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High-Resolution Mass Spectrometry Identification of Secondary Metabolites in Four Red Grape Varieties Potentially Useful as Traceability Markers of Wines

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Abstract: Liquid chromatography coupled to high-resolution mass spectrometry (LC-Q/TOF) is a powerful tool to perform chemotaxonomic studies through identification of grape secondary metabolites. In the present work, the metabolomes of four autochthonous Italian red grape varieties including the chemical classes of anthocyanins, flavonols/flavanols/flavanones, and terpenol glycosides, were studied. By using this information, the metabolites that can potentially be used as chemical markers for the traceability of the corresponding wines were proposed. In Raboso wines, relatively high abundance of both anthocyanic and non-anthocyanic acyl derivatives, is expected. Potentially, Primitivo wines are characterized by high tri-substituted flavonoids, while Corvina wines are characterized by higher di-substituted compounds and lower acyl derivatives. Negro Amaro wine's volatile fraction is characterized by free monoterpenes, such as α-terpineol, linalool, geraniol, and Ho-diendiol I. A similar approach can be applied for the traceability of other high-quality wines.

Keywords: wine; grape; traceability; metabolomics; high-resolution mass spectrometry; Amarone; Recioto; Raboso; Primitivo; Negro Amaro

1. Introduction

Amarone della Valpolicella and Recioto are two red DOCG wines (controlled and guaranteed designation of origin) produced in Northeast Italy (Verona province, Veneto) by using a blend of autochthonous red grape varieties, such as Corvina Veronese and Corvinone. Types and percentages of grape varieties that can be used are stated in the disciplinary of production of the wines (approved by Ministerial Decree 24 March 2010), which defines the municipalities allowed for the cultivation, the maximum yield per hectare, and the winemaking practices allowed. The main variety used is Corvina Veronese, which has to account for 45–95% of the grape blend.

Raboso Piave is another red grape variety cultivated in the Veneto region, whose grapes are characterized by high polyphenolic content, used to produce the high-quality reinforced wine *Raboso Passito* DOCG [1].

Primitivo and Negro Amaro are two red grape varieties cultivated in Southern Italy. In general, these grapes are characterized by high sugar and polyphenolic content and the corresponding wines by high alcohol and color [2–4].

Despite the measures in place to regulate and guarantee the authenticity and geographical traceability of wines, different kinds of fraud (e.g., mislabeling, blending with wines of a lesser quality and/or without denomination of origin, etc.) has been reported [5]. In this context, over the last years

a growing interest in developing analytical methods for wine authentication has been observed [6,7]. For the characterization of wine origin and variety, as well as the grape growing and winemaking practices used, the chemical characterization of wines is generally based on the characterization of the polyphenolic compounds, such as anthocyanins, flavones, flavonols, hydroxycinnamic acids, as well as of aroma compounds, such as terpenols, norisoprenoids, and benzenoids [8–14].

Among the metabolomic methods available, liquid chromatography coupled to high-resolution mass spectrometry (HRMS) is very effective by providing the identification of several hundred metabolites in grape and wine in just two analyses performed in positive and negative ionization modes [15–19]. Recently, an approach of HRMS-suspect screening metabolomics in grape was proposed and it allowed identification of new grape compounds belonging to the chemical classes of stilbenes, flavonols, anthocyanins, and glycoside terpenes [20–22].

In the present study this method was used to investigate the metabolome of Corvina, Raboso Piave, Primitivo, and Negro Amaro grapes. In particular, the profiles of flavonols, flavanols and flavanones, glycoside terpenols, procyanidins, stilbenes, and anthocyanins of each variety were determined, and the peculiar metabolites, which can be used as traceability markers of the corresponding wines, were identified.

2. Materials and Methods

2.1. Samples and Standards

Grape samples of *Vitis vinifera* Corvina Veronese, Primitivo, and Negro Amaro were harvested in 2016, while Raboso Piave grapes were collected in 2013. All samples were sourced from the vine Germoplasm Collection of the CREA-Viticulture & Enology sited in Susegana (Veneto, Italy). For each variety, 100 berries were collected at the technological maturity (maximum soluble solid content in the juice) from five different plants using randomized criteria, and kept frozen at −20 °C until analysis.

Standards of kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, myricetin-3-*O*-glucoside, malvidin-3-*O*-glucoside, kaempferol-3-*O*-glucuronide, (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, procyanidin B1, procyanidin B2, tamarixetin, syringetin, and rutin were purchased from Extrasynthese (Genay, France); quercetin, myricetin, kaempferol, *trans*-resveratrol, *trans*-piceid, piceatannol, *E*-piceid, isorhamnetin, and 4′,5,7-trihydroxy flavanone from Sigma-Aldrich (Milan, Italy). δ-viniferin was provided by CT Chrom (Marly, Switzerland). *Z*-piceid was produced by photoisomerization of the *E* isomer as reported for the isomerization of *trans*-resveratrol (around 80% conversion yield) [23]. *E*-ε-viniferin was extracted from lignified vine cane as proposed by Pezet and coworkers [24].

2.2. Sample Preparation

Sample preparation for analysis was performed using 20 grape berries. After removing the seeds, pulp and skins were ground under liquid nitrogen using an ultra-turrax (IKA, Staufen, Germany). Pure methanol was added to the resulting powder in a ratio 2:1 (*v*/*w*), and the extraction was carried out for 20 min. After the addition of 200 µL of 4′,5,7-trihydroxyflavanone solution (520 mg/L) as internal standard, samples were centrifuged (2957 rcf, 18 °C, 12 min), the supernatant was filtered by using an Acrodisc GHP 0.22 µm filter (Waters, Milford, MA, USA) and LC/MS analysis of the solution was performed. For each variety (Corvina, Primitivo, Negro Amaro, and Raboso Piave), two grape samples were studied.

2.3. UHPLC-Q/TOF Analysis

An analytical system composed by Ultra-High Performance Liquid Chromatography (UHPLC) Agilent 1290 Infinity coupled to Agilent 1290 Infinity G4226A autosampler and accurate-mass Quadrupole-Time of Flight (Q/TOF) Mass Spectrometer Agilent 6540 (nominal resolution 40000) with Agilent Dual Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA, USA), was used.

Data acquisition software: Agilent MassHunter version B.04.00 (B4033.2). Chromatographic separation was performed by Zorbax reverse-phase column (RRHD SB-C18 3 × 150 mm, 1.8 µm) (Agilent Technologies, Santa Clara, CA, USA) using solvent A 0.1% (*v*/*v*) aqueous formic acid and solvent B 0.1% (*v*/*v*) formic acid in acetonitrile, and the following elution gradient program: 5% B isocratic for 8 min, from 5% to 45% B in 10 min, from 45% to 65% B in 5 min, from 65% to 90% in 4 min, 90% B isocratic for 10 min; flow rate 0.4 mL/min. Sample injection 5 µL; column temperature 35 °C. False positives were checked by analyzing a blank between each pair of samples. For each sample, two repeated analyses in both positive and negative ionization mode were performed.

Q/TOF conditions: sheath gas nitrogen 10 L/min at 400 °C; drying gas nitrogen 8 L/min at 350 °C; nebulizer pressure 60 psig, nozzle voltage 0 kV (negative ionization mode) and 1 kV (positive ionization mode), capillary voltage ±3.5 kV in positive and negative ion modes, respectively. Signals in the *m*/*z* 100–1700 range, were recorded. Mass calibration was performed with standard mix G1969-85000 (Supelco Inc.) and had residual error for the expected masses between ±0.2 ppm. Lock masses: TFA anion at *m*/*z* 112.9856 and HP-0921(+formate) at *m*/*z* 966.0007 in negative-ion mode, purine at *m*/*z* 121.0509 and HP-0921 at *m*/*z* 922.0098 in positive-ion mode.

Data analysis was performed by Agilent MassHunter Qualitative Analysis software version B.05.00 (5.0.519.0). Compound identification was based on accurate mass and isotope pattern and expressed as "overall identification score" computed as weighted average of the isotopic pattern signal (W_{mass} = 100, $W_{abundance}$ = 60, $W_{spacing}$ = 50, mass expected data variation 2.0 mDa + 5.6 ppm, mass isotope abundance 7.5%, mass isotope grouping peak spacing tolerance 0.0025 *m*/*z* + 7.0 ppm).

Targeted data analysis was performed by using the algorithm '*Find by Molecular Formula*'. Compounds were identified by using the in-house constructed HRMS database *GrapeMetabolomics*. Identifications were confirmed by performing autoMS/MS of the precursor ions in the *m*/*z* 100–1700 range (collision energy 20–60 eV, acquisition rate 2 spectra/s) and using the standards available.

2.4. Statistical Analysis

Multivariate analysis was performed by using the $[M-H]^-$ or $[M]^+$ ion peak area normalized to the internal standard.

Tukey's test was performed by PAST 3.01 software (Paleontological statistics software package for education and data analysis; Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001) using the intensity of the normalized recorded signals. The data with different letters were significantly different for $p < 0.01$.

Principal component and Cluster analyses (Ward method, Euclidean distance) were performed by MetaboAnalyst, version 4.0 (http://www.metaboanalyst.ca, last visited on 26 July 2018, Xia and Wishart 2016) [25]. Data were normalized (sum), transformed (log), and scaled (mean-centered by SD of each variable).

3. Results and Discussion

3.1. Identification of the Metabolites

By performing ultra-high performance liquid chromatography quadrupole-time of flight mass spectrometry (UHPLC-Q/TOF) in negative ionization and the identification of metabolites using the grape and wine database *GrapeMetabolomics* [21], on average 350–400 compounds were putatively identified for each of the four grape varieties. The identity of the metabolites belonging to the chemical classes of flavonols/flavanones, glycoside terpenols (aroma precursors), flavanols and procyanidins, and stilbenes, was successively confirmed by multiple mass spectrometry (MS/MS), and their potential as wine varietal markers was evaluated.

Among them, a $[M-H]^-$ signal at *m*/*z* 285.068 corresponding to the molecular formula $C_{16}H_{13}O_5$, was observed in all the samples (mass error 1.4 ppm). This compound, eluting at 22.32 min, showed as main MS/MS fragments the signals at *m*/*z* 270.052 corresponding to the $C_{15}H_{10}O_5$ ion formed by $\bullet CH_3$ loss (mass error −2.9 ppm), at *m*/*z* 243.066 corresponding to $C_{14}H_{11}O_4$ ion formed by CH_2CO

loss (mass error −0.8 ppm), and as mass spectrum base peak the signal at m/z 164.011 corresponding to the $C_8H_4O_4$ ion (mass error −2.4 ppm) (Figure 1). This compound was putatively identified as a methyl-naringenin isomer.

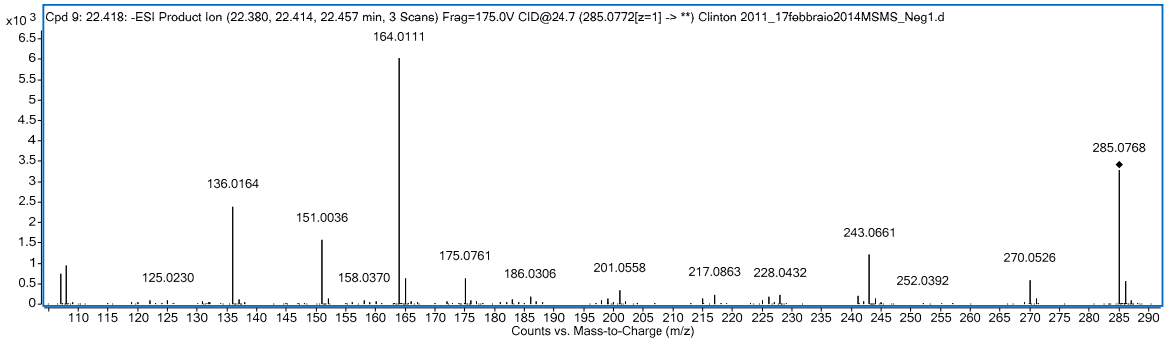

Figure 1. Ultra-high performance liquid chromatography quadrupole-time of flight multiple mass spectrometry (UHPLC-Q/TOF) spectrum of putative methyl-naringenin isomer identified in the grape.

Positive-MS analysis provided the identification of the grape anthocyanins, in particular delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv) glucoside, alongside with their acetylglucoside and *p*-coumaroylglucoside derivatives, and of Mv-caffeoylglucoside.

A total of 92 metabolites were identified in the samples, including 35 flavonols/flavanones, 16 anthocyanins, 11 glycoside monoterpenes, 11 flavanols/procyanidins, and 19 stilbenes. The potential for these metabolites to be used as a marker of the corresponding wines was then investigated.

3.2. Potential Flavonoid Markers of the Wine Varieties

Polyphenolic biosynthesis is regulated by genetic factors and several chemotaxonomic studies have shown that grape varieties can be differentiated on the basis of their anthocyanin and flavonol profiles [8,26–28]. In fact, despite that their amounts in grape are affected by environmental and agronomical factors, the profiles mainly depend on the cultivar characteristics [29]. In particular, while the F3′H enzyme is always active, the activity of the flavonoid 3′5′hydroxylase enzyme (F3′5H) varies depending on the grape variety [30]. Therefore, even if the phenolic parameters can be affected by the winemaking techniques and wine aging conditions used [6], anthocyanins and flavonols and their derivatives can be probably evaluated as potential variety traceability markers of wines [28,30–32].

Figure 2 shows the biplot of principal component analysis (PCA) calculated by using as variables the flavonols and flavanones identified in the grape varieties. Results indicate that the first two components account for 72.4% of the total variance, first component 37.0% and second component 35.4%.

The PCA clearly visualizes the separation among the varieties based on the non-anthocyanic flavonoids. The separation of the second component is driven by high contents of methyl-naringenin, myricetin (Mr), isorhamnetin (Iso), a tetrahydroxy-dimethoxy flavanone hexoside, three *p*-coumaroyl derivatives (kaempferide-*p*-coumaroylhexoside, isorhamnetin-*p*-coumaroylglucoside, and dihydrokaempferide-*p*-coumaroylhexoside) which were found in Raboso Piave. Tukey's test ($p < 0.01$) confirmed the statistical significance of these differences towards the other varieties (Table 1). Also statistically significant is the difference for the concentration of quercetin (Q) glucuronide, that was the lowest in Raboso Piave.

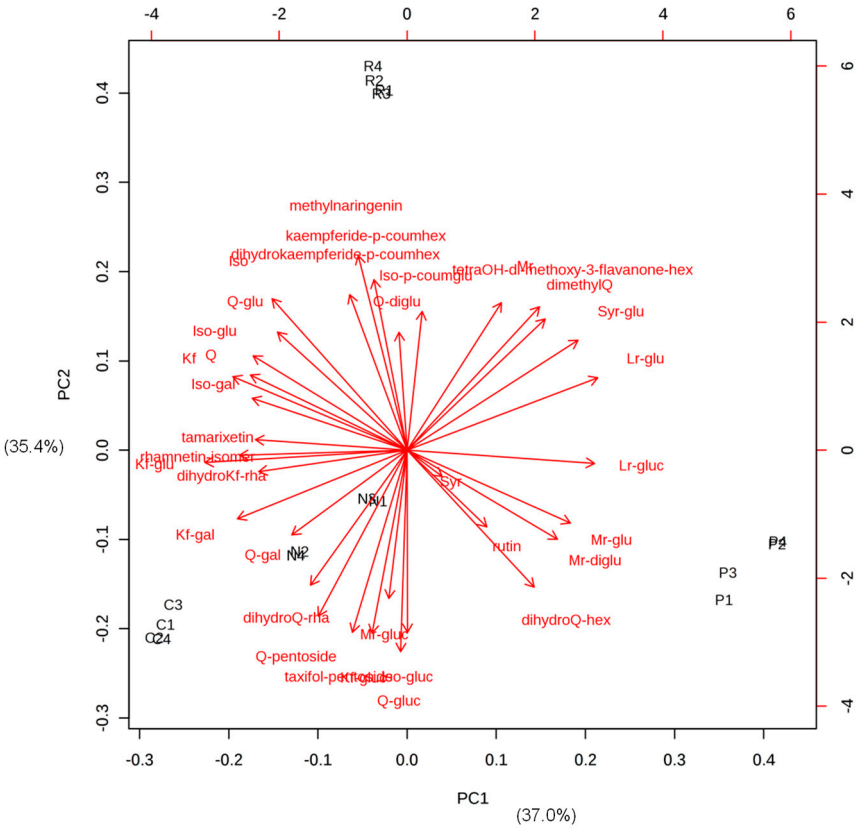

Figure 2. Biplot of the normalized UHPLC-Q/TOF signal intensities of non-anthocyanic flavonoids identified in the four grape varieties. P: Primitivo, C: Corvina, N: Negro Amaro, R: Raboso Piave. Mr, myricetin; Iso, isorhamnetin; Q, quercetin; Syr, syringetin; Lr, laricitrin; Kf, kaempferol; glu, glucoside; diglu, diglucoside; gluc, glucuronide; hex, hexoside; gal, galactoside; rha, rhamnoside; p-coumhex, *p*-coumaroylhexoside.

Primitivo grapes had contents of laricitrin (Lr) glucoside and Lr-glucuronide, as well as dihydroquercetin hexoside, that were significantly higher than in the other varieties.

The PCA also highlights in Primitivo high signals of Mr-glucoside and its diglucoside derivative, which are, however, not significantly different from those found in Negro Amaro. This variety also showed particularly low contents of tamarixetin, Iso, and kaempferol (Kf) derivatives.

Corvina is characterized by higher signals of taxifolin-pentoside and dihydroquercetin rhamnoside, the difference of which was statistically significant. As shown in Figure 2, lower signals of Lr and Syr derivates were observed in this variety.

Lastly, Negro Amaro showed significantly higher levels of Mr-glucuronide, Q- and Iso-galactosides, and tamarixetin (Table 1 and Figure 2).

Figure 3 shows the biplot of PCA of the four varieties calculated using the anthocyanins as variables. The first two components accounted for 78.5% of the total variance, with the first component 51.4% and the second component 27.1% of the variance. The four varieties were clearly separated also by their anthocyanin content. In particular, Raboso had significantly higher content of acetyl derivatives, in particular Dp, Cy, Pt, and Pn acetylglucosides, and Cy-*p*-coumaroylglucoside ($p < 0.01$, Tukey's test in Table 2). Significantly higher levels of Mv derivatives and Dp-*p*-coumaroylglucoside were found in Primitivo. Conversely, this variety had the lowest level of Cy-glucoside when compared to the other three varieties. In Corvina, a statistically significant low signal of Pt-glucoside was observed. Negro Amaro was mainly characterized by significantly higher Dp-glucoside levels, and the signals of acyl-anthocyanins had low intensities (as visualized by PCA), however they were not significantly different from the other varieties.

Table 1. Tukey's test calculated using the normalized UHPLC-Q/TOF signal intensities of flavonols and flavanones identified in the four grape varieties (n = 4). The data with different letters are significantly different for $p < 0.01$. n.f., signal not found.

Flavonols/Flavanones	$p < 0.01$			
	Corvina	**Primitivo**	**Negro Amaro**	**Raboso**
dihydrokaempferol-rhamnoside	b	a	a	c
dihydroquercetin-hexoside	a	c	ab	b
dihydroquercetin-rhamnoside	b	a	a	a
dimethylquercetin	a	b	a	c
isorhamnetin	a	n.f.	a	b
isorhamnetin-galactoside	a	b	c	a
isorhamnetin-glucoside	b	c	a	a
isorhamnetin-glucuronide	ab	ab	a	b
kaempferol	ab	a	ab	b
kaempferol-galactoside	a	b	ab	ab
kaempferol-glucoside	a	b	a	a
kaempferol-glucuronide	a	ab	ab	b
laricitrin-glucoside	a	b	a	c
laricitrin-glucuronide	a	c	b	ab
methylnaringenin	a	a	a	b
myricetin	a	b	c	d
myricetin-diglucoside	a	b	b	a
myricetin-glucoside	a	b	b	a
myricetin-glucuronide	a	a	b	a
quercetin	ab	b	c	ac
quercetin-diglucoside	a	a	b	b
quercetin-galactoside	a	b	c	ab
quercetin-glucoside	ab	a	bc	c
quercetin-glucuronide	a	b	a	c
quercetin-pentoside	a	a	a	a
rhamnetin-isomer	a	b	a	a
rutin	a	a	a	a
syringetin	a	ab	b	ab
syringetin-glucoside	b	a	c	a
tamarixetin	a	b	c	a
taxifolin-pentoside	b	a	a	a
tetrahydroxy-dimethoxyflavanone hexoside	b	a	a	c
kaempferide-*p*-coumaroylhexoside	a	a	a	b
isorhamnetin-*p*-coumaroylglucoside	a	a	b	c
dihydrokaempferide-*p*-coumaroylhexoside	a	a	a	b

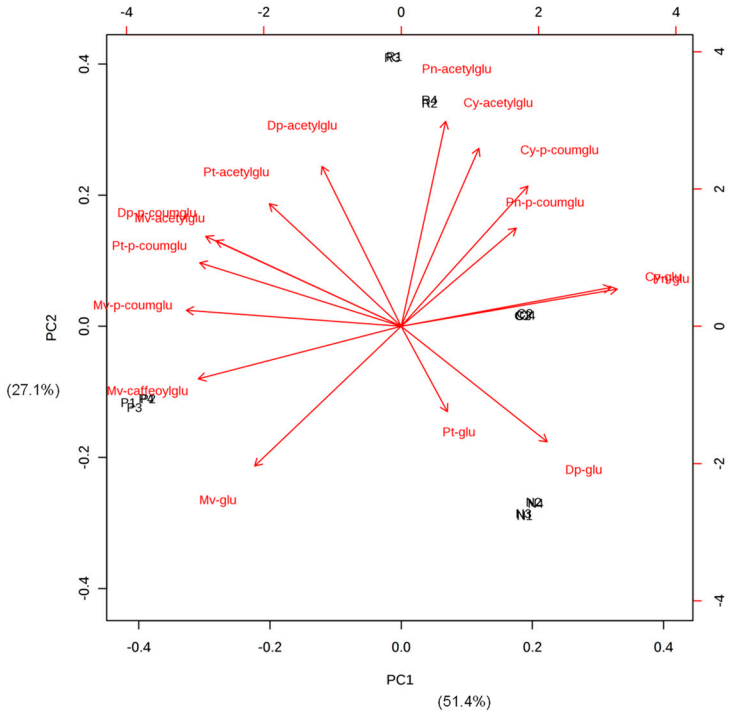

Figure 3. Biplot of the normalized UHPLC-Q/TOF anthocyanin signal intensities in the four grape varieties studied. P: Primitivo, C: Corvina, N: Negro Amaro, R: Raboso Piave. Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin. glu, glucoside; p-coumglu, *p*-coumaroylglucoside; acetylglu, acetylglucoside.

Table 2. Tukey's test calculated using the normalized UHPLC-Q/TOF signal intensities of anthocyanins in the four grape varieties ($n = 4$). The data with different letters are significantly different for $p < 0.01$. Cy, cyanidin; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pt, petunidin.

Anthocyanins	$p < 0.01$			
	Corvina	**Primitivo**	**Negro Amaro**	**Raboso**
Cy-acetylglucoside	a	a	a	b
Cy-*p*-coumaroylglucoside	b	a	a	c
Cy-glucoside	b	c	a	a
Dp-acetylglucoside	a	a	a	b
Dp-*p*-coumaroylglucoside	a	b	a	c
Dp-glucoside	a	a	b	ab
Mv-acetylglucoside	a	b	a	b
Mv-caffeoylglucoside	a	b	a	a
Mv-*p*-coumaroylglucoside	a	b	a	c
Mv-glucoside	b	c	a	a
Pn-acetylglucoside	a	a	a	b
Pn-*p*-coumaroylglucoside	b	a	a	b
Pn-glucoside	a	c	ab	b
Pt-acetylglucoside	a	b	a	c
Pt-*p*-coumaroylglucoside	a	b	a	c
Pt-glucoside	a	ab	b	b

By performing Liquid chromatography coupled to high-resolution mass spectrometry (LC-Q/TOF) metabolomic analysis, also flavan-3-ols and procyanidins in pulp and skins, were identified. Table 3 reports the normalized signal intensities of flavan-3-ol monomers, dimers, and trimers identified in the berries of the samples after seeds had been removed. Corvina grapes had the highest procyanidin content, which was almost 4-fold higher than that of Raboso Piave and 2-fold than that of both Primitivo and Negro Amaro. Corvina also had the highest signals of (+)-catechin and

procyanidin dimers. In a previous study, procyanidin B1 and B2 resulted determinant in discriminating the wines in terms of variety and origin [33].

Table 3. Normalized UHPLC-Q/TOF signal intensities of flavan-3-ols and procyanidin dimers and trimers identified in the berries removed by the seeds of the four grape varieties. CV%, coefficient of variance (SD × 100/mean, n = 4). In the last line, the percentages of total signal normalized to Raboso Piave are reported in bold. n.f., signal not found.

Procyanidins	Normalized $[M-H]^-$ Signal Area							
	Corvina		Primitivo		Negro Amaro		Raboso	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
(−)-epicatechin	1,687,850	19	851,612	12	1,784,264	17	1,294,547	25
(+)-catechin	5,299,475	16	2,007,948	55	2,028,302	10	1,571,897	8
(−)-epigallocatechin	446,924	1	653,619	17	442,091	4	448,730	14
(−)-epicatechin gallate	984,163	24	445,224	25	729,303	21	51,256	14
procyanidin (B3/B4/B5)	1,314,453	15	477,508	8	882,147	18	289,062	2
procyanidin B1	6,813,192	4	2,778,443	7	2,501,480	8	839,493	6
procyanidin B2	111,031	15	n.f.		55,704	26	95,472	22
procyanidin T2/T3(T4)/C1	927,837	6	256,004	6	261,175	13	58,481	18
procyanidin T2/T3(T4)/C1	280,969	6	67,896	39	104,278	14	28,399	12
procyanidin T2/T3(T4)/C1	312,539	15	120,654	6	264,665	20	45,451	18
prodelphinidin T2/T3	134,478	10	51,808	26	89,307	9	41,249	17
Sum	18,312,911	(384%)	7,710,716	(162%)	9,142,716	(192%)	4,764,039	(100%)

In our study seeds were not analyzed, therefore their contribution to the wine procyanidin profile was not evaluated. Hence, these data just show the differences among the grape varieties but cannot be used for a wine traceability model.

In the biosynthesis of anthocyanins, the enzymes 3′methyltransferase (3′OMT) and flavonoid-3′,5′-hydroxylase (F3′5′H) transform Cy into Pn and into Dp, respectively. Higher F3′5′H activity increases the levels of trihydroxylated anthocyanins by affecting the dihydroxy/trihydroxy ratios, while 3′OMT induces methylation of Dp with formation of Pt and Mv [32,34]. A study on the F3′H and F3′5′H genes' expression showed a close relationship between the biosynthetic pathways of flavonols and anthocyanins [35].

With regard to our varieties, Primitivo grape is dominated by the presence of tri-substituted flavonoids, such as Lr, Mr, and Syr, as well as high content of tri-substituted anthocyanins, such as Pt and Mv derivatives. On the other hand, Corvina and Negro Amaro were found to be richer in di-substituted compounds. Raboso is characterized by a significant presence of both anthocyanic and non-anthocyanic acyl derivatives.

A study of Sangiovese wines showed that the wine anthocyanic pattern recognition is linked to the grape variety and the pigments formed during aging, such as vitisin B-like and vitisin A-like compounds, and ethyl-linked and direct-linked flavanol-anthocyanin derivatives [36]. The structures of these pigments are shown in Figure 4.

Taking these findings into consideration, one would expect to find in Primitivo wines higher amounts of the pigments formed by Pt and Mv, while in Raboso young wines, higher acyl anthocyanins are expected. Moreover, Primitivo and Raboso young wines can have significant *p*-coumaroyl anthocyanins, different from Negro Amaro, Corvina, or Sangiovese wines [36], and Raboso also high acetyl anthocyanins. However, the simple grape anthocyanins and their acyl derivatives that are usually present in large quantities in young wines, gradually decrease during aging due to degradation processes and reactions leading to the formation of more stable pigments. For example, vitisin A-like and vitisin B-like pigments are more stable than the corresponding grape anthocyanins [37], and Pinotin A-like pigments were found to increase with wine ageing [38].

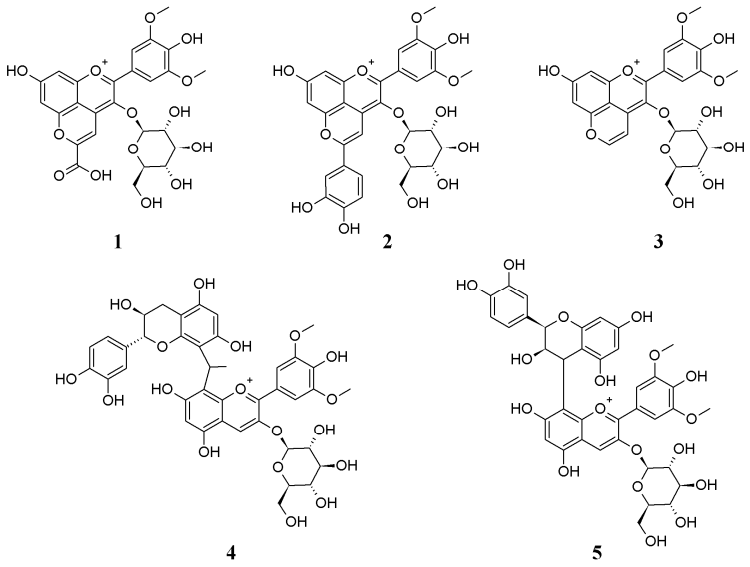

Figure 4. Pigments formed during wine aging: (**1**) vitisin A; (**2**) pinotin A; (**3**) vitisin B; (**4**) ethyl-linked catechin-Mv glucoside; (**5**) direct-linked catechin-Mv glucoside.

A study performed on Primitivo wines showed that the presence of Mv-*p*-coumaroylglucoside persists also in 2-year old wines [39]. The high Mv-glucoside content we found in Primitivo grapes indicated that aged wines probably have important content of Mv derivatives, such as pyranoanthocyanidins and flavanol-anthocyanin adducts. This assumption was confirmed by the study of Dipalmo et al., who identified the presence of many Mv-pigments in the 2-year old Primitivo wines, such as Mv-glucoside-4-vinyl-phenol, Mv-glucoside-4-vinyl-(epi)catechin, Mv-glucoside-8-ethyl-(epi)catechin, Mv-(*p*-coumaroyl)-glucoside-8-ethyl-(epi)catechin, (epi)-catechin -Mv-glucoside, di(epi)catechin-Mv-glucoside, Mv-acetylglucoside-4-vinyl-di(epi)catechin, Mv-(*p*-coum aroyl)-glucoside-4-vinyl-(epi)catechin, Mv-glucoside-8-ethyl-(epi)catechin, Mv-glucoside-4-vinyl-tri (epi)catechin, Mv-(caffeoyl)-glucoside-4-vinyl-di(epi)catechin, and Mv-(*p*-coumaroyl)-glucoside-4-vin yl-di(epi)catechin [39].

The high contents of Pn-glucoside and (+)-catechin found in Corvina grape suggest, during wine ageing, the formation of Pn-catechin derivatives which can be both direct-linked and ethyl-linked.

In general, flavonol and flavanone aglycones are present in wines as a result of the hydrolysis of corresponding glycosides occurring during winemaking [40]. Conversely, during wine aging, flavonols show different evolution patterns, a behavior that in some cases was observed and is dependent on the grape variety studied [41]. A study on red wines stabilized for 5 months showed a significant decrease of glycoside flavonols as result of the sugar moiety hydrolysis, and a significant decrease of total flavonol content due to their oxidation and co-pigmentation with anthocyanins [42,43].

It can be hypothesized that Primitivo grapes, characterized by high tri-substituted flavonols, produce wines richer in Lr, Mr, and Syr (aglycones or glycosides). In previous studies, the ratios between the total content of single flavonols were used to differentiate wine varieties. For example, the Q/Mr ratio was used to distinguish between Carménère and Merlot wines [44]. In Primitivo and Raboso wines, higher Mr/Q and Mr/Kf ratios are expected, being driven by the higher Mr and the lower Q and Kf in grapes. Primitivo wines can be also characterized by high Lr/Q and Lr/Kf ratios. On the contrary, lower Mr/Q and Mr/Kf ratios are expected in Corvina wines, being this variety characterized by lower Mr, Lr, and Syr and higher Kf and Q.

The abundance of Q and Kf glucuronides and galactosides, Q and taxifolin pentosides, and dihydroquercetin-rhamnoside could characterize the Negro Amaro and Corvina wines.

3.3. Monoterpene Glycosides (Aroma Precursors)

Wine aroma can be influenced by many factors, such as grape variety, climate, fermentation condition, yeast strains, winemaking process, aging, and storage conditions [45–47].

Glycoside monoterpenols are precursors responsible, in particular, for the aroma of aromatic and semi-aromatic grapes, e.g., Muscat and Malvasia varieties, Glera, Riesling, etc. Study of these secondary metabolites is also performed for grape chemotaxonomy aims [10,11,22,48], and wines from different varieties have been successfully differentiated on the basis of their terpene contents (e.g., nerol, β-santalol, 4-carene) [49].

A PCA performed using the monoterpene glycosides identified in the four grape varieties as variables, is shown in biplot Figure 5. The first two components accounted for 85.7% of the total variance, with the first component being 60.6% and the second component 25.1%. Results of the Tukey's test ($p < 0.01$) are reported in Table 4.

Table 4. Tukey's test calculated using the normalized UHPLC-Q/TOF signal intensities of monoterpene glycosides identified in the four grape varieties ($n = 4$). The data with different letters are significantly different for $p < 0.01$. n.f., signal not found.

Monoterpene Glycosides	$p < 0.01$			
	Corvina	Primitivo	Negro Amaro	Raboso
α-terpineol pentosyl-hexoside	n.f.	n.f.	a	b
linalool pentosyl-hexoside	n.f.	n.f.	a	b
geraniol pentosyl-hexoside	b	c	a	a
Ho-diendiol I pentosyl-hexoside	b	c	a	a
Ho-diendiol I rhamnosyl-hexoside	b	c	a	a
trans/*cis* 8-hydroxylinalool pentosyl-hexoside	a	a	b	c
trans/*cis* furan/pyran linalool oxide pentosyl-hexoside	a	a	b	n.f.
3,7-dimethyl-1-octen-6-one-3,7-diol pentosyl-hexoside 1	a	n.f.	a	b
3,7-dimethyl-1-octen-6-one-3,7-diol pentosyl-hexoside 2	a	a	a	b
3,7-dimethyl-1-octen-6-one-3,7-diol rhamnosyl-hexoside 1	a	a	b	c
3,7-dimethyl-1-octen-6-one-3,7-diol rhamnosyl-hexoside 2	b	a	c	a

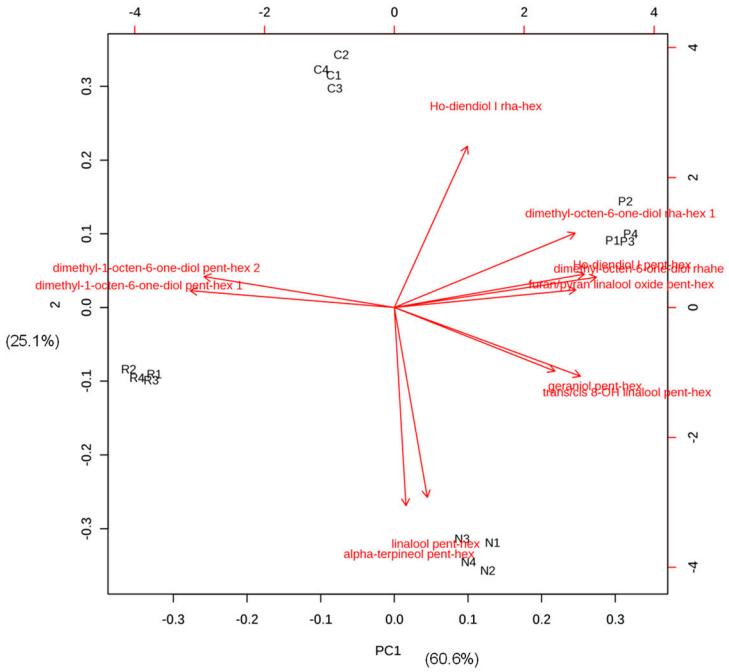

Figure 5. Biplot of the normalized UHPLC-Q/TOF signal intensities of monoterpene glycosides identified in the four varieties studied. P: Primitivo, C: Corvina, N: Negro Amaro, R: Raboso Piave. Pent-hex, pentosyl-hexoside; rha-hex, rhamnosyl-hexoside.

As observed for the anthocyanins and flavonols, the profiles of monoterpenol glycosides discriminate the four grape varieties. The separation along the second component was mainly driven by the high signals of linalool and α-terpineol pentosyl-hexosides, which were significantly lower in Raboso and were not detected in Corvina and Primitivo. Also, geraniol pentosyl-hexoside signal was very low in Corvina and Primitivo in respect to the other varieties. Negro Amaro and Raboso showed also a higher content of the Ho-diendiol I glycosides, and Raboso had a statistically significant high content of 3,7-dimethyl-1-octen-6-one-3,7-diol pentosyl-hexosides (Table 4). The high content of monoterpene glycosides found in Negro Amaro is in agreement with previous studies [50].

3.4. Other Metabolites

In addition to the compounds discussed above, the profiles of stilbenes in the four samples were detected and the normalized signal intensities are reported in Table 5.

Table 5. Normalized LC-Q/TOF signal intensities of resveratrol derivatives identified in the grape varieties studied. CV%, coefficient of variance (SD × 100/mean, $n = 4$). In the last line, the percentages of the total signal normalized to Raboso Piave samples are reported in bold. n.f., signal not found.

Stilbenes	Normalized $[M - H]^-$ Signal Area							
	Corvina		Primitivo		Negro Amaro		Raboso	
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
trans-resveratrol	283,900	53	22,549	17	59,096	10	807,651	15
piceatannol	269,846	45	106,655	49	205,075	30	1,400,281	4
cis-piceid	1,270,082	10	429,127	9	1,026,866	19	1,691,503	8
trans-piceid	177,040	17	99,308	14	96,404	30	313,008	15
E-astringin	67,013	15	47,798	10	68,551	9	44,347	10
Z-astringin	34,428	15	29,354	41	38,666	25	45,557	15
pallidol	246,754	8	71,397	30	42,236	26	172,312	18
resveratrol dimer 2	60,835	12	26,747	21	13,466	30	265,081	24
Z-ε-viniferin	169,259	2	63,423	15	72,224	24	1,771,218	18
E-ε-viniferin	187,388	10	110,183	13	68,958	4	990,456	14
Z-ω-viniferin	66,891	3	40,938	14	28,140	18	703,006	29
δ-viniferin	70,332	5	10,490	17	23,925	14	137,580	41
caraphenol	22,200	25	11,504	23	4690	10	126,287	43
pallidol-3-*O*-glucoside	33,095	12	29,203	9	14,455	12	91,436	11
α-viniferin	7699	53	59,992	37	16,784	83	113,833	57
Z-miyabenol C	25,288	4	18,977	17	11,579	21	342,147	23
E-miyabenol C	62,101	25	118,412	24	39,757	24	1,256,874	35
tetramer resveratrol 1	60,484	65	11,364	50	8958	14	152,028	49
tetramer resveratrol 2	31,491	27	12,509	48	8728	25	1,129,196	18
Sum	3,146,127	(27%)	1,319,930	(11%)	1,848,558	(16%)	11,553,801	(100%)

Several differences among the samples were found. In particular, the total signal of stilbenes in Raboso was up to 1–2 magnitude order higher than the other samples, *trans*-resveratrol was over 30-fold than Primitivo and 10-fold than Negro Amaro. A similar trend was also observed for piceatannol and the resveratrol oligomers.

Stilbenes accumulation in grape is induced by genetic factors, but viniferins and resveratrol oligomers are phytoalexins which can be synthetized as "inducible" compounds through the activation of the stilbene synthase gene (STS) under the elicitation of biotic and/or abiotic agents [51,52]. As a consequence, these compounds can hardly be considered as pure variety markers and were not evaluated for wine traceability in this study.

4. Conclusions

LC-Q/TOF *suspect screening analysis* provided the identification and relative quantification of metabolites belonging to the main chemical classes in the four grape varieties. This grape chemotaxonomy approach allowed the identification of several potential variety markers, which are likely to be found also in the resulting wines.

In Raboso wines, relatively high Mr/Q and Mr/Kf ratios (around 1 and 4, respectively) and a high abundance of both anthocyanic and non-anthocyanic acyl derivatives (in particular acetyl anthocyanins in young wines), are expected. The volatile fraction of these wines is probably characterized by the presence of 3,7-dimethyl-1-octen-6-one-3,7-diol and Ho-diendiol I.

Primitivo wines potentially have high contents of tri-substituted flavonoids, such as Lr, Mr, and Syr, and lower Iso and Kf derivatives. High Mr/Q and Mr/Kf ratios (around 1 and 6, respectively) and relatively high Lr/Q and Lr/Kf ratios (0.1 and 0.3, respectively), are expected. Wine color is characterized by high Pt and Mv pigments, with a significant presence of *p*-coumaroyl anthocyanins in young wines, and Pt and Mv pyranoanthocyanidins and flavanol-anthocyanin adducts in aged wines.

In general, Corvina wines are likely to have higher level of di-substituted compounds and lower acyl derivatives, with significant presence of taxifolin and dihydroquercetin, and low Lr and Syr. Young wines can be characterized by the presence of Q and Kf glucuronides and galactosides, Q and taxifolin pentosides and dihydroquercetin-rhamnoside, and low Mr/Q and Mr/Kf ratios (around 0.2 and 1, respectively). In aged wines, the presence of Pn-flavanol derivatives can be expected.

Negro Amaro wines have a non-anthocyanic flavonoid profile similar to Corvina, with higher di-substituted compounds, lower acyl derivatives, and a significant presence of Q, Kf, taxifolin, and dihydroquercetin. The volatile fraction will likely present peculiarly high levels of monoterpenols, such as α-terpineol, linalool, geraniol, and Ho-diendiol I.

It is worthy to note that the samples studied were collected from the same vine collection in just one vintage. Consequently, these findings do not take into account key variables such as vineyard location and vintage. However, this approach can potentially be applied to different study models and other high-quality wines. Despite the alcoholic fermentation impacts on the metabolites profile of a wine, generally the products partially maintain the varietal profiles. By comparing our findings and the previous results, the traceability markers here proposed can be probably applied to the wines. Future studies conducted on wines can confirm the hypotheses proposed.

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