols were found in the hydrolysates after extensive GC×GC analysis shows that sesquiterpene alcohols are rather by-products of sesquiterpene hydrocarbon synthases that are responsible for the biosynthesis of structurally diverse carbon skeletons. Glycosylated sesquiterpene alcohols are probably stored in the vacuole of the grape berry cell as water-soluble flavor precursors, similar to glycosylated monoterpene alcohols [37].

It should also be noted that both, numerous monoterpene hydrocarbons and numerous monoterpene alcohols are present in grape berries. In the case of sesquiterpenes, hydrocarbons dominate the volatile profile without the corresponding sesquiterpene alcohol analogues. However, these grape-derived glycosidically bound sesquiterpene alcohols, in particular farnesol and nerolidol, represent another important source of aroma precursors that may influence the wine aroma.

Table 2 Identified terpene aglycones enzymatically hydrolyzed from terpene glycosides in Gewürztraminer grapes (*Vitis vinifera* subsp. *vinifera*) using SPE–HS–SPME–GC×GC–TOF–MS

| No. | Terpene aglycone | Molecular formula | Percentage ^c | Identification criteria | | | |
|-------------------------|--|--|-------------------------|--------------------------------|--------------------------------|---------------------------|-------------------|
| | | | | RI _{exp} ^d | RI _{lit} ^e | MS (MF, RMF) ^f | STD ^g |
| Sesquiterpene aglycones | | | | | | | |
| 1 | (<i>E</i>)-Nerolidol ^a | C ₁₅ H ₂₆ O | 0.47 ± 0.31% | 2040 | 2040 [38] | 805, 852 | a. ⁱ |
| 2 | Farnesol ^a | C ₁₅ H ₂₆ O | 2.68 ± 1.71% | 2357 | 2354 [39] | 832, 835 | a. ⁱ |
| 3 | Drimenol ^b | C ₁₅ H ₂₆ O | 0.19 ± 0.03% | 2505 ^h | 2494 [40] | 800, 816 | n.a. ^j |
| Monoterpene aglycones | | | | | | | |
| 20 | (<i>Z</i>)-Furan linalool oxide ^b | C ₁₀ H ₁₈ O ₂ | 3.05 ± 0.72% | 1454 | 1454 [41] | 842, 850 | n.a. ^j |
| 21 | (<i>E</i>)-Furan linalool oxide ^b | C ₁₀ H ₁₈ O ₂ | 3.39 ± 1.02% | 1483 | 1483 [42] | 804, 814 | n.a. ^j |
| 10 | Linalool ^a | C ₁₀ H ₁₈ O | 13.84 ± 0.84% | 1547 | 1547 [43] | 797, 808 | a. ⁱ |
| 11 | Hotrienol ^b | C ₁₀ H ₁₆ O | 0.13 ± 0.04% | 1608 | 1613 [44] | 729, 763 | n.a. ^j |
| 12 | Menthol ^a | C ₁₀ H ₂₀ O | 0.86 ± 0.13% | 1645 | 1644 [45] | 856, 874 | a. ⁱ |
| 24 | (<i>E</i>)-Pinocarveol ^b | C ₁₀ H ₁₆ O | 1.19 ± 0.19% | 1666 | 1659 [46] | 815, 831 | n.a. ^j |
| 4 | Dihydrocitronellol ^a | C ₁₀ H ₂₂ O | 0.17 ± 0.02% | 1669 | 1666 [47] | 732, 748 | a. ⁱ |
| 14 | α-Terpineol ^a | C ₁₀ H ₁₈ O | 15.70 ± 1.58% | 1703 | 1703 [48] | 848, 889 | a. ⁱ |
| 13 | Dihydrocarveol ^b | C ₁₀ H ₁₈ O | 10.43 ± 0.64% | 1709 | 1702 [49] | 724, 757 | n.a. ^j |
| 22 | (<i>Z</i>)-Pyran linalool oxide ^b | C ₁₀ H ₁₈ O ₂ | 2.05 ± 0.45% | 1744 | 1744 [50] | 774, 801 | n.a. ^j |
| 5 | α-Citronellol ^b | C ₁₀ H ₂₀ O | 0.25 ± 0.03% | 1759 | 1767 [47] | 883, 924 | n.a. ^j |
| 23 | (<i>E</i>)-Pyran linalool oxide ^b | C ₁₀ H ₁₈ O ₂ | 0.52 ± 0.51% | 1764 | 1763 [51] | 828, 839 | n.a. ^j |
| 6 | β-Citronellol ^a | C ₁₀ H ₂₀ O | 11.33 ± 2.02% | 1767 | 1767 [52] | 846, 859 | a. ⁱ |
| 7 | Nerol ^a | C ₁₀ H ₁₈ O | 20.45 ± 3.91% | 1805 | 1803 [53] | 813, 821 | a. ⁱ |
| 9 | (<i>Z</i>)-Iso-geraniol ^b | C ₁₀ H ₁₈ O | 0.17 ± 0.04% | 1835 | 1849 [54] | 767, 789 | n.a. ^j |
| 8 | Geraniol ^a | C ₁₀ H ₁₈ O | 11.50 ± 0.44% | 1848 | 1848 [55] | 804, 809 | a. ⁱ |
| 15 | <i>p</i> -Mentha-1-en-9-ol ^b | C ₁₀ H ₁₈ O | 0.17 ± 0.03% | 1943 | 1948 [56] | 814, 868 | n.a. ^j |
| 16 | Perillyl alcohol ^b | C ₁₀ H ₁₆ O | 0.03 ± 0.01% | 2000 | 2004 [57] | 693, 762 | n.a. ^j |
| 19 | Cuminol ^b | C ₁₀ H ₁₄ O | 0.08 ± 0.01% | 2106 | 2101 [58] | 763, 804 | n.a. ^j |
| 17 | Thymol ^a | C ₁₀ H ₁₄ O | 0.84 ± 0.19% | 2182 | 2183 [59] | 876, 898 | a. ⁱ |
| 18 | Carvacrol ^a | C ₁₀ H ₁₄ O | 0.54 ± 0.17% | 2213 | 2212 [60] | 905, 916 | a. ⁱ |

^aIdentified by retention index, mass spectrum and authentic standard

^bTentatively identified by retention index and mass spectral data

^cPercentage of aglycone determined by the sum of peak areas. The mean value and the standard deviation resulting from the integration of three biological replicates are given

^dExperimental retention indices on a polar DB-WAX Ultra Inert column calculated according to the method of van Den Dool and Kratz for temperature-programmed gas chromatography

^eRetention indices reported in the literature for equivalent capillary GC columns

^fMean mass spectral match quality. The mean values of the match factors (MF) and reverse match factors (RMF) for the respective terpene aglycones are listed

^gLiquid injection of an authentic standard for compound identification

^hRI_{exp} value has been extrapolated

ⁱAn authentic standard was commercially available (a.) and was measured using GC×GC–TOF–MS. The purity of this reference material was documented in the “Materials and methods”

^jAn authentic standard material was not available (n.a.)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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