HPLC/MS Application to Anthocyanins of *Vitis vinifera* L.

Alessandro Baldi,[†] Annalisa Romani,[†] Nadia Mulinacci,[†] Franco F. Vincieri,*[,†] and Bruno Casetta[‡]

Dipartimento di Scienze Farmaceutiche, Universitá degli Studi di Firenze, via Gino Capponi 9, 50121 Firenze, Italy, and Perkin-Elmer Holding GmbH, Langen, Frankfurt, Germany

An HPLC/MS application to anthocyanic compounds of *Vitis vinifera* L. is described. A series of liquid–liquid and liquid–solid extractions yielded an extract containing anthocyanic compounds only. This extract was analyzed by HPLC/DAD and HPLC/MS, and UV–vis and MS spectra of each compound were obtained. An API ion-spray interface allowed coupling between the chromatographic system and a mass spectrometer. This interface allowed application of the chromatographic conditions normally used in the HPLC analysis of anthocyanic compounds, which led to the identification of the 3-glucosides, the 3-acetylglucosides, and the 3-*p*-coumaroylglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, already known in the literature. Two 3-caffeoylglucoside derivatives were identified too, and it has been possible to identify, for the first time, some 3,5-diglucosides that are known to be present in *Vitis* sp. but not in *Vitis vinifera* L. The investigated cultivars showed the same anthocyanic profile but dramatic quantitative differences.

Keywords: *Anthocyanin; Vitis vinifera; HPLC/MS*

INTRODUCTION

Mass spectrometry has proved to be effective and of considerable diagnostic value when used as a supporting technique in the characterization of flavonoids. Among the different techniques, FAB is the most commonly used for the mass spectrometric study of anthocyanins (Strack and Wray, 1994). Usually it allows one to obtain the signal corresponding to the molecular peak and excellent fragmentation, thus providing relevant information on the aglycon and sugars, as well as useful structural information about the examined compound. If the FAB analytical procedure is to yield such information, it must necessarily be preceded by the purification of the compound and its solubilization in a polar matrix. Direct analysis of an anthocyanic mixture is also possible (Laing and Cormier, 1991); in this case a series of molecular peaks is obtained, and using MS/MS techniques it is possible to obtain information on the substituents of some compounds. Nevertheless, prior chromatographic separation is a useful prerequisite for any mass spectrometry analytical method used to test anthocyanic mixtures. When mass spectrometry is coupled to a chromatographic separation method, both the retention characteristics and the mass spectra provide structural information without the necessity of isolating the individual compounds (Glaβgen et al., 1992a). The first applications of this coupling to anthocyanic compounds were GC/MS techniques. Gas chromatographic analysis of anthocyanins necessarily requires the derivatization of the compounds either by TMCS or HMDS to increase their volatility. This implies a considerable increase in their molecular weight and makes the interpretation of their mass spectra more difficult. Bombardelli et al. (1977) developed a GC/MS analytical technique applied to an anthocyanic extract from blueberry (*Vaccinium mirtillus* L.). However, HPLC is a better separation method for anthocyanins. The interfacing of a HPLC apparatus with a mass spectrometer (HPLC/MS) has been optimized in several applications (Thompson et al., 1982; Whitehouse et al., 1985; Bruins et al., 1987). Such a technique has been little used to analyze anthocyanins (Glaβgen et al., 1992b) because of their low volatility and high polarity, which requires the use of RP columns and acid eluents having a high proportion of water. So, the low volatility of the eluents, their high polarity, and the chemical characteristics of the compounds for analysis make the choice of the interface between the chromatographic system and the mass spectrometer absolutely crucial for this procedure. This application and the characteristics of the involved molecules require an interface that operates at low temperatures, to avoid degradation of analytes, and permits the use of highly polar eluents under acidic pH conditions, to keep molecules unfragmented until they enter the mass spectrometer. An interface fitting these requirements is the API ion spray interface, which allows ambient pressure ionization and the use of any aqueous eluent.

The pool of anthocyans contained in the berry skins of *Vitis vinifera* L. was taken as a research model to investigate the possible application of HPLC/MS to anthocyanins. The anthocyanic content of the fruits of *V. vinifera* L. was determined (Riberau-Gayon, 1968) long ago for the presence of the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, as well as their relative 3-acetylglucosides and 3-*p*-coumaroylglucosides.

This paper aims at providing both a scientific contribution to the knowledge of the anthocyanins contained in the grapes of *V. vinifera* L. and a practical–analytical contribution in the development of a HPLC/MS method. Results have been obtained using a HPLC apparatus coupled with a mass spectrometer equipped with an API (atmospheric pressure ionization) ion spray interface. The use of this technique made it possible to obtain the mass spectra of all the anthocyanic compounds present in the extracts under investigation, even those occurring in traces or some coeluted ones.

* Author to whom correspondence should be addressed [telephone (39) 55-2757-288; fax (39) 55-240776; e-mail VINCIERI@farmfi.chim.unifi.it].

[†] Universitá degli Studi di Firenze.

[‡] Perkin-Elmer.

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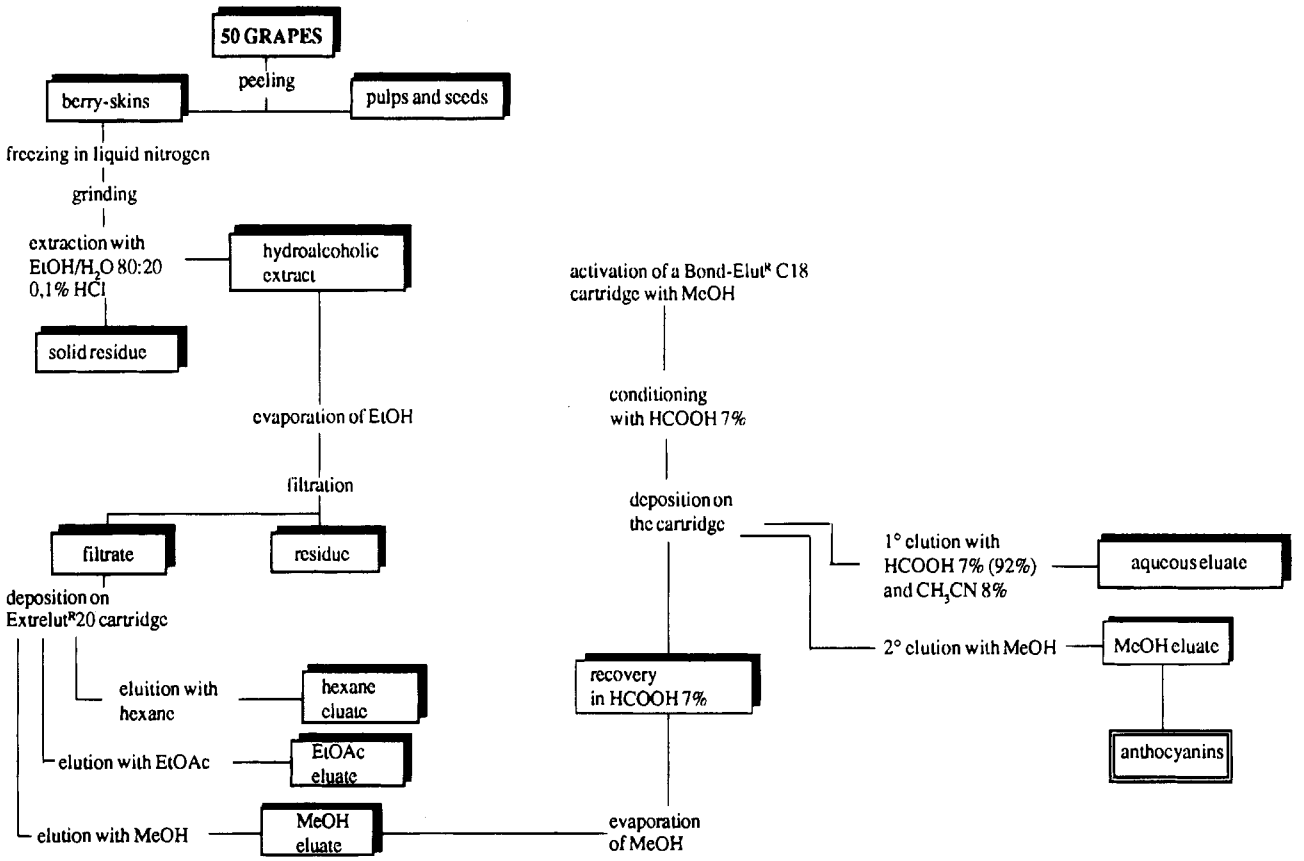

Figure 1. Extraction and fractionation method.

To be sure about the origin and genetic characteristics of the materials used, this research was carried out on registered clones of *V. vinifera* L. Clones belonged to two varieties commonly used for the production of the Chianti DOCG red wine. The first, known as Sangiovese (clone SS-F9-A5-48), is the variety most widely used for wine production in Tuscany, and the second one, Colorino (clone Nipozzano 6), is used because it is particularly rich in anthocyanic compounds and is therefore important in obtaining a good degree of color saturation in the wine. We have already investigated the anthocyanic fraction of these two varieties in a paper that proposed a chemotaxonomic classification (Baldi et al., 1993).

MATERIALS AND METHODS

Sample Preparation. The samples were obtained from the berries of Sangiovese SS-F9-A5-48 and Colorino Nipozzano 6 clones, cultivated by the Department of Horticulture of the University of Florence, in an experimental field on the hills of the Chianti region in the Siena area. The samples were harvested in September 1993 at technological ripening and immediately frozen at −23 °C.

Extraction and Fractionation Method. Peels were manually separated from fruit pulp, dipped in liquid N_2, and then blended in an Osterizer. The powder obtained was then extracted three times with 80% EtOH acidified to pH 2 with HCOOH. The hydroalcoholic extract was then concentrated by evaporation of the EtOH (25 °C, low pressure) and resolubilized with 7% HCOOH. Then the sample was deposed in an Extrelut 20 cartridge (Merck, Darmstadt, Germany) which allows one to perform liquid−liquid extractions on a solid matrix.

First of all, elutions with hexane to eliminate lipophilic compounds such as chlorophylls and carotenoids, and with EtOAC to eliminate polyphenolic compounds such as flavonols and phenolic acids, were performed. Then anthocyanins and hydrophilic molecules, such as sugars and organic acids, were desorbed using MeOH. The methanolic eluate was evaporated, resolubilized with 7% HCOOH, and applied to a Bond Elut C_{18} cartridge (Analytichem International Harbor City, CA) that had been previously activated with MeOH and conditioned with 7% HCOOH. After elution with a 92% solution of 7% HCOOH and 8% CH_3CN, elution was performed with MeOH and a fraction containing mainly anthocyanic compounds was obtained. This solution after evaporation and resolubilization in 7% HCOOH, was ready to be analyzed by HPLC/DAD and HPLC/MS techniques (Figure 1).

Acid hydrolysis was performed to evaluate the occurrence of phenolic acids as esters of some anthocyanic compounds as follows: the extract was concentrated and re-solubilized with 6 N HCl, heated at 100 °C for 60 min, cooled in ice, and extracted with EtOAc. The EtOAc extract was evaporated and the residue resolubilized in 7% HCOOH/CH_3CN (92:8) and analyzed by HPLC acquiring chromatograms at 280 and 320 nm.

Instrumental Apparatus. *HPLC/DAD Analysis.* The HPLC/DAD analyses were performed by means of a liquid chromatograph HP 1090A equipped with a diode array detector HP 1040 A managed by a HP 9000 workstation (Hewlett-Packard, Palo Alto, CA). Anthocyanins were separated on an Aquapore RP300 (Brownlee Laboratory Santa Clara, CA) column, 7 μm; 250 × 4.6 mm, equipped with a precolumn.

HPLC/MS Analysis. The HPLC/MS analyses were carried out using a Perkin-Elmer LC-250 with a UV−vis detector PE LC-95 and a triple-quadrupole mass spectrometer PE-Sciex API-III (PE Sciex, Toronto, Canada) equipped with an ion spray interface.

Analytical Conditions. *HPLC/DAD Analysis.* Anthocyanic products were separated using the following mobile phase: solution A, H_2O/HCOOH (93:7); solution B, H_2O/CH_3CN/MeOH/HCOOH (47:23:23:7); three-step linear gradient starting from 0 up to 50% solution B within 60 min. The flow rate was 1.5 mL/min. The UV−vis spectra were recorded between 250 and 600 nm, and chromatograms were acquired at 535, 330, 310, and 280 nm.

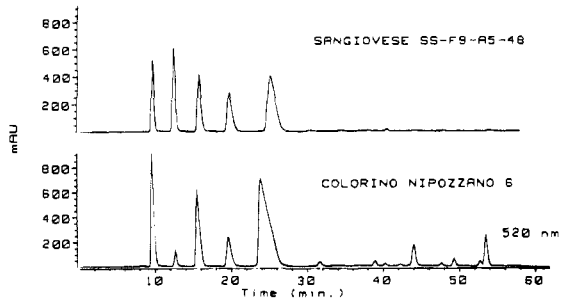

Figure 2. HPLC separation of the anthocyanic fraction of two cultivars of *V. vinifera* L.

HPLC/MS Analysis. The use of the API ion spray interface allows one to use the same analytical conditions as in the above-mentioned HPLC/DAD analysis. Chromatograms were recorded at 535 nm using a UV–vis detector in series with the mass spectrometer. Flow to the mass spectrometer was split 1:25 to have a 60 μL/min flow rate in the interface through a fused-silica capillary (length 80 cm; 75 μm i.d.). Ion spray voltage was 5 kV and gas pressure in the sprayer 80 psi. Due to the chemical characteristics of the anthocyanins (analyzed in the flavilium cationic form), the mass spectrometer was operated in positive ion mode. Spectra were acquired between m/z 200 and 700 using a 0.1 amu step size.

RESULTS AND DISCUSSION

The extraction and fractionation method allows one to obtain a fraction containing exclusively anthocyans. Such finding was confirmed from data of acquired chromatograms at different wavelengths and UV–vis and mass spectra. This result made the characterization of anthocyanins easier and more effective. The pH value of ca. 2, the use of a "soft" acidifier, such as formic acid, and the low temperatures characterizing each stage of the experiment prevented the occurrence of degradation and hydrolysis events. It thus allows one to obtain an extract representing the pool of anthocyanins contained in the berry skins of *V. vinifera* L. (Figure 2). The API ion spray interface also allows one to maintain the same analytical conditions used in the HPLC/DAD analysis. The relatively soft ionization technique, conducted at atmospheric pressure and ambient temperature, provided information on the compounds without the interference of signals due to the formation of degradation compounds. This fact smoothed the interpretation of data and allowed comparison of the present results with earlier values obtained under the same chromatographic conditions.

Anthocyanin 3-*O*-Glucosides. The occurrence of 3-*O*-glucoside derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin in the extracts obtained from the berry skins of *V. vinifera* L. has been known for a long time. The presence of the five 3-*O*-glucosides was confirmed, and the mass spectra acquired showed a signal corresponding to the quasi-molecular ion $[M + H]^+$, as well as the fragment resulting from the loss of a sugar $[M - 162]^+$. So the mass spectrum of the peak at Rt 13.5 min showed $[M + H]^+ = 465$ and the fragment $[(M + H) - 162]^+ = m/z$ 303 corresponding to the loss of a glucose; similar patterns of fragmentation were shown by the other 3-glucosides (Figure 3). Therefore, the five products at issue were the following: delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside. So the mass spectrum of each of the five 3-glucosides consisted of two major signals, the peak corresponding to the quasi-molecular ion $[M + H]^+$ and

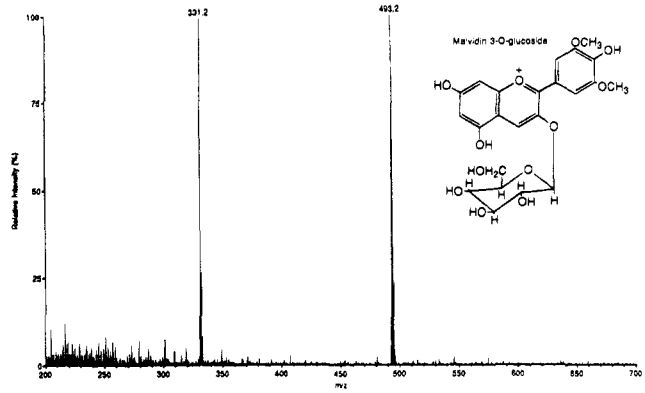

Figure 3. Mass spectrum of malvidin 3-*O*-glucoside.

the $[M + H]^+$ of the related aglycon. It is possible to sum up this set of data effectively by means of the three-dimensional map shown in Figure 4. Here the m/z values are reported on the y-axis, time is reported on the x-axis, and signal intensity is shown by the varying tones of gray. It is easy to realize the elution sequence of the 3-*O*-glucoside derivatives and their fragmentation. This map also provides useful information on the purity of the extract under investigation; no other product, within the range m/z 200–700 is eluted in this interval of the analytical process.

Anthocyanin 3-*O*-(6-*O*-Acetyl)glucosides. In most cultivars of *V. vinifera* L., acetylated derivatives of the 3-*O*-glucosides are the least frequent anthocyanins. Their concentrations lie below the detection limit as far as Sangiovese grapes are concerned, while they may be detected and quantified in the cultivar Colorino. The HPLC/DAD analysis allows one to separate the products having a higher Rt from the 3-*O*-glucosides whose UV–vis spectrum is analogous to that of the related 3-*O*-glucosides. The mass spectrum of this group of molecules showed two signals corresponding to the quasi-molecular ion $[M + H]^+$ and to the fragment $[(M + H) - 204]^+$. The m/z 204 value corresponded to the "acetylglucoside" acyl. The fragment $[(M + H) - 204]^+$ therefore corresponded to the $[M + H]^+$ of the related aglycon (Figure 5). The values are reported in Table 1.

Anthocyanin 3-*O*-(6-*O*-*p*-Coumaroyl)glucosides. It has been widely stated in the literature that *p*-coumaroyl derivatives of the anthocyanic 3-*O*-glucosides are present in *V. vinifera* L. They may be identified by comparing the UV–vis spectrum with the spectrum of their related 3-*O*-glucosides. The 3-*O*-(6-*O*-*p*-coumaroyl)glucoside derivatives showed higher absorbance values in the range between 280 and 340 nm (due to the absorbance of the *p*-coumaric acid) as compared to the corresponding 3-*O*-glucosides (Figure 6). Moreover, the acid hydrolysis of these products indicated the presence of the corresponding aglycons, and the EtOAc of the hydrolyzed fraction contained *p*-coumaric acid. The presence of esters of the *p*-coumaric acid was further confirmed by the mass spectra of these products recorded during the HPLC/MS analysis. The acquired mass spectra showed the $[M + H]^+$ peak and the fragment related to $[(M + H) - 308]^+$ (Figure 7). This fragment has derived from the loss of a glucose esterified with *p*-coumaric acid. In particular, the peak with Rt 60.4 min showed the spectrum in Figure 8. The four signals were consistent with the quasi-molecular ions and $[M + H]^+$ of the relative aglycons of peonidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside (A) and malvidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside (B). So, even if this chromatographic peak was not pure, it has been possible to

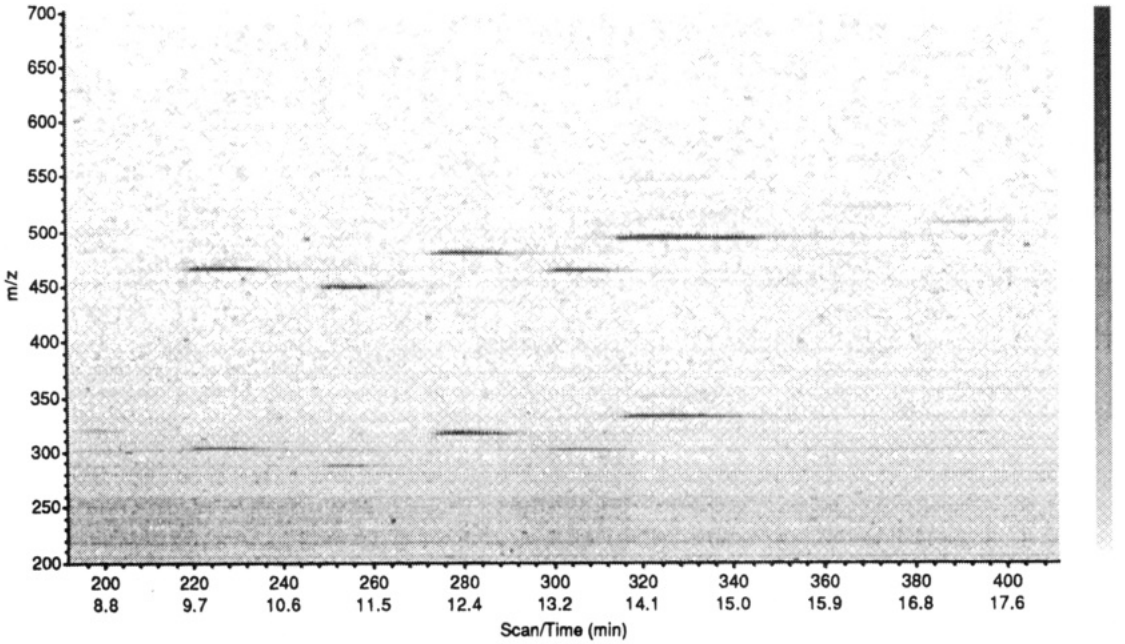

Figure 4. Three-dimensional map of signals related to the 3-*O*-glucoside derivatives.

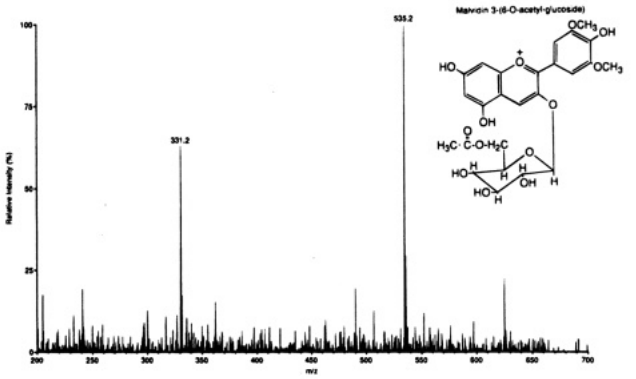

Figure 5. Mass spectrum of malvidin 3-*O*-acetylglucoside.

identify the two coeluted compounds. The data relative to the *p*-coumaroyl derivatives are reported in Table 1.

Anthocyanin 3-*O*-(6-*O*-Caffeoyl)glucosides. Moreover, the application of HPLC/MS allows one to identify in the extracts two anthocyanins esterified with caffeic acid: peonidin 3-*O*-(6-*O*-caffeoyl)glucoside and malvidin 3-*O*-(6-*O*-caffeoyl)glucoside. The latter was identified in several anthocyanic extracts from *V. vinifera* L. (Roggero et al., 1988), while the presence of peonidin 3-*O*-(6-*O*-caffeoyl)glucoside was assumed by many authors but never ascertained. Only Lofty et al. (1989) demonstrated the presence of peonidin 3-*O*-(6-*O*-caffeoyl)glucoside in grape cell cultures of *V. vinifera* L. cv. Gamay. The occurrence of caffeic acid was confirmed by analyzing the EtOAc extract of the hydrolyzed fraction and referring to the compounds having Rt higher than 30 min. The compound at Rt 50.2 min actually showed a signal at m/z 625 corresponding to the $[M + H]^+$ of the peonidin 3-*O*-(6-*O*-caffeoyl)glucoside. After normalization, the UV−vis spectrum showed a higher absorption at ca. 320 nm compared to the related glucoside. This compound is coeluted with another one with Rt 50.8 min, showing a signal at $[M + H]^+$ m/z 535 [corresponding to the $[M + H]^+$ of malvidin 3-*O*-(6-*O*-acetyl)glucoside]. Its occurrence was detected by careful observation of the three-dimensional map, which revealed the presence of a signal at m/z 625 shifted about 10 s compared to the peak with Rt 50.8 min. Background noise suppression produced the mass spectrum shown in Figure 9, where the peak at m/z 625 corresponding to the $[M + H]^+$ also displayed the isotopic peaks ^{13}C and $^{13.2}C$. The compound with Rt 54.1 min, showed a signal at m/z 655 corresponding to the $[M + H]^+$ of the malvidin 3-*O*-(6-*O*-caffeoyl)glucoside. The signal appearing on the chromatogram recorded at 535 nm was very weak, but free from any extra signal of other coeluted molecules. The mass spectrum obtained after background noise suppression appears to be well-defined and indicated the quasi-molecular ion at m/z 655 and the related isotopic peaks ^{13}C and $^{13.2}C$.

Anthocyanin 3,5-*O*-Diglucosides. The high sen-

Table 1. Identified Compounds

Rt (min)	$[M + H]^+$	compound
11.5	627	delphinidin 3,5-*O*-diglucoside
18.9	641	petunidin 3,5-*O*-diglucoside

Rt (min)	$[M + H]^+$	$[(M + H) - 162]^+$	compound
13.5	465	303	delphinidin 3-*O*-glucoside
17.2	449	287	cyanidin 3-*O*-glucoside
21.0	479	317	petunidin 3-*O*-glucoside
27.5	463	301	peonidin 3-*O*-glucoside
31.5	493	331	malvidin 3-*O*-glucoside

Rt (min)	$[M + H]^+$	$[(M + H) - 204]^+$	compound
38.8	507	303	delphinidin 3-*O*-acetylglucoside
40.8	491	287	cyanidin 3-*O*-acetylglucoside
45.7	521	301	petunidin 3-*O*-acetylglucoside
49.0	505	271	peonidin 3-*O*-acetylglucoside
50.8	535	331	malvidin 3-*O*-acetylglucoside

Rt (min)	$[M + H]^+$	$[(M + H) - 308]^+$	compound
51.5	611	303	delphinidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside
55.0	595	287	cyanidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside
56.8	625	317	petunidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside
60.1	609	301	peonidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside
60.5	639	331	malvidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside

Rt (min)	$[M + H]^+$	compound
50.2	625	peonidin 3-*O*-(6-*O*-caffeoyl)glucoside
54.1	655	malvidin 3-*O*-(6-*O*-caffeoyl)glucoside

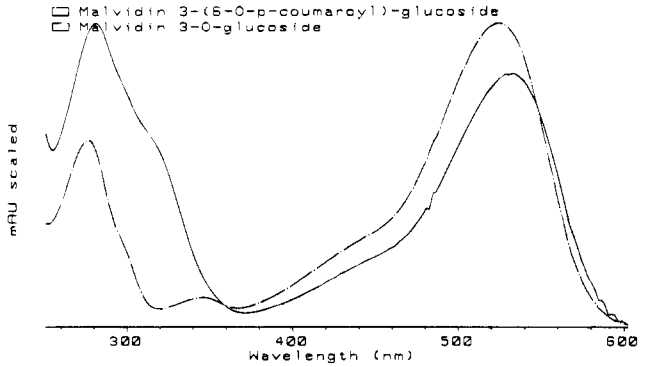

Figure 6. Comparison of UV–vis spectra of malvidin 3-*O*-glucoside and malvidin 3-(6-*O*-*p*-coumaroyl)glucoside.

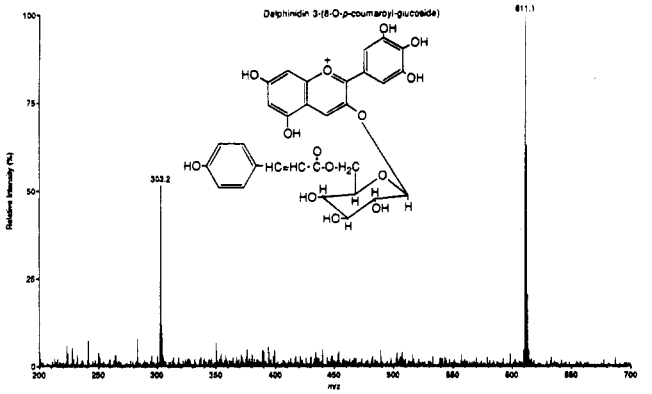

Figure 7. Mass spectrum of delphinidin 3-(6-*O*-*p*-coumaroyl)-glucoside.

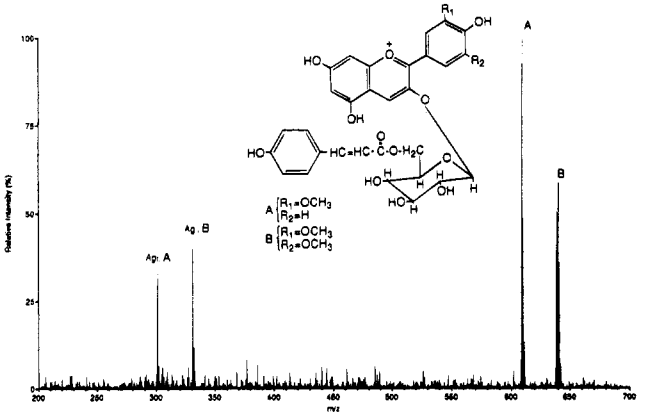

Figure 8. Signals corresponding to the peak with Rt 60.4 min.

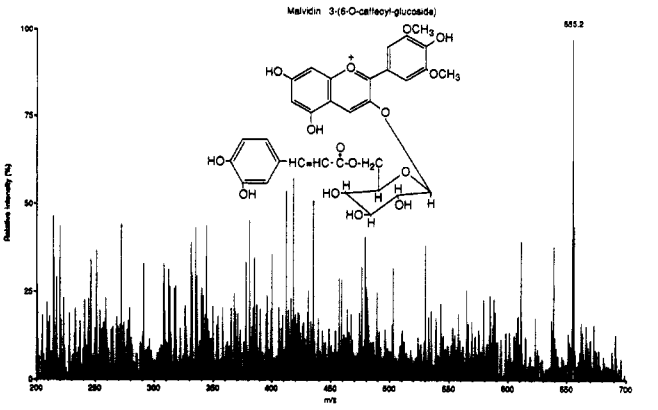

Figure 9. Mass spectrum of malvidin 3-(6-*O*-caffeoyl)glucoside.

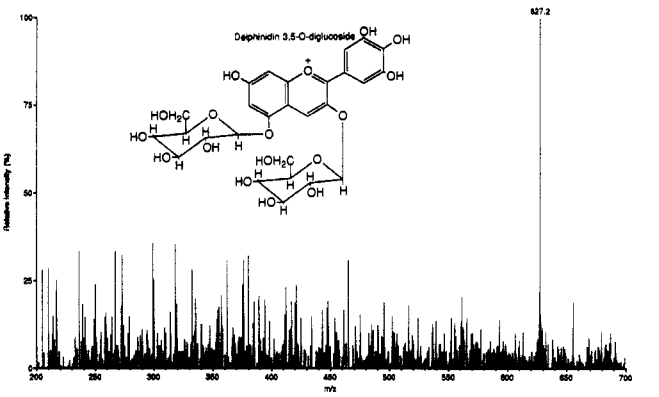

Figure 10. Mass spectrum of delphinidin 3,5-*O*-diglucoside.

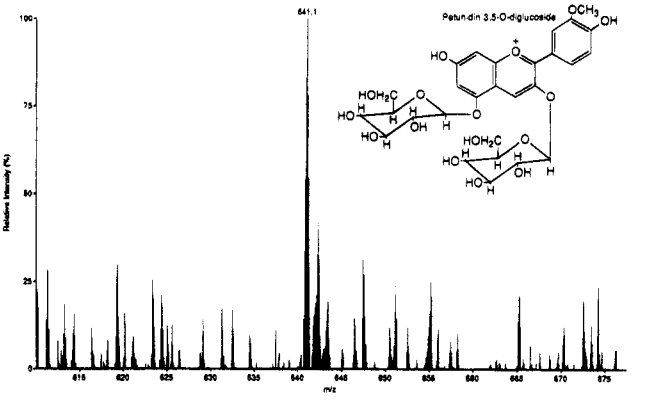

Figure 11. Mass spectrum of petunidin 3,5-*O*-diglucoside.

sitivity of this HPLC/MS application allows one to identify some diglucosidic derivatives present in the extracts. In particular, the compound with Rt 11.5 min showed a signal at *m*/*z* 627 that may be ascribed to the quasi-molecular ion of delphinidin 3,5-*O*-diglucoside (Figure 10). The compound appears to be well separated from the related 3-*O*-glucoside, and its UV–vis spectrum is consistent with data in the literature. A weak signal at Rt 18.9 min is present on the chromatogram recorded at 535 nm. Although the retention time of this compound is intermediate and very similar to that of two 3-*O*-glucoside derivatives (cyanidin and petunidin), its mass spectrum showed a signal at *m*/*z* 641, which may be ascribed to the quasi-molecular ion of petunidin 3,5-*O*-diglucoside (Figure 11). The presence of these two products was confirmed by spiking a mixture of diglucosidic derivatives extracted from hybrid grapes. These products had the same retention times under the same analytical conditions and UV–vis spectra comparable to those of the products found in the analyzed samples.

As far as we know, this is the first time that diglucosidic derivatives have been found in extracts from fruits of *V. vinifera* L. Only Tamura and Sugisawa (1991) reported the presence of 3,5-diglucosides of cyanidin, peonidin, and malvidin in extracts of anthocyanins from cell cultures of Muscat Bailey A. Two other peaks with a chromophore similar to that of anthocyanic molecules were observed on the chromatogram at retention times of 38.6 and 55.4 min. The mass spectra of these compounds displayed a signal at *m*/*z* 651 and another one at *m*/*z* 639. The compound having the highest Rt seems to be a derivative of malvidin because of its fragment at *m*/*z* 331, which may be ascribed to the $[M + H]^+$ of the related aglycon. Additional studies aimed at the full characterization of the investigated molecules are in progress.

Conclusions. The application of HPLC/MS by means of a Sciex API ion spray interface coupled with a quadrupole mass spectrometer made it possible to obtain the mass spectra of all the anthocyanic deriva-

tives present in the analyzed extracts. The development of a fractionation method that produced a fraction containing only anthocyanic molecules made it possible to work on compounds sharing the same chemical characteristics. The choice of a proper chromatographic separation procedure and the use of eluents aimed at achieving adequate molecular ionization enhanced the potential technical qualities of the interfacing system and of the detectors employed. As a result, it was also possible to obtain information on compounds occurring in traces and/or coeluted with other compounds, which could instead be present in considerable amounts. The use of a three-dimensional map allows one to recognize the signals related to compounds having similar retention times, by recording their molecular peaks and possible fragments. The ionization technique employed made it possible to obtain a fragmentation which allows one to identify the signals of $[M + H]^+$ and the fragment $[M + H]^+$ related to the aglycon. All of these achievements helped to confirm the structure of the anthocyanic derivatives present in the berry skin extracts of *V. vinifera* L. and whose occurrence had been determined long ago. Moreover, it was possible to ascertain the presence of two caffeoyl derivatives and to note for the first time the presence of diglucosidic anthocyanins in this species. Both cultivars analyzed showed the presence of the compounds given in Table 1, even if the cultivar Sangiovese showed a lower amount of the acylated compounds. This analytical application may therefore be useful as a supporting technique for the structural investigation of the polyphenolic compounds of different cultivars used in the production of red wines.

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