20

Analytical Methodology for Characterization of Grape and Wine Phenolic Bioactives

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CONTENTS

Introduction

Phenolic compounds, as very important constituents of grape and essential components for wine quality, have been extensively studied in recent years. Scientists became very interested in the nutritional and beneficial health effects of polyphenolic compounds. Red and white grapes and wines have different phenolic compositions and characteristics for each variety. The polyphenolic content of a finished wine depends on grape variety, but also on different wine-making procedures (Nagel and Wulf 1979; Gil-Muñoz et al. 1999; Gómez-Plaza et al. 2000; Monagas et al. 2003; Bautista-Ortín et al. 2007; Ivanova et al. 2009). Red wine production includes the procedure of maceration, which is not applied in white wine production (i.e., white wines are produced without grape mash, having no contact with the grape skins or maceration is kept to a minimum).

Various assay methods for analysis of phenolic compounds have been developed in order to study the concentration of total phenolics, specific subgroups or specific phenolic compounds. Among the analytical techniques, reversed phase high-performance liquid chromatography (RP-HPLC) is commonly employed for separation of the complex mixtures of phenolic compounds (McMurrough and McDowell 1978; Wulf and Nagel 1978; Betés-Saura et al. 1996; Palomino et al. 2000; Viñas et al. 2000; Careri et al. 2003; Monagas et al. 2003; Tsao and Yang 2003; Castillo-Muñoz et al. 2007; Gómez-Alonso et al. 2007). HPLC requires costly equipment and consumables and is not available in the wineries and spectrophotometric determinations, as easier and faster assays, could be used for routine quality control during the grape ripening and wine production. Thus, a number of chromatographic and spectrophotometric methods have been developed and applied for analysis of phenolic compounds in grapes and wine. Since different parameters influence the extraction of phenolic compounds from grapes and wine—such as their chemical nature, extraction method, sample storage time, and conditions—different previous purification steps and extraction procedures have been proposed as suitable for the extraction of phenolics from grapes and wine.

Sample Preparation Procedures

The separation methods have to be capable of determining most of the compounds of interest. The complexity of grapes and wine requires the use of sample preparation and/or clean-up procedures. When analyzing phenolic compounds from grape seeds and skins, an initial extraction step must be performed, including milling of grape solids in the presence of a suitable extraction solvent. Most of the procedures for extraction of polyphenols from grapes use aqueous methanol, acetone, or ethanol as a solvent for extraction, mostly acidified with HCl or with formic acid. Thus, Marinova et al. (2005) used 80% aqueous methanol (v/v) for phenolic and flavonoid compounds extraction. Montealegre et al. (2006) used methanol, water, and formic acid (50:48.5:1.5, v/v/v) for extraction of phenolic compounds from lyophilized grape skins and seeds. Kennedy et al. (2002) extracted the phenolics from grape skins with 66% aqueous acetone during 24 hours at 20°C and evaporated the solvent after filtration of the extract. Ivanova et al. (2009) tested the efficiency of methanol and acetone (80% aqueous solutions, v/v, containing 0.1% HCl that do not cause degradation as 1% HCl may induce hydrolysis of acetylated anthocyanins [Revilla et al. 1998]) observing that an overall slightly better extraction efficiency of the phenolics was achieved using acetone, mainly evident for extraction of flavan-3-ols and flavonols from grape skin and seed. This can be attributed to the more efficient dissolution of seed's lipidic external layer caused by acetone, which is less polar and thus a better solvent for lipids than methanol, yielding the largest amounts of polyphenols. Also, one step is not enough for total extraction of the analyzed components from the skins, seeds, and pulp and therefore, two subsequent extractions are usually performed.

Separation and fractionation of phenolic compounds from wine and grapes can be performed by solid-phase extraction (SPE) as an efficient method for separation of different fractions of phenolics that offers the advantages of reduction of solvents and time, increased speed and selectivity, and improved recoveries. Among the numerous applications of SPE, C18 Sep-Pak cartridges are mostly used for fractionation of phenolics prior to HPLC analysis. Elution with ethyl acetate or diethyl ether allows isolation of flavan-3-ol monomers and oligomers, followed by elution with methanol to isolate polymeric proanthocyanidins and anthocyanins. Successful fractionation of phenolic compounds from red wine belonging to different groups: phenolic acids, flavonols, anthocyanin monomers and polymers, procyanidins and catechins has been performed by Oszmianski et al. (1998) in four fractions using C18 cartridges and elution with methanol, 16% acetonitrile and ethyl acetate. In addition, the sample purification has been performed using column chromatography on Sephadex LH-20 or on Toyopearl 40 (S) or 50 (F) eluting the components with methanol/water or ethanol/water that allows phenolic fractionation and analysis of wine pigment composition (Mateus et al. 2002).

Spectrophotometry

Spectrophotometric methods, more affordable for routine analyses and particularly because of their speed and simplicity, are widely used for the determination of various subgroups belonging to polyphenols. Based on different principles, these assays have been used to quantify total polyphenols (Slinkard and Singleton 1977; Di Stefano and Cravero 1989; Ivanova et al. 2009, 2010), anthocyanins (Di Stefano and Cravero 1989; Burns et al. 2000; Ho et al. 2003), flavonoids (Mazza et al. 1999; Zhishen et al. 1999; Kim et al. 2003; Marinova et al. 2005), flavan-3-ols (Di Stefano and Cravero 1989) in wine and fruits, as well as color intensity and hues of wine (Glories 1984).

The Folin–Chiocalteu method (Slinkard and Singleton 1977) is most widely used for determination of total phenolics in grapes and wine. It is based on a redox reaction of phosphomolybdic-phosphotungstic acid (Folin–Chiocalteu reagent) to a blue-colored complex in an alkaline solution in the presence of phenolic compounds and shows maximum absorbance at a wavelength of 765 nm. For determination of total flavonoids, the colorimetric method with $AlCl_3$ is usually applied to analyze wines (Zhishen et al. 1999). This method is based on the formation of stable complexes with the C-4 carboxyl group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, exhibiting maximum absorbance at 510 nm. Determination of flavan-3-ols can be performed using the *p*-DMACA (*p*-dimethylaminocinnamaldehyde)

method that was first reported by Thies and Fisher in 1971. This method is based on formation of a colored product from the reaction between tannins and the aldehyde reagent (Thies and Fischer 1971). Applying this method, monomeric procyanidins ((+)-catechin and (–)-epicatehicn)) are determined, reacting with *p*-DMACA reagent and measuring the absorbance of the formed adducts at 640 nm. An important feature of this method is that it theoretically responds only to the units that are not substituted in the A-ring, and thus only one unit per chain, regardless of its length unless there is some branching. In addition, the vanilin assay, which is more sensitive to polymeric tannins than to momoneric flavan-3-ols, is also a widely used method for quantification of proanthocyanins in grapes and wine, as well as in other food samples (Goldstein and Swain 1963; Burns 1971; Deshpande and Cheryan 1987). Simple and fast anthocyanin analysis of wine and grape extracts can be performed by the method proposed by Di Stefano and Cravero (1989) based on dillution of the sample in a mixture of ethanol, water, and HCl in appropriate amounts (ethanol/water/HCl = 70/30/1) and measuring the absorbance at 540 nm.

The color intensity depends on the content and structure of the anthocyanins present in wine and it is usually determined as sum of the absorbances at 420 nm, 520 nm, and 620 nm (Glories 1984). The ratio A_{420}/A_{520} defines the hue (tint) of the wine and gives a measure of the wine redness. A direct measurement of undiluted wine at 420, 520, and 620 nm can be carried out using 1 or 2 mm optical path and the color intensity (CI), hue (H), proportion of red color (% Rd), proportion of blue color (% Bl), and proportion of yellow color (% Ye) are calculated using the following equations:

$$CI = A_{420} + A_{520} + A_{620} \qquad H = A_{420}/A_{520}$$

$$\%\ Ye = A_{420}/CI \cdot 100 \qquad \%\ Rd = A_{520}/CI \cdot 100 \qquad \%\ Bl = A_{620}/CI \cdot 100,$$

where % Ye is the percentage of yellow color in the overall color, % Rd is the percentage of red color, and % Bl is the percentage of blue color in the overall wine color.

The values of the color intensity vary between 0.3 and 1.8 depending on the variety, while the hue values of young wines are 0.5–0.7 and increase throughout wine aging. The results for the color of wine, measured at these three wavelengths, are easy to interpret, which is of great importance for the winemakers to control the wine making and wine aging stages.

High Performance Liquid Chromatography (HPLC)

Reverse phase liquid chromatography (HPLC) coupled to UV-Vis (ultraviolet-visible) detection is the standard method for analysis of various classes of polyphenolic compounds (Wulf and Nagel 1978) using C18 column, a binary solvent system with a polar acidified solvent, such as aqueous formic acid, acetic acid, phosphoric acid, or perchloric acid solution (solvent A) and an organic modifier such as methanol or acetonitrile, possibly acidified (solvent B), (McMurrough and McDowell 1978; Wulf and Nagel 1978; Betés-Saura et al. 1996; Palomino et al. 2000; Viñas et al. 2000; Monagas et al. 2003; Careri et al. 2003; Tsao and Yang 2003; Castillo-Muñoz et al. 2007; Gómez-Alonso et al. 2007). Phenolic compounds exhibit characteristic absorbtion in the UV-Vis region enabling the distinction of the various classes: anthocyanins have an absorbance maximum around 520 nm, as well as in the UV range around 280 nm, flavonols at around 360 nm, and hydroxycinnamic acids can be detected at their absorption maximum at 320 nm. Flavan-3-ols exhibit the maximum absorbance around 280 nm and these substances possess fluorescence properties that the other wine polyphenols do not have that enable their more specific detection and determination. Figure 20.1 shows the UV-Vis spectra of anthocyanin monoglucosides. A typical chromatogram obtained for a wine sample, recorded at 520 nm is presented in Figure 20.2.

Liquid chromatography coupled to mass spectrometry (LC/MS) is applicable to a wide range of compounds and, in recent years, it has become the most sophisticated technique for analysis of phenolic compounds in wine and grapes and for studying the structure of compounds formed by the reaction of anthocyanins with other compounds. Especially, this technique is very effective for studies of glycoside compounds, allowing characterization of the aglycone and sugar moiety (Pérez-Magariño et al. 1999; de Villiers et al. 2004; Monagas et al. 2005).

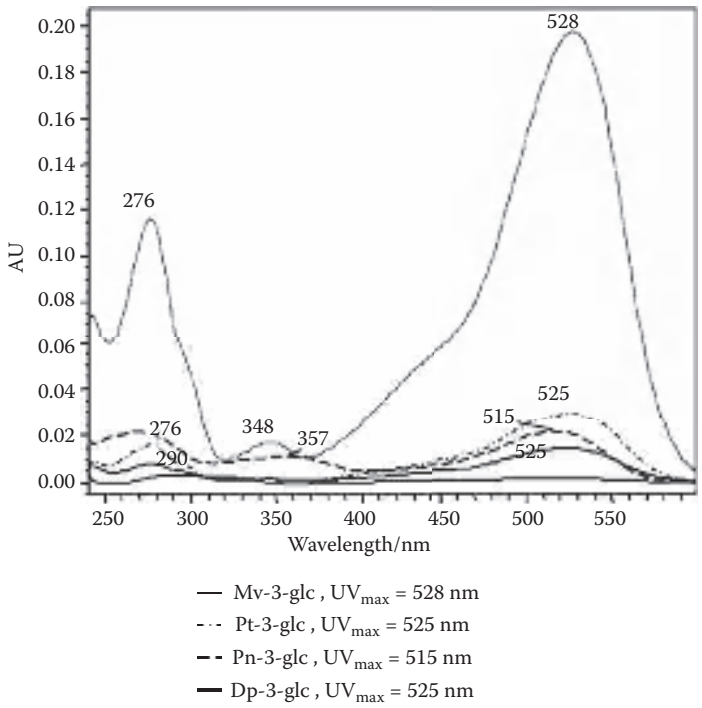

FIGURE 20.1 UV-Vis spectra of anthocyanin monoglucosides. (Mv-malvidin, Pt-petunidin, Pn-peonidin, Dp-delphinidin, and glc-glucoside)

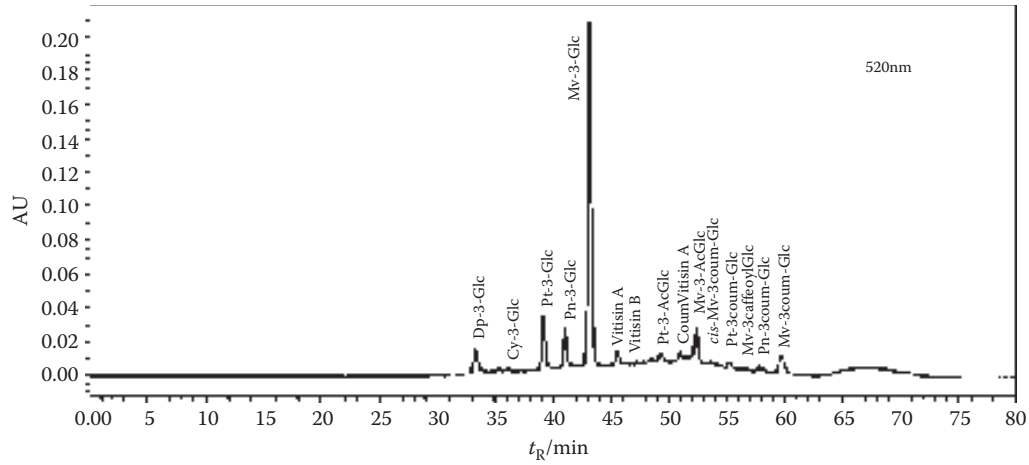

FIGURE 20.2 Chromatogram obtained for a wine sample monitored at 520 nm for separation of anthocyanins (Dp-delphinidine, Cy-cyanidine, Pt-petunidine, Pn-peonidine, Mv-malvidine, Glc-glucoside, Coum-coumaroyl, Ac-acetyl). Chromatographic conditions: C18 column (250 mm × 2.1 mm i.d., 5 μm packing, Waters, Milford, MA) protected by a guard column of the same material (20 × 2.1 mm i.d.; Waters, Milford, MA); mobile phase consisting of water/formic acid (95:5; solvent A), and acetonitrile/water/formic acid (80:15:5; solvent B) at a flow rate of 0.25 mL/min at 38°C. Gradient program of solvent B: isocratic for 2 min with 0%; 2–5 min, 0–2%; 5–12 min, isocratic with 2%; 12–15 min, 2–3%; 15–25 min, 3–8%; 25–40 min, 8–20%; 40–45 min, 20–25%; 45–55 isocratic with 25%, 55–70 min, 25–65% and 70–75 min, 65–0%. (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

Coupling HPLC to mass spectrometry allows characterization and structure elucidation of the anthocyanin pigments present in trace amounts in grapes and wine (Baldi et al. 1995; Cameira-dos-Santos et al. 1996; Wang and Sporns 1999; Revilla and González-San José 2001; Núñez et al. 2004; La Torre et al. 2006). This especially counts for the ion trap mass spectrometers, which enable several subsequent fragmentation steps thus allowing identification of complex molecules in a step-by-step removal of the various groups. However, distinguishing between glucoside and galactoside in flavonoid glycosides, or catechin and epicatechin units is not possible with mass spectrometry (MS). In that case, enzymatic reactions, applying appropriate enzymes (e.g., ß-glucosidase) can be used in order to identify the sugars in glycosides. In addition, an acid-catalyzed cleavage of the glycosidic bond can be performed for sugar determination. The acid-catalyzed cleavage is also successfully applied for determination of proanthocyanidin composition in the presence of a nucleophilic agent. Examples of chromatograms recorded with a diode array detector at 280 nm and fluorimetric detector (excitation wavelength of 275 nm and emission wavelength of 322 nm) are reported in Figure 20.3 (phloroglucinol used as a nucleophilic reagent for depolymerization).

Analytical features of LC/MS, such as sensitivity, selectivity, speed of analysis, cost, and effectiveness have been continually improved and are focused on separation, detection, and structural characterization of novel compounds in wine. LC/MS provides the capability to analyze the phenolic compounds either in the positive or in the negative ion mode, generating cations ($[M + H]^+$, $M + Na]^+$) or anions ($[M–H]^-$, $[M–Cl]^-$. The positive ion mode has been proven as efficient for anthocyanin analysis, as well as for flavan-3-ols detection, while the negative ion mode is more suitable for phenolic acids, flavonoids, and can be successfully applied for flavan-3-ols identification. One of the first applications of mass spectrometry for grape polyphenols identification was published in 1990 by Lee and Jaworski where positive and negative ion modes were applied for analysis of extracts from grape samples and three compounds have been detected: catechin–gallate ($[M–H]^-$ at m/z 441, fragment ions at m/z 151 and 137), catechin–catechin–gallate ($[M–H]^-$ at m/z 729, fragment ion at m/z 577) and gallocatechin–gallate ($[M–H]^-$ at m/z 459).

The LC/MS system equipped with an electrospray ionization (ESI), giving access to molecular weights of different species present in the samples, has been successfully applied for studying different groups of polyphenols in wine and grapes, such as nonflavonoids (phenolic acids and stilbenes) and flavonoids (flavonols, dihydroflavonols, anthocyanins, flavan-3-ols: oligomers and polymers). A list of nonanthocyanin compounds detected by LC/ESI-MS in red wine samples is reported in Table 20.1 also containing UV and MS data.

Liquid chromatography/mass spectrometry analysis of phenolic acids is usually performed in negative ion mode. With regard to hydroxybenozoic acids, all of them are detected as $[M–H]^-$ showing a characteristic fragmentation $[M–H\text{-}44]^-$, which corresponds to elimination of CO_2 group from the carboxylic acid and production of the corresponding fragment ions (Monagas et al. 2005), as shown in Table 20.1. The common compounds present in grapes and wine belonging to the group of hydroxycinnamic acid derivatives are caffeoyltartaric (caftaric) acid at m/z 311 (fragment ions: m/z 179, 149), *p*-coumaroyltartaric (coutaric) acid at m/z 295 (fragment ion at m/z 163), and feruloyltartaric (fertaric) acid at m/z 325 (fragment ion at m/z 193), which have a characteristic fragmentation pattern $[M–H\text{-}132]^-$ that corresponds to a loss of tartaric acid residue (Baranowski and Nagel 1981; Baderschneider and Winterhalter 2001; Monagas et al. 2005). The phenolic acid GRP (2-*S*-glutathionylcaffeoyltartaric acid), detected in wine at m/z 616 (fragment ions at m/z 484, 440 and 272; Boselli et al. 2006) was also confirmed to be present in wine (Singleton et al. 1985, 1986).

For analysis of flavan-3-ols, both positive and negative ion modes can be applied. Figures 20.4 and 20.5 present extracted ion chromatograms relative to the analysis of wine flavan-3-ols in negative-ion mode. Since MS does not distinguish flavan-3-ol monomers (+)-catechin ($[M–H]^-$ = m/z 289) and (–)-epicatechin ($[M–H]^-$ = m/z 289), further identification of these compounds can be achieved by coelution with the corresponding standard compounds. A characteristic feature of flavan-3-ols, compared to the other phenolic compounds, is the Retro-Diels-Alder (RDA) rearrangement on the C-ring of catechin and epicatechin derivatives followed by the elimination of 152 Da fragment (Table 20.1).

The glucoside derivatives of flavonols (myricetin, quercetin, laricitrin, and syringetin) known to be the predominant flavonol hexosides in grapes and wine are identified on the basis of their $[M–H]^-$ signals

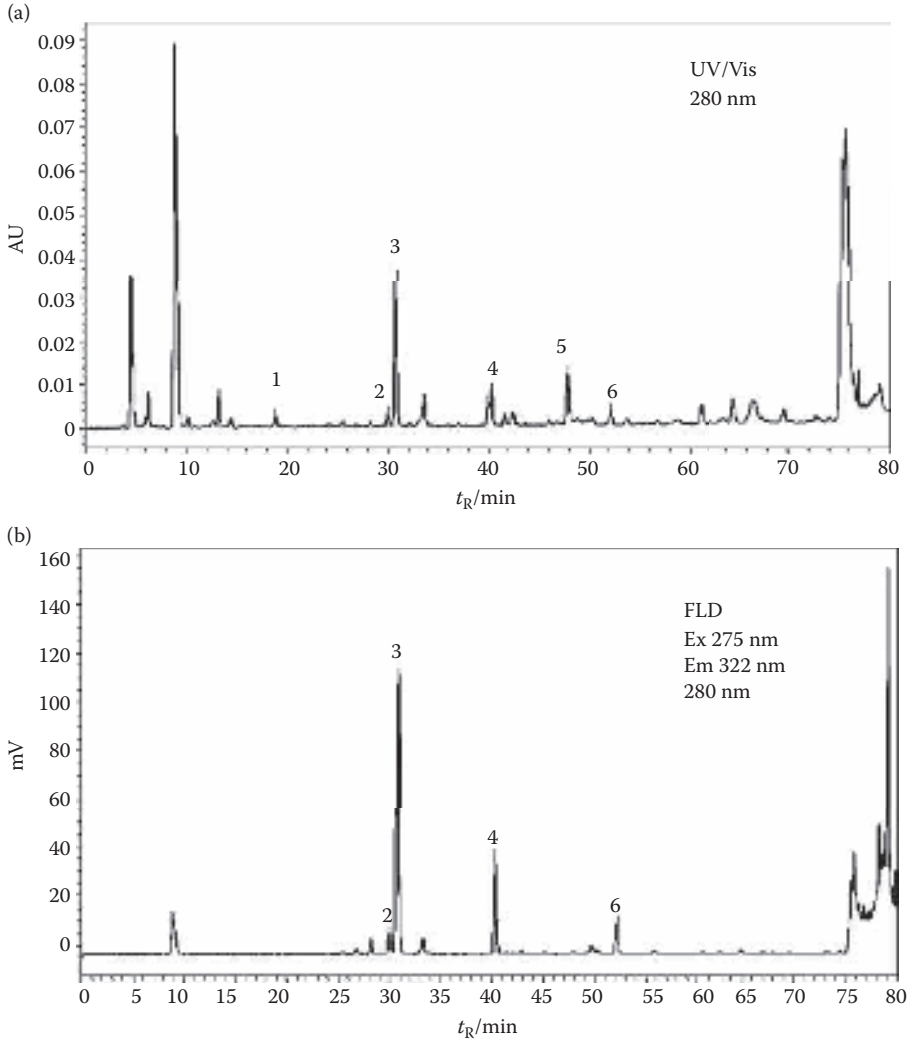

FIGURE 20.3 Chromatograms obtained for wine sample recorded with (a) diode array detector at 280 nm and (b) fluorimetric detector, Ex-275 nm, and Em-322 nm for analysis of flavan-3-ols (terminal units) and phloroglucinol adducts (extension subunits) after acid-catalyzed depolymerization with phloroglucinol. Peak identification: 1-Epigallocatechin-(4α→2)-phloroglycinol, 18.8 min; 2-Catechin-(4α→2)-phloroglucinol, 29.9 min; 3-Epicatechin-(4α→2)-phloroglucinol, 30.7 min; 4-Catechin, 40.2 min; 5- Epicatechin-3-*O*-gallate-(4α→2)-phloroglucinol, 47.8 min; 6-Epicatechin, 52.1 min. Chromatographic conditions: C18 column (250 mm × 4.6 mm i.d., 5 μm packing Waters, Milford, MA) protected by a guard column of the same material (20 × 4.6 mm i.d.; Waters, Milford, MA). A binary solvent consisted of water/formic acid (98:2; solvent A) and acetonitrile/water/formic acid (80:18:2; solvent B) at a flow rate of 1 mL/min at 30°C: Gradient program of solvent B: isocratic for 5 min with 0%; 5–35 min, 0–10%; 35–70 min, 10–20%; 70–75 min, 20%–100%; and 75–80 min, 100–0%. (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

and formed fragment ions ([M–H–162]$^-$), which correspond to the elimination of a glucose molecule (Ribereau-Gayon 1964; Mattivi et al. 2006; Castillo-Muñoz et al. 2007), while the glucuronide derivatives (e.g., myricetin-3-*O*-glucuronide, quercetin-3-*O*-glucuronide) are characterized by a loss of 176 Da ([M–H–176]), corresponding to the elimination of a glucuronide group (Cheynier and Rigaud 1986). Previous studies have shown the presence of astilbin (dihydroquercetin-3-*O*-rhamnoside, [M–H]$^-$ = m/z 449) and engeletin (dihydrokaempferol-3-*O*-rhamnoside, [M–H]$^-$ = m/z 433) in skins of white grapes and

TABLE 20.1

UV and MS Spectral Data for Nonanthocyanin Phenolics Detected in Vranec[a] Wines

Phenolic Acids	**λ max/nm**	**[M-H]⁻**	**Fragments (m/z)**
Gallic acid	272.3	169	125
Protocatechuic acid	293.7, 255.8	153	109
Caftaric acid	328	311	179, 149
cis-Coutaric acid	310.4	295	163
trans-Coutaric acid	310	295	163
GRP		616	
Fertaric acid	312.8	325	193
Syringic acid	272.3	197	**153**
Flavan-3-ols	**λ max/nm**	**[M–H]⁻**	**Fragments (m/z)**
Procyanidin B3	284.2	577	559, 451, 425, 407, 289, 245
Catechin	276.5	289	245, 205, 179
Procyanidin B1	264.7	577	559, 451, 425, 407, 289, 245
Procyanidin B4	264.7	577	559, 451, 425, 407, 289, 245
Epicatechin	276.5	289	245, 205, 179
Procyanidin B2	264.7	577	559, 451, 425, 407, 289, 245
Flavan-3-ols	**λ max/nm**	**[M+H]⁺**	**Fragments (m/z)**
Catechin	276.5	291	273, 165, 139, 123
Epicatechin	276.5	291	273, 165, 139, 123
Dihydroflavonols	**λ max/nm**	**[M–H]⁻**	**Fragments (m/z)**
Dihydromyricetin-3-*O*-rha		465	339, 301
Astilbin		449	303, 285
Engeletin		433	287, 269
Flavonols	**λ max/nm**	**[M+H]⁺**	**Fragments (m/z)**
Myricetin-3-glcA	343.5	495	319
Myricetin-3-glc	343.5	481	319
Quercetin-3-glcA	353.4	479	303
Quercetin-3-glc	353.4	465	303
Laricitrin-3-glc	364.2	495	333
Syringetin-3-glc	358.2	509	481, 392, 347
Quercetin	368.7	303	

Source: Adapted from Monagas, M., Suárez, R., Gómez-Cordovés, C., and Bartolomé, B., *Am. J. Enol. Vitic.*, 56, 139–47, 2005.

Labels: GRP = grape reaction product, which is 2-*S*-glutathionylcaffeoyltartaric acid, glc: glucoside, glcA: glucuronide, rha: rhamnoside.

[a] Vranec is a red grape variety used for production of quality wines typical for the Balkan Peninsula.

in white wines (Trousdale and Singleton 1983) showing characteristic fragmentation that corresponds to the elimination of the rhamnoside group (–164 amu; Souquet et al. 2000). Another compound, dihydromyricetin-3-*O*-rhamnoside at m/z 465, shows the same fragmentation pattern (Vitrac et al. 2001). The characteristic MS fragmentations of astilbin and engelitin detected in wine samples are given in Figure 20.6.

Structural identification and characterization of grape anthocyanins: glucosides, acetylglucosides, and *p*-coumaroylglucoside derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin as the main colored compounds are achieved by electrospray ionization in the positive mode and MS^n analyses, as a highly effective tool for compound differentiation. They have similar fragmentation patterns: their mass spectra contain two signals, the molecular ion, M^+ and the fragment ions $[M\text{-}162]^+$, $[M\text{-}204]^+$, or $[M\text{-}308]^+$ due to elimination of glucose, acetylglucose, or *p*-coumaroylglucose groups, respectively (Baldi et al. 1995; Vivar-Quintana et al. 2002; Salas et al. 2004; Kelebek et al. 2007). The λ_{max} values for cyanidin-3-glucoside and peonidin-3-glucoside are lower than λ_{max} for the other three glucosides and the acylated derivatives with either *p*-coumaric acid or caffeic acid absorb in the 300–340 nm region, as

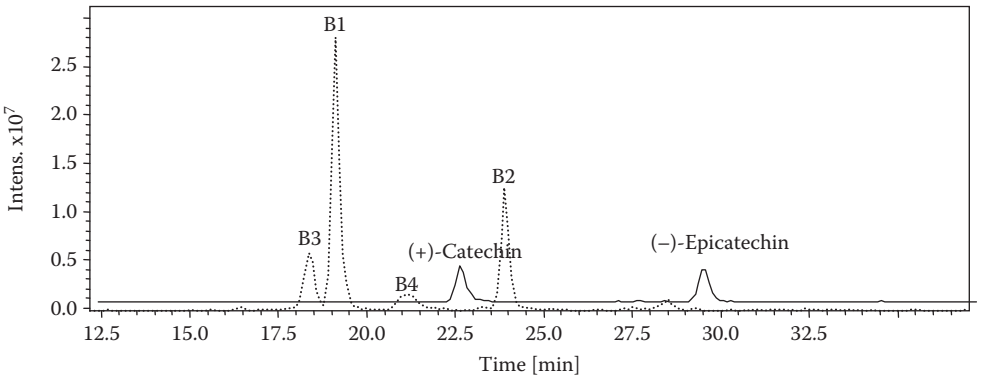

FIGURE 20.4 Negative ion-mode extracted ESI-MS chromatograms obtained from wine analysis in the m/z range 200–1200. Extracted m/z values correspond to the ions of (+)-catechin (m/z 289), (–)-epicatechin (m/z 289), and procyanidins: B1 (m/z 577), B2 (m/z 577), B3 (m/z 577), and B4 (m/z 577). (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

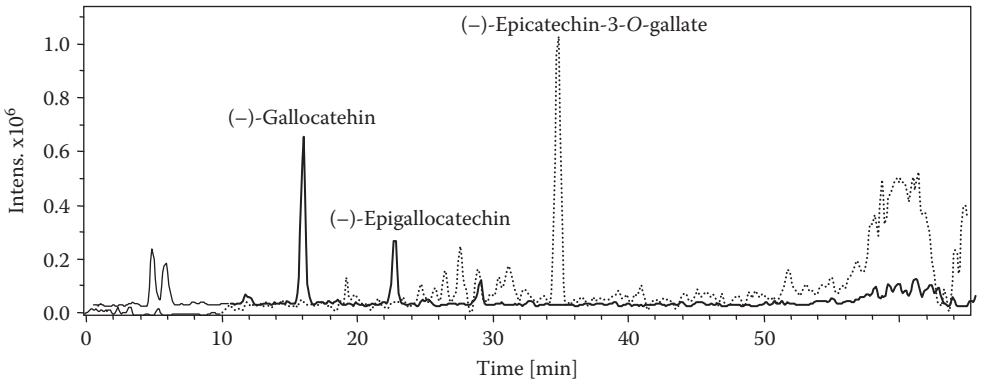

FIGURE 20.5 Negative ion-mode extracted ESI-MS chromatograms obtained from wine analysis in m/z range 200–1200. Extracted m/z values correspond to the ions of ion chromatograms extracted at *m/z* values corresponding to the negative ions of (–)-gallocatechin (m/z 305), (–)-epigallocatechin (m/z 305), and (–)-epicatechin-3-gallate (m/z 441). (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

previously described by Wulf and Nagel (1978) and in agreement with other published data (Piovan et al. 1998; de Villiers et al. 2004). Typically for reversed-phase liquid chromatography, the components elute in order of their polarity: (1) delphinidin-3-glucoside, (2) cyanidin-3-glucoside, (3) petunidin-3-glucoside, (4) peonidin-3-glucoside, and (5) malvidin-3-glucoside. Acetyl and *p*-coumaroyl derivatives elute in the same order as anthocyanin-3-monoglucosides. In this way, the order of elution of the anthocyanins is monoglucoside < acetylmonoglucoside < coumaroylmonoglucoside (Vivar-Quintana et al. 2002; Kelebek et al. 2007). Fragmentations and λ_{max} values of different groups of pigments are listed in Table 20.2.

The MS/MS analyses are successfully used for structural characterization of anthocyanin derivatives formed during maceration and wine aging, in particular the pyranoanthocyanins, which arise from reactions of anthocyanins with pyruvic acid (Bakker et al. 1997; Revilla et al. 1999; Hayasaka and Asenstorfer 2002; Heier et al. 2002; Morata et al. 2003; Alcalde-Eon et al. 2004, 2006; Chinnici et al. 2009). All these molecules have the same main fragment ion that corresponds to carboxy-pyrano-

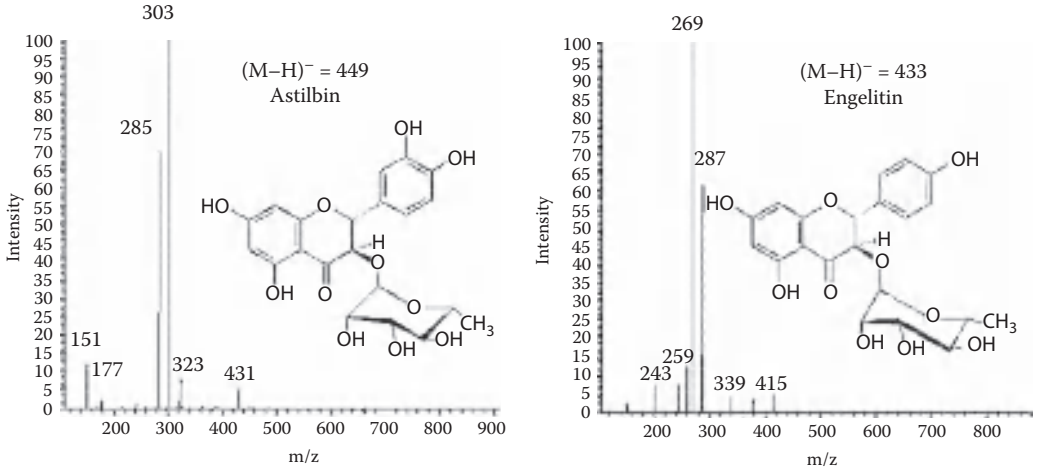

FIGURE 20.6 MS/MS fragmentations of (a) astilbin and (b) engelitin detected in wine under the negative ESI mode. (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

anthocyanin aglycone, released after loss of 162, 204, and 308 mass units (Table 20.2; Fulcrand et al. 1998; Mateus et al. 2003). A series of pyranoanthocyanins formed by reactions of anthocyanins with acetaldehyde (pyrano-anthocyanins) have also been shown to be present in wines (Table 20.2). Other pyranoanthocyanins, formed by a reaction of acetaldehyde, flavanols and anthocyanins have been detected in wine samples (He et al. 2006) and have been identified as flavanyl-pyranoanthocyanins. An example of the fragmentation of catechin-pyrano-malvidin-3-glucoside [M + H]+ at m/z 805 is reported in Figure 20.7. As shown in Table 20.2, the compound detected with a molecular mass of 805 unites corresponds to (epi)catechin-pyrano-malvidin-3-glucoside (fragment ions: 643, 491) and those with m/z 1093 (fragment ions: m/z 931, 803) and 1135 (fragment ions: m/z 931, 845) to procyanidin dimer-pyrano-malvidin-3-glucoside and procyanidin dimer-pyrano-malvidin-3-acetylglucoside (Francia-Aricha et al. 1997).

Anthocyanins that can condense either directly or by mediation of acetaldehyde with flavan-3-ols, forming anthocyanin–flavanol direct condensation pigments or ethyl-bridged pigments as a result of reactions that take place spontaneously during the maceration and aging of wine, have been detected in wines (Table 20.2). Thus, the compounds with m/z 779, 925, and 795 have been identified as (epi) catechin-ethyl-peonidin-3-glucoside, (epi)catechin-ethyl-peonidin-3-*p*-coumaroylglucoside and (epi) catechin-ethyl-petunidin-3-glucoside, respectively. An example of fragmentation of (epi)catechin)-(epi)catechin-malvidin-3-glucoside (m/z 1069) is shown in Figure 20.8.

Matrix-Assisted Laser Desorption/Ionization (MALDI)

The determination of molecular masses of polyphenols by mass spectrometry could be performed using the electrospray ionization (ESI; Cheynier et al. 1997; Hayasaka and Asenstorfer 2002), the atmospheric pressure chemical ionization (APCI) or the matrix-assisted laser desorption/ionization (MALDI; Sugui et al. 1998, 1999; Wang and Sporns 1999; Robards 2003; Reed et al. 2005; Es-Safi et al. 2006; Tholey and Heinzle 2006; Carpentieri et al. 2007). MALDI was first demonstrated by Karas et al. (1987) and originally developed for large biomolecules analyses. MALDI is usually coupled with a time of flight (TOF) analyzer, one of the oldest and simplest mass analyzers (Sporns and Wang 1998), since the timing of the ionization is very precise using a short nanosecond laser pulse and the initial ion velocities are remarkably consistent. The TOF gives access to a theoretically unlimited mass range and also

TABLE 20.2

UV-Vis and MS Spectral Data of Anthocyanins and Their Derivatives Detected in Wine Samples

Pigment Compounds	λ_{max}/nm	$M^+/[M+H]^+$	Fragments (m/z)	References
Dp-3-glc	525	465	303	*, **, ***, ****
Cy-3-glc	516	449	287	*, **, ***, ****
Pt-3-glc	525	479	317	*, **, ***, ****
Pn-3-glc	516	463	301	*, **, ***, ****
Mv-3-glc	528	493	331	*, **, ***, ****
Dp-3-acetylglc	527	507	303	*, **, ***, ****
Cy-3-acetylglc	523	491	287	*, **, ***, ****
Pt-3-acetylglc	529	521	317	*, **, ***, ****
Pn-3-acetylglc	522	505	301	*, **, ***, ****
Mv-3-acetylglc	530	535	331	*, **, ***, ****
Dp-3-*p*-coumglc	530	611	303	*, **, ***, ****
Cy-3-*p*-coumglc	520	595	287	*, **, ***, ****
Pt-3-*p*-coumglc	532	625	317	*, **, ***, ****
Pn-3-*p*-coumglc	526	609	301	*, **, ***, ****
Mv-3-*p*-coumglc	532	639	331	*, **, ***, ****
Dp-3-caffeoylglc	532	627	303	**
Pt-3-caffeoylglc	531	641	317	**
Pn-3-caffeoylglc	525	625	301	**
Mv-3-caffeoylglc	534	655	331	**
Dp-3,7-diglc	523	627	303	**
Pt-3,7-diglc	522	641	317	**
Pn-3,7-diglc		625	301	**
Mv-3,7-diglc	526	655	331	**
Dp-3-glc + L(+)lactic acid		537		**
Pt-3-glc + D(-)lactic acid		551		**
Pt-3-glc + L(+)lactic acid		551	317	**
Pn-3-glc + D(-)lactic acid		535		**
Pn-3-glc + L(+)lactic acid		535	301	**
Mv-3-glc + D(-)lactic acid		565	331	**
Mv-3-glc + L(+)lactic acid		565	331	**
Carboxy-pyrano-Dp-3-glc	507	533	371	**
Carboxy-pyrano-Pt-3-glc	508	547	385	*, **
Carboxy-pyrano-Pn-3-glc	503	531	369	*, **
Carboxy-pyrano-Mv-3-glc (Vitisin A)	510	561	399	*, **
Carboxy-pyrano-Dp-3-acetylglc		575	371	****
Carboxy-pyrano-Pt-3-acetylglc		589	385	****
Carboxy-pyrano-Pn-3-acetylglc		573		****
Carboxy-pyrano-Mv-3-acetylglc (acetylVitisin A)		603	399	*, ****
Carboxy-pyrano-Dp-3-*p*-coumglc		679	371	****
Carboxy-pyrano-Pt-3-*p*-coumglc		693		**, ****
Carboxy-pyrano-Pn-3-*p*-coumglc	508	677	369	**, ****
Carboxy-pyrano-Mv-3-*p*-coumglc (*p*-coumVitisin A)	514	707	399	*, ****
Pyrano-Dp-3-glc		489	327	**, ****
Pyrano-Pt-3-glc	492	503	341	**, ****
Pyrano-Pn-3-glc		487	325	**, ****
Pyrano-Mv-3-glc (Vitisin B)	490	517	355	*, ****
Pyrano-Mv-3-glc-dimer		1093	931, 803	*

TABLE 20.2 (CONTINUED)

UV-Vis and MS Spectral Data of Anthocyanins and Their Derivatives Detected in Wine Samples

Pigment Compounds	λ_{max}/nm	$M^+/[M+H]^+$	Fragments (m/z)	References
Pyrano-Pn-3-acetylglc		529	325	**
Pyrano-Mv-3-acetylglc (acetylVitisin B)	494	559	355	**
Pyrano-Mv-3-*p*-coumglc (*p*-coumVitisin B)	497	663	355	****
Dp-3-glc-4-vinylphenol	503	581	419	**
Cy-3-glc-4-vinylphenol		565		**
Pt-3-glc-4-vinylphenol	502	596	433	**
Pn-3-glc-4-vinylphenol	500	579	417	**
Mv-3-glc-4-vinylphenol	504	609	447	**
Mv-3-acetylglc-4-vinylphenol	505	651	447	**
Dp-3-*p*-coumglc-4-vinylphenol		727	419	**
Pt-3-*p*-coumglc-4-vinylphenol	504	741	433	**
Pn-3-*p*-coumglc-4-vinylphenol	501	725	417	**
Mv-3-*p*-coumglc-4-vinylphenol	505	755	447	**
Dp-3-glc-4-vinylcatechol	509	597	435	**
Pt-3-glc-4-vinylcatechol	510	611	449	**
Pn-3-glc-4-vinylcatechol	506	595	433	**
Mv-3-glc-4-vinylcatechol	510	625	463	**
Mv-3-acetylglc-4-vinylcatechol	513	667	463	**
Dp-3-*p*-coumglc-4-vinylcatechol		743	435	**
Pt-3-*p*-coumglc-4-vinylcatechol		757	449	**
Mv-3-*p*-coumglc-4-vinylcatechol		771	463	**
Dp-3-glc-(epi)cat		753	591	**
Cy-3-glc-(epi)cat		737	575	**
Pt-3-glc-(epi)cat		767	605	**
Pn-3-glc-(epi)cat		751	589	**
Mv-3-glc-(epi)cat		781	619	**
(epi)cat-(epi)cat-Mv-3-glc		1069	907, 619	*
Mv-3-acetylglc-(epi)cat		823	619	*
Mv-3-acetylglc-flavan-3-ol-dimer		1135	931, 845, 641, 435	*
Dp-3-*p*-coumglc-(epi)cat		899	591	**
Cy-3-*p*-coumglc-(epi)cat		883		**
Pt-3-*p*-coumglc-(epi)cat		913	605	**
Pn-3-*p*-coumglc-(epi)cat		897	589	**
Mv-3-*p*-coumglc-(epi)cat		927	619	**
(epi)cat-pyrano-Mv-3-glc		805		*
Dp-3-glc-gallcat	531	769	607	**
Cy-3-glc-gallcat	282	753	591	**
Pt-3-glc-gallcat	279	783	621	**
Pn-3-glc-gallcat		767	605	**
Mv-3-glc-(epi)gallcat	281	797	635	**
Dp-3-*p*-coumglc-gallcat		915	607	**
Cy-3-*p*-coumglc-gallcat		899		**
Pt-3-*p*-coumglc-gallcat		929		**
Pn-3-*p*-coumglc-gallcat		913	605	**
Mv-3-*p*-coumglc-(epi)gallcat		943	635	**
Dp-3-glc-ethyl-(epi)cat		781	329	**

(*Continued*)

TABLE 20.2 (CONTINUED)

UV-Vis and MS Spectral Data of Anthocyanins and Their Derivatives Detected in Wine Samples

Pigment Compounds	λ_{max}/nm	$M^+/[M+H]^+$	Fragments (m/z)	References
Cy-3-glc-ethyl-(epi)cat		765		**
Pt-3-glc-ethyl-(epi)cat		795	343	**
Pn-3-glc-ethyl-(epi)cat		779	327	**
Mv-3-glc-ethyl-(epi)cat		809	357	**
Mv-3-glc-ethyl-flavan-3-ol dimer		1097		*
Mv-3-acetylglc-ethyl-(epi)cat		851	357	**
Mv-3-*p*-coumglc-ethyl-(epi)cat		955	357	**
Dp-3-glc-ethyl-(epi)gallcat		797	329	**
Cy-3-glc-ethyl-(epi)gallcat		781		**
Pt-3-glc-ethyl-(epi)gallcat		811	343	**
Pn-3-glc-ethyl-(epi)gallcat		795		**
Mv-3-glc-ethyl-(epi)gallcat		825	357	**
Mv-3-acetylglc-ethyl-(epi)gallcat		867		**
Mv-3-glc-ethyl-Mv-3-glc		1012		*

Labels: Dp: delphinidin, Cy: cyanidin, Pt: petunidin, Pn: peonidin, Mv: malvidin, glc: glucoside, acetylglc: acetylglucoside, *p*-coumglc: *p*-coumaroylglucoside, (epi)cat: (epi)catechin, gallcat: gallocatechin.

* Adapted from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD thesis, Ss Cyril and Methodius Univ ersity, Skopje, Republic of Macedonia, 2009.

** Adapted from Alcalde-Eon, C., Escribano-Bailón, M. T., Santos-Buelga, C., and Rivas-Gonzalo, J. C., Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study, *Anal. Chim. Acta,* 563, 238–54, 2006.

*** Adapted from Alcalde-Eon, C., Escribano-Bailón, M. T., Santos-Buelga, C., and Rivas-Gonzalo, J. C., Separation of pyranoanthocyanins from red wine by column chromatography. *Anal. Chim. Acta,* 513, 305–18, 2004.

**** Adapted from Boido, E., Alcalde-Eon, C., Carrau, F., Dellacassa, E., and Rivas-Gonzalo, J. C., Aging effect on the pigment composition and color of *Vitis vinifera* L. Cv. Tannat wines. Contribution of the main pigment families to wine color. *J. Agric. Food Chem.,* 54, 6692–704, 2006.

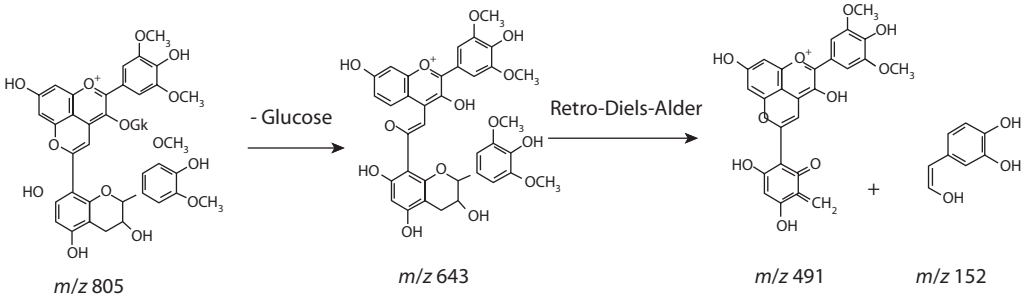

FIGURE 20.7 Fragmentation of catechin-pyrano-malvidin-3-glucoside with m/z 805 under positive ESI-MS mode. (Adapted from He, J., Santos-Buelga, C., Mateus, N., and De Freitas, V., *J. Chromatogr. A*., 1134, 215, 2006.)

provides high resolution giving access to accurate mass determination. A basic characteristic of MALDI is the application of the analyte with a suitable matrix substance and irradiation of this mixture by a pulsed laser, including ablation of the sample and matrix molecules accompanied by ionization of the analytes. MALDI can be operated in the positive or negative ion mode and it yields mostly singly charged ions, generating cations such as $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ or anions such as $[M–H]^–$ and $[M + Cl]^–$ (Fulcrand et al. 1998). Recent investigations demonstrated that MALDI as a sensitive and efficient technique could be used for characterization of different molecules in food science: anthocyanins in red wine and fruit juice (Wang and Sporns 1999), carotenoids in crop plants (Fraser et al. 2007),

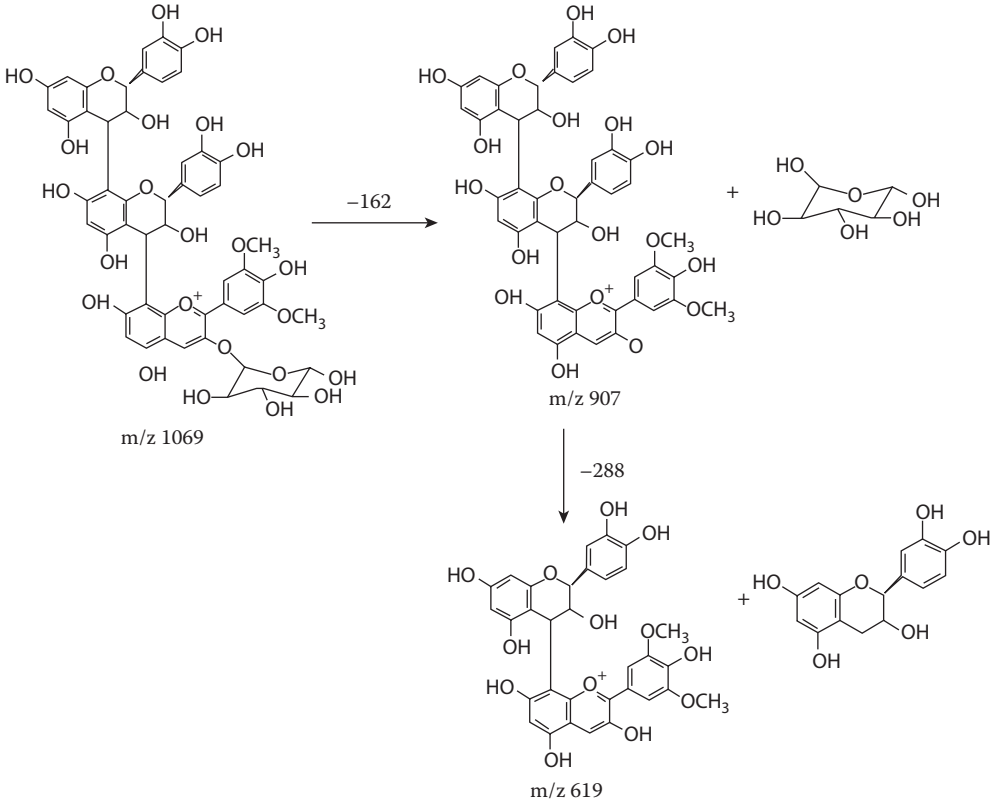

FIGURE 20.8 Fragmentation of (epi)catechin-(epi)catechin-malvidin-3-glucoside (m/z 1069). (Adapted from Francia-Aricha, E. M., Guerra, M. T., Rivas-Gonzalo, J. C., and Santos-Buelga, C., *J. Agric. Food Chem.*, 45, 2262–66, 1997.)

plant oligosaccharides (Lerouxel et al. 2002), black tea stain consisting of polyphenols (Yamada et al. 2007), and polygalloyl polyflavan-3-ols in grape seed extracts (Krueger et al. 2000). This technique has become the routine method of choice for peptide and protein characterization (Marentes and Grusak 1998; Schiller et al. 2004; Joss et al. 2006; Piraino et al. 2007; Sheoran et al. 2007; Zhang et al. 2008). The advantages of MALDI-TOF-MS over the other methodologies include the ease of use, speed of analysis, high sensitivity, wide applicability combined with a good tolerance toward contaminants, as well as the ability to analyze complex mixtures (Karas 1996).

The most widely used sample preparation methods for MALDI-TOF-MS analysis are: *the dried droplet* technique when a mixed solution of the sample with analyte(s) and matrix is deposited onto a sample plate and allowed to dry, and in *the sandwich* method the sample is placed "in a sandwich" between two matrix layers. Two critical concerns for successful MALDI analyses are the choice of the matrix and the sample preparation (Sugui et al. 1999). A number of substances have been tested as possible MALDI matrices and applied as well. In general, there are no rules for predicting the suitability of a substance as a matrix, with the exception of the absolute requirement for absorption of UV laser energy. The most frequently used MALDI matrices are derivatives of benzoic acid (e.g., 2,5-dihydroxybenzoic acid [2,5-DHB]), which is suitable for analysis of low molecular weight compounds, and analysis of proteins and glycoproteins (Juhasz et al. 1993) and derivatives of cinnamic acid (e.g., α-cyano-4-hydroxycinnamic acid [CHCA] or synapic acid [SA]). Carpentieri et al. (2007) used 2,5-DHB for fast fingerprinting of red wines. Fullerenes have also been used as matrices (Ugarov et al. 2004), but there are no published data for their application for anthocyanins analyses in wine and grape samples even though these compounds have been attracting scientists all over the world working extensively on their syntheses and properties studies. The first use of fullerenes for laser desorption of biomolecules involved the application of a protein sample solution directly onto the predeposited

fullerene film (Michalak et al. 1994). The fullerene matrix has then been used for MALDI-TOF analysis of small hydrophobic molecules (e.g., steroids, fatty acids; Mernyak et al. 2008). Figure 20.9 illustrates the positive-ion MALDI-TOF-MS spectra of different tested matrices: CHCA, SA, 2,5-DHB, and C_{70} fullerene, and it is shown that the obtained quasimolecular, fragment and adduct ion peaks for CHCA, SA and 2,5-DHB have relatively high intensity in the range of m/z 100–700 compared to the peaks of C_{70} fullerene matrix (Ivanova 2009).

Taking into account the molecular mass of fullerene (m/z 840), it is expected that the obtained signal peaks would have very low intensity in the range of interest for the anthoycanin identification, from m/z 100 to 700 and they would not interfere the identification of the sample peaks in this low mass range. So, applying the fullerene as a matrix, all five anthocyanins (malvidin, peonidin, petunidin, delphinidin, and cyanidin) have been identified in their aglycone forms since applying higher laser energy, needed for ionization of fullerene molecules, causes fragmentation of the glucosides. In Figure 20.10, an example of application of different MALDI matrices, CHCA, SA, 2,5-DHB, and C_{70} fullerene, for MALDI-TOF-MS analysis of grape skin extract is presented (Ivanova 2009).

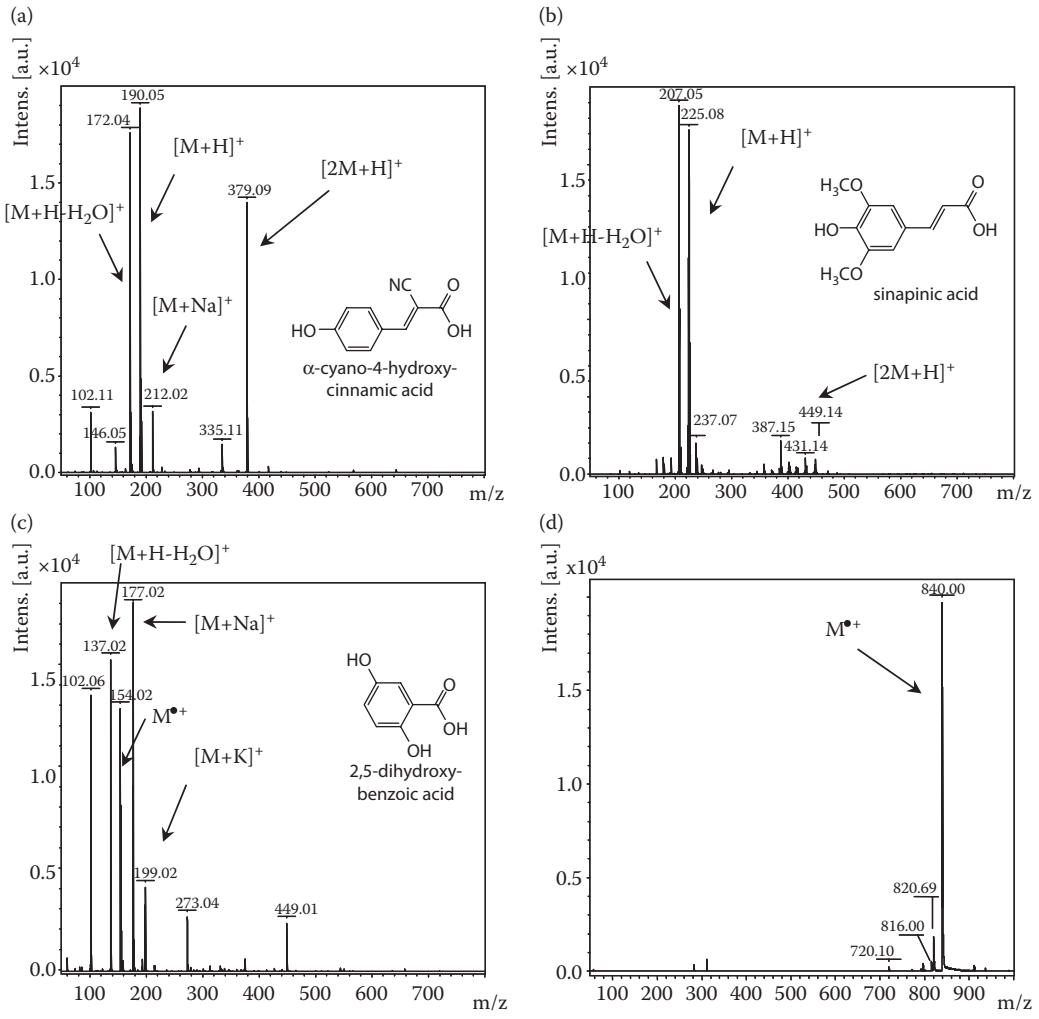

FIGURE 20.9 Positive-ion MALDI TOF mass spectra of the matrices (a) CHCA, (b) SA, (c) 2,5-DHB, and (d) C_{70} fullerene. (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

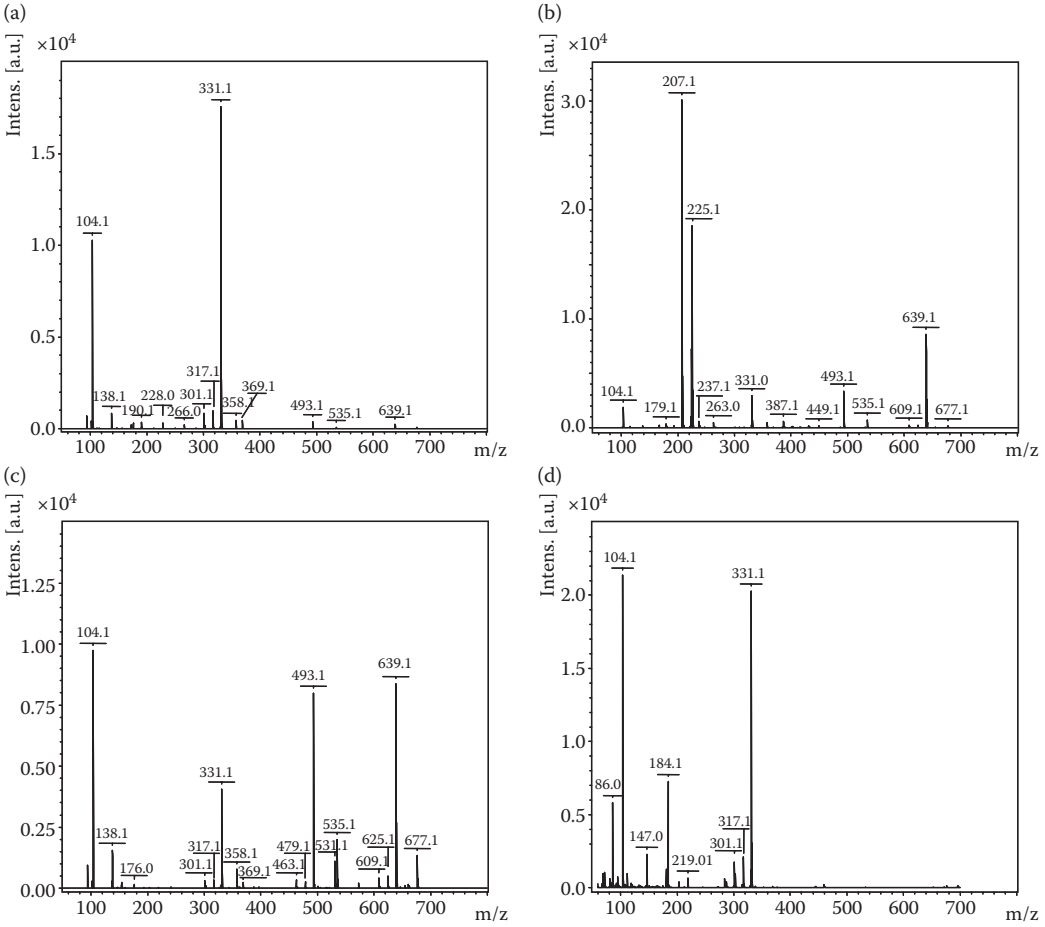

FIGURE 20.10 Positive-ion MALDI TOF mass spectra of the skin extract of the Vranec grape (red grape variety) in the presence of different MALDI matrices: (a) CHCA, (b) SA, (c) 2,5-DHB, and (d) C_{70} fullerene "sandwich." (Data from Ivanova, V., Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry, PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia, 2009.)

For better identification of anthocyanin glycosides (acetyl and *p*-coumaroyl derivatives) in wine and grapes further investigations are needed to be performed, in order to test, for example, the effect of the derivatized and/or acidified fullerenes as possible matrices. The ability to use a lower laser power is thus one advantage of these matrices. There are a number of other advantages that this new class of matrices possesses: high analyte ionization efficiency, small molar ratios (less than 1) of matrix/analyte, and a broader optical absorption spectrum, which should obviate specific wavelength lasers for MALDI acquisitions (Uragov et al. 2004).

REFERENCES

Alcalde-Eon, C., Escribano-Bailón, M. T., Santos-Buelga, C., and Rivas-Gonzalo, J. C. 2004. Separation of pyranoanthocyanins from red wine by column chromatography. *Anal. Chim. Acta* 513:305–18.

Alcalde-Eon, C., Escribano-Bailón, M. T., Santos-Buelga, C., and Rivas-Gonzalo, J. C. 2006. Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study. *Anal. Chim. Acta* 563:238–54.

Baderschneider, B., and Winterhalter, P. 2001. Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* 49:2788–98.

Bakker, J., Bridle, P., Honda, T., Kuwano, H., Saito, N., Terahara, N., and Timberlake, C. F. 1997. Identification of anthocyanin occurring in some red wines. *Phytochemistry* 44:1375–82.

Baldi, A., Romani, A., Mulinacci, N., Vincieri, F. F., and Casetta, B. 1995. HPLC/MS application to anthocyanins of Vitis-vinifera L. *J. Agric. Food Chem.* 43:2104–9.

Baranowski, J. D., and Nagel, C. W. 1981. Isolation and identification of the hydroxycinnamic acid derivatives in white Riesling wine. *Am. J. Enol. Vitic.* 32:5–13.

Bautista-Ortín, A. B., Fernández-Fernández, J. I., López-Roca, J. M., and Gómez-Plaza, E. 2007. The effects of enological practices in anthocyanins, phenolic compounds and wine colour and their dependence on grape characteristics. *J. Food Compos. Anal.* 20:546–52.

Betés-Saura, C., Andrés-Lacueva, C., and Lamuela-Raventós, R. M. 1996. Phenolics in white free run juices and wines from Penedés by high-performance liquid chromatography: changes during vinification. *J. Agric. Food Chem.* 44:3040–6.

Boselli, E., Minardi, M., Giomo, A., and Frega, N. G. 2006. Phenolic composition and quality of white d.o.c. wines from Marche (Italy). *Anal. Chim. Acta* 563:93–100.

Burns, J., Gardner, P. T., O'Neil, J., Crawford, S., Morecroft, I., McPhail, D. B., Lister, C., et al. 2000. Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *J. Agric. Food Chem.* 48:220–30.

Burns, R. E. 1971. Methods for estimation of tannin in grain sorghum. *Agron. J.* 63:511–2.

Cameira-dos-Santos, P. J., Brillouet, J. M., Cheynier V., and Moutounet, M. 1996. Detection and partial characterisation of new anthocyanin-derived pigments in wine. *J. Sci. Food Agric.* 70:204–8.

Careri, M., Corradini, C., Elvir, L., Nicoletti, I., and Zagnoni, I. 2003. Direct HPLC analysis of quercetin and trans-resveratrol in red wine, grape, and winemaking byproducts. *J. Agric. Food Chem.* 51:5226–31.

Carpentieri, A., Marino, G., and Amoresano, A. 2007. Rapid fingerprinting of red wines by MALDI mass spectrometry. *Anal. Bioanal. Chem.* 389:969–82.

Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., and Hermosín-Gutiérrez, I. 2007. Flavonol profiles of *Vitis vinifera* red grapes and their single-cultivar wines. *J. Agric. Food Chem.* 55:992–1002.

Cheynier, V., Doco, T., Fulcrand, H., Guyot, S., Le Roux, E., Souquet, J. M., Rigaud, J., and Moutounet, M. 1997. ESI-MS analysis of polyphenolic oligomers and polymers: New methods for analysing old plant polyphenols. *Analusis* 25:M32–37.

Cheynier, V., and Rigaud, J. 1986. HPLC separation and characterization of flavonols in the skins of Vitis vinifera var. Cinsault. *Am. J. Enol. Vitic.* 37:248–52.

Chinnici, F., Sonni, F., Natali, N., Galassi, S., and Riponi, C. 2009. Colour features and pigment composition of Italian carbonic macerated red wines. *Food Chem.* 113:651–7.

Deshpande, S. S., and Cheryan, M. 1987. Determination of phenolic compounds of dry beans using vanillin, redox and precipitation assays. *J. Food Sci.* 52:332–4.

de Villiers, A., Vanhoenacker, G., Majek, P., and Sandra, P. 2004. Determination of anthocyanins in wine by direct injection liquid chromatography-diode array detection-mass spectrometry and classification of wines using discriminant analysis. *J. Chromatogr. A* 1054:195–204.

Di Stefano, R., and Cravero, M. C. 1989. I composti fenolici e la natura del colore dei vini rossi. *L'enotecnico Ottobre* 81–7.

Es-Safi, N. E., Guyot, S., and Ducrot, P. H. 2006. NMR, ESI/MS, and MALDI-TOF/MS analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *J. Agric. Food Chem.* 54:6969–77.

Francia-Aricha, E. M., Guerra, M. T., Rivas-Gonzalo, J. C., and Santos-Buelga, C. 1997. New anthocyanin pigments formed after condensation with flavanols. *J. Agric. Food Chem.* 45:2262–6.

Fraser, P. D., Enfissi, E. M. A., Goodfellow, M., Eguchi, T., and Bramley, P. M. 2007. Metabolite profiling of plant carotenoids using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *The Plant Journal* 49:552–64.

Fulcrand, H., Benabdeljalil, C., Rigaud, J., Cheynier, V., and Mountounet, M. 1998. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* 47:1401–7.

Gil-Muñoz, R., Gómez-Plaza, E., Martínez, A., and López-Roca, J. M. 1999. Evolution of phenolic compounds during wine fermentation and post-fermentation: Influence of grape temperature. *J. Food Compos. Anal* 12:259–72.

Glories, Y. 1984. La couleur des vins rouges II, Connaissance de la vigne et du vin. *Vigne vin* 18:253–71.

Goldstein, J. L., and Swain, T. 1963. Changes in tannins in ripening fruits. *Phytochemistry* 2:371–83.

Gómez-Alonso, S., García-Romero, E., and Hermosin-Gutiérrez, I. 2007. HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence. *J. Food Compos. Anal.* 20:618–26.

Gómez-Plaza, E., Gil-Nuñoz, R., López-Roca, J. M., and Martínez, A. 2000. Color and phenolic compounds of a young red wine. Influence of wine-making techniques, storage temperature, and length of storage time. *J. Agric. Food Chem.* 48:736–41.

Hayasaka, Y., and Asenstorfer, R. E. 2002. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.* 50:756–61.

He, J., Santos-Buelga, C., Mateus, N., and De Freitas, V. 2006. Isolation and quantification of oligomeric pyranoanthocyanin-flavanol pigments from red wines by combination of column chromatography techniques. *J. Chromatogr. A* 1134:215–25.

Heier, A., Blaas, W., Droß, A., and Wittkowski, R. 2002. Anthocyanin analysis by HPLC/ESI-MS. *Am. J. Enol. Vitic.* 53:78–84.

Ho, P., Da Conceição, M., Silva, M., and Hogg, T. A. 2003. Changes in the colour of the phenolic composition during the early stages of maturation of port in wood, stainless steel and glass. *J. Sci. Food Agric.* 81:1269–80.

Ivanova, V. 2009. Development of methods for identification and quantification of phenolic compounds in wine and grape applying spectrophotometry, liquid chromatography and mass spectrometry. PhD Thesis, Ss Cyril and Methodius University, Skopje, Republic of Macedonia.

Ivanova, V., Stefova, M., and Chinnici, F. 2010. Determination of polyphenol contents in Macedonian grapes and wines assessed by standardized spectrophotometric methods. *J. Serb. Chem. Soc.* 75:45–59.

Ivanova, V., Stefova, M., and Vojnoski, B. 2009. Assay of the phenolic profile of Merlot wines from Macedonia: Effect of maceration time, storage, SO_2 and temperature of storage. *Maced. J. Chem. Chem. Eng.* 28:141–9.

Joss, J. L., Molloy, M. P., Hinds, L. A., and Deane, E. M. 2006. Evaluation of chemical derivatisation methods for protein identification using MALDI MS/MS. *Int. J. Pept. Res. Ther.* 12:225–35.

Juhasz, P., Costello, C. E., and Biemann, K. 1993. Matrix-assisted laser desorption ionization mass spectrometry with 2-(4-hydroxyphenylazo)benzoic acid matrix. *J. Am Soc. Mass Spectrom.* 4:399–409.

Karas, M. 1996. Matrix-assisted laser desorption ionization MS: A progress report. *Biochem. Soc. Trans.* 24:897–900.

Karas, M., Bachmann, D., Bahr, U., and Hillenkamp, F. 1987. Matrix-assisted ultraviolet laser desorption of nonvolatile compounds. *Int. J. Mass Spectrom. Ion Processes* 78:53–68.

Kelebek, H., Canbas, A., and Selli, S. 2007. HPLC-DAD-MS analysis of anthocyanins in rose wine made from cv. Öküzgözü grapes, and effect of maceration time on anthocyanin content. *Chromatographia* 66:207–12.

Kennedy, J. A., Matthews, M. A., and Waterhouse, A. L. 2002. Effect of maturity and vine water status on grape skin and wine flavonoids. *Am. J. Enol. Vitic.* 53:268–74.

Kim, D., Chun, O. K., Kim, Y. J., Moon, H., and Lee, C. Y. 2003. Quantification of polyphenols and their antioxidant capacity in fresh plums. *J. Agric. Food Chem.* 51:6509–15.

Krueger, C. G., Dopke, N. C., Treichel, P. M., Folts, J., and Reed, J. D. 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.* 48:1663–7.

La Torre, G. L., Saitta, M., Vilasi, F., Pellicanò, T., and Dugo, G. 2006. Direct determination of phenolic compounds in Sicilian wines by liquid chromatography with PDA and MS detection. *Food Chem.* 94:640–50.

Lee, C. Y., and Jaworski, A. W. 1990. Identification of some phenolics in white grapes (research note). *Am. J. Enol. Vitic.* 41:87–9.

Lerouxel, O., Choo, T. S., Séveno, M., Usadel, B., Faye, L., Lerouge, P., and Pauly, M. 2002. Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. *Plant Physiol.* 130:1754–63.

Marentes, E., and Grusak, M. 1998. Mass determination of low-molecular-weight proteins in phloem sap using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Exp. Bot.* 49:903–11.

Marinova, D., Ribarova, F., and Atanassova, M. 2005. Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *J. Univ. Chem Tech Metall.* 40:255–60.

Mateus, N., Carvalho, E., Carvalho, A. R. F., Melo, A., Gonzlez-Params, A. M., Santos-Buelga, C., Silva, A. M. S., and de Freitas, V. 2003. Isolation and structural characterization of new acylated anthocyanin-vinyl-flavanol pigments occurring in aging red wines. *J. Agric. Food Chem.* 51:277–82.

Mateus, N., de Pascual-Teresa, S., Rivas-Gonzalo, J. C., Santos-Buelga, C., and de Freitas, V. 2002. Structural diversity of anthocyanin-derived pigments in port wines. *Food Chem.* 76:335–42.

Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., and Velasco, R. 2006. Metabolite profiling of grape: Flavonols and anthocyanins. *J. Agric. Food Chem.* 54:7692–7702.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B., and Ewert, B. 1999. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *J. Agric. Food Chem.* 47:4009–17.

McMurrough, I., and McDowell, J. 1978. Chromatographic separation and automated analysis of flavanols. *Anal. Biochem.* 91:92–100.

Mernyak, E., Bikadi, Z., Hazai, E., Mark, L., Schneider, G., and Wolfling, J. 2008. Steroidal delta-alkenyl oximes as ambident nucleophiles: Electrophile-induced formation of oxazepane derivatives in the bis-estrone series. *Lett. Org. Chem.* 5:17–21.

Michalak, L., Fisher, K., Alderdice, D., Jardine, D., and Willett, G. 1994. C60-assisted laser desorption-ionization mass-spectrometry. *Org. Mass Spectrom.* 29:512–5.

Monagas, M., Mez-Cordoveä, C. G., Bartolomeä, B., Laureano, O., and Ricardo da Silva, J. M. 2003. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from *Vitis Vinifera* L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *J. Agric. Food Chem.* 51:6475–81.

Monagas, M., Suárez, R., Gómez-Cordovés, C., and Bartolomé, B. 2005. Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. *Am. J. Enol. Vitic.* 56:139–47.

Montealegre, R. R., Peces, R. R., Vozmediano, J. L. C., Gascueña, J. M., and Romero, E. G. 2006. Phenolic compounds in skin and seeds in ten grape *Vitis Vinifera* varieties grown in a warm climate. *J. Food Compos. Anal.* 19:687–93.

Morata, A., Gómez-Corovés, M. C., Suberviola, J., and Bartolomé, B. 2003. Adsorption of anthocyanins by yeast cell walls during the fermentation of red wines. *J. Agric. Food Chem.* 51:4084–8.

Nagel, C. W., and Wulf, L. W. 1979. Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. *Am. J. Enol. Vitic.* 30:111–6.

Núñez, V., Monagas, M., Gómez-Cordovés, C., and Bartolom, B. 2004. *Vitis vinifera* L. cv. Graciano grapes characterized by its anthocyanin profile. *Postharvest Biol. Technol.* 31:69–79.

Oszmianski, J., Ramos, T., and Bourzeix, M. 1998. Fractionation of phenolic compounds in red wine. *Am. J. Enol. Vitic.* 39:259–62.

Palomino, O., Gómez-Serranillos, M. P., Slowing, K., Carretero, E., and Villar, A. 2000. Study of polyphenols in grape berries by reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* 870:449–51.

Pérez-Magariño, S., Revilla, I., González-San José, M. L., and Beltrán, S. 1999. Various applications of liquid chromatography-mass spectrometry to the analysis of phenolic compounds. *J. Chromatogr. A* 847:75–81.

Piovan, A., Filippini, R., and Favretto, D. 1998. Characterization of the anthocyanins of Catharanthus roseus (L.) G. Don in vivo and in vitro by electrospray ionization ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* 12:361–7.

Piraino, P., Upadhyay, V. K., Rossano, R., Riccio, P., Parente, E., Kelly, A. L., and McSweeney, P. L. H. 2007. Use of mass spectrometry to characterize proteolysis in cheese. *Food Chem.* 101:964–72.

Reed, J. D., Krueger, C. G., and Vestling, M. M. 2005. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry* 66:2248–63.

Revilla, I., and González-San José, M. L. 2001. Evolution during the storage of red wines treated with pectolytic enzymes: New anthocyanin pigment formation. *J. Wine Res.* 12:183–97.

Revilla, I., Pérez-Magariño, S., González-San José, M. L., and Beltrán, S. 1999. Identification of anthocyanin derivatives in grape skin extracts and red wines by liquid chromatography with diode array and mass spectrometric detection. *J. Chromatogr. A* 847:83–90.

Revilla, E., Ryan, J. M., and Martín-Ortega, G. 1998. Comparison of several procedures used for the extraction of anthocyanins from red grapes. *J. Agric. Food Chem.* 46:4592–7.

Robards, K. 2003. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A* 1000:657–91.

Ribereau-Gayon, P. 1964. Les composes phénoliques du Raisin et du Vin. *Ann. Physiol. Végét* 2:119–47.

Salas, E., Atanasova, V., Poncet-Legrand, C., Meudec, E., Mazauric, J. P., and Cheynier, V. 2004. Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions. *Anal. Chim. Acta* 513:325–32.

Schiller, J., Süß, R., Arnhold, J., Fuchs, B., Leßig, J., Müller, M., Petković, H., Spaltehoz, H., Zschörnig, O., and Arnold, K. 2004. Matrix-assisted laser desorption and ionisation time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. *Prog. Lipid Res.* 43:449–88.

Sheoran, I. S., Ross, A. R. S., Olson, D. J. H., and Sawhney, V. K. 2007. Proteomic analysis of tomato (Lycopersicon esculentum) pollen. *J. Exp. Bot.* 58:3525–35.

Singleton, V. L., Salgues, M., Zaya, J., and Trousdale, E. 1985. Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. *Am. J. Enol. Vitic.* 36:50–6.

Singleton, V. L., Zaya, J., and Trousdale, E. 1986. Compositional changes in ripening grapes: Caftaric and coutaric acids. *Vitis* 25:107–17.

Slinkard, K., and Singleton, V. L. 1977. Total phenol analysis: Automation and comparison with manual methods. *Am. J. Enol. Vitic.* 28:49–55.

Souquet, J. M., Labarbe, B., Le Guernevé, C., Cheynier, V., and Moutounet, M. 2000. Phenolic composition of grape stems. *J. Agric. Food Chem.* 48:1076–80.

Sporns, P., and Wang, J. 1998. Exploring new frontiers in food analysis using MALDI-MS. *Food Res. Int.* 31:181–9.

Sugui, J. A., Bonham, C., Lo, S. C., Wood, K. V., and Nicholson, R. L. 1998. MALDI-TOF analysis of mixtures of 3-deoxyanthocyanidins and anthocyanins. *Phytochemistry* 8:1063–6.

Sugui, J. A., Wood, K. V., Yang, Z. Y., Bonham, C. C., and Nicholson, R. L. 1999. Matrix assisted laser desorption ionization mass spectrometry analysis of grape anthocyanins. *Am. J. Enol. Vitic.* 50:199–203.

Thies, M., and Fischer, R. 1971. New reaction for microchemical detection and the quantitative determination of catechins. *Mikrochim. Acta* 1:9–13.

Tholey, A., and Heinzle, E. 2006. Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry-applications and perspectives. *Anal. Bioanal. Chem.* 386:24–37.

Trousdale, E. K., and Singleton, V. L. 1983. Astilbin and engeletin in grapes and wine. *Phytochemistry* 22:619–20.

Tsao, R., and Yang, R. 2003. Optimization of a new mobile phase to know the complex and real polyphenolic composition: Towards a total phenolic index using high-performance liquid chromatography. *J. Chromatogr. A* 1018:29–40.

Ugarov, M. V., Egan, T., Khabashesku, D. V., Schultz, J. A., Peng, H., Khabashesku, V. N., Furutani, N., et al. 2004. MALDI matrices for biomolecular analysis based on functionalized carbon nanomaterials. *Anal. Chem.* 76:6734–42.

Viñas, P., López-Erroz, C., Marín-Hernández, J. J., and Hernández-Córdoba, M. 2000. Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection. *J. Chromatogr. A* 871:85–93.

Vitrac, X., Castagnino, C., Waffo-Téguo, P., Delaunay, J. C., Vercauteren, J., Monti, J. P., Deffieux, G., and Mérillon, J. M. 2001. Polyphenols newly extracted in red wine from southwestern France by centrifugal partition chromatography. *J. Agric. Food Chem.* 49:5934–8.

Vivar-Quintana, A. M., Santos-Buelga, C., and Rivas-Gonzalo, J. C. 2002. Anthocyanin-derived pigments and colour of red wines. *Anal. Chim. Acta* 458:147–55.

Wang, J., and Sporns, P. 1999. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. *J. Agric. Food Chem.* 47:2009–15.

Wulf, L. W., and Nagel, C. W. 1978. High-pressure liquid chromatography separation of anthocyanins of *Vitis Vinifera*. *Am. J. Enol. Vitic.* 29:42–9.

Yamada, K., Abe, T., and Tanizawa, Y. 2007. Black tea stain formed on the surface of teacups and pots. Part 2—Study of the structure change caused by aging and calcium addition. *Food Chem.* 103:8–14.

Zhang, J., Ma, H., Feng, J., Zeng, L., Wang, Z., and Chen, S. 2008. Grape berry plasma membrane proteome analysis and its differential expression during ripening. *J. Exp. Bot.* 59:2979–90.

Zhishen, J., Mengeheng, T., and Jianming, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64:555–9.