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Identification of wine aroma precursors in *Moscato Giallo* grape juice: A nuclear magnetic resonance and liquid chromatography–mass spectrometry tandem study

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ABSTRACT

In this work, several aroma precursors present in *Moscato Giallo* grape juice were identified and characterized using LC–MS and NMR techniques. A preliminary separation of various fractions was obtained using adsorption on Amberlite® XAD resin and HPLC chromatography on a reverse phase column. Subsequently, U-HPLC with mass spectrometry allowed the identification of some compounds corresponding to mono- and disaccharides linked to terpenes. The MS–MS fragmentation step indicated which kind of glycosides, the moiety sequence and sometimes which kind of terpene were present. NMR enabled the correct identification of glycosides and terpene when the fraction analyzed was sufficiently concentrated and with few components. Twelve glycosidically bound terpenes were characterized: (E) and (Z)-furanosyl-linalooloxide-7-O-[α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], (E)-furanosyl-linalooloxide-7-O-[1-β-D-glucopyranoside], (Z)-8-hydroxylinalool-8-O-[1-β-D-glucopyranoside], 1,2-dihydroxylinalool-1-O-[1-β-D-glucopyranoside], linalool-3-O-[α-L-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside], linalool-3-O-[α-L-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], linalool-3-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside], nerol-1-O-α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside, geraniol-1-O-[α-L-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside], geraniol-1-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside], and a geranic acid disaccharide derivative. It is the first time that this kind of compounds are directly detected and identified in a mixture with these two techniques.

1. Introduction

The aroma of grapes such as Muscat of Alexandria, Yellow Muscat, Muscat Frontignan is conferred by the presence of terpenes, as terpenols in various oxidation states, and of terpenoid polyols [1,2] that accumulate in the berry during ripening [3,4].

According to Ribereau-Gayon et al. [5] also hotrienol, and furan and pyran forms of linalool oxides give Muscat its characteristic aroma. They hypothesized also an increment in the aroma of wine because terpenes may be present as such or as aroma precursors, in the form of glycosylated disaccharides such as α-L-arabinofuranosyl-β-D-glucopyranosides or α-L-rhamnofuranosyl-β-D-glucopyranosides, the aglycone of which can be a terpenol, a terpene diol, 2-phenylethanol or benzyl alcohol. It was found that total bound terpenols, after enzymatic hydrolysis, are about 2–3 times more abundant than the free ones. They were prevalently geraniol, linalool, nerol, α-terpineol, citronellol, both free and glycosylated [6]. During vinification, they may undergo enzymatic hydrolysis by endogenous or exogenous enzymes [7–16].

Among the Muscat grapes, *Moscato Giallo* is characterized by a high presence of terpenes [17]. In particular, the terpenes identified as characteristic, found in the volatile fraction in elevated quantity are: linalool, geraniol, trace of oxylinalool, limonene, α-terpineol, citronellol and myrcene [18]. Williams et al. [19] state that their precursors are a mixture of disaccharides.

Free and bound monoterpenes were determined directly and after enzymatic hydrolysis, respectively, by Wilson et al. [20]. Also Karagiannis et al. [21] found nerol oxide, linalool, α-terpineol, citronellol, nerol, and geraniol among the volatile aromas.

Sánchez-Palomo et al. [22] used and compared different extraction methods for volatile compounds in Muscat: liquid–liquid extraction, solid-phase extraction and a simultaneous steam distillation-solvent extraction technique.

Wirth et al. [23] isolated the glycoconjugates from Muscat and Shiraz leaves and grape berries, by adsorption on Amberlite® XAD-2 resin, and analyzed the enzymatically released aglycons by GC-FID and GC–MS.

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Many authors used GC–MS to analyze and quantify free and bound terpenes and other aromas, after hydrolysis in grape juice and wines [6,24–26]. The limit of this method in the case of glycosylated terpenes is the hydrolysis step. It is documented that acid hydrolysis may cause modification and rearrangement in terpenes [27], but it does not allow the identification of glycosides, unless subsequently derivatized [28–30]. Recently, some other authors used GC–MS to analyze and quantify free and bound terpenes and other aromas, after hydrolysis of grape juice and various wine components [29]. Dziadas and Jeleń [30] analyzed terpenes in white wines using a SPE–SPME-GC/MS approach. On the other hand, Tamborra and Esti [31] identified authenticity markers in Aglianico, uva di Troia, Negroamaro and Primitivo grapes.

Very few papers deal with the LC–MS analysis of aroma precursors because glycosidic terpenes are usually extracted, hydrolyzed and identified with GC–MS through their hydrolyzed products. One of the first studies with LC–MS deals with the application of fast atom bombardment tandem mass spectrometry (FAB MS/MS) in positive and negative ion modes to the characterization of bound terpenes in Riesling wine. FAB MS/MS assignments were supported by ^{1}H NMR data on isolated and derivatized glycosides, which allowed the characterization of various terpene disaccharides [32]. Using also other techniques, the authors found five terpene-disaccharides, three of which were identified as 2,6-dimethylocta-2,7-diene-1,6-diol-API-GLU, 2,6-dimethylocta-2,6-diene-1,8-diol-API-GLU, and 3,7-dimethyloct-2-ene-l,7-diol-ARA-GLU. Another paper reported the use of ESI-MS–MS together with two-dimensional ^{1}H and ^{13}C NMR spectra for the identification of two linalool disaccharides: 3(S)-linalool-3-O-β-D-glucopyranosyl-β-D-apiofuranoside and 3(S)-linalool-3-O-β-D-glucopyranosyl-α-L-arabinopyranoside in green coffee beans [33]. Baltenweck-Guyot et al. isolated two β-D-glucopyranosides and two 6-O-β-D-apiofuranosyl-β-D-glucopyranosides of (E)-6,9-dihydroxymegastigma-4,7-dien-3-one from *Vitis vinifera* cv. Gewürztraminer wine, and characterized their structures by NMR spectroscopy [34].

Villena et al. quantified aroma precursors in grape, must and wine with a method based on the isolation of terpenyl-β-D-glycosides by reversed-phase HPLC, followed by hydrolysis to release terpenes and saccharides. The latter were measured using an enzyme analysis kit [35].

Nasi et al. identified free and bound volatile compounds as typicity and authenticity markers of non-aromatic Falanghina grapes and wines by combining various mass spectrometric techniques, such as GC/MS, LC/ESI-MS, MALDI-TOF-MS [36]. The GC/MS analysis of TMS-derivatives of terpene glycosides showed the presence of glucosides, arabinosylglucosides, and rhamnosylglucosides of linalool and geraniol.

The scarceness of studies on intact aroma precursors prompted us to investigate a potential protocol to identify and possibly even quantify these compounds with as little sample treatment as feasible. In this work, aroma precursors from *Moscato Giallo* grape juice (mono and oligosaccharide terpene derivatives) are characterized in their integrity by LC–MS–MS and NMR spectroscopy, without hydrolysis or derivatization. This is a first step toward a protocol that would be a help to understand the developing of wine aroma and also to improve winemaking.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Methanol, ammonia and acetic acid were purchased from Sigma-Aldrich. Pure water was obtained with a Millipore MilliQ system apparatus.

2.2. General procedures

Analytical grade solvents were used for extractions and flash chromatography. Ethanol was HPLC-grade (VWR International, Fontenay-sous-Bois, France). Amberlite® XAD-2 resin was purchased from Fluka Chemie (Büchs, CH) and lead acetate, $Pb(OAc)_2$, from Merck (Steinheim, Germany). Stationary phases cyanopropyl silica (LiChroprep® CN 40–63 μm, Merck, Darmstadt) and ODS (LiChroprep® RP-18 40–63 μm, Merck, Darmstadt) were employed for flash chromatography. The preparative HPLC system consisted of a Merck Hitachi pump model L-7100, an L-7400 UV detector, a D-7500 integrator and a Rheodyne manual injector equipped with a 200 μL loop. The HPLC column was a Synergi Hydro (150 × 10 mm^2, 4 μm particle size, 80 Å pore size; Phenomenex, Torrance, CA, USA).

2.3. Preparation of the glycosidic fraction

The grapes used were *Moscato Giallo* (yellow Muscat) of the Colli Euganei harvested at a good level of ripeness and in perfect health as deduced from the absence or limited presence of metabolites typical of Botrytis.

Fresh grape juice (3 L) was centrifuged at 3000 rpm for 15 min at room temperature. The supernatant (in batches of about 1 L) was leached three times through a column packed with Amberlite® XAD-2 Fluka Chemie Büchs (CH), 25 × 4 cm i.d. The column was washed with water (2 L) and ethanol was used for elution (2 L). The three elutes were pooled and concentrated to dryness in vacuo to give about 2 g of organic material.

2.4. Isolation of the glycosidic fractions

Aliquots of dry extract were subjected to flash chromatography (8 × 4 cm i.d.) on a cyanopropyl silica column (LiChroprep® CN 40–63 μm, Merck, Darmstadt) with stepwise elution of 60% (200 mL, fr. 1) and 100% (400 mL, fr. 2) ethyl acetate in hexane, then 5% (200 mL, fr. 3) and 100% (200 mL, fr. 4) ethanol in ethyl acetate. The combined flash fractions 2 and 3 (850 mg) were dried and dissolved in 40 mL of methanol and added of lead acetate (2.5 g). The mixture was stirred for two hours and then centrifuged at 3000 rpm for 15 min. The precipitate was washed with methanol and discarded. ODS (LiChroprep® RP-18 40–63 μm, Merck, Darmstadt) stationary phase was added to the clear solution and the solvent was evaporated. The desiccated slurry was applied to a column (8 × 4 cm i.d.) and chromatographed with a gradient of methanol in water. The central fractions, eluted with methanol ranging from 15% to 70% (240 mg), were dried and dissolved in ethanol and purified by reversed phase HPLC with a Synergi Hydro column (150 × 10 mm, 4 μm particle size, 80 Å pore size; Phenomenex, Torrance, CA, USA) and the detection wavelength set at 210 nm. The eluents were ethanol:water 50:50 v/v (B) and water (A). The gradient was changed linearly from 60% to 80% B in 30 min. Peaks marked as A, B, C, D, E, and F eluted at RT = 4.5 min (8.0 mg), 6.8 min (3.5 mg), 9.4 min (2.0 mg), 12.5 min (2.2 mg), 16.2 min (4.5 mg) and 17.1 min (7.0 mg), respectively.

2.5. NMR spectroscopy

NMR spectra were recorded with a Bruker AVANCE DMX 600 spectrometer operating at 600 MHz, with the following experimental parameters. ^{1}H-1D: 32 scans, TD 32,768 points, spectral width 14 ppm, relaxation delay 1 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at its frequency during the recycle delay. ^{13}C-1D: 65,600 scans, TD 65,536 points, spectral width 220 ppm, relaxation delay 2.5 s. ^{11}H COSY: 112–174 scans, 2048 × 400 data points,

spectral width between 6 and 13 ppm, relaxation delay 1 s. ^{11}H TOCSY: 80–128 scans, 2048 × 512 data points, spectral width between 6 and 13 ppm, relaxation delay 1.5 s. ^{11}H NOESY: 200–216 scans, 2048 × 512 data points, spectral width between 6 and 14 ppm, relaxation delay 1.5 s. ^{113}C HMQC: 180–304 scans, 2048 × 128 data points, spectral width between 8 and 13 ppm for proton and between 170 and 220 ppm for carbon, relaxation delay 2 s; ^{113}C folded HMQC: same parameters as HMQC, except for spectral width between 8 and 11 ppm for proton and between 20 and 50 ppm for carbon. ^{113}C HMBC: 256–360 scans, 2048 × 128 data points, spectral width between 7 and 13 ppm for proton and between 150 and 220 for carbon, relaxation delay 1.85 s.

All NMR spectra were taken at a probe temperature of 298 K using a 5 mm standard tube. The solvent used was CD_3OD, 600 μL (Aldrich ≥99%). Chemical shifts are quoted in parts per million (reference: tetramethylsilane). Multiplicities are abbreviated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet.

2.6. HPLC

The U-HPLC system was an Ultra Pressure 1200 Agilent (Santa Clara, CA, USA). Flow rate was 0.2 mL min^{-1}. Analytical U-HPLC was performed with a column core shell Kinetex, C18 100 × 2.1 mm, 2.6 μm (Phenomenex, Torrance, CA, USA). The eluents were: water (A) and methanol (B) both with 5 mM ammonium acetate. The gradient was: 0–5 min 20% B; 5–25 min, from 20% to 100% B; 25–30 min, 100% B; 30–31 min, from 100% to 20% B; a column conditioning step from 31 to 36 min at 20% of B. Flow rate was 0.2 mL min^{-1}, column temperature 30 °C, and injection volume 10 μL.

2.7. MS

The LC–MS–MS instrument was an Agilent (U-HPLC 1200 equipped with photodiode array detector (DAD) and Q-TOF 6520 MS–MS detector with Electro-Spray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photochemical Ionization (APPI) sources). MS operating conditions: ESI mode, positive; capillary voltage 3500 V; nebulizer gas 35 psig; drying gas 11 L min^{-1}, 350 °C; fragmentor (declustering) 120 V. In MS–MS modality: collision gas nitrogen; CID collision voltage: 5–10 V. TOF (reflectron) was characterized by medium-high resolution (FWHM 18,000, sulphadimethoxine *m/z* 310 amu) and good mass accuracy (Δ*m* 2 and 4 ppm, respectively, in MS and MS–MS mode). Mass calibration was performed externally with an Agilent standard mixture every day, and internally monitoring 2–3 specific masses.

Linalool-glucoside, nerol-glucoside and citronellol-glucoside were used as standards: their retention times, the precursor ion and fragmentation pattern in ammoniate form were analyzed with HPLC and positive ESI MS.

3. Results and discussion

The fractions obtained from the preparation steps, marked as A, B, C, D, E, and F, according to the elution order, were analyzed by NMR and LC–MS techniques. Usually, this kind of compounds is studied by GC–MS and NMR techniques after a derivatization step or hydrolysis. In this work, the fractions were analyzed without any preparative chemical reaction. Mass and NMR spectra showed that each fraction contained several glycosylated terpenes. Most of these compounds have very similar structures, composed of terpenes with a common molecular formula linked to a monosaccharide or a disaccharide.

The total ion current (TIC) chromatogram of each fraction evidenced several peaks so that the identification of some specific mass to recognize terpene-glycosides was mandatory. We focused our attention on C5 and C6 glycosides and on the basic terpene formula $C_{10}H_{18}O$. Ammoniate adducts, that more easily generate fragments in positive ionization mode, were especially searched for. Some peaks showed a mass spectrum with many characteristic *m/z* values, for example 153 ($C_{10}H_{18}O^+$) and 135 ($C_{10}H_{16}^+$), typical of free terpenes. They were present also as secondary ions in many chromatographic peaks.

The 1D-^{1} spectra of all the fractions in methanol are reported in Fig. 1. Three spectral regions were easily identified: 0–2.5 ppm, containing overall terpene aliphatic signals; 3–5 ppm, containing most sugar backbone signals; 5–6.1 ppm, containing olefinic terpene signals. Because of several signals overlap in 1D spectra, detailed structural determination was achieved using several 2D experiments.

In general, 2D-homonuclear correlation spectroscopy and 1D-selective TOCSY experiments were used to assign all the ^{1}H spin systems present in the mixture while 2D-heteronuclear experiments (^{1}H/^{13}C) and NOESY spectra were used to connect the various spin systems belonging to the same molecule. The same methodology was used to identify all the compounds. Below, we describe in detail the assignment strategy for the aroma precursor **a′** (Fig. 2). For the other molecules, only important correlations will be highlighted in the text. In Table 4, the complete resonance assignment is reported. Atom numbers are referred to Fig. 2.

In the ^{1}H-1D spectrum of fraction A, three different doublets of doublets are present at 5.91, 5.92 and 6.01 ppm, as shown in Fig. 1. They correspond to protons belonging to three terminal double bonds. Starting from these signals, the elucidation of the structures of the three most concentrated compounds was reached owing to the correlations found in 2D spectra.

Compound **a′** was the most concentrated species present in this fraction. In the TOCSY spectrum, its proton at 5.92 ppm (H-2), belongs to a monosubstituted olefinic spin system; *J* values and also NOE cross-peaks reveal the correct geometry of the three vinyl protons while the HMQC spectrum allows the assignment of the ^{13}C chemical shifts. The HMBC and NOESY spectra revealed the connectivities beyond the quaternary carbon (C-3), which stops the proton spin system: its substituents are a methyl group, an –OH or –OR group, and an R chain that starts with an aliphatic carbon. Further analysis of the HMQC and TOCSY spectra allowed the identification of a furanosidic heterocycle. The terpene skeleton was completed finding a dimethylmethanolic group: the terpene was identified as furanosyl-linalooloxide. Finally, an NOE cross-peak between the olefinic protons and H-6 (and the absence of NOE correlations between H-10 and H-6) demonstrated that this isomer is “E” with respect to the pseudo molecular plane generated by the ring. The absolute configuration of the terpene chiral carbons was not determined; rather, the results by Weckerle et al. [33] were used.

The presence of a glycosidic linkage was deduced from the NOE observed between the anomeric H-1′ of the sugar and H-6, H-8 and H-9 of the aglycone and also from the HMBC cross-peak between the anomeric proton H-1′ of the sugar and C-7 of terpene (shown in Fig. 3).

The entire spin system of the sugar was found and identified as that of glucose in the TOCSY spectrum. The correct order of protons was established using COSY cross-peaks and also correlations in heteronuclear experiments. The β-configuration of the glucose moiety was proved by the coupling constant of the anomeric proton $^3J_{1,2}$=7.7 Hz. Intense connectivities were observed in the HMBC spectrum, between the anomeric proton of the second sugar moiety and the C-6′ carbon of the glucose

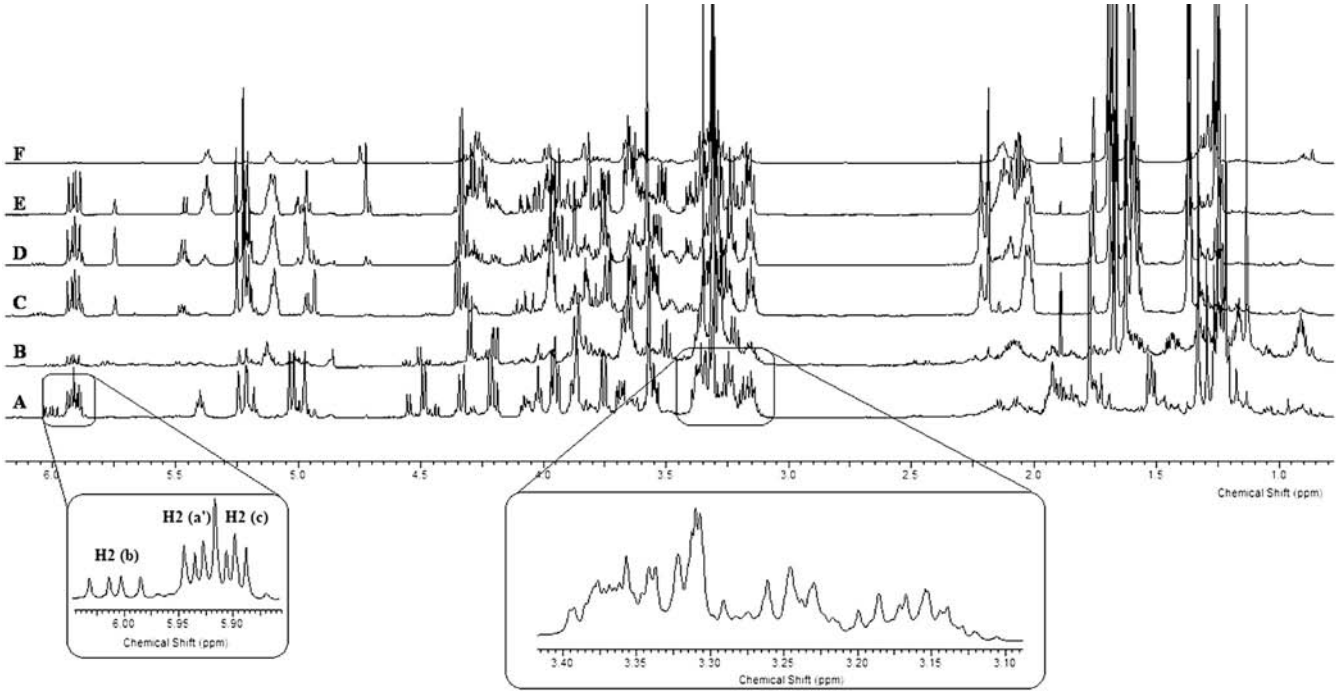

Fig. 1. ^{1}H NMR spectra of the fractions in methanol-d_4 (from the bottom: A–F). In the bottom of the figure, terpenic and sugar regions are zoomed.

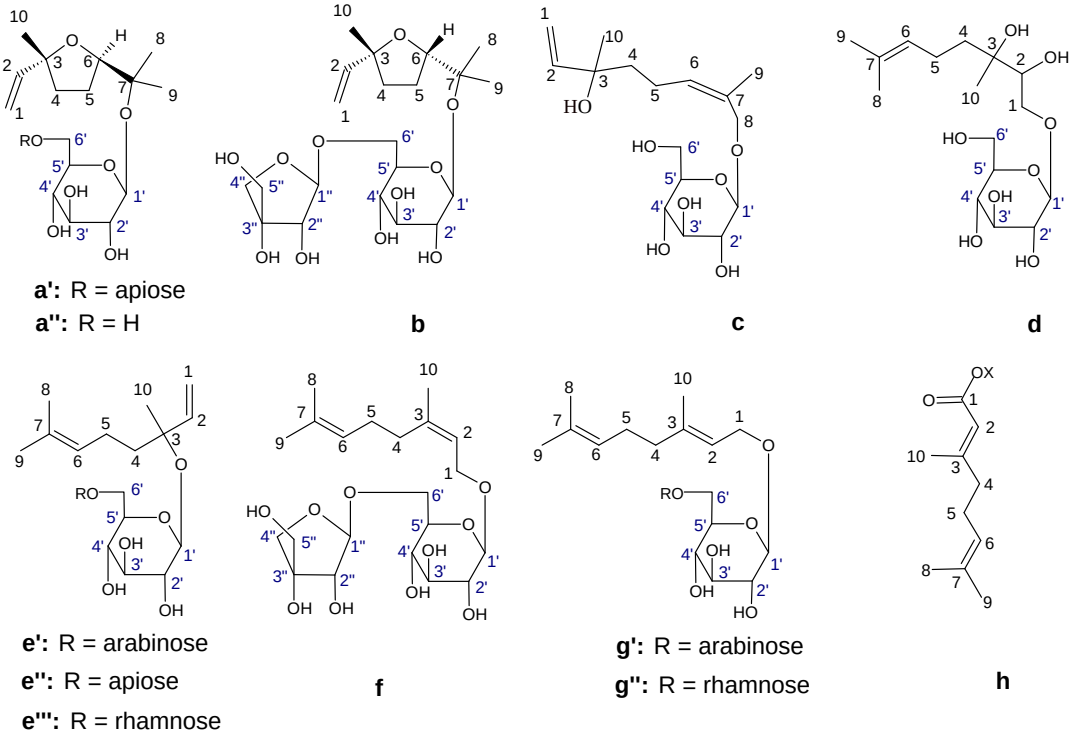

Fig. 2. Structures of aroma precursors identified in *Moscato Giallo* grape juice.

moiety. The second sugar was unambiguously identified by 2D spectra correlations as the pentose, apiose. A coupling constant of 2.3 Hz between the anomeric H-1″ and the H-2″ protons of the terminal apiose unit indicated a β-configuration [10,33]. The compound was therefore fully characterized as **a**′, (E)-furanosyl-linalooloxide-7-O-[β−D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside].

The analysis of the different fractions and the identification of several compounds is described below.

3.1. Fraction A

A first direct infusion in MS without additives produced prevalently the ion *m*/*z* 487. A chromatogram of fraction A, reported in Fig. 4a, shows the presence of eight peaks. Table 1 shows the corresponding masses. The MS spectrum of the peak at 15.30 min, obtained in the presence of ammonium acetate, showed a group of peaks at *m*/*z* 482, 487, and 503, differing by

5 and 16 amu, respectively, that represent the ammonium, sodium and potassium adducts of a molecule with *m/z* 464. This mass corresponds to $C_{21}H_{36}O_{11}$ ($C_{10}H_{18}O_2$+$C_6H_{12}O_6$+$C_5H_{10}O_5$–$2H_2O$) and may be a terpene linked to a disaccharide composed by glucose (GLU) and apiose (API) or arabinose (ARA). Also ions 153 and 135, identified by accurate mass as terpene ion and dehydrated terpene ion, were present in the mass spectrum. Their presence may be explained by assuming a partial fragmentation of the molecular ion **a**′ (Fig. 5a).

The Extracted Ion Chromatogram (EIC) of ion *m/z* 482 corresponded to four isomers eluted at 13.89, 15.40, 16.28 and 16.72 min, with relative intensities 0.1:2:3:0.05. The fragmentation spectrum obtained in MS–MS at 10 eV for the highest peak is reported in Fig. 5b. It shows that the principal fragment corresponds to the dehydrated oxylinalool ion (*m/z* 153). Other peaks are justified in Table 2. The fragmentation reported above is also obtained using a lower fragmentation energy (5 eV), because these compounds are particularly labile (the sodium adducts do not give any fragment). The NMR analysis of this fraction allowed us to identify the exact structure of the two molecules with *m/z* 464: compound **a**′ (described above) and compound **b**, (Z)-furanosyl-linalooloxide-7-O-[β-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], an isomer of **a**′, with the only difference of the "Z" form of the terpene, proved by NOE correlations. Isomers **a**′

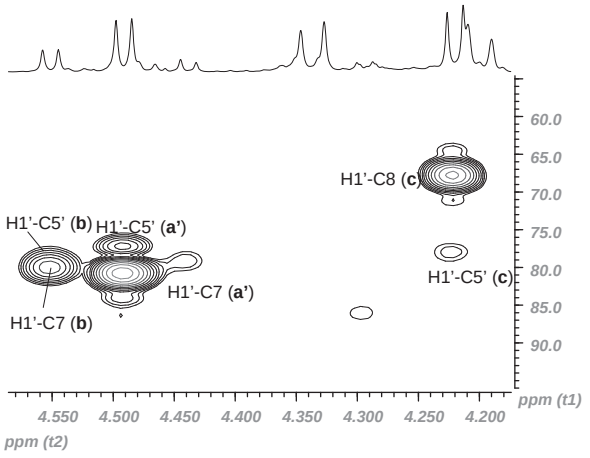

Fig. 3. Expanded region of the HMBC spectrum of fraction A. The correlations between glucose anomeric protons and C5′ and terpene carbons are shown.

Table 1
Ions relative to chromatographic peaks in Fig. 4a.

	RT (min)	*m/z* Ion	Δ*m/z* (ppm)*	Ion	Molecule
1	15.30	482.2615	4	$M+NH_4^+$	$C_{21}H_{36}O_{11}$
		487.2172	4	$M+Na^+$	
		503.1918	6	$M+K^+$	
		355.1742	4	M_1+Na^+	$C_{16}H_{28}O_7$
2	16.28	482.2617	4	$M+NH_4^+$	$C_{21}H_{36}O_{10}$
		487.2175	5	$M+Na^+$	
3	17.12	496.2773	4	$M+NH_4^+$	$C_{22}H_{38}O_{11}$
		501.2331	5	$M+Na^+$	
4	20.89	320.2166	–	n.i.	–
5	24.86	Contaminant			
6	25.9	Contaminant			
7	26.00	Contaminant			
8	26.63	310.2390	–	n.i.	–
9	30.5	Contaminant			

* Difference from exact mass.

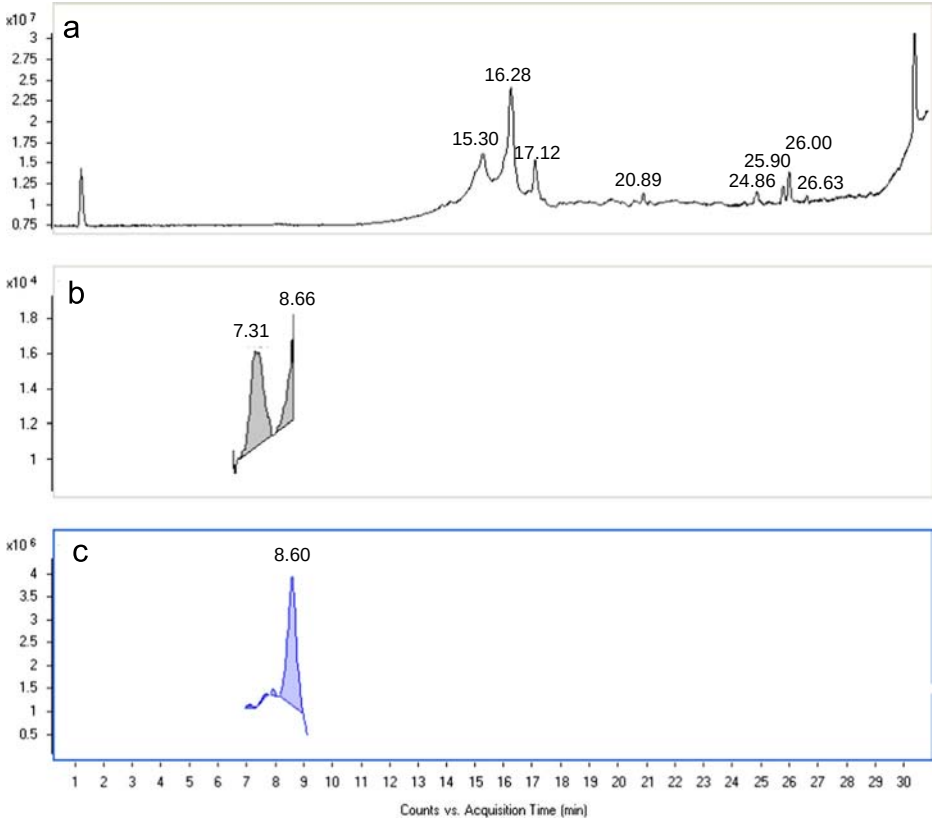

Fig. 4. (a) chromatogram in Total Ion Current (TIC) of fraction A. See conditions in the text; (b) EIC chromatogram for *m/z* 368 in fraction B; (c) EIC chromatogram for *m/z* 466 in fraction E.

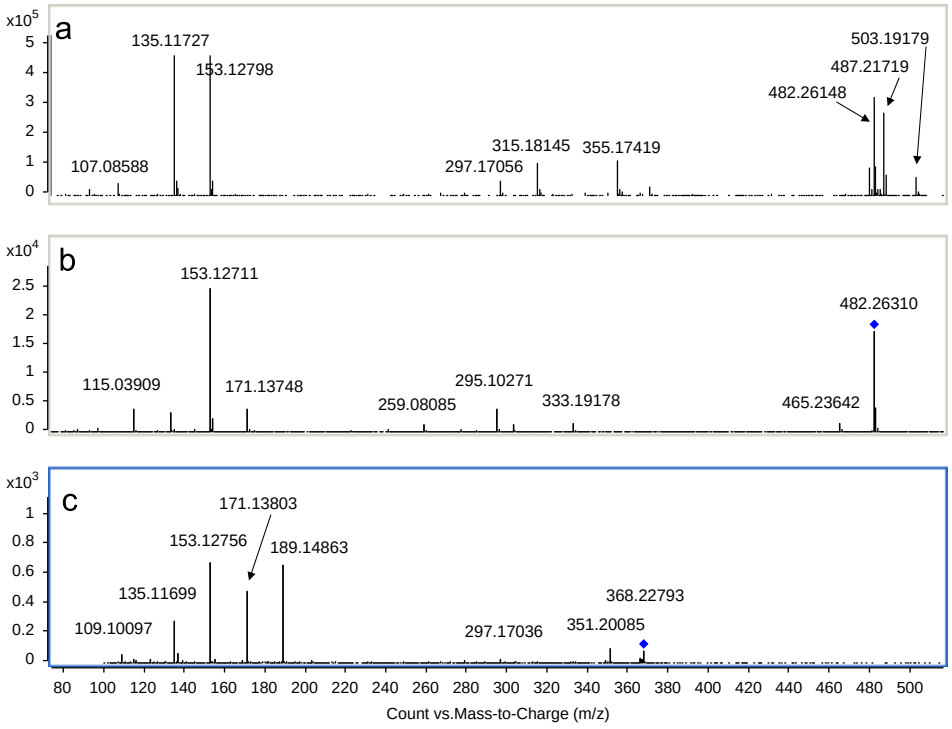

Fig. 5. (a) mass spectrum relative to peak at 15.30 min of Fig. 4a; (b) MS–MS fragmentation spectrum obtained for ion *m*/*z* 482 at 16.28 min; (c) MS–MS fragmentation spectrum obtained for ion *m*/*z* 368 of peak at 7.31 min in Fig. 4b.

Table 2
Fragments obtained in MS–MS for ion 482 and 496, fraction A, RT 16.28 and 17.12 min, respectively.

Precursor	*m*/*z*	Δ*m*/*z* (ppm)*	Ion	Loss
482.2631	465.2364	7	$C_{21}H_{37}O_{11}^+$	NH_3
	333.1918	3	$C_{16}H_{29}O_7^+$	Apiose
	295.1027	1	$C_{11}H_{19}O_9^+$	Linalool oxide
	259.0808	2	$C_{11}H_{15}O_7^+$	Linalool oxide and 2 H_2O
	171.1375	3	$C_{10}H_{19}O_2^+$ linalool oxide	–
	153.1271	2	$C_{10}H_{17}O^+$ dehydrated linalool oxide	–
	133.0492	2	$C_5H_9O_4^+$ dehydrated apiose	
	115.0391	1	$C_5H_7O_3^+$	H_2O from 133
496.2726	479.2471	3	$C_{22}H_{39}O_{11}^+$	NH_3
	317.1944	5	$C_{16}H_{29}O_6^+$	Dehydrated glucose
	309.1177	1	$C_{12}H_{21}O_9^+$	Linalool oxide
	273.0938	11	$C_{12}H_{17}O_7^+$	Linalool oxide and $2H_2O$
	171.1357	13	$C_{10}H_{19}O_2^+$ linalool oxide	–
	153.1265	6	$C_{10}H_{17}O^+$dehydrated linalool oxide	–
	147.0625	18	$C_6H_{11}O_4^+$ dehydrated glucose minus O	Glucose–linalool oxide
	129.0537	7	$C_6H_9O_3^+$	H_2O from 147
	85.0283	1	$C_4H_5O_2^+$	

* Difference from exact mass. Fragments with Δ*m*/*z* higher than 5 ppm are not certainly identified.

and **b** contain a terpene with 2 stereocenters. Assuming no configurational changes in the saccharides, four diastereoisomers are possible, as highlighted by mass analysis, but only two (**a**′, **b**) were present in the NMR spectra in relative concentration 3:2, in agreement with the relative MS intensities.

Accurate mass and NMR data identified mass 355 (molecule 332 amu) as compound **c**, (Z)-8-hydroxylinalool-8-O-[1-β-D-glucopyranoside], with only a GLU bound through the oxygen in position 8. The glycoterpene is present as the sodium adduct and, less intense, the potassium adduct. In MS–MS experiments, it did not fragment, typical of sodium adducts. The prevalence of the sodium adduct with respect to the ammonium one may be due to different charge distribution and coordination capacity of the two cations and to the spatial structure of the molecule. Compound **c** contains only one stereocenter in the terpene moiety; again, assuming no configurational changes in the saccharide, we could in principle find two diastereoisomers, but in NMR and MS spectra only one seemed to be present. While it was not possible to determine the absolute configuration at carbon 3, the chemical shifts and the NOESY patterns are in agreement with a *Z* configuration at the double bond (Table 4). This compound has already been reported in the literature as betulalbuside B [37].

The molecular ion with *m*/*z* 496 (ammonium adduct, RT 17.12 min, Table 1) was supposed to be oxylinalool-GLU-GLU

Table 3
ESI-MS–MS fragmentations of ion 466 in fraction D.

Precursor	Fragment *m/z*	Δ*m/z* (ppm)*	Loss *m/z*	Fragment formula	Loss formula
$C_{21}H_{40}NO_{10}^+$ 466.2652	330.1403	2	136.1249	$C_{11}H_{24}NO_{10}^+$	$C_{10}H_{16}$
	295.1034	3	171.1618	$C_{11}H_{19}O_9^+$	$C_{10}H_{18}O$ and NH_3
	277.0926	3	189.1726	$C_{11}H_{17}O_8^+$	H_2O from 295
	259.0821	3	207.1831	$C_{11}H_{15}O_7^+$	H_2O from 277
	241.0713	2	225.1939	$C_{11}H_{13}O_6^+$	H_2O from 259
	223.0611	4	243.2041	$C_{11}H_{11}O_5^+$	H_2O from 241
Apiose$^+$	163.0610	6	303.2042	$C_6H_{11}O_5^+$	$C_{15}H_{27}O_6$
	154.1600	6	312.1052	$\mathbf{C_{10}H_{20}N^+}$	
	145.0505	3	321.2147	$C_6H_9O_4^+$	H_2O from 163
	137.1336	8	329.1316	$\mathbf{C_{10}H_{17}^+}$	
	133.0507	9	333.2145	$C_5H_9O_4^+$	
	127.0401	2	339.2251	$C_6H_7O_3^+$	
	115.0404	9	351.2248	$C_5H_7O_3^+$	H_2O from 133
	97.0302	19	369.2350	$C_5H_5O_2^+$	H_2O from 115
	85.0303	22	381.2349	$C_4H_5O_2^+$	
	81.0719	25	385.1933	$\mathbf{C_6H_9^+}$	

Terpene fragments in bold.

* Difference from exact mass. Fragments with Δ*m/z* higher than 5 ppm are not certainly identified.

($C_{22}H_{40}O_{11}$) by mass and literature data [18]. The confirmation by NMR was not possible because its concentration was too low. Table 2 reports the fragmentation of the proposed molecular ion. The analogies with ion 482 are evident. Ions reported in Table 1 with *m/z* 320 and *m/z* 310 were not identified.

3.2. Fraction B

In fraction B, a peak was found with mass 368 (molecule 350 amu) with also the characteristic *m/z* at +5 and +16 (ammonium, sodium and potassium adducts, of which the ammonium was the second in abundance) and another peak with mass 350 (molecule 332 amu). The first ion corresponded to the formula $(C_{16}H_{30}O_8)NH_4^+$, a monoglucoside linked to a terpene that contains three oxygen atoms: $C_{10}H_{20}O_3$-GLU. Two chromatographic peaks with the same *m/z* were found indicating the presence of two isomers (Fig. 4b). Fig. 5c shows the relative mass spectrum where is evident a partial fragmentation of ammonium adduct. All fragments were justified and fragment 109, not found in preceding fragmentations, corresponded to the carbocation $C_8H_{13}^+$ coming from the terpene (accurate mass).

The complete identification of these two aroma precursors was reached by means of NMR that confirms the presence of the following monosaccharide terpenes: **a″**, (E)-furanosyl-linalool-oxide-7-O-[1-β-D-glucopyranoside] (molecule 332 amu) and **d**, 1,2-dihydroxylinalool-1-O-[1-β-D-glucopyranoside] (molecule 350 amu), reported in Fig. 2. The first one is identical to compound **a′**, except for the absence of the API moiety. Only one isomer of each

Table 4
^1H and ^{13}C chemical shift values.

Compound	**a′**		**a″**		**b**		**c**	
Position	H	C	H	C	H	C	H	C
1	*x*: 5.03, d (10.8) *y*: 5.23, dd (17.2, 1.3)	112.25	*x*: 5.02, dd (1.4, 10.8) *y*: 5.22, dd (1.4, 17.3)	112.27	*x*: 4.99, d (1.5) *y*: 5.22, dd (17.4, 1.5)	112.08	*x*: 5.03, d (10.9) *y*: 5.19, dd (17.3, 1.3)	112.30
2	5.92, dd (17.2, 10.8)	144.96	5.91, dd (10.8, 17.3)	144.98	6.01, dd (17.4, 10.8)	145.56	5.91, dd (17.3, 10.9)	146.91
3	–	85.06	–	85.07	–	84.60	–	73.92
4	*x*: 1.75, m *y*: 1.92, m	38.21	*x*: 1.74, m *y*: 1.90, m	38.21	*x*: 1.80, m *y*: 1.86, m	38.82	*x*: 1.50, m *y*: 1.53, m	43.71
5	*x*: 1.85, m *y*: 1.93, m	28.25	*x*: 1.84, m *y*: 1.94, m	28.27	*x*: 2.01, m *y*: 1.87, m	28.16	*x*: 2.07, m *y*: 2.15, m	23.59
6	4.02, dd (7.2, 6.9)	87.17	4.02, t (7.1)	87.13	4.07, dd (7.2, 6.9)	85.37	5.40, t (7.4)	131.56
7	–	80.69	–	80.81	–	80.20	–	132.78
8	1.25, s	20.92	1.25, s	20.93	1.27, s	23.87	*x*: 4.20, d *y*: 4.33, d (11.3, both)	67.89
9	1.21, s	23.92	1.22, s	23.92	1.23, s	22.79	1.77, s	22.01
10	1.33, s	26.94	1.33, s	26.92	1.29, s	26.01	1.24, s	27.79
1′	4.49, d (7.7)	98.92	4.50, d (7.8)	98.92	4.55, d (7.7)	98.52	4.22, d (7.7)	102.55
2′	3.15, m	75.27	3.15, dd (7.8, 9.3)	75.30	3.14, m	75.49	3.19, m	75.17
3′	3.35, m	78.20	3.34, m	78.09	3.37, m	78.20	3.34, m	78.20
4′	3.25, m	72.06	3.24, m	71.89	3.26, m	71.95	3.23, m	71.81
5′	3.38, m	76.69	3.27, m	77.78	3.39, m	76.70	3.31, m	78.04
6′	*x*: 3.55, m *y*: 3.94, m	69.01	*x*: 3.66, m *y*: 3.85, m	62.96	*x*: 3.56, m *y*: 3.95, m	68.95	*x*: 3.69, dd (11.8, 5.4) *y*: 3.88, dd (11.8, 2.1)	62.88
1″	4.97, d (2.3)	111.11			4.97, d (2.3)	111.16		
2″	3.86, d (2.3)	78.28			3.87, d (2.3)	78.28		
3″	–	80.86			–	80.86		
4″	*x*: 3.75, d *y*: 3.96, d (9.7 both)	75.11			*x*: 3.76, d *y*: 3.96, d (9.7 both)	75.12		
5″	3.57, s	65.81			3.57, s	65.81		

Compound	d		e′		e″		e‴	
Position	H	C	H	C	H	C	H	C
1	*x*: 3.49, dd (9.1, 10.0) *y*: 4.19, dd (2.2, 10.0)	72.72	*x*: 5.21, dd (1.0, 11.0) *y*: 5.23, d (17.6)	116.06	*x*: 5.21, dd (1.0. 11.0) *y*: 5.23, dd (1.0. 17.8)	116.01	*x*: 5.21, dd (0.8, 10.9) *y*: 5.24, dd (0.8, 17.7)	116.12
2	3.65	77.21	5.91, dd (11.0, 17.6)	144.52	5.91, dd (11.0. 17.8)	144.57	5.91, dd (10.9, 17.7)	144.48
3	–	75.45	–	81.69	–	81.64	–	81.64
4	*x*: 1.42, ddd (5.3, 11.9, 13.6) *y*: 1.58, ddd (5.0, 11.9, 13.6)	40.27	*x*: 1.57, m *y*: 1.60, m	42.81	1.58, m	42.83	*x*: 1.58, m *y*: 1.60, m	42.84
5	*x*: 2.06, m *y*: 2.10, m	22.93	2.02, m	23.81	2.03, m	23.81	2.02, m	23.80
6	5.12, m	125.98	5.09, t (7.2)	125.84	5.10, t (7.2)	125.86	5.09, t (7.2)	125.81
7	–	132.26	–	132.29	–	133.68	–	132.31
8	1.62, s	17.85	1.59, s	17.87	1.59, s	17.87	1.59, s	17.87
9	1.67, s	26.02	1.66, s	26.00	1.66, s	26.00	1.66, s	26.01
10	1.13	22.45	1.36, s	23.38	1.37, s	23.40	1.36, s	23.44
1′	4.29, d (7.8)	105.16	4.35, d (7.9)	99.70	4.33, d (7.9)	99.66	4.33, d (7.8)	99.70
2′	3.22, dd (7.8, 9.2)	75.45	3.15, dd (7.9, 8.9)	75.35	3.15, dd (7.9. 9.0)	75.35	3.16, m	75.36
3′	3.37, t (8.9)	78.07	3.30, m	78.34	3.30, m	78.38	3.30, m	78.43
4′	3.26, m	71.76	3.26, m	72.21	3.24, d (8.9)	72.03	3.23, d (9.0)	72.04
5′	3.29, m	78.15	3.34, m	76.48	3.29, m	76.67	3.28	76.68
6′	*x*: 3.67, m *y*: 3.86, dd (1.6, 12.1)	62.86	*x*: 3.54, dd (6.1, 11.0) *y*: 3.97, m	68.34	*x*: 3.53, dd (6.4, 11.1) *y*: 3.93, dd (1.5, 11.1)	68.95	*x*: 3.51, dd (6.6, 11.0) *y*: 3.94, dd (1.7, 11.0)	68.23
1″			4.93, s	110.11	4.97, d (2.3)	111.14	4.72, d (1.2)	102.28
2″			3.97, m	83.38	3.87, d (2.3)	78.22	3.81, m	72.37
3″			3.81, dd (3.1, 5.7)	79.18	–	80.71	3.65, m	72.52
4″			3.96, m	86.06	*x*: 3.75, d *y*: 3.96, d (9.7 both)	75.13	3.35, m	74.21
5″			*x*: 3.63, dd (5.3, 11.9) *y*: 3.74, dd (3.3, 11.9)	63.21	3.57, s	65.85	3.64, m	69.91
6″							1.25, d (6.2)	18.18

Compound	f		g′		g″		h	
Position	^{1}H	^{13}C	^{1}H	^{1}H	^{1}H	^{13}C	^{1}H	^{13}C
1	4.19, m 4.27, m	66.39	4.24, m 4.31, m	66.49	4.25, m 4.28, m	66.13	–	164.52
2	5.37, t (6.7)	122.61	5.36, t (6.7)	121.57	5.37, t (6.4)	121.46	5.74, s	116.01
3	–	142.07	–	142.27	–	142.52	–	nd
4	2.12, m	33.18	2.06, m 2.12, m	40.86	2.06, t (7.0)	40.87	2.22, m	42.16
5	2.09, m	27.85	2.10, m	27.58	2.13, m	27.58	2.20, m	27.22
6	5.11, m	125.16	5.11, m	125.22	5.11, t (6.7)	125.22	5.11, m	124.22
7	–	133.00	–	132.67	–	132.13	–	133.68
8	1.61, s	17.91	1.61, s	17.87	1.61, s	17.93	1.62, s	17.88
9	1.68, s	26.12	1.68, s	26.06	1.68, s	26.07	1.68, s	26.01
10	1.75, s	23.86	1.69, s	16.68	1.70, s	16.66	2.18, s	19.38
1′	4.25, d (7.5)	103.03	4.28, d (7.8)	102.79	4.27, d (7.9)	102.50		
2′	3.16, m	75.13	3.17, m	75.17	3.17, t (7.9)	75.18		
3′	nd	nd	3.33, m	78.20	3.33, m	78.30		
4′	nd	nd	3.29, m	72.12	3.28, m	71.88		
5′	nd	nd	3.40, ddd (2.3, 6.0, 9.6)	76.91	3.35, m	77.04		
6′	3.56, m 3.89, m	67.46	3.61, dd (6.1, 11.0) 4.24, dd (2.3, 11.0)	68.23	3.60, dd (6.2, 11.1) 3.99, dd (1.3, 11.1)	68.18		
1″	4.99, d (2.4)	111.10	4.96, d (0.9)	110.12	4.75, d (0.9)	102.37		
2″	3.89, m	78.25	3.99, m	83.39	3.84, m	72.36		
3″	–	80.71	3.82, m	79.10	3.67, m	72.53		
4″	3.74, m 3.96, m	75.13	3.96	86.02	3.36, m	74.19		
5″	3.58, s	65.85	3.64, m 3.74, m	63.22	3.66, m	69.95		
6″					1.26, d (6.2)	18.19		

In each compound, proton "1a" is in position *cis* in respect of proton "2" and proton "1b" is in position *trans* in respect of proton "2".

compound is present in the NMR spectra in sufficient amounts to be identified.

The terpene moiety of the second molecule is a new linalool derivative, in which the double bond in position 1 is oxidized to a diol, probably through an epoxidic intermediate [38]. The linked saccharide was demonstrated to be GLU, as expected. HMBC spectrum demonstrated that the glycosidic bond is in position 1.

3.3. Fraction C

In this fraction, two molecules with *m*/*z* 466 (molecule 448 amu) and *m*/*z* 480.24 (molecule 462 amu), respectively, were found.

The NMR analysis of this fraction allowed the identification of the ion with *m*/*z* 466 as compound **e′**, linalool-3-O-[α-L-arabino-furanosyl-(1→6)-1-β-D-glucopyranoside]. Several other compounds, all in lower concentration (less than 30% of **e′**) were visible in the spectrum. The aglycon structure of one of these was recognizable as that of geranic acid, but there was not enough information to describe the glycosidic part of the molecule. The presence of geranic acid was confirmed by the typical MS fragmentation pattern (*m*/*z* 169, 151 and 123 amu) characteristic of the presence of a carboxylic group reported in Fig. 2. It can be assumed to be a compound with geranic acid linked to a GLU and an ARA or an API, with molecular mass 462 amu, **h** (Fig. 6). The fragmentation on the right of the figure is

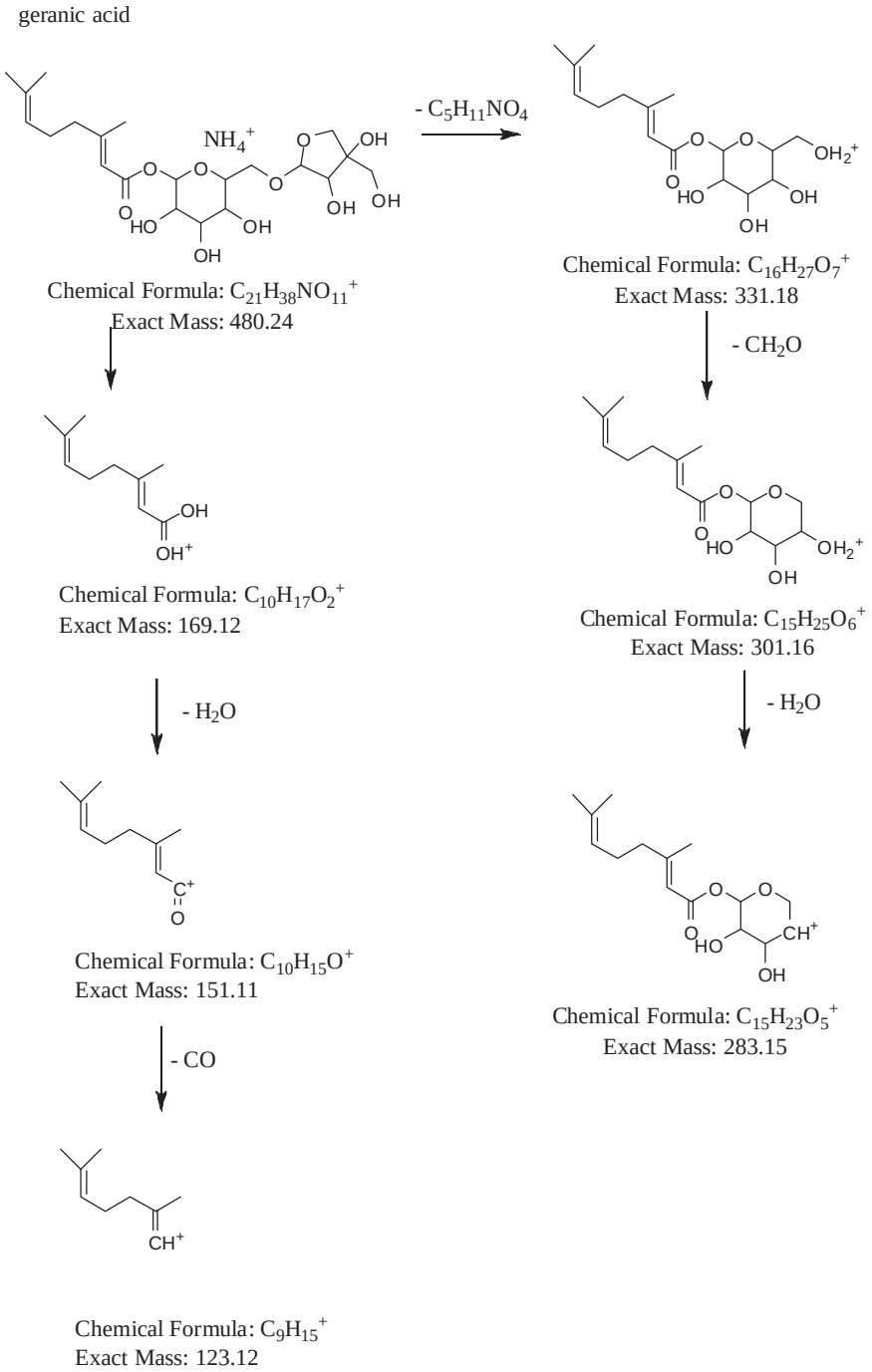

Fig. 6. Fragmentation pattern for geranic acid-GLU-API, *m*/*z* 480.24.

justified by fragments obtained with the loss of the assumed ARA (or API).

3.4. Fraction D

The NMR analysis of fraction D identified molecule **e″**, a linalool derivative, as linalool-3-O-[α-L-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], corresponding to ion *m/z* 466 (molecule mass 448 amu). The MS fragmentation of the ion is reported in Table 3.

3.5. Fractions E and F

In fraction E, three glycoterpenes corresponding to *m/z* values 466 (molecule 448 amu), 480.24 and 480.28 were found. The first consisted of four isomers (four partially resolved chromatographic peaks), while the second and the third consisted of four and two isomers, respectively (see Figs. 4c and 7). *m/z* 466 is in accordance with the canonic formula of a terpene, $C_{10}H_{18}O$, linked to a disaccharide containing a hexose and a pentose unit, probably glucose and apiose or arabinose [33]. NMR spectrometry allowed the identification of two of the four molecules with the same mass of 448 amu: nerol-1-O-α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside (**f**) and geraniol-1-O-[α-L-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside] (**g′**). Distinction between nerol and geraniol was possible through NOE cross-peaks.

Concerning the ions with nominal mass 480, it must be pointed out that, although well separated by chromatography, only high mass resolution and good mass accuracy allowed the distinction between the two ions 480.24 and 480.28. *m/z* 480.24 was recognized as constituted of a pentose, a hexose and a terpene with formula $C_{10}H_{16}O_2$ ($C_{21}H_{34}O_{11}$, $C_{10}H_{16}O_2$-GLU-API). Similar to the compounds previously described, the GLU moiety is not terminal, because it is never present as single fragment. *m/z* 480.28, less polar than 480.24 (longer retention time), differs from it for a "CH_4" in place of an oxygen, a difference in mass of 0.036 amu (75 ppm). The NMR study allowed the identification of the exact structure of the molecule with mass of 480.28. This compound is a differently glycosylated linalool, with a hexose instead of a pentose. The COSY spectrum revealed a terminal methyl group that identified this sugar as rhamnose. Ultimately the compound was identified as linalool-3-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside] (**e‴**). The fragmentation pattern confirms this hypothesis.

In a similar way, geraniol-1-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside] (**g″**) was identified in fraction F.

Unidentified peaks with *m/z* 466 could be due to nerol-1-O-α-D-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside and geraniol-1-O-α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside. Similarly, nerol-1-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside] could be the compound responsible for an unidentified peak with *m/z* 480.28.

4. Conclusions

In the present study, samples extracted from grape juice were analyzed with the aim to discover glycosylterpenes, aroma precursors of aromatic Moscato wine. They were separated and detected with U-HPLC–MS. The same fractions, more concentrated, were analyzed by NMR to better characterize the compounds identified. This is one of the few times that LC–MS is used in place of GC–MS, enabling direct analysis without hydrolysis and/or derivatization.

The fractions analyzed contained mono and especially disaccharides linked to terpenes. The study identified the presence of twelve aroma precursors in *Moscato Giallo* grape juice. Specifically, we found (E) and (Z)-furanosyl-linalooloxide-7-O-[α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], (E)-furanosyl-linalooloxide-7-O-[1-β-D-glucopyranoside], (Z)-8-hydroxylinalool-8-O-[1-β-D-glucopyranoside], 1,2-dihydroxylinalool-1-O-[1-β-D-glucopyranoside], linalool-3-O-[α-L-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside], linalool-3-O-[α-L-apiofuranosyl-(1→6)-1-β-D-glucopyranoside], linalool-3-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside], nerol-1-O-α-D-apiofuranosyl-(1→6)-1-β-D-glucopyranoside, geraniol-1-O-[α-L-arabinofuranosyl-(1→6)-1-β-D-glucopyranoside], geraniol-1-O-[α-L-rhamnopyranosyl-(1→6)-1-β-D-glucopyranoside], and a geranic acid disaccharide derivative.

Most of these compounds were already known, although not necessarily from grape juice; for example, compound **e″** was identified in green coffee [33]. To the best of our knowledge, compound **d**, 1,2-dihydroxylinalool-1-O-[1-β-D-glucopyranoside], had never been found in any food matrix.

This work demonstrates that MS and MS–MS can rapidly provide reliable structural information on the molecular mass of glycosides, the number of sugars involved, the sequence in which

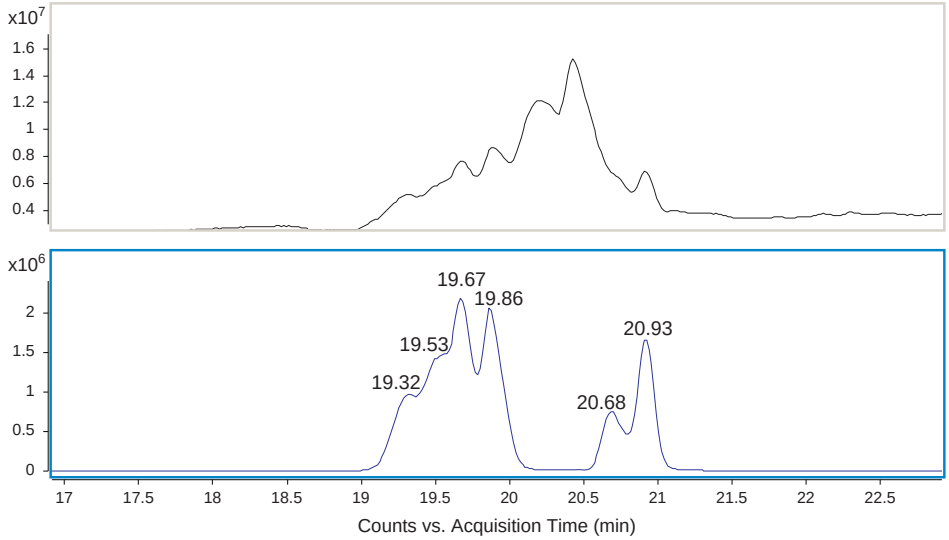

Fig. 7. TIC and EIC chromatogram for *m/z* 480 in fraction E.

the sugars are attached, and information about the aglycon moieties. On the other hand, data provided by LC–MS and LC–MS–MS experiments alone allow only tentative structural assignments, and confirmation of the initial assignments is possible only through further characterization by NMR spectroscopy. Homonuclear and heteronuclear correlations observed in 2D NMR spectra indeed allowed the correct identification of the aglycon and sugar moieties of many compounds in complex mixtures. The strategy present here requires reduced sample preparation time for the analysis by avoiding the necessity for chemical derivatizations, and provides time efficiency because the analyses were performed directly on mixtures.

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