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Differentiation of *Vitis vinifera* L. and Hybrid Red Grapes by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Analysis of Berry Skin Anthocyanins

Gianluca Picariello,*[,†] Pasquale Ferranti,[†,‡] Lina Chianese,[‡] and Francesco Addeo[†,‡]

[†]Istituto di Scienze dell'Alimentazione (ISA), CNR, Via Roma 64, 83100 Avellino, Italy

[‡]Dipartimento di Scienza degli Alimenti, University of Naples "Federico II", Parco Gussone, Portici (NA) 80055, Italy

ABSTRACT: Among the methods that have been developed for anthocyanin characterization, matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF MS) offers several analytical advantages in terms of speed, minimal sample handling, specificity, and reliability, without requiring any previous chromatographic separation. This study used MALDI-TOF MS to profile the anthocyanins from the berry skins of 23 red grape varieties clustered as (i) authentic *Vitis vinifera* grapes, (ii) American hybrid cultivars, and (iii) Casavecchia cultivars, previously characterized as functional crosses of *V. vinifera* with nondefined hybrid grapevines. Anthocyanin profiling demonstrated evidence of several varietal traits that enabled the differentiation of authentic *V. vinifera* from hybrid cultivars on a molecular basis. In particular, acyl 3,5-*O*-diglucoside anthocyanins were established as easily monitored molecular markers of the hybrid varieties. It was also demonstrated that MALDI-post source decay MS is a powerful tool to differentiate isobaric 3,5-*O*-diglucosides and their derivatives, which prevail in hybrid cultivars, from acylated 3-*O*-glucoside anthocyanins.

KEYWORDS: *anthocyanins, MALDI-TOF mass spectrometry, Vitis vinifera, hybrid grapevines, varietal differentiation*

INTRODUCTION

The variety of grapes can be determined by DNA analysis[1] or by evaluating various wine components such as proteins,[2] peptides,[3] polyphenols,[4,5] and amino acids and aromatic compounds.[6,7] Anthocyanins and their relative abundance also have been proposed as species for validating the identity of the red grapes used for winemaking. In fact, the anthocyanin profile is characteristic of the variety and can enable the chemotaxonomic differentiation of red grapes.[8] Grape anthocyanins are essentially composed by five aglycones, that is, cyanidin (Cy), delphinidin (De), peonidin (Pn), petunidin (Pe), and malvidin (Mv), which occur either monoglucosylated at the 3-*O*-H or diglucosylated at both the 3-*O*H and the 5-*O*H position. Minor amounts of pelargonidin (Pg), occurring as both 3-*O*-monoglucoside and 3,5-*O*-diglucoside along with their acylated derivatives, have been detected in *Vitis vinifera* and *Vitis rupestris* grapes.[9,10] The occurrence of trace amounts of Pg glucosides in Cabernet Sauvignon and Pinot noir *V. vinifera* berry skins has been recently confirmed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS).[11]

The 3,5-*O*-diglucosides, although present only in trace amounts in the berry skins of *V. vinifera* grapes, are greater in abundance than 3-*O*-monoglucosides in both *Vitis riparia* and *V. rupestris* grapes. Therefore, diglucosides are considered to be markers of both non-*V. vinifera* species and interspecies hybrids.

The acylation of the 3-*O*-glucoside moiety by several acids (mainly acetic, *p*-coumaric, and caffeic) greatly increases the heterogeneity of the basic anthocyanins, thereby complicating their comprehensive analysis.

Consequently, analytical tools for the authentication of anthocyanins include high-resolution chromatographic techniques such as reversed phase high-performance liquid chromatography (RP-HPLC) often coupled to identification techniques such as photodiode array (PDA) detection. Due to the complexity of the anthocyanin fraction, congener molecules belonging to different subclasses can coelute or overlap to a certain degree,[12,13] rendering PDA detection often ineffective to discriminate among these compounds that have similar spectroscopic characteristics. Therefore, mass spectrometry (MS) detection has become the method of choice for monitoring phenolics in grapes and wines. In previous studies liquid chromatography (LC) combined with ESI-MS and direct flow injection MS-based approaches has been used to profile anthocyanins from different *Vitis* cultivars.[12] Anthocyanin MS patterns allowed for the simultaneous differentiation of authentic *V. vinifera*, non-*V. vinifera*, and hybrid red grapevine cultivars at the molecular level. Furthermore, the ESI-MS/MS analysis has been applied successfully to the characterization of anthocyanin pentosides as signature glycosides of hybrid grape varieties. In agreement with previous findings,[14] acetyl and *p*-coumaroyl esters of 3,5-*O*-diglucosides were confirmed to be biosynthesized specifically by hybrid grapevines, pointing to these compounds as potential analytic targets for varietal differentiation. Due to the analytical advantages of profiling wine and grape anthocyanins by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, research interest in this method is increasing.[15−17] The MALDI-MS analysis of anthocyanins is fast, robust, relatively easy to conduct, and provides excellent responses in terms of

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sensitivity, specificity, and reliability. MALDI-MS is moderately tolerant toward the presence of contaminants and requires minimal sample handling. Furthermore, because of its unique features, MALDI-MS fits well to the direct analysis of complex mixtures of biomolecules without requiring time-consuming chromatographic separation.[9] As early as the late 1990s, Sugui et al. determined the anthocyanin profiles of five French hybrid red grape varieties by MALDI-TOF MS;[18] the five basic anthocyanins were identified as 3-*O*-glucosides, acetyl-glucosides, *p*-coumaroyl-glucosides, 3,5-*O*-diglucosides, and *p*-coumaroyl-diglucosides. More recently, MALDI-TOF MS profiling of grape seed proteins has been proposed as a tool that is complementary to DNA analysis for the differentiation of *V. vinifera* grape cultivars.[20,21]

In this study, we describe the use of MALDI-TOF MS to determine rapidly the anthocyanin fingerprinting of different grape varieties to discriminate between *V. vinifera* and non-*V. vinifera* grapes without performing a previous chromatographic separation. In addition, MALDI-post source decay (PSD) MS was utilized as an alternative technique to distinguish diglucoside species from isobaric acylated monoglucosides on the basis of the fragmentation pattern of anthocyanins.

MATERIALS AND METHODS

Chemicals. Solvents and chemicals were of the highest commercially available purity and were used without further purification. Methanol and concentrated HCl were provided by J. T. Baker (Deventer, The Netherlands). The MALDI matrices were purchased from Sigma (Milan, Italy).

Grape Samples. Overall, 23 red grape samples were screened in this study. Grapes were sampled in vineyards located in the Campania region (Italy) at the complete maturation stage. Maturity was considered to be reached when the sugar content measured as total soluble solids, as assessed by a PCE-032 optical refractometer (PCE Instruments, U.K.), was steadily ∼22%. Approximately 3 kg of grape bunches, carefully inspected to exclude visible diseases, were manually harvested from different grapevines of each variety. According to the varieties, they were clustered in three groups: (i) eight samples of certified *V. vinifera* grape varieties that included Pallagrello, Piedirosso (two different vineyards), Nero d'Avola, Cabernet Sauvignon, Aglianico (two different vineyards), and Primitivo cultivars; (ii) seven samples of Casavecchia red grape clones collected in different vineyards and two suspected hybrids, that is, Olivella (or Livella) and Suricillo varieties; and (iii) six hybrid samples that included grapes from Tintoria (Teinturier-derived), Kober 5BB rootstock (*Vitis berlandieri* × *Vitis riparia*), and four Italian Isabella clones (French Seibel-derived, *V. vinifera* × *Vitis labrusca*) from different vineyards.

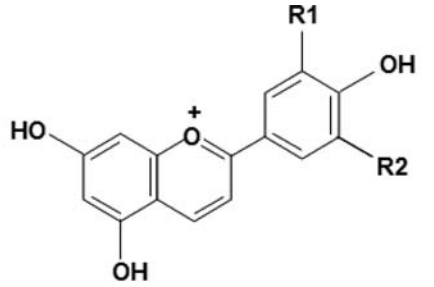
Anthocyanin Extraction. The anthocyanin extraction was carried out within 3 days after grape harvesting. Grape berries were randomly sampled from the bunches of each variety and squeezed. The skins were thoroughly washed with distilled water to eliminate pulp residues. The extraction of polyphenols was carried out according to the method of Takeoka et al.[22] A skin sample aliquot (20 g) was dried with filter paper, suspended in 40 mL of methanol containing 0.5% (v/v) 12 N HCl, and continuously stirred for 6 h at room temperature. Fine particles in the suspension were removed by applying the extracts to an anhydrous sodium sulfate column, and the methanol was evaporated at room temperature using a rotary evaporator (Heidolph, U.K.). The residue was adjusted to 20 mL with water. The methanol-free filtrate (3 mL) was loaded onto a Sep-Pak C_{18} cartridge (Water Corp., Milford, MA, USA) preconditioned by sequential washing with 5 mL of methanol and 5 mL of water, adapting the producer's instructions. The cartridge was washed with 10 mL of water and, finally, anthocyanins were eluted with 70% (v/v) aqueous methanol. Extracts were stored at −20 °C until use.

MALDI-TOF MS Analysis. MALDI-TOF MS experiments were carried out using a Voyager DE-PRO instrument (PerSeptive Biosystems, Framingham, MA, USA) equipped with an N_2 laser (337 nm, 3 ns pulse width). Mass spectrometry was conducted in the reflector positive ion mode with an accelerating voltage of 20 kV. The m/z 400−1200 range was explored, and typically 250 laser shots were acquired for each spectrum. To minimize the in-source fragmentation, the laser power was kept at a value not higher than 10% above the threshold. External mass calibration was performed through a separate acquisition of a mixture of standard anthocyanins (Sigma) that included Mv aglycone, Mv 3-*O*-glucoside, and Cy and Mv 3,5-*O*-diglucosides. Standards were dissolved in 50% acetonitrile (v/v) contaning 0.1% TFA at the concentration of 10 μg/mL. Under routine operating conditions, resolution at the full width/half-maximum of the peaks was normally in the 5500−7000 range. Sample solutions (1 μL) were mixed with the matrix solutions (1 μL) directly on the stainless steel target and air-dried. Sinapinic acid, α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid, all prepared by dissolving the crystalline powders (10 mg/mL) in 50% aqueous acetonitrile (v/v) containing 0.1% TFA, were tested as the matrices. At least three replicate analyses of the extracts from different batches were carried out to check for repeatability. Mass spectra were processed using the Data Explorer 4.0 software (PerSeptive BioSystems). PSD analysis was carried out after isolation of the precursor ions using a timed ion selector set at an ion gate width of 1 Da. The PSD mass spectra were divided into seven segments; the laser power and the guide wire voltage were varied for each segment to optimize fragmentation and data collection. Approximately 200 laser shots were acquired for each segment. Fragmented ions were refocused onto the final detector by stepping down the voltage applied to the reflector. Finally, the individual segments were stitched together using the software purchased with the instrument.

RESULTS AND DISCUSSION

A acidic pH (pH ∼1−2) converts anthocyanins almost exclusively to the corresponding flavylium (2-phenylbenzopyrylium) ions, which is well described as a structure carrying a net positive charge on the oxygen of the six-membered pyrylium ring in the C6−C3−C6 skeleton. Thus, in the positive ion mode, anthocyanins and their derivatives are detected in the aromatic oxonium ion form $[M^+]$. The chemical structures and molecular weights of the six common grape anthocyanidins are shown in Figure 1.

To define the operative conditions for the profiling of grape anthocyanins, several matrices were tested, including α-cyano-4-hydroxycinnamic and 2,5-dihydroxybenzoic acids. We found



aglycone	R1	R2	MW
Pelargonidin (Pg)	H	H	271
Cyanidin (Cy)	OH	H	287
Peonidin (Pn)	OCH_3	H	301
Delphinidin (Dp)	OH	OH	303
Petunidin (Pt)	OH	OCH_3	317
Malvidin (Mv)	OCH_3	OCH_3	331

Figure 1. Chemical structures and molecular weights (MWs) of the six common grape anthocyanidins.

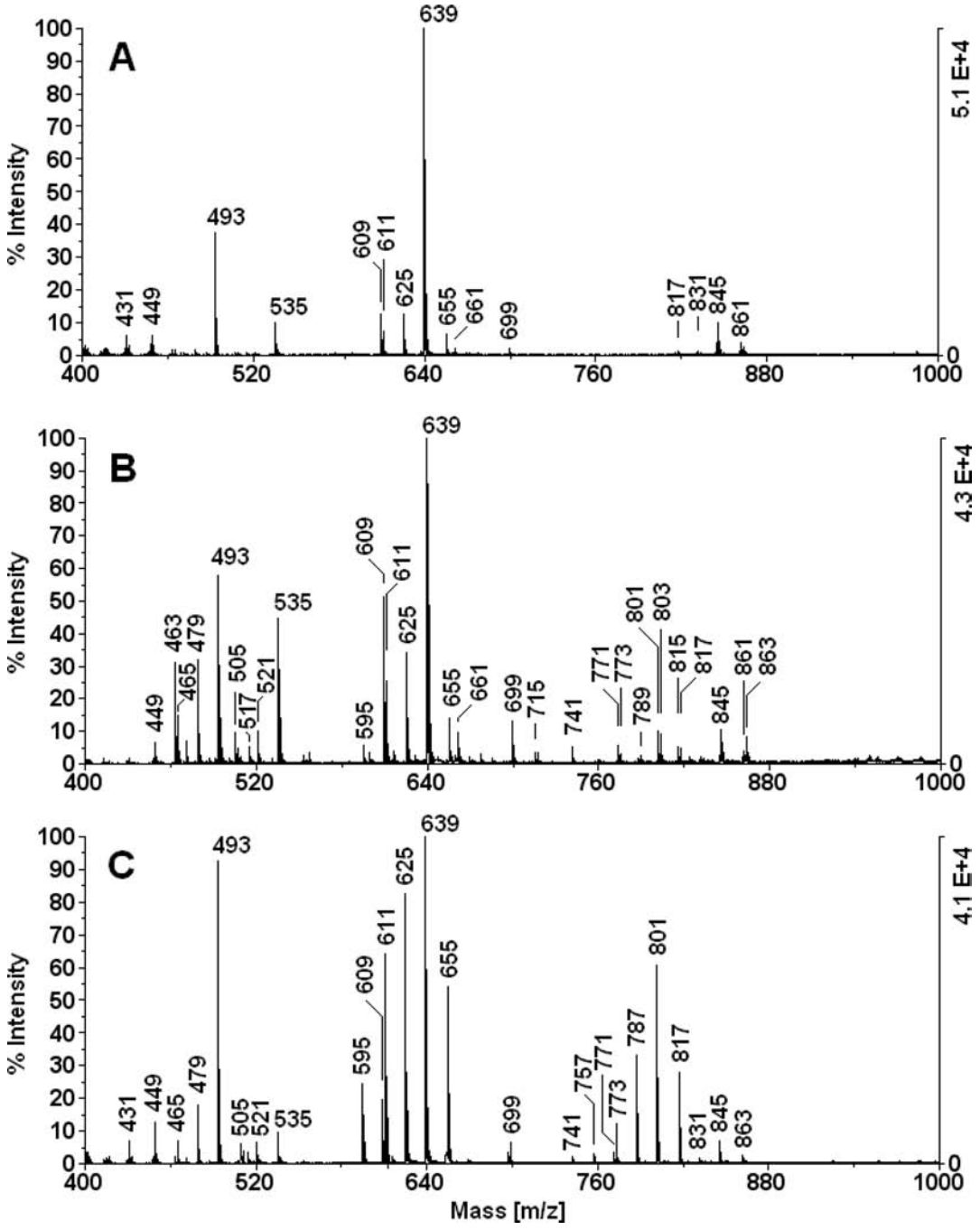


Figure 2. Comparative MALDI-TOF MS anthocyanin profiles of samples that are representative of the three grape varieties investigated in this study: (A) authentic *V. vinifera* grapes (Pallagrello variety); (B) Casavecchia grapes (Casavecchia Gentile variety); (C) hybrid grapes (Kober 5BB clone).

that the sinapinic acid matrix performed best in terms of improved signal-to-noise ratios, mass resolution, and clarity of the spectra at the low *m*/*z* range.

In the current investigation, we analyzed nonfractionated methanolic extracts from the skins of 23 varieties of red grapes; among these varieties, 8 were authentic *V. vinifera*, 6 were previously catalogued as hybrid red grapes, 2 were suspected hybrid cuntivars, and 7 belonged to the Casavecchia cultivar, which had been designated provisionally as *V. vinifera* according to the ampelographic characteristics. In contrast, the LC-ESI MS and MS/MS characterizations of the anthocyanin profiles have already demonstrated that the Casavecchia samples were not classifiable as *V. vinifera* but that they were late-generation crosses between *V. vinifera* and hybrid grapevines that are yet to be identified.[12] To average fluctuations of the molecular compositions, grapes were sampled from plants of different ages from seven vineyards in different sites of the Italian Campania region.

In Figure 2 are compared representative MALDI-TOF MS spectra of anthocyanins from a certified *V. vinifera* grape variety (Pallagrello, Figure 2A), from one of the Casavecchia samples (Figure 2B), and from a hybrid grape sample (Kober 5BB clone, Figure 2C). The molecular components can be clustered into families that roughly fall into the following three *m*/*z*

spectral regions: (1) the m/z 400−500 low range, which encompasses the five simple anthocyanins; (2) the intermediate m/z 500−700 range, which is dominated by acylated monoglucoside anthocyanins in *V. vinifera* and by 3,5-*O*-diglucosides in hybrid cultivars; and (3) the higher m/z range of 700−900, where the acylated 3,5-*O*-diglucosides fall, providing phenotypic traits that distinguish hybrid cultivars from *V. vinifera* grapes.

The identity of the components summarized in Table 1 was consitent with the structural characterization performed by ESI-MS/MS analyses, as previously described.[12] To investigate the reliability of the MALDI-MS-based approach, we confirmed the identity of the components by PSD fragmentation.

The approximate relative signal intensity recorded in the MALDI spectra can be considered to be indicative of the relative abundance because the various anthocyanin congeners ionize with very similar efficiencies.[9,18,19] Thus, the relative ion intensity approximately reflects the amount of the single species. In Table 1 the anthocyanins are considered to be present if the related S/N ratio was >3 (means of three replicates). For S/N ratios lower than 10 (means of three replicates) the corresponding abundance is considered to be "low".

For all of the varieties analyzed, oenin (Mv 3-*O*-monoglucoside) was the most abundant basic anthocyanin, as expected for both *V. vinifera* and hybrid grapes.[23] We did not detect the other monoglucoside anthocyanins in the grape skins of *V. vinifera* with the exception of Cy 3-*O*-monoglucoside (m/z 449), which was found in relatively minor amounts (Figure 2A). Similarly, whereas the 3-*O*-(6-*O*-acetyl)glucosides of Mv (m/z 535), Pn (m/z 505), Dp (m/z 507), and Pt (m/z 521) were clearly detected in both Casavecchia (Figure 2B) and hybrid samples (Figure 2C), only the acetylated Mv monoglucoside was detected in *V. vinifera* grapes. Even though we did not specifically investigate this issue, the level of 3-*O*-(6-*O*-acetyl)glucoside anthocyanins might be a characteristic trait of hybrid grapes, considering that these compounds are completely missing in some of the most widespread pure *V. vinifera* varieties such as Pinot noir and Cabernet Sauvignon.[23−25]

Previous studies have demonstrated that the relative amounts of the anthocyanin families can change significantly depending on the cultivar and within a specific cultivar on endogenous and exogenous factors that include plant age, cultivation, vintage, and weather and climate conditions.[26−28] Therefore, the possible utilization of 3-*O*-(6-*O*-acetyl)glucoside anthocyanins as varietal markers has to be investigated further, taking into consideration the variability factors. The condensation product of Mv 3-*O*-monoglucoside and acetaldehyde (vitisin B, m/z 517), which is found in the juices of hybrid grapes,[29] was detected in trace amounts in the hybrid and Casavecchia cultivars.

In the intermediate m/z range, the acylated monoglucoside anthocyanins (*p*-coumaroyl, caffeoyl, and feruloyl derivatives) were detected in the three sample typologies. Among the acylated derivatives, the *p*-coumaroyl-3-*O*-glucosides, especially that of malvidin (m/z 639), were dominant. The pattern of the relative ion signal intensities for the acylated monoglucosides is characteristic of the cultivar and, therefore, might be considered to be one of the distinctive varietal traits.

Particularly in hybrid varieties, multiple molecular species can contribute to the final intensity of an ion signal in the mass spectrum. For instance, the m/z values of 3-*O*-caffeoyl-

Table 1. Anthocyanin Components and Molecular Ions Detected by MALDI-TOF MS Analysis of the Methanol Extract from Representative Varieties of *V. vinifera* (Eight Varieties), Casavecchia (Clones from Seven Vineyards), and Hybrid (Eight Samples) Red Grapes[a]

anthocyanin	m/z [M$^+$]	*V. vinifera* L.	Casavecchia clones	hybrids
Cy 3-*O*-glucoside	449	Y	Y	Y
Pn 3-*O*-glucoside	463	Y	Y	Y
Dp 3-*O*-glucoside	465	Y	Y	Y
Pt 3-*O*-glucoside	479	Y	Y	Y
Mv 3-*O*-glucoside	493	Y	Y	Y
Mv 3-*O*-glucoside-acetaldehyde (vitisin B)	517		Y	Y
Cy 3-*O*-(6-*O*-acetyl)glucoside	491			
Pn 3-*O*-(6-*O*-acetyl)glucoside	505		Y	Y
Dp 3-*O*-(6-*O*-acetyl)glucoside	507		Y	
Pt 3-*O*-(6-*O*-acetyl)glucoside	521		Y	Y
Mv 3-*O*-(6-*O*-acetyl)glucoside	535	Y	Y	Y
Cy 3-*O*-(6-*O*-*p*-coumaroyl)glucoside	595		Y	Y
Pn 3-*O*-(6-*O*-*p*-coumaroyl)glucoside	609	Y	Y	Y
Dp 3-*O*-(6-*O*-*p*-coumaroyl)glucoside/Cy 3,5-*O*-diglucoside/Cy 3-*O*-(6-*O*-*p*-caffeoyl)glucoside	611	Y	Y	Y
Pt 3-*O*-(6-*O*-*p*-coumaroyl)glucoside/Pn 3,5-*O*-diglucoside/Pn 3-*O*-(6-*O*-*p*-caffeoyl)glucoside	625	Y	Y	Y
Mv 3-*O*-(6-*O*-*p*-coumaroyl)glucoside	639	Y	Y	Y
Dp 3,5-*O*-diglucoside/Dp 3-*O*-(6-*O*-*p*-caffeoyl)glucoside	627			low
Pt 3,5-*O*-diglucoside/Pt 3-*O*-(6-*O*-*p*-caffeoyl)glucoside	641			low
Mv 3,5-*O*-diglucoside/Mv 3-*O*-(6-*O*-*p*-caffeoyl)glucoside	655	Y	Y	Y
?	699	Y	Y	Y
Pg 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside[b]	741		Y	Y
Cy 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	757			Y
Pn 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	771		Y	Y
Dp 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside/Cy 3-*O*-(6-*O*-*p*-caffeoyl)-5-*O*-diglucoside	773		Y	Y
Pt 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	787		low	Y
Dp 3-*O*-(6-*O*-*p*-caffeoyl)-5-*O*-diglucoside	789		Y	Y
Mv 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	801		Y	Y
Pt 3-*O*-(6-*O*-*p*-caffeoyl)-5-*O*-diglucoside/Dp 3-*O*-(6-*O*-feruloyl)-5-*O*-diglucoside	803		Y	Y
Dp 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-acetyl diglucoside[b]	815		Y	
Mv 3-*O*-(6-*O*-*p*-caffeoyl)-5-*O*-diglucoside	817	low	Y	Y
Mv 3-*O*-(6-*O*-feruloyl)-5-*O*-diglucoside	831	low	low	Y
Mv 3-*O*-(6-*O*-sinapoyl)-5-*O*-diglucoside	861	low	low	low
?	845	Y	Y	Y
?	863	low	Y	low

[a]Y indicates the presence of the component; blank indicates the absence of the component. [b]Structural assignment to be confirmed.

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monoglucosides are indistinguishable from those of possible 3,5-*O*-diglucosides. Similarly, the 3-*O*-*p*-coumaroyl-monoglucosides of Dp (*m*/*z* 611) and Pt (*m*/*z* 625) are isobaric with the 3-*O*-caffeoyl-monoglucosides and the 3,5-*O*-diglucosides of Cy and Pn, respectively. The definite structural assignment of the isobaric anthocyanins can be decisive for establishing the occurrence of diglucoside derivatives in a sample as varietal markers of non-*V. vinifera* varieties. The resolution of MALDI-TOF MS is far beyond the capability to discriminate among isobaric acylated monoglucoside and diglucoside anthocyanins. Thus, in analogy with the already demonstrated capability of ESI-MS/MS,[8,12,30] we investigated the potential of MALDI-PSD MS to discriminate diglucosides from acetylated monoglucosides. In Figure 3, the PSD spectra of the ion signal *m*/*z* 655 from the berry skins of Pallagrello (Figure 3A), Casavecchia (Figure 3B), and Kober 5BB hybrid grapes (Figure 3C) are compared. The PSD spectra of anthocyanins generally exhibit the M^+ form of aglycones as the main daughter ions due to the cleavage of the labile glucoside bond(s). Figure 3A shows the exclusive loss of the caffeoyl-glucoside through the detection of Mv aglycon (*m*/*z* 331), without detection of additional fragments. On the contrary, signals at *m*/*z* 493 $[M - 162]^+$, indicative of the stepwise loss of an additional glucose moiety due to an additional glucoside bond, were detected in the PSD spectra of *m*/*z* 655 from both the Kober 5BB hybrid and Casavecchia grapes. This finding demonstrates that the *m*/*z* 655 signal is the result of two different ion contributions, those of Mv 3-*O*-(6-*O*-caffeoyl)glucoside that prevail in *V. vinifera* and those of Mv 3,5-*O*-diglucoside that dominate in hybrid grapes. The PSD spectrum of *m*/*z* 655 of the standard Mv 3,5-*O*-diglucoside (Figure 3D) showed *m*/*z* 493 as the base peak, confirming the tendency of diglucosides to easily undergo a neutral loss of glucose. The high intensity of the *m*/*z* 611, 625, and 655 ion signals in the MALDI-TOF spectrum of Figure 2C suggests that the 3,5-*O*-diglucosides significantly contributed to the anthocyanin fraction of hybrid grapes in addition to the *p*-coumaroyl-3-*O*- and 3-*O*-(6-*O*-caffeoyl)-glucosides, as confirmed by PSD analysis. More specifically, the PSD spectrum of the *m*/*z* 625 ion for the Kober 5BB hybrid grape (Figure 4) showed a fragmentation pattern in which three different aglycones were produced, demonstrating that the parent ion signal arose from the contributions of at least four different isobaric compounds, as follows: (1) Pn 3-*O*-(6-*O*-caffeoyl)glucoside; (2) Pn 3,5-*O*-diglucoside, of which the primary loss of glucose generated the fragment *m*/*z* 463; (3) Pt 3-*O*-(6-*O*-*p*-coumaroyl)glucoside, of which the one-step loss of the acetyl-glucoside produced the M^+ of Pt aglycon (*m*/*z* 317); and (4) Mv 3-*O*-glucoside-5-*O*-pentoside. The occurrence of pentoside derivatives in minor amounts, as demonstrated by the ESI-MS/MS for Casavecchia cultivars,[12] is a typical phenotypic trait of hybrid grapes. Among the other occurring species, the loss of a pentoside moiety is evident for the presence of the fragment ion $[M - 132]^+$ in Figure 4.

The high *m*/*z* spectral region of authentic *V. vinifera* grapes did not contain detectable acylated 3,5-*O*-diglucoside anthocyanins, except for trace amounts of Mv 3-*O*-(6-*O*-*p*-caffeoyl)-5-*O*-diglucoside (*m*/*z* 817), Mv 3-*O*-(6-*O*-feruloyl)-5-*O*-diglucoside (*m*/*z* 831), and Mv 3-*O*-(6-*O*-sinapoyl)-5-*O*-diglucoside (*m*/*z* 861). In contrast, the acylated 3,5-*O*-diglucoside anthocyanins were clearly detectable in Casavecchia grapes and were particularly abundant in the other hybrid cultivars. The PSD spectrum of the signal at *m*/*z* 801, which was assigned to Mv 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside (Figure 5) by the loss of both glucose (*m*/*z* 639) and 3-*O*-(6-*O*-*p*-coumaroyl)glucose (*m*/*z* 493), illustrates the identification of one of the components that are characteristic of Casavecchia and hybrid grapes.

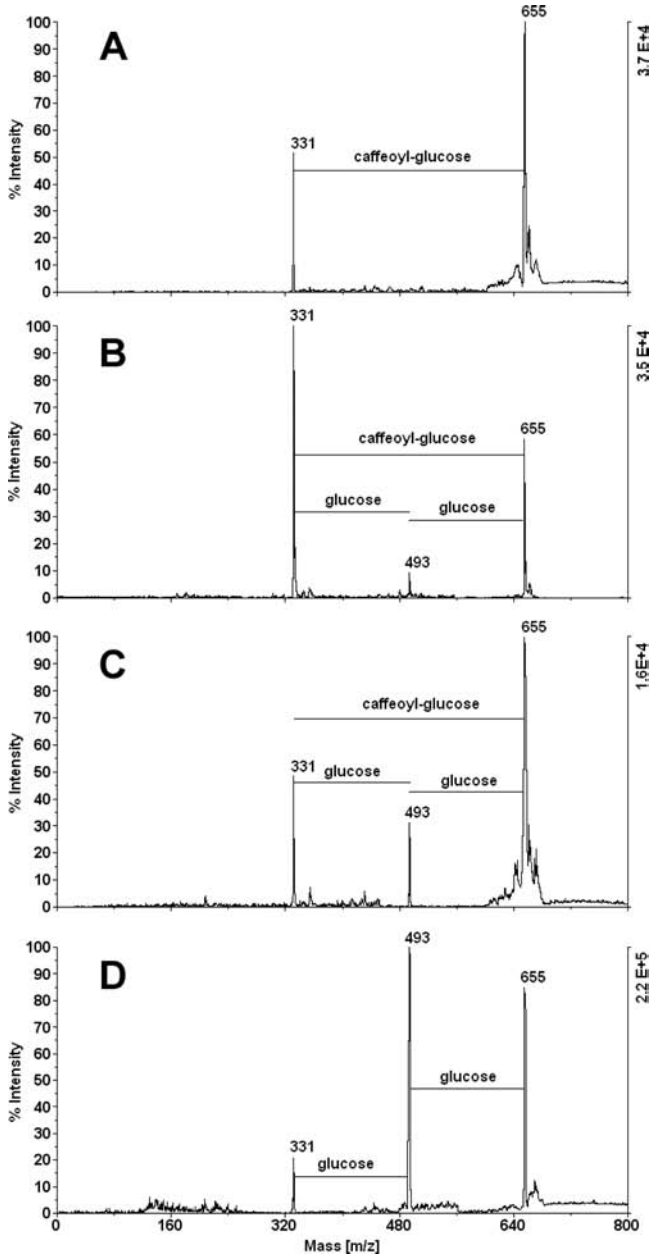

Figure 3. MALDI-PSD MS spectrum of the ion signal *m*/*z* 655 detected from the berry skins of Pallagrello (A), Casavecchia (B), and Kober 5BB hybrid (C) grapes. The increased contribution of Mv 3,5-*O*-diglucoside with respect to Mv 3-*O*-(6-*O*-caffeoyl)glucoside is related to the increment of the fragment at *m*/*z* 493 $[M^+ - 162]$, indicating the primary loss of a glucose moiety. The signal at *m*/*z* 493 is the base peak in the PSD fragmentation spectrum of pure Mv 3,5-*O*-diglucoside (D).

The signal ion at *m*/*z* 845 was detected in almost all of the cultivars analyzed, including *V. vinifera*. It was assigned by MALDI-PSD analysis (Figure 6) to a doubly substituted Mv derivative that most likely carries a (6-*O*-*p*-coumaroyl)-*O*-glucoside moiety $[M - 308]^+$ and a further glucoside that is acylated by an unknown group of 44 Da $[M - 206]^+$. To the best of our knowledge, this compound was detected for the first

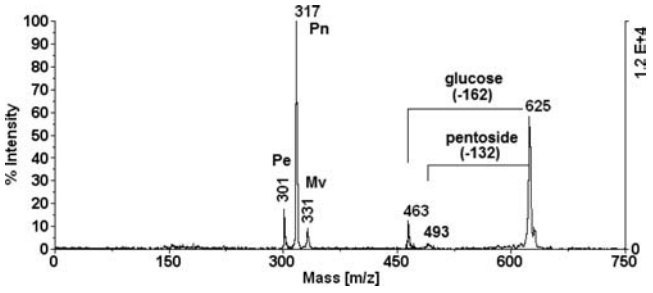

Figure 4. MALDI-PSD MS spectrum of the *m/z* 625 ion from the Kober 5BB hybrid grape. The fragmentation pattern showed evidence of the occurrence of three different aglycones, thereby demonstrating that the parent ion signal arose from the contributions of four different isobaric compounds (see text).

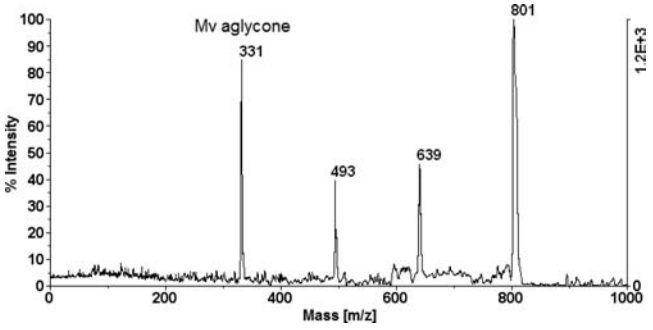

Figure 5. MALDI-PSD MS structural characterization of Mv 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside (*m/z* 801) that was detected in the anthocyanin profile of Casavecchia and Kober 5BB hybrid grapes.

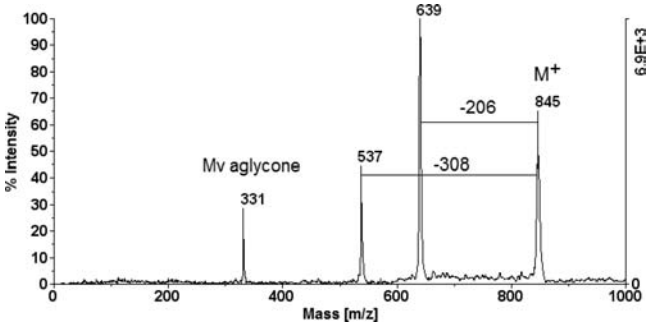

Figure 6. Signal ion at *m/z* 845 detected in all grape samples analyzed was identified as an anthocyanin derivative of Mv 3-*O*-(6-*O*-*p*-coumaroyl)glucoside carrying an additional acylated glucose. The MALDI-PSD MS fragmentation was not definitive for a complete structural assignment.

time in red grape skins, even though it was not possible to definitively assign its structure by MS-based techniques alone (including MALDI-PSD and ESI-MS/MS, data not shown). This pigment is most likely structurally correlated to the compound at *m/z* 699, from which it differs for the *p*-coumaroyl moiety (mass difference = 146), that was found in all of the grape samples as well. Other signals, such as *m/z* 831, 861, and 863 that occurred either in *V. vinifera* or in hybrid grapes, did not receive a definite assignment.

In the analysis of Casavecchia grapes and in two of the potentially hybrid cultivars (Suricillo and French teinturier), the *m/z* 741 signal was assigned to pelargonidin 3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside solely on the basis of the molecular mass. Other authors have assigned the *m/z* 741 signal to the 4-vinylphenol adducts of Pt acetyl-glucoside.[31] The PSD analysis of this component was not conclusive, likely due to the low ion intensity and, therefore, its structure still needs to be confirmed.

An almost twice as high content of acylated diglucoside anthocyanins has been reported for Clinton (*V. labrusca* × *V. riparia*) with respect to the Isabella hybrids,[32] which, as expected, preserves in part the phenotypic traits of *V. vinifera*. In agreement with these results, we found that the signal intensity of the acylated diglucoside anthocyanins from the Kober 5BB (*V. berlandieri* × *V. riparia*) was significantly enhanced with respect to the Isabella and Tintoria grapes (not shown). The anthocyanin pattern and the presence of intense signals of acylated diglucosides confirmed that Olivella and Suricillo varieties were indeed hybrids associable with the Seibel-derived (Isabella) clutivars. Within the sample typologies, including *V. vinifera*, Casavecchia, and Isabella groups of samples, we observed a slight variability in the MALDI profile that was restricted only to differences in the signal intensity of anthocyanins. The signal intensity fluctuations were in any case not higher than 15% with respect to the mean intensities.

These results can thus be accommodated in a model where the anthocyanin-specific profile is extremely similar for the red-skinned grapes belonging to the cultivars of the same genus (Table 1).

In general, the MALDI MS-based anthocyanin profiles of hybrid red grapes are more complex compared with those of *V. vinifera*. The anthocyanin profile arises from cultivar-specific metabolic assets that could be monitored, in principle, with other "omic" technologies, such as the proteomic, transcriptomic, or genomic platforms. A possible target of this approach is the 5-*O*-glucosyltransferase (5GT) or its encoding gene. In fact, a recent investigation seeking to explain the inability of most European *V. vinifera* cultivars to biosynthesize 3,5-*O*-diglucosides has demonstrated that because of inherited nucleotide mutations, *V. vinifera* grapevines encode an inactive C-terminally truncated isoform of 5GT that also contains five amino acid substitutions. These mutations, in particular the V121→L that occurs spatially close to the catalytic site, render 5GT functionally inactive, in contrast to the active enzymatic isoforms of the American *Vitis* species.[33]

MALDI-TOF MS was demonstrated to be a powerful technique for detecting, profiling, and monitoring the phenotypic expression of anthocyanins, with the consideration that the non-*V. vinifera* signature compounds occur in an interference-free spectral region. In addition, MALDI-PSD MS enabled the definition of structural details, permitting discrimination between isobaric acylated 3-*O*-monoglucoside and diglucoside anthocyanins.

The possibility of exploiting a late-generation hybrid as a cross-cultivar model of grapevines was tested by distinguishing hybrid and *V. vinifera* varieties through the use of MALDI-TOF MS. From the results reported in Table 1, the levels of either nonacylated or acylated 3,5-*O*-diglucosides in hybrid grapes were significantly higher compared with those of Casavecchia grapes, thus confirming that the latter could derive from second- or late-generation interspecific crosses between *V. vinifera* and other species of the *Vitis* genus. Acylated derivatives of 3,5-*O*-diglucosides were confirmed as analytical molecular markers of non-*V. vinifera* cultivars that can be easily monitored. This finding provides a method through which a pure cultivar can be selected for characteristics that easily distinguish it from any other known cultivars, such as the

absence of 3,5-*O*-diglucosides that can be maintained in a uniform and stable manner under repeated propagation. In this study, we did not determine the threshold value for the signal intensity of acylated diglucosides to establish a variety as a hybrid; further study is necessary to tune the strategy in order to validate the identification of hybrid or suspected hybrid cultivars of grape berries.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 0825 299111. Fax: +39 0825 782599. E-mail: picariello@isa.cnr.it.

Notes

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