JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 6779−6786 **6779**

Anthocyanin Quantification and Radical Scavenging Capacity of Concord, Norton, and Marechal Foch Grapes and Wines

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The anthocyanin content and the radical scavenging capacity of three non-*Vitis vinifera* grapes (Marechal Foch, Norton, and Concord varieties) were determined. Analyses of anthocyanins in the skin (S) and wine (W) of these grape varieties were performed by spectrophotometry, HPLC with electrochemical detection, and matrix-assisted laser desorption ionization (MALDI). The total anthocyanin contents of S samples were 258 ± 37 mg/100 g of wet weight for Foch, 888 ± 78 mg/100 g for Norton, and 326 ± 5.9 mg/100 g for Concord grapes. The malvidin 3,5-diglucoside content quantified by HPLC indicated that Norton S had the highest amount of the compound (327 ± 110 mg/100 g). The MALDI mass spectrometric analysis indicated an abundance of malvidin glucosides in W of Foch grapes and in S and W of Norton grapes and of cyanidin aglycon in S and W of Concord grapes. S samples were subjected to a radical scavenging capacity test using the 2,2-diphenyl-1-picrylhydrazyl radical and compared to Trolox. The radical scavenging capacity for Foch S was 0.78 mM Trolox equiv, that of Concord S, 0.80 Trolox equiv, and that of Norton S was highest at 0.95 Trolox equiv. The higher concentrations of malvidin 3,5-diglucoside in S of grape varieties were associated with greater radical scavenging capacity.

KEYWORDS: Anthocyanins; grapes; malvidin 3,5-diglucoside; DPPH; high-performance liquid chromatography

INTRODUCTION

Anthocyanins are flavonoid pigments present in and responsible for many red, violet, and blue colors in fruits and flowers (*1*, *2*). Anthocyanins are a normal component of the daily diet when certain fruits and vegetables are consumed. Regular intake of foods that contain flavonoids may help reduce the risk of cardiovascular disease (*3*, *4*). It is estimated that the traditional Western diet provides 1 g/day of mixed flavonoids (*5*). Grapes and berries are the chief dietary sources of anthocyanins (*6*). The anthocyanin content in red grapes ranges from 72 to 1708 mg/L as reported by Frankel et al. (*7*), and the average content of anthocyanins in red wines is estimated at 26 mg/L (*8*). The estimated content of anthocyanins in the skin of grapes ranges from 200 to 5000 mg/kg of fresh whole grape (*9*). Malvidin is the predominant anthocyanin in grapes and is the reddest of all anthocyanins, providing the characteristic color of young red wines (*10*).

It is well recognized that diets rich in fruits and vegetables afford protective effects against the development of chronic diseases such as cardiovascular disease and some cancers (*3*). Recent attention has been given to understanding the potential health benefits of polyphenolic compounds present in plants; although found in small quantities, these substances offer significant antioxidant capacity, especially the flavonoids. For example, studies indicate that the polyphenolic fraction of wine, and not the ethanol constituent, seems to be responsible for the reduction of cardiovascular disease risk (*8*, *11*−*13*). Moreover, the polyphenolic fraction possesses the antioxidant properties (*12*, *14*, *15*), which may explain the anticarcinogenic or cardioprotective actions of wine and related fruits (*16*).

Polyphenols are multifunctional, and the antioxidant activity may be due to their capacity to act as reducing agents by donating a hydrogen, by quenching singlet oxygen, or by acting as chelators (*16*−*18*). The free radical scavenging capacity of an antioxidant can be estimated using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) test (*19*). On the basis of this method for testing potential antioxidant activity, the scavenging effect of the compound relative to DPPH is thought to be due to the donation of a hydrogen. The antioxidant capacity of polyphenols is generally related to their hydroxyl group on the B benzene ring and the presence of a second hydroxyl group in the ortho or para positions (*16*, *18*, *20*).

Grapes and wines used for studies on the anthocyanin composition and/or antioxidant activity are generally from the *Vitis vinifera* species (*8*, *13*, *14*, *21*, *22*). The *V. vinifera* grapes

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10.1021/jf040087y CCC: $27.50
Published on Web 10/07/2004

are commonly used for wine production around the world; however, in the United States other grape species are also used and include species such as *Vitis labrusca*, *Vitis riparia*, *Vitis aestivalis*, *Vitis rupestris*, and *Vitis rotundifolia*. Concord is an example of the *V. labrusca* species, and this grape has also been the subject of several studies (*7*, *23*, *24*) because of its abundance in the United States and consumption as juice and jellies. Cyanidin 3-monoglucoside and delphinidin 3-monoglucoside are major anthocyanins in Concord grapes. Hrazdina (*24*) reported that increased methylation of anthocyanin pigments was associated with greater stability of the Concord pigments. Frankel et al. (*7*) found that Concord juice has a protective action in vitro against oxidation of human low-density lipoprotein (LDL). With regard to other grape species, Talcott and Lee (*25*) found that the processing method used to make wine from Muscadine grapes (*V. rotundifolia*) affected the initial and stored contents of flavonoids found in the wines. Wines fermented with the grape skins (on-hull) had higher initial antioxidant polyphenolic concentrations as determined by the oxygen radical absorbance capacity (ORAC). In contrast, the wines fermented on-hull for 5 and 7 days had greater polyphenolic losses during storage than the wines fermented on-hull for 3 days and from hot press.

The analysis of anthocyanins is important for differentiating the quality and health aspects of the grapes and red wines derived from the grapes. However, it is often difficult to make a direct correlation between the amount and composition of anthocyanins present in the grapes and wine (*26*) to their health benefits. Differences in the manufacturing process and aging also influence the amount and type of anthocyanins in the wine (*2*, *21*, *25*−*27*).

In this study, three non-*V. vinifera* Indiana-grown grapes, Marechal Foch, Norton, and Concord, were evaluated for the types and amounts of anthocyanins. Concord is a *V. labrusca* grape, Marechal Foch is a hybrid of *V. rupestris* and *V. vinifera*, and Norton is a *V. aestivalis* grape (*28*). It is hypothesized that these species of *Vitis*, other than *V. vinifera*, or hybrids of these grapes would contain different amounts and types of anthocyanins. Furthermore, the wines made from Marechal Foch, Norton, and Concord grapes would vary in anthocyanin content and maintain a correlation with content present in the raw material. In addition, the radical scavenging capacities of these grapes and wines would be different due to the amount and composition of anthocyanins present in the grapes.

MATERIALS AND METHODS

Extraction of Anthocyanins from Grapes. Marechal Foch, Norton, and Concord grapes were obtained from the Purdue University Research Farms (Purdue University, West Lafayette, IN) in the harvest season of 2001. The anthocyanins were extracted from skins (S) of the grapes following the method described by Revilla et al. (*29*). The grape varieties were made into wine (W) (three replicates of 25 kg of grapes per wine batch). An aliquot of 25 grapes from each replicate of winemaking was used to extract anthocyanins from the S. The mean weights for whole fresh grapes (25 pieces) were 33.63, 28.33, and 82.64 g for Foch, Norton, and Concord, respectively, whereas the mean weights for the skins alone were 15.10, 8.77, and 23.95 g, respectively. Anthocyanins from the S were extracted three times (4, 12, and 24 h) using 60 mL of a 1% 12 N HCl solution in methanol at room temperature. The sample was centrifuged (Avanti J-25 I, Beckman Instruments, Inc., Palo Alto, CA) at 5000*g* for 15 min at 15 °C. The solvent extracts from S were combined and brought to a volume of 200 mL.

Isolation of Anthocyanins from Wine. Anthocyanins from bottled wine were extracted using solid-phase extraction following the method described by Hong and Wrolstad (*30*) with some modifications. One milliliter of bottled wine was passed through a phenyl column cartridge (Phenomenex, Torrance, CA). Anthocyanins were retained by the column and eluted with a 1% 12 N HCl solution in methanol. To obtain the wine extract (W) the total volume was brought to 10 mL in a volumetric flask. The W extract was performed in triplicate for each wine obtained from the aliquots of grape varieties.

High-Performance Liquid Chromatography (HPLC): Separation and Detection of Anthocyanins. The anthocyanin contents of S and W samples were measured using an HPLC configured with two solvent delivery pumps (model 582), an autosampler (model 542 outfitted with a refrigerated sample tray), a column (amide C16 column, 25 cm × 4.6 mm, and particle size = 5 μm, Supelco, Bellefonte, PA), a 75 μL sample loop, and a 12-channel CoulArray detector (model 5600A, ESA, Inc., Chemsford, MA). Malvidin 3,5-diglucoside (Mv-diglu) was used as a standard (Indofine Chemical Co., Inc., Sommerville, NJ). The two mobile phases consisted of (A) 100 mM sodium phosphate buffer, pH 3.0, methanol (99:1, v/v) and (B) 100 mM sodium phosphate buffer, pH 3.45, acetonitrile, and methanol (30:60:10, v/v/v). All solvents were of HPLC grade and obtained from Mallinckrodt Baker, Inc. (Paris, KY). The gradient system was 0% B for 2 min, increased to 80% B by 40 min, held at 80% B until 45 min, and decreased to 0% B at 55 min; the column was re-equilibrated with 0% B for 5 min for a total separation time of 60 min. The column temperature was 46 °C, injection volume was 10 μL, flow rate was 0.7 mL/min, and cell potentials were set at 100, 140, 200, 240, 280, 330, 380, and 420 mV.

S and W samples were analyzed with a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Voyager, PerSeptive Biosystems, Framingham, MA) in the Mass Spectrometry Laboratory at Purdue University. The instrument utilized a nitrogen laser (337 nm UV laser) for ionization with a time-of-flight mass analyzer and detection limit for anthocyanins estimated at 10 pg. The anthocyanin samples and matrix (α-cyano-4-hydroxycinnamic acid) were mixed in a ratio of 1:1 (v/v) on the sample plate. This mixture (the matrix is present in a large relative excess in comparison to the sample amount) was allowed to air-dry prior to analysis. Crystallization of the sample within the matrix is an important component for successful MALDI analysis. The ions formed during laser irradiation are then accelerated into a time-of-flight mass analyzer for mass analysis.

Total Anthocyanin Analysis. Total anthocyanins were quantified using the method described by Wrolstad (*31*) with modification. A 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Sunnyvale, CA) was used for the measurements. Two aliquots of 0.5 mL of either S or W were dried under nitrogen gas. Both dried samples were redissolved with 0.5 mL of methanol, one aliquot was then diluted with 100 μM sodium phosphate buffer (pH 1.0) until color absorbance was not higher than 1.3 optical density for the spectrophotometric reading at 520 nm (maximum absorbance wavelength for malvidin 3,5-diglucoside) and at 700 nm (to account for turbidity). The other sample was diluted to the same volume with sodium acetate buffer (pH 4.5) and a spectrophotometric reading taken at the same wavelengths. The absorbance (A) was then calculated using the following formula:

$$A = (A_{520\text{nm, pH=1}} - A_{700\text{nm ,pH=1}}) - (A_{520\text{nm ,pH=4.5}} - A_{700\text{nm,pH=4.5}})$$

Later, anthocyanin concentration (milligrams per liter) was calculated with the formula

$$C\ (\text{mg/L}) = \frac{A \times \text{MW} \times \text{dilution factor}}{\epsilon l}$$

where ϵ = molar absorbance (mol/L) and l = path length (cm).

A standard curve for malvidin 3,5-diglucoside was made to check the linear response given by the equipment, obtaining an $R^2 = 0.99$. The smaller concentration tested was 5 mg/L, and the absorbance calculation and the actual concentration were between 2 and 6%.

DPPH Radical Scavenging Capacity Test. The DPPH antioxidant test was performed following the method of Brand-Williams et al. (*19*) with modifications. A solution of DPPH radical at a concentration of 1×10^{-4} M (in methanol) was prepared. In a 96-well plate, 220 μL of DPPH solution was combined with 10 μL of S sample. The decrease

in absorbance at 515 nm was monitored for 10 min using a spectrophotometer (Spectra MAX 190, Molecular Devices).

The DPPH solution was made daily and the concentration confirmed by the following standard curve:

$$A_{515\text{nm}} = 6342.2[\text{DPPH}] + 0.043$$

The DPPH concentration was plotted against time, and a nonlinear regression line was fitted using the following formula (*32*):

$$[\text{DPPH}] = [\text{DPPH}]_0 \, e^{-k_{\text{obsd}}t}$$

The previous formula can be transformed into

$$\ln [\text{DPPH}] = \ln [\text{DPPH}]_0 - k_{\text{obsd}}t$$

where $[\text{DPPH}]_0$ is the initial concentration of DPPH, k_{obsd} is the slope of the line or pseudo-first-order rate constant, [DPPH] is the concentration of DPPH at a specific time, and t is the time in minutes.

To obtain the radical scavenging capacity of the samples, the method of Arnous et al. (*33*) was followed with slight modifications. Trolox (Sigma, Milwaukee, WI) was used as a reference antioxidant. An equation that related Trolox with the decrease in DPPH was obtained by fitting a linear regression curve to the plotted data of percentage change in $A_{515\text{nm}}$ versus Trolox concentration (0.05−1.0 mM). The following equation was obtained:

$$\text{radical scavenging capacity} = \frac{(\%\text{ change in absorbance} - 1.883)}{89.112}$$

Radical scavenging capacity results were expressed as millimolar Trolox equivalents. The percentage change in absorbance was obtained using the following equation:

$$[(A_{515(t=0)} - A_{515(t=5\text{min})})/(A_{515(t=0)})] \times 100$$

Statistical Analysis. The data collected were analyzed using a two-way multivariate analysis of variance (MANOVA). The type of grape or wine and the aliquot number were used as predictor values in the statistical design, and the total anthocyanins and malvidin 3,5-diglucoside concentrations served as the output values. A Tukey's multiple-comparison test was performed in conjunction with each MANOVA (*34*). Results were presented as means of measurements and their corresponding standard deviations.

RESULTS AND DISCUSSION

In this study, the anthocyanin type and content of three non-*V. vinifera* grape S and W samples were measured, and their antioxidant capacities were compared. The measurements included total anthocyanin content, followed by a mass spectrometric analysis of the species of anthocyanins present in the extract, and HPLC quantification analysis of malvidin 3,5-diglucoside. A radical scavenging capacity test was performed on the anthocyanin extract from S samples.

Grapes from species other than *V. vinifera* have a significant amount of malvidin 3,5-diglucoside, and grape samples are usually analyzed for malvidin 3,5-diglucoside equivalents, which accounted for at least 94% of the total anthocyanins present in the samples (*35*). Anthocyanins have a maximum absorbance at a wavelength of 520 nm at a pH of 1.0. At this pH, anthocyanins are in the flavylium cation form (*22*, *31*) and present a red color; however, at pH 4.5 a proton is lost from the anthocyanin, resulting in a carbinol pseudobase structure, which is colorless (*36*). Any color given at pH 4.5 is not due to anthocyanins; therefore, an adjustment of the original absorbance measurement was made.

The statistical analysis indicated significant differences in the total anthocyanin contents of the three aliquots of S and W samples for each type of grape (**Table 1**). The extracts of S from Norton (888 ± 78 mg/100 g of skins) contained more total anthocyanins than the amount in Concord S (326 ± 5.9 mg/100 g of skins) and Foch S (258 ± 37 mg/100 g of skins). Our findings are consistent with the dark color of the grape skin of Concord and Norton varieties compared with the color and content in Foch grape skins. The analysis of anthocyanins in W of these grapes revealed that Norton (881 ± 18 mg/L) produced the highest amount when the grapes were processed into wine (**Table 1**).

MALDI analysis yielded more specific data on the anthocyanin composition of S and W extracts of each grape type (**Table 2**). Anthocyanins were identified by comparing the molecular weights of the analytes identified to the values reported by Sugui et al. (*37*). Although MALDI analytical results do not provide exact quantities of compounds identified, they do show the relative abundance of the different anthocyanins present in the samples (**Table 2**). The relative amounts of anthocyanins in the S and W samples of the different grapes were identified as follows: most abundant with the letter "a", second or third most abundant with the letter "b", and present in the sample with the letter "c". Concentrations for the MALDI

Table 1. Total Anthocyanin Content of Grape Skin and Wine Extracts Measured by Spectrophotometric Method[a]

	Foch	Norton	Concord
total anthocyanins (mg/100 g) in skins[b]	260 ± 40B	890 ± 80A	330 ± 6B
total anthocyanins (mg/L) in wine	140 ± 20B	880 ± 20A	170 ± 20B

[a] Values (mean ± SD, $n = 3$) in rows having different letters (A, B) are significantly different ($p < 0.05$). [b] Mean total anthocyanins values calculated for fresh grapes were 116 mg/100 g for Foch, 275 mg/100 g for Norton, and 95 mg/100 g for Concord.

Table 2. Identification of Anthocyanins in Skin (S) and Wine (W) Extracts by Mass Spectrometry[a]

		Foch		Norton		Concord	
anthocyanin species	*m/z*	S	W	S	W	S	W
cyanidin aglycon	287					a	a
peonidin aglycon	301						c
delphinidin aglycon	303			c	c	c	a
petunidin aglycon	317	c	c				b
malvidin aglycon	331	c	a	b	b	c	b
cyanidin monoglucoside	449	c				c	c
peonidin monoglucoside	463	c					c
delphinidin monoglucoside	465	c		c		c	c
petunidin monoglucoside	479	c				c	c
malvidin monoglucoside	493	c	b	b	c	c	c
delphinidin acetylglucoside	507						c
petunidin acetylglucoside	521	c		c			
malvidin acetylglucoside	535	c	c		c		
cyanidin coumaroylglucoside	595					c	c
petunidin coumaroylglucoside	625	c		c	c		c
malvidin coumaroylglucoside	639	c		c	c		c
cyanidin diglucoside	611					c	
peonidin diglucoside	625						
petunidin diglucoside	641	c					
malvidin diglucoside	655	c	c	a	b		b
cyanidin coumaroyldiglucoside	757					b	b
petunidine coumaroyldiglucoside	787	c					
malvidin coumaroyldiglucoside	801	c	c	b	a	b	

[a] Values represent the analysis of $n = 3$; detection limit estimated at 10 pg; a = most abundant anthocyanin in the sample; b = second or third most abundant anthocyanin in the sample; c = anthocyanin present in the sample, but not abundant.

Table 3. Malvidin 3,5-Diglucoside Content of Grape Skin and Wine Extracts Measured by HPLC[a]

	Foch	Norton	Concord
Mv-3,5-diglucoside (mg/L) in skins[b]	15.4 ± 7A	140 ± 50A	45.0 ± 14B
Mv-3,5-diglucoside (mg/L) in wine	2.0 ± 0.1C	58 ± 5A	5.0 ± 0.1B

[a] Values (mean ± SD, $n = 3$) in rows having different letters (A–C) are significantly different ($p < 0.05$). [b] Mean total malvidin 3,5-diglucoside values calculated for fresh grapes were 9.2 mg/100 g for Foch, 101 mg/100 g for Norton, and 10.9 mg/100 g for Concord.

analysis are usually expressed in terms of picomoles per microliter. To obtain a detection limit for MALDI, a known amount of the compound of interest was continually diluted until the ion corresponding to that compound was no longer identified.

Results of MALDI in **Table 2** indicate that Foch S and Concord S and W samples contained different anthocyanin compounds; however, the anthocyanins in Foch S were relatively low in abundance, as denoted with the letter "c" in **Table 2**. The Foch grape variety has a light red skin most likely due to the content of malvidin anthocyanins and the presence of the anthocyanins in a pseudobase form, which is colorless. The low abundance but diverse anthocyanin content found by MALDI analysis (**Table 2**) agreed with the lower anthocyanin content found in Foch S samples (**Table 1**).

The data presented in **Table 2** indicate that malvidin-containing anthocyanins are the most abundant flavonoids in the Foch W and Norton S and W samples. Analysis of Norton S samples revealed that malvidin 3,5-diglucoside was the most plentiful anthocyanin present (**Table 2**). Moreover, the HPLC analysis of Norton S and W indicated that these samples contained the highest concentrations of this anthocyanin compared to the S and W samples from the other two grapes evaluated (**Table 3**). The primary malvidin-containing anthocyanins in Norton S samples were malvidin-3,5-diglucoside, malvidin coumaroyldiglucoside, malvidin monoglucoside, and malvidin aglycon. Our results corroborate the findings of Jackson (*10*) that malvidin is the most abundant anthocyanin in red grapes.

In contrast, Concord S and W samples contained significant amounts of cyanin-containing anthocyanins, specifically cyanidin monoglucoside (*37*). These grapes have deep blue-black skins. The abundance of cyanidin and delphinidin compounds (**Table 2**) in the Concord samples is consistent with the total amount of anthocyanins in the S extract (**Table 1**) and the findings of Ingalsbe et al. (*38*). The abundant content of malvidin compounds measured in Norton S and W (**Table 2**) samples substantiates the deep bluish color characteristic of these grapes and the higher total anthocyanin content for this grape variety (**Table 1**). In general, the results of total anthocyanin content of S and W samples agree with the relative abundance based on the MALDI measurements of the different grape varieties.

The 3-glycosides or 3,5-diglycosides are naturally occurring anthocyanins (*5*), and anthocyanins found in many foods exist as glycosides with one or more substituted sugar residues (*39*). There are two explanations for which aglycon compounds listed in **Table 2** were identified. First, others have reported that anthocyanins fragment to some degree to their aglycon when subjected to the MALDI method (*37*). Second, because hydrochloric acid was used to extract the anthocyanins prior to analysis by MALDI, the acidic environment may have cleaved the glycosidic linkage, hence resulting in more aglycons that contributed to an increased amount of total anthocyanidins. Therefore, it is possible that the data in **Table 2** reflect a higher amount of aglycons than what has generally been reported.

A representative HPLC chromatogram of Norton W extract is presented in **Figure 1**. The order of anthocyanin elution and the results indicated by MALDI analysis was used to identify the anthocyanin peaks. **Figure 1** shows that the malvidin aglycon eluted ahead of the glycosylated derivatives, and diglucoside anthocyanins eluted before the monoglucosides, following the pattern previously reported by da Costa et al. (*26*). In addition, the acylated derivative of malvidin eluted after the nonacetylated molecules, which is consistent with results reported previously by HPLC methods (*26*).

Malvidin 3,5-diglucoside was used as a peak identification reference standard as well as a response standard (serial dilution standard curve). This particular anthocyanin is characteristic of non-*V. vinifera* grapes (*35*), and on the basis of the relative amount of malvidin 3,5-diglucoside in S and W samples (**Table 2**), it would be an appropriate reference standard for quantifying the amounts by HPLC. The malvidin 3,5-diglucoside was present in all samples, but the concentrations in Norton grape samples were significantly higher compared with the other two grape varieties analyzed (**Table 3**). Norton had the greatest amount of this anthocyanin in S (144 ± 48 mg/L or 101 ± 34 g/100 g grapes) and W (58.3 ± 5.0 mg/L) samples, which is consistent with the abundance observed by our MALDI analysis (**Table 2**).

The HPLC analytes were identified by retention times and electrochemical behavior. The electrode of the detection system produces a current and induces a redox reaction of each analyte compound by generating a voltage: positive voltages receive electrons, whereas negative voltages donate electrons. The detector quantifies the amount of electrons given off by the analyte at each reference voltage as an oxidation−reduction reaction occurs. The electrons given off by the anthocyanin being oxidized are amplified, and the resulting response appears as a peak in the chromatogram. The CoulArray electrochemical detector system has some capacity to predict the chemical structure of a compound on the basis of its electrochemical signature. Therefore, the opposite may also be said, by knowing the chemical structure of a compound, the electrochemical behavior may be predicted. The degree of hydroxylation and methoxylation pattern in the B ring of flavonoids influences the antioxidant capacity of these compounds. It is interesting that the two cyanidin compounds identified by this method are detected in a lower channel than the malvidin compounds, as they have a diphenyl structure in the B ring. This indicates that cyanidin has a higher capacity for releasing electrons when exposed to a redox environment, which suggests that it would be a better antioxidant. In support of this observation, Rice-Evans et al. (*16*) reported a much higher Trolox equivalent antioxidant activity (TEAC) value for cyanidin aglycon (4.4 ± 0.11 mM) than for malvidin 3,5-diglucoside (2.06 ± 0.1 mM), thus supporting the premise that the cyanidin compounds may indeed be better antioxidants.

DPPH is a stable free radical compound and will exhibit a strong absorption at 515 nm. This method provides information on the reactivity of the anthocyanins with a stable free radical (*40*). When an anthocyanin extract is added to DPPH, the free electron is paired up and color is lost, which is measured as a decrease in absorbance. There is a stoichiometric relationship with the color lost and the number of electrons taken up (*40*); thus, the concentration of DPPH, measured by absorbance, will decrease over time as indicated in **Figure 2**. This decrease in

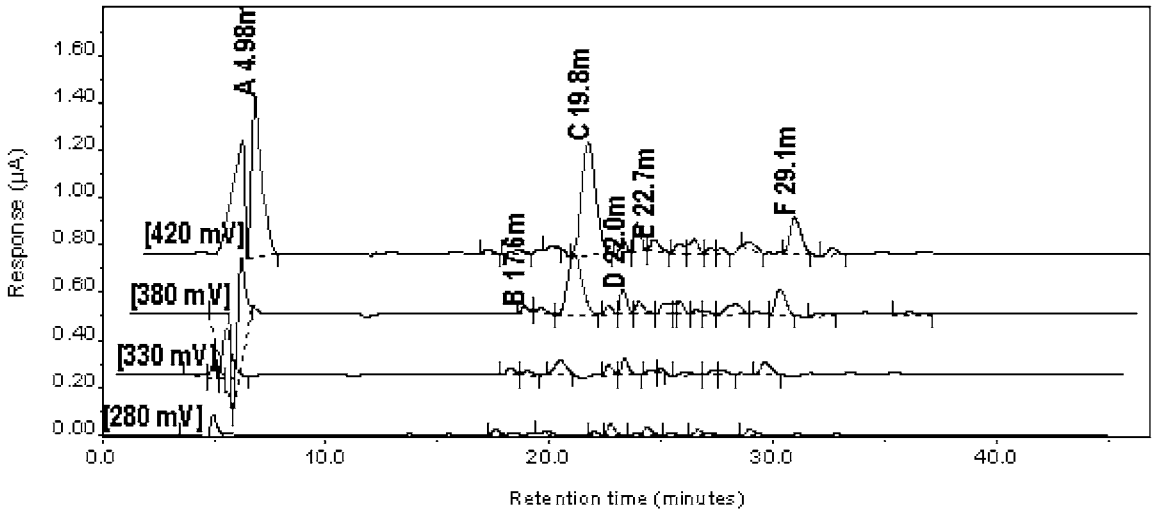

Figure 1. HPLC analysis of anthocyanins in Norton wine extract. The letters on the chromatogram peaks correspond to the following confirmed anthocyanins: A = malvidin aglycon; B = cyanidin 3,5-diglucoside; C = malvidin 3,5-diglucoside; D = cyanidin monoglucoside; E = malvidin monoglucoside; F = malvidin coumaroylglucoside.

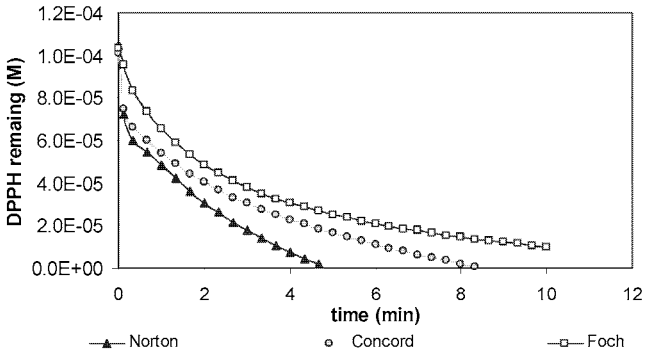

Figure 2. Loss of DPPH radical over time after the addition of different grape skin extracts.

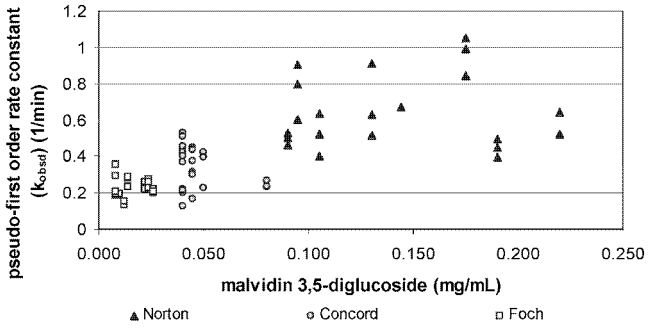

Figure 3. Rate (k_{obsd}) of free radical scavenging activity of grape skin extracts compared to malvidin 3,5-diglucoside content.

concentration indicates that the anthocyanin had scavenged the free radical from DPPH, thus promoting a color loss.

DPPH changes color from purple to yellow as the antioxidant scavenges its proton. Anthocyanins are red in color and absorb at 520 nm. DPPH is purple, and the maximum absorbance is at 515 nm, but the absorption spectra of DPPH and anthocyanins overlap at 515 nm. Therefore, the red color of grape S samples would affect the absorbance of DPPH, overexpressing the absorbance of DPPH. To eliminate that error, the molar absorbance (ϵ), 20350 (mol/L) of malvidin 3,5-diglucoside, was calculated at 515 nm. This molar absorbance is lower than the 37700 proposed for malvidin 3,5-diglucoside at 520 nm (*35*) because anthocyanins will have less absorbance at a wavelength of 515 nm. To adjust for the assay, absorbance of each anthocyanin extract was calculated at 515 nm and subtracted from the DPPH absorbance. This new DPPH absorbance was used to calculate the DPPH remaining over time when the extract was present.

A plot of k_{obsd} versus antioxidant concentration yields information on how quickly the sample extract works in scavenging radicals from DPPH. Espin et al. (*32*) found a linear relationship between the initial concentration of different anthocyanin extracts obtained from natural sources and the pseudo-first-order rate constant, k_{obsd}. This relationship was found when the same extract was diluted at different concentrations and the DPPH test was performed. The grape S samples were evaluated at only one concentration; however, it was noted that as the concentration of the malvidin 3,5-diglucoside of the sample increased, the k_{obsd} increased. This relationship is shown in **Figure 3**; although there was a tendency for a positive association, the response was not linear over the range tested. The higher the concentration of malvidin 3,5-diglucoside, the higher the k_{obsd}; thus, the radical scavenging capacity would be higher. Our results are consistent with Stintzing et al. (*41*), who stated that higher cyanidin concentrations of anthocyanin extracts showed higher antioxidant capacities when measured by ORAC.

Table 4. Radical Scavenging Capacity Measurement of Grape Skin Measured by DPPH Method[a]

	Foch	Norton	Concord
mean % change in absorbance of DPPH	72 ± 5B	88 ± 9A	73 ± 4B
mean Trolox (mM) equivalents	0.8B	0.9A	0.8B

[a] Values (mean ± SD, n = 3) in rows having different letters (A, B) are significantly different ($p < 0.05$).

The radical scavenging capacity of the S samples is presented in **Table 4**. Foch S had a radical scavenging capacity of 0.78 mM Trolox equiv, Concord S had 0.80 mM Trolox equiv, and Norton had 0.95 mM Trolox equiv. Arnous et al. (*33*) reported the radical scavenging capacities for aged red Greek wines to be between 0.82 and 1.60 mM Trolox equiv. The lower values obtained for the S samples in our study could be due to the fact that only the skin extract was used for the analysis, whereas Arnous et al. (*33*) used wine samples. Wine may also contain other polyphenolic compounds besides those present in the skin,

such as those extracted from the seeds, thus increasing the radical scavenging capacity. Nevertheless, our values are within the range of those reported by Arnous et al. (*33*). On the basis of our analysis, Norton S samples had the highest radical scavenging capacity compared to the values for S samples from Foch and Concord grapes. This means that the drop in absorbance of the DPPH was faster with Norton S samples as shown in **Figure 2**. These results suggest that Norton grapes would have a greater antioxidant capacity.

Pietta (*18*) suggested that anthocyanins are equipotent antioxidants to quercetin due to the similarity in their structures. Although the DPPH and ORAC tests give different results, it is reasonable to compare the significance of the results obtained by ORAC with those obtained by DPPH, as they both show the same trend in antioxidant potential.

Anthocyanins have been found to have antioxidant properties measured by analytical techniques such as ORAC, DPPH, and FRAP (*16*, *33*, *40*, *41*). In a study during which 300 mL of red wine was given to elderly women, an increase in the antioxidant capacity of blood, measured by ORAC and FRAP, was observed (*42*). This result supports the hypothesis of the French paradox, as red wine increased the overall antioxidant capacity did so in serum. When Concord grape juice was given orally (10 mL/kg/day) to subjects, an ORAC test revealed a significant increase in the antioxidant activity of the plasma. The researchers suggested that the flavonoids in the juice were absorbed, conjugated, and retained for some time in the body in order to produce the increase in antioxidant activity of the plasma (*43*). At the given dose, there was no indication of prooxidant activity of the Concord grape juice, which contained 350−500 ppm of malvidin.

The antioxidant activity of anthocyanin may be involved in reducing oxidative stress in vivo as observed in rat-feeding studies and cell culture. In a study during which rats were fed 2 g/kg of cyanidin 3-glucoside for 14 days, there was a suppression in the changes caused by hepatic ischemia/reperfusion (I/R), which is an oxidiative stress model (*44*). In a different experiment rats were fed a vitamin E-deficient diet for 12 weeks to enhance susceptibility to oxidative damage, followed by the repletion of the rations with a highly purified anthocyanin-rich extract (1 g/kg). The results showed that there was a significant improvement in the plasma antioxidant capacity, as well as a decrease in the vitamin E deficiency-enhanced hydroperoxides and 8-oxodeoxyguanosine concentrations in the liver (*45*). Endothelial cells enriched with elderberry anthocyanins (1 mg/mL) incorporated the compounds into the plasma membrane and the cytosol. In addition, these cells had significant protection against oxidative stressors such as hydrogen peroxide and $FeSO_4$/ascorbic acid (*46*).

The bioavailability of a compound needs to be considered to determine the potential activity it may have in vivo (*47*). Reports on the bioavailability of malvidin 3,5-diglucoside are extremely limited. Although it is generally believed that after the consumption of flavonoids the body only absorbs those in the aglycon form, recent studies have shown the contrary. A study conducted by Miyazawa et al. (*48*) demonstrated that there was a direct intestinal uptake of red anthocyanins, cyanin 3-glucoside and cyanin 3,5-diglucoside, observed by the increase in plasma concentrations and delivery to liver in rats given fruit extracts. Furthermore, Hollman et al. (*49*) found flavonoid glycosides in human plasma after subjects consumed onions (225 ± 43 μmol of quercetin) and apples (325 ± 7 μmol of quercetin).

The mechanism by which anthocyanins are absorbed and metabolized in the body is currently unclear. Cao et al. (*50*) suggested that anthocyanin glycosides are absorbed via the interaction with sodium-dependent glucose transport receptors in the mucosal epithelium, implying they follow the same mechanism as quercetin glucosides (*51*) due to their similar basic structure. Terao et al. (*52*) orally administrated (−)-epicatechin and quercetin to rats, finding an increase in the antioxidant plasma activity and the accumulation of glucoronide and sulfate conjugates in plasma, thus indicating that flavonoids were absorbed and transformed in blood. Until now, no toxicological evidence on anthocyanins has been shown since their approval as food colorants (*53*).

Apart from the reported antioxidant actions of anthocyanins, it has been suggested that these compounds may interfere with metabolism of normal and cancerous cells by interacting with the production of metabolites and with cellular receptors. In LPS/IDN-γ-activated RAW264.7 mouse macrophages, anthocyanins had strong inhibitory effects on the production of nitric oxide (NO), a metabolite of an inflammatory condition (*54*). Cyanidin, pelargonidin, and delphinidin tested in human breast cancer cells (MCF-7) showed a weak but significant estrogenic activity (*55*). Cyanidin and delphinidin were potent inhibitors of the epidermal growth-factor receptor in the human vulva carcinoma cell line A431 (*56*), contributing to the growth-inhibitory properties of these compounds. In human promyelocytic leukemia cells (HL-60), anthocyanins caused cells to generate intracellular hydrogen peroxide, triggering apoptotic cell death, possibly through an oxidative stress-involved JNK signaling pathway (*57*).

On the basis of the studies done by other researchers in which the anthocyanins are absorbed by the body and metabolized, it is suggested that anthocyanins possibly are bioactive compounds in vivo. It is reasonable to conclude that Norton grapes may serve as a better source of potential antioxidants than Concord and Foch due to its higher content of total anthocyanins and greater radical scavenging capacity observed in the present study. Future research on the analysis of other red grapes and resultant wines including those from the *V. vinifera* type is warranted. A natural extension of this investigation is to determine plasma levels of Norton grape anthocyanins in animal models consuming these grapes and to examine the antioxidant properties of these compounds in cell cultures.

ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; HCl, hydrochloric acid; HPLC, high-pressure liquid chromatography; LDL, low-density lipoprotein; MALDI, matrix-assisted laser desorption ionization; ORAC, oxygen radical absorbance capacity; S, skin; TEAC, Trolox equivalent antioxidant activity; FRAP, ferric reducing/antioxidant power; W, wine.

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Received for review February 24, 2004. Accepted August 6, 2004. Competitive grant support from the 21st Century Science and Technology Fund of Indiana and the resources of the Center for Enhancing Foods to Protect Health. Approved as Journal Paper 16959 of the Purdue Agricultural Experiment Station.

JF040087Y