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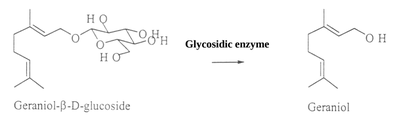
UHPLC/QToF of monoterpene glycosides of *White Muscat, Glera* and *Riesling Italico* grapes



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Introduction

Monoterpene glycosides are the main grape aroma precursors of the aromatic (e.g., Moscati, Malvasie, Gewurztraminer) and semi-aromatic (Riesling, Glera, …) grape varieties. Hydrolysis of glycoside terpenes in winemaking is very important to increase aroma of wines and knowledge of these aroma precursors can be determinant for choosing the suitable oenological practices which favor the enzymatic hydrolysis during winemaking.[1] Traditional methods to study monoterpene glycosides are perform hydrolysis of the grape extract by using glycosidase enzymes and GC/MS analysis. The method provides identification of the aglycones, but not information on the sugar moiety, and can be affected by hydrolytic artifacts. UHPLC/QTOF analysis was used to study the monoterpene glycosides profile of three grape varieties: Moscato bianco, Glera and Riesling Italico.[2] Compounds were identified by overlapping various analytical approaches, in agreement with the indications recommended in MS-based metabolomics, such as accurate mass and isotopic pattern, MS/MS fragmentation, correlation between fragments observed and putative structures and between LC/MS and GC/MS signals. By this approach,17 monoterpene glycosides were identified in the grape extracts.[3]

Experimental

Samples. 100 berries of White Muscat, Riesling Italico and Glera grapes were harvested in 2011 and 2012 at full ripeness (maximum sugar content) from the CREA-VIT grapevine Germoplasm Collection (Susegana, Veneto, Italy). Berries were picked randomly from five different plants and immediately frozen at -20 °C.

Skins of 50 berries were extracted with 35 mL of methanol, the solution was homogenized, centrifuged, concentrated to 10 mL by rotary evaporator, finally the residue was adjusted to 100 mL by water. Polyphenols and tannins were removed by using 1 g Polyclar AT under stirring for 20 min then centrifuged. Pulps were added of 50 mg sodium metabisulphite, the solution was homogenized, centrifuged, the volume was adjusted to 100 mL by water and the solution was clarified by pectolytic enzyme Pectazina DC then centrifuged.

Sample preparation for LC/MS. 10 mL of skin extract were combined with an equal volume of limpid pulp juice and added of IS heptyl glucoside. The solution was passed through a 1-g Sep-Pak C18 cartridge. After cartridge washing by 5 mL water and 5 mL dichloromethane, the glycoside compounds fraction was recovered with 5 mL methanol. For each variety two samples were analyzed.

Sample preparation for GC/MS. 90 mL of skin extract was combined with an equal volume of pulp juice, added of IS 1-heptanol and the solution was passed through a 10-g Sep-Pak C18 cartridge. After washing the cartridge with 50 mL water and 50 mL dichloromethane, the fraction of glycosides was recovered with 30 mL methanol. The solvent was evaporated until dry, the residue was dissolved in 5 mL of citrate-phosphate buffer and added of 100 mg of AR 2000 enzyme. Reaction was carried out overnight at 40 °C, the solution was passed through a 1-g Sep-Pak C18 cartridge and the aglycones were recovered with 6 mL dichloromethane.

UHPLC/QToF. Agilent UHPLC 1290 Infinity ultra-high performance-liquid chromatography system coupled to1290 Infinity Autosampler (G4226A) and 6540 accurate-mass Q-ToF Mass Spectrometer (nominal resolution 40,000) with Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA). Chromatography: Zorbax reverse-phase column (RRHD SB-C_{18} 3×150 mm, 1.8 µm) and mobile phase composed of A) 0.1% v/v aqueous HCOOH, and B) CH_3CN containing 0.1% v/v HCOOH. QToF conditions: sheath gas nitrogen 10 L/min at 400 °C; drying gas nitrogen 8 L/min at 350 °C; nebulizer pressure 60 psi, nozzle voltage 1 kV, capillary voltage 3.5 kV. Signals in the *m/z* 100-1700 range, were recorded.

GC/MS. 6850 gas chromatograph coupled with HP 5975C mass spectrometer and 7693A sampler injector (Agilent Technologies, Santa Clara, CA, US) and equipped with a fused silica HP-Innowax polyethylene glycol (PEG) capillary column (30 m×0.25 mm, 0.25 mm i.d.). Oven temperature program: 40 °C isothermal for 1 min, increase 2 °C/min until 160 °C, 3 °C/min until 230 °C, 230 °C isothermal for 15 min. Injector temperature, 230 °C; carrier gas helium at constant flow, 1.2 mL/min; sample volume, 1 mL; splitless injection mode; transfer line temperature, 250 °C; quadrupole temperature, 150 °C; mass range, *m/z* 20-550. Compounds were identified by searching in NIST08 and CREA-VIT database ESTRATTI.

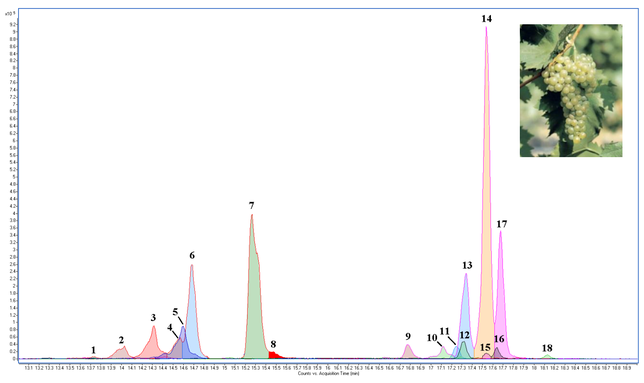

Figure 1. EIC of LC/QToF of putative monoterpene glycoside $[M-H]^-$ signals of White Muscat grape extract (harvest 2011). Peak numbers correspond to compounds tentatively assigned in Table 1.

peak	RT (min)		Formula	[M-H]⁻ experimental mass	[M-H]⁻ theoretical mass	Error ppm	Id. Score
		Monoterpendiols pentosyl-hexoside					
1	13.72	*p* -menthenediol I	$C_{21}H_{36}O_{11}$	463.2192	463.2185	1.5	82.44
2	13.96	furan / pyran linalool oxides	$C_{21}H_{36}O_{11}$	463.2187	463.2185	0.4	83.06
3	14.28	furan / pyran linalool oxides	$C_{21}H_{36}O_{11}$	463.2188	463.2185	0.6	82.62
4	14.53	furan / pyran linalool oxides	$C_{21}H_{36}O_{11}$	463.2188	463.2185	0.6	81.68
5	14.59	7-hydroxygeraniol / 7-hydroxynerol	$C_{21}H_{36}O_{11}$	465.2341	465.2341	0.0	98.18
6	14.66	diendiol I	$C_{21}H_{36}O_{11}$	463.2193	463.2185	1.7	81.81
7	15.24	*cis* / *trans* 8-hydroxylinalool	$C_{21}H_{36}O_{11}$	463.2195	463.2185	2.2	94.63
8	15.39	isomeric compound	$C_{21}H_{36}O_{11}$	463.2190	463.2185	1.1	92.09
		Monoterpenols pentosyl-hexoside					
9	16.75	α-terpineol	$C_{21}H_{36}O_{10}$	447.2233	447.2236	-0.7	99.27
10	17.09	isomeric compound	$C_{21}H_{36}O_{10}$	447.2247	447.2236	2.5	96.36
11	17.25	heptyl glucoside (internal standard)	$C_{13}H_{26}O_6$	277.1660	277.1657	1.1	99.42
13	17.32	linalool	$C_{21}H_{36}O_{10}$	447.2238	447.2236	0.4	98.57
14	17.51	nerol	$C_{21}H_{36}O_{10}$	447.2243	447.2236	1.6	98.77
17	17.65	geraniol	$C_{21}H_{36}O_{10}$	447.2245	447.2236	2.0	97.63
18	18.08	citronellol	$C_{21}H_{38}O_{10}$	449.2397	449.2392	1.1	98.69
		Monoterpenols rhamnosyl-hexoside					
12	17.29	linalool / nerol / geraniol	$C_{22}H_{38}O_{10}$	461.2396	461.2392	0.9	82.79
15	17.51	isomeric compound	$C_{22}H_{38}O_{10}$	461.2393	461.2392	0.2	82.33
16	17.61	isomeric compound	$C_{22}H_{38}O_{10}$	461.2383	461.2392	-2.0	85.33

Table 1. Putative monoterpene glycosides identified. Confidence of molecular formula (Id. Score) calculated on accurate mass measurements of $[M-H]^-$ ion and isotopic pattern. Numbers correspond to peaks Figure 1.

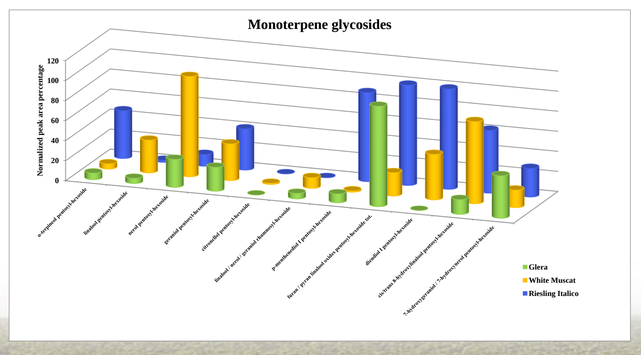

Figure 3. Monoterpene glycoside profiles of the three grape varieties studied.

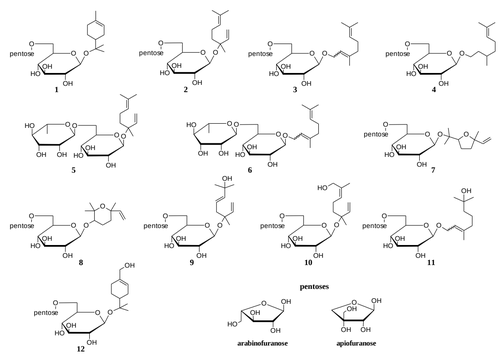

Figure 2. Structures of monoterpene glycosides identified.

Compound	r-Pearson	*p*
p -menthenediol I	0.999	0.03
Linalool oxides tot.	0.997	0.05
8-hydroxylinalool tot.	1.000	0.00
7-Hydroxygeraniol / 7-Hydroxynerol	0.982	0.12
Linalool tot.	0.999	0.02
α-terpineol	0.944	0.21
Nerol tot.	1.000	0.02
Geraniol tot.	0.999	0.02
Diendiol I	1.000	0.00

Table 2. Correlation between GC/MS signal intensity of the aglycones after enzymatic hydrolysis and the sum of their LC/MS $[M-H]^-$ signals in the three grape samples.

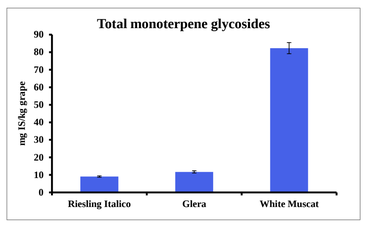

Figure 4. Total monoterpene glycoside contents in the three grape varieties calculated on $[M+HCOO]^-$ adduct signals and expressed as mg heptyl glucoside/Kg grape (harvests 2011 and 2012, two samples per harvest).

Results

Compounds identification. Figure 1 shows the EIC of $[M-H]^-$ ions of putative glycoside monoterpenes identified, the structures are shown in Figure 2. Initial identification was based on accurate mass measurements of the monoisotopic signal and isotopic pattern of the compounds. Molecular formulae of more abundant signals were identifiable with confidence score >90%. For the lower signals identification had lower score due to low signal intensity of the lower isotopic ions. In any event, accurate mass measurement of these monoisotopic ion signal was achieved with error maximum 2 ppm (Table 1). MS/MS identified sugar moiety: the pentosyl-hexoside derivatives have the fragments at *m/z* 161.0455 and 119.0350, the former characteristic of hexose and the latter of pentose; the rhamnosyl-hexoside derivatives the rhamnose fragment at *m/z* 145.0506. Further identification of aglycones was achieved by the correlation between the GC/MS signal intensity of the compounds liberated by enzymatic hydrolysis and that of $[M-H]^-$ ions recorded in LC/MS analysis was studied by calculating r-Pearson coefficients. For the nine aglycones identified, the correlation between GC/MS area signal was correlated with the sum of signal intensity of the corresponding glycosides recorded by LC/MS in the three grape samples (Table 2).

Samples profile. Significant difference of ionization yield in producing the $[M-H]^-$ ion between the internal standard and the analytes was observed, due probably to the standard is a saturated molecule. By contrast, formation of intense $[M+HCOO]^-$ adduct was observed for both the internal standard and the analytes. Consequently, the semi-quantitative analysis was performed on signal intensity of $[M+HCOO]^-$ ions. Figure 3 shows the monoterpene glycoside profiles of the three varieties; the total contents are reported in Figure 4. White Muscat had the highest rhamnosyl-hexoside derivatives. Riesling Italico showed a peak at retention time 15.39 min with molecular formula $C_{21}H_{36}O_{11}$ (MW 463.2185) not found in the other samples, which was putatively assigned to a pentosyl-hexoside diol, and Diendiol I pentosyl-hexoside and total pyran linalool oxides were the more abundant compounds. As expected, White Muscat had the highest contents of monoterpene glycosides, particularly linalool, geraniol, and nerol in pentosyl-hexoside and rhamnosyl-hexoside forms. Riesling Italico and Glera (the grape variety used to produce Prosecco wine) showed minor but significant contents of monoterpenols and confirmed their semi-aromatic character.

Conclusions

This is the first detailed study of these aroma precursors carried out by direct LC/MS analysis. As all samples had been grown in the same vineyard, they were fairly affected by cultural or environmental variables, and the differences found are essentially due to their varietal expression. Due to the lack of standards commercially available, just a semi-quantitative study was possible. Anyway, this method is effective for comparative studies, e.g.: a) characterize grape varieties according to the profiles of secondary metabolites; b) study climate change and cultural variable effects; c) improve and refine enological techniques (monitoring grape compound extraction in wine-making and study of the evolution of aroma precursors during aging); d) estimate the aromatic potential of various kinds of grapes.

Literature

1. **A. Rapp, H. Mandery, H. Niebergall. Vitis, 1986, 23, 79.**
2. **Y.Z. Gunata, C. L. Bayonove, R. L. Baumes, R. E. Cordonnier. J. Sci. Food Agric. 1985, 36, 857.**
3. **R. Flamini, M. De Rosso, A. Panighel, A. Dalla Vedova, F. De Marchi, L. Bavaresco. J. Mass Spec. 2014, 49(12), 1214.**