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A Simple Extraction and Chromatographic System for the Simultaneous Analysis of Anthocyanins and Stilbenes of *Vitis* Species

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A separation system has been developed to permit the simultaneous analysis of major anthocyanins and stilbenes from berries of *Vitis* species in <1 h. The system makes use of separation by HPLC and detection by UV−visible absorption and fluorescence, for anthocyanins and stilbenes, respectively, with the two detection systems linked in series. Monitoring the absorption at 520 nm permits ready identification and quantification of major anthocyanins. Chromatograms derived from fluorescence (330 nm excitation/374 nm emission) produce less clear data for the piceids and resveratrols, which are present in much lower amounts; peaks for these compounds can be verified by means of external or internal standards.

KEYWORDS: Anthocyanins; stilbenes; HPLC; *Vitis vinifera*

INTRODUCTION

The anthocyanins, source of the red/blue hues of flowers and fruits of higher plants, are valued not only for their aesthetic contributions but for their roles in attracting the insects and birds needed for plant pollination and seed dispersal (*1*), their protection of plants from ultraviolet light (*2*), and their inhibition of insect predation and fungal infection (*3*, *4*) and as a source of colorants for human dyes and foods (*5*). A second class of polyphenols characteristic of grape berries and wines are the stilbenes, derived from the same biochemical pathway but of more limited occurrence. They have been of interest particularly for their roles in the response of grape plants to infection (*6*−*8*). The anthocyanins and stilbenes are of increasing interest for a multitude of reported contributions to human health, including activity against allergies, viruses, hypertension, arthritis, mutagenesis, and cancer (e.g., ref *9*).

Although >200 naturally occurring anthocyanins have been identified (*10*), berries of *Vitis vinifera* produce a relatively small number, characterized by simple side groups. In addition to the anthocyanin 3-monoglucosides depicted in **Figure 1A**, *vinifera* berries from most varieties produce acetate and coumarate derivatives of the 3-monoglucosides (*11*, *12*). Malvidins constitute the major class of anthocyanins produced in berries of *V. vinifera* (*12*), and monoglucosides are found at much higher levels than 3-acetylglucosides and 3-*p*-coumarylglucosides. Pinot Noir produces neither of the derivative forms of the 3-monoglucosides. Diglucosides are found primarily, and some argue exclusively, in North American species of *Vitis* (*12*).

The early means for the separation of anthocyanins, thin-layer chromatography (TLC), was supplanted several years ago by high-pressure liquid chromatography (HPLC) (*13*, *14*). Currently HPLC is the most commonly used method, with capillary electrophoresis of increasing interest (*15*). The usual acid solvents are acetic, formic, or phosphoric acid in water/MeOH or water/acetonitrile, all of which separate the anthocyanins as flavylium cations with absorbance maxima in the 500−550 nm range (*10*). The separated anthocyanins can be readily detected and measured with an on-line absorbance monitor.

An increasing number of stilbenes are reported to be present in extracts of grape leaves and berries (*16*−*18*); the best characterized are *trans*-resveratrol, produced enzymatically, and its simple derivative *trans*-piceid, along with the cis isomers of both (**Figure 1B**). Procedures used for the extraction of stilbenes from grape commonly involve methanol, ethanol, formic acid, and ethyl acetate in various combinations (*18*−*21*). As with anthocyanins, stilbenes are analyzed by HPLC, gas chromatography, and capillary electrophoresis, with detection based on fluorometry or absorption in the ultraviolet range (e.g., refs *19* and *21*−*23*).

Traditionally, laboratories have focused on either anthocyanins or stilbenes, and the methods for analysis developed independently. Our studies, encompassing the genetics and biochemistry of both anthocyanin and stilbene production, stood to benefit from the development of a method for the simultaneous analysis of the two. To be useful, this required appropriate extraction buffer and solvents and a rapid system for clean separations of both sets of phenolics in concentration ranges suitable for the quantification of both. We report here a simple method for the extraction and simultaneous HPLC-based analysis of anthocyanins and stilbenes from berries of *V. vinifera*.

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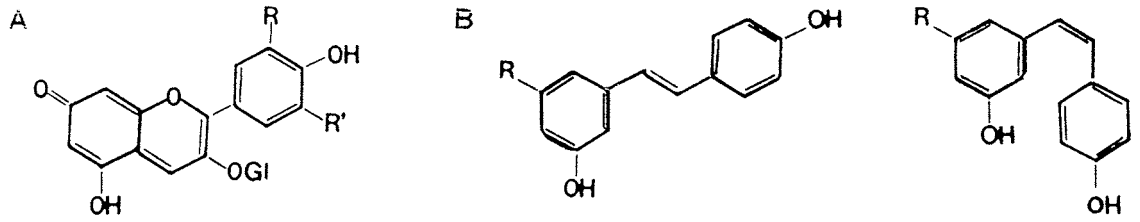

A Anthocyanin 3-monoglucosides

	R	R'
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

B Stilbenes

	R
Resveratrol	OH
Piceid	OGl

Figure 1. Structures of anthocyanins (**A**) and stilbenes (**B**) characteristic of *V. vinifera.* In structure **B**, the trans isomer is illustrated at the left and the cis isomer at the right. "Gl" designates a glucose moiety.

MATERIALS AND METHODS

Anthocyanin standards were the generous gift of Geza Hrazdina, Department of Food Science and Technology, Cornell University. The eight anthocyanin standards consisted of diglucosides of delphinidin, peonidin, and malvidin, monoglucosides of delphinidin, cyanidin, petunidin, and malvidin (the latter containing small amounts of peonidin monoglucoside and malvidin 3-acetylglucoside), and malvidin 3-*p*-coumarylglucoside. Solutions were prepared by dissolution in extraction buffer [formic acid/water/methanol (2:28:70), *24*] at concentrations ranging from 100 to 200 μg/mL. Individual or combined standards (5−10 μL) in extraction buffer were subjected to chromatography.

trans-Resveratrol (MW 228.2) was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved to the desired concentration in extraction buffer. Polydatin, composed primarily of *trans*-resveratrol and *trans*-piceid, with small amounts of *cis*-resveratrol and *cis*-piceid, was kindly provided by George Soleas, Liquor Control Board of Ontario. Increased levels of cis isomers were generated by exposing polydatin at a concentration of ~0.5 mg/mL in a clear glass vial to light from a 34 W gro-lux bulb at a distance of 20 cm for 16 h. Routinely, 5−10 μL of a 1/10 dilution of this solution was injected into the chromatographic system.

Quantification of anthocyanin and stilbene peaks relied on the generation of standard curves. These were produced by the integration of absorption or fluorescence peaks generated from chromatography of dilution series of malvidin 3′-monoglucoside and *trans*-resveratrol solutions. Malvidin 3-monoglucoside was prepared at a concentration of 0.6 mg/mL (1 mM) and *trans*-resveratrol at 0.38 mg/mL (1.67 mM), both in extraction buffer. Prior to chromatography, these stocks were diluted in extraction buffer as needed such that 10 μL contained from 60 to 600 ng of anthocyanin or 100−1000 ng of *trans*-resveratrol. The areas under the major malvidin 3-monoglucoside peak along with the minor peonidin 3-monoglucoside and malvidin 3-acetylglucoside peaks were summed to compute the anthocyanin extinction coefficient; the *trans*-resveratrol sample produced the expected single peak of fluorescence.

Berries were obtained from mature vines planted in vineyards in the Niagara peninsula of southern Ontario. Berries were harvested and weighed and the skins removed. After weighing, skins were quick-frozen in liquid nitrogen before storage at −80 °C. Skin samples from one to three berries were homogenized in dim light using a chilled mortar and pestle and minimal extraction buffer. Homogenates were brought to a final volume of 6 or 8 mL in the same buffer. Water in the skins resulted in final solvent compositions of formic acid/water/methanol in ratios between 2:28:70 and 2:29:69. After storage in the dark at 4 °C for 24 h, samples were centrifuged at 7000*g* for 20 min and then filtered using a PTFE 0.45 μm membrane syringe filter (Corning, Inc.) Filtered extracts of samples and standards were stored in foil-wrapped glass vials at −20 °C.

Reversed phase HPLC separations relied upon a Hewlett-Packard HP1100 system with Agilent Zorbax SB-C18 analytical (5 μm, 250 mm × 3.6 mm) and guard (5 μm, 12.5 mm × 4.6 mm) columns. A flow rate of 1.5 mL/min was used for all chromatographies. Sample volumes ranged from 5 to 25 μL. Anthocyanins were detected using a diode array system with absorbance at 520 nm; stilbenes were detected by fluorescence, using excitation and emission settings of 330 and 374 nm, respectively. Major peaks were identified by comigration with standards and assignments confirmed by mixing experiments, in which known amounts of standards were added to samples prior to injection.

RESULTS

Standards were initially chromatographed according to the method of Kalt et al. (*24*), which relied on a 5% formic acid/100% methanol step gradient for separation. Several standard anthocyanin monoglucosides coeluted in this system (data not shown), which was subsequently modified through a number of steps to a gradient employing 5% formic acid in water (solvent A) and methanol/acetonitrile/water 33:60:70 (solvent B). This method, which was used for all separations reported here, relies on a solvent system initially composed of 90% A and 10% B and increases as follows: 6 min, 11% B; 17 min, 12% B; 21 min, 14% B; 27 min, 23% B; 37 min, 47% B; 39 min, 85% B; and 40−48 min, 100% B. The column was then washed with 90%A:10%B until no trace of absorbing or fluorescing material was detectable in the eluate.

Figure 2 presents anthocyanin and stilbene standard chromatographs generated by this method. Approximately 400 ng of mixed anthocyanins or 200 ng of light-treated polydatin was applied to the chromatographic column. Simple 3-monoglucosides of anthocyanins eluted between 10 and 30 min and the four stilbenes, between 25 and 40 min. Standards were used to verify the identities of sample peaks.

With anthocyanins detected by light absorption at 520 nm (**Figure 2A**), the diglucoside standards eluted at approximately 5.5 (delphinidin), 16.8 (peonidin), and 20.6 min (malvidin). Monoglucosides eluted at roughly 11.9 (delphinidin), 18.0 (cyanidin), 23.8 (petunidin), 27.3 (peonidin), and 28.6 min (malvidin). The acetylated and coumarylated forms of malvidin eluted at approximately 33.8 and 36.3 min. The overlapping peaks for peonidin 3,5-diglucoside and cyanidin 3-monoglucoside were not considered to be a problem for two reasons: our interest lay in commercial *vinifera* varieties, which do not produce diglucosides (*9*), and a smaller sample volume would have allowed resolution, if needed.

The same elution system permitted clean resolution of piceids and resveratrols, detected by fluorescence at 374 nm (**Figure 2B**). Individual peak identities were established by comparison

Table 1. Anthocyanin Profiles for Chromatograms Depicted in **Figure 3**

variety	% total anthocyanins D3m[a]	C3m[a]	Pt3m[a]	Pn3m[a]	M3m[a]	Ace[a]	Cou[a]	% Malv[b]	total anthocyanins, mg/bs[c]
Cabernet Sauvignon	18.3	8.6	7.0	13.6	22.4[d]	20.0	9.8	30.9	5.01
Merlot	14.7	1.1	8.3	6.2	38.9	21.5	9.4	58.7	2.25
Pinot Noir	4.2	<1	9.4	14.8	71.4	nd[e]	nd	71.4	1.79
Cabernet Franc	5.2	<1	5.2	4.5	39.5	28.8	16.6	70.9	2.60

[a] D3m = delphinidin 3-monoglucoside, etc.; Ace = 3-acetylglucosides; Cou = 3-*p*-coumarylglucosides. [b] Percentage malvidin, all forms. [c] Milligrams of anthocyanins per berry skin. [d] The average level of malvidin 3-monoglucoside over all Cabernet Sauvignon berry skins sampled was 32.2%, significantly higher than in this sample. [e] Not detectable.

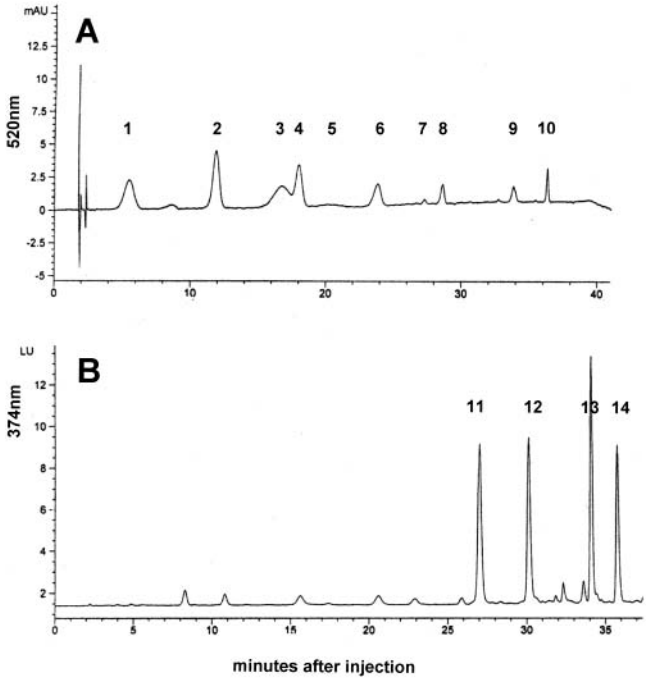

Figure 2. Chromatographic separation of anthocyanin (**A**) and stilbene (**B**) standards using the solutions and chromatography conditions described in the text. Peaks of anthocyanin standards in panel **A** represent delphinidin 3,5-diglucoside (1), delphinidin 3-monoglucoside (2), peonidin 3,5-diglucoside (3), cyanidin 3-monoglucoside (4), malvidin 3,5-diglucoside (5), petunidin 3-monoglucoside (6), peonidin 3-monoglucoside (7), malvidin 3-monoglucoside (8), malvidin 3-acetylglucoside (9), and malvidin 3-*p*-coumarylglucoside (10). The unnumbered peak at ~9 min represents a contaminant of the delphinidin 3-monoglucoside standard. Identified stilbene peaks in panel **B** represent *trans*-piceid (11), *cis*-piceid (12), *trans*-resveratrol (13), and *cis*-resveratrol (14). *Y* axis values designate absorbance at 520 nm (mAU) and fluorescence at 374 nm (LU).

to the profile of pure *trans*-resveratrol and the patterns of original polydatin before conversion of trans isomers to their cis forms. *trans*- and *cis*-piceid eluted at approximately 27.0 and 30.1 min, respectively, and the corresponding resveratrols at approximately 34.1 and 35.8 min. The methanol/acetonitrile solvent system thus produced clear patterns for both anthocyanins and stilbenes in a reasonable time, <40 min.

Results obtained with an extract from the pooled skins of three mature Cabernet Franc berries, depicted in **Figure 3A**,**E**, confirm the applicability of this method for the analysis of experimental samples. Thirteen peaks are distinguishable in the A_{520} absorption profile (**Figure 3A**); >20 can be seen in the A_{374} fluorescence profile (**Figure 3E**). To confirm piceid and resveratrol peaks, samples were chromatographed a second time, after the addition of polydatin.

Table 2. Stilbene Profiles for Chromatograms Depicted in **Figure 3**

variety	% total piceids and resveratrols *trans*-piceid	*cis*-piceid	*trans*-resveratrol	*cis*-resveratrol	total stilbenes, μg/bs[a]
Cabernet Sauvignon	23.4	11.3	65.2	nd[b]	8.69
Merlot	57.5	nd	42.5	nd	1.95
Pinot Noir	86.0	nd	64.0	nd	2.65
Cabernet Franc	58.2	6.7	19.3	15.8	1.69

[a] Micrograms per berry skin. [b] Not detectable.

Dilution series of malvidin 3-glucoside and *trans*-resveratrol were used to assess the linearity of absorbance or fluorescence over a wide range of sample concentrations. The results of two series of HPLC chromatography are shown in **Figure 4**. These standard curves were also used to quantify levels of specific anthocyanins and resveratrols in tissue extracts, assuming similar extinction coefficients for major contributors to the profiles. Malvidin 3-monoglucoside itself is the primary contributor to anthocyanins in *V. vinifera*, and the other major anthocyanins are very close in molecular weight. For the conversion of anthocyanins from absorbance to concentration, then, the malvidin monoglucoside figure was used directly. The molecular weight of the piceids, in contrast, is 135% that of resveratrol. For quantification of piceids we multiplied the apparent masses by 1.35. The assumption of similar extinction coefficients has been used previously in such analyses (*25*, *26*).

As additional controls, volumes of test samples ranging from 5 to 25 μL were chromatographed and shown to produce proportionally greater areas under peaks. Analysis of serial extractions of individual samples, continued until no color was visible in berries or extract, confirmed that the extraction method releases >95% of the total anthocyanins and resveratrols (data not shown).

Figure 3 presents chromatograms of the anthocyanins and stilbene profiles from individual mature berries of three additional commercial *vinifera* cultivars (Cabernet Sauvignon, Merlot, and Pinot Noir). Calculated levels of anthocyanins and stilbenes of these samples, plus those derived from Cabernet Franc, are shown in **Tables 1** and **2**. The data are expressed in mass per berry skin. The conversions from berry skin to grams for these samples are as follows: Cabernet Franc, 0.173 g/bs; Cabernet Sauvignon, 0.134 g/bs; Merlot, 0.126 g/bs; and Pinot Noir, 0.085 g/bs.

DISCUSSION

This work verifies the utility of HPLC for relatively rapid, qualitative, and quantitative analysis of stilbenes and anthocyanins from *Vitis*. Detection requires a dual monitoring system: an absorbance monitor for anthocyanins and a fluorescence monitor for stilbenes.

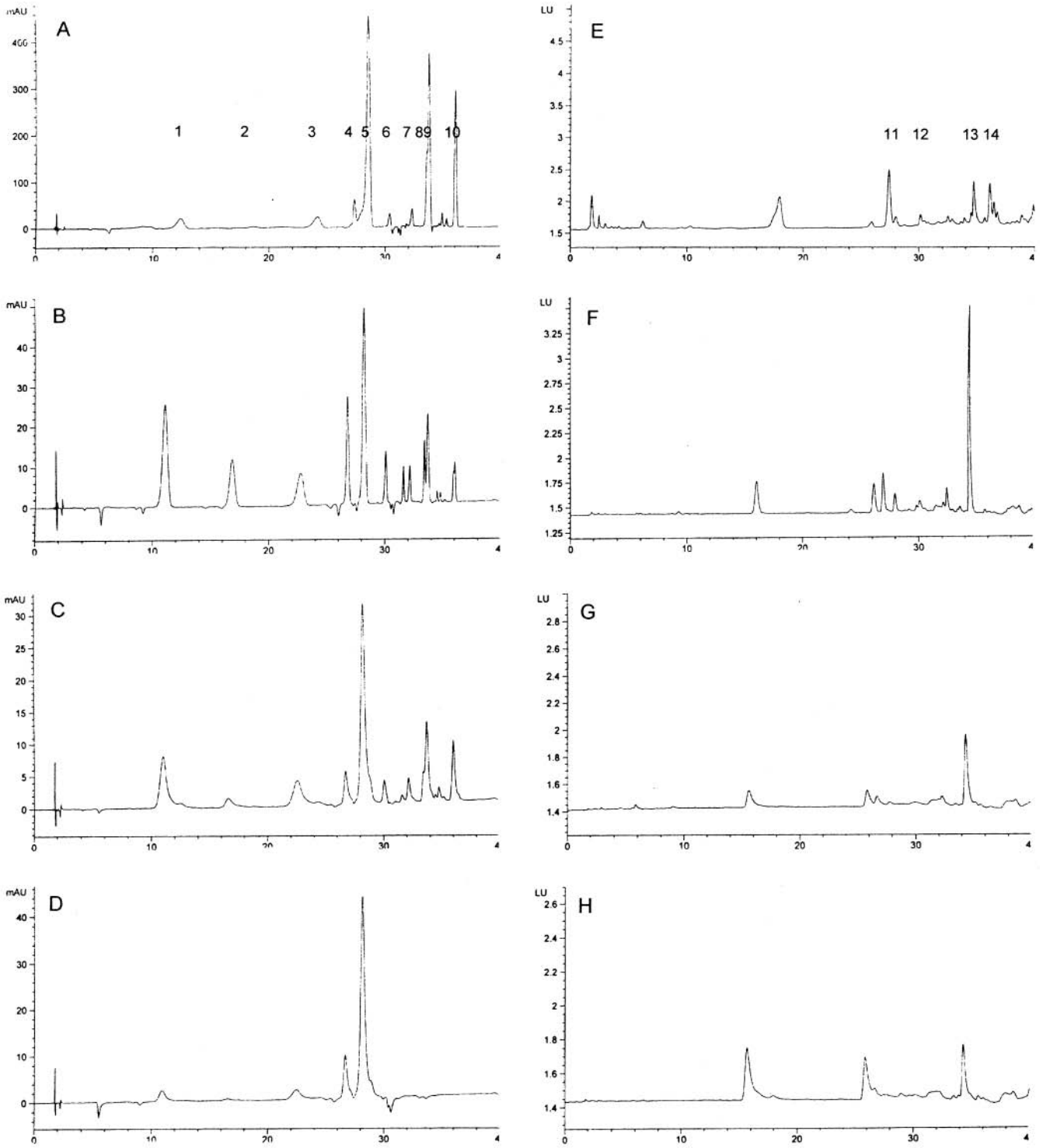

Figure 3. Absorbance (**A**–**D**) and fluorescence (**E**–**H**) of chromatographed extracts from one to three mature berries of Cabernet Franc (**A**, **E**), Cabernet Sauvignon (**B**, **F**), Merlot (**C**, **G**), and Pinot Noir (**D**, **H**): (**A**) anthocyanins detected by absorbance at 520 nm [delphinidin (1), cyanidin (2), petunidin (3), peonidin (4), and malvidin (5) 3-monoglucoside; delphinidin (6), petunidin (7) peonidin (8), and malvidin (9) 3-acetylglucoside; and malvidin 3-*p*-coumarylglucoside (10)]; (**E**) stilbenes detected by fluorescence, with excitation and emission at 330 and 374 nm, respectively [*trans*-piceid (11), *cis*-piceid (12), *trans*-resveratrol (13), and *cic*-resveratrol (14)].

All of the 3-*p*-coumarylglucosides eluted in <3 min, sufficient for our purposes but not well enough separated to distinguish individual components. Better separation, at the cost of chromatography time, can undoubtedly be achieved by increasing the number of gradient steps in the later stages of chromatography.

Availability of the polydatin standard assured correct identification of the piceids and resveratrols. A characteristic peak at 16−17 min has not been identified, but the elution profiles presented by Ribeiro de Lima et al. (*17*) suggest that this peak may represent *trans*-astringen, which differs from piceid by the addition of a hydroxyl group on the 3′-carbon of the B-ring. Pterostilbene, another stilbene reported to be present in *vinifera* extracts (*19*), would be expected to pass through the columns more slowly than *trans*-resveratrol (cf. ref *19*); its levels are apparently not very high in our samples.

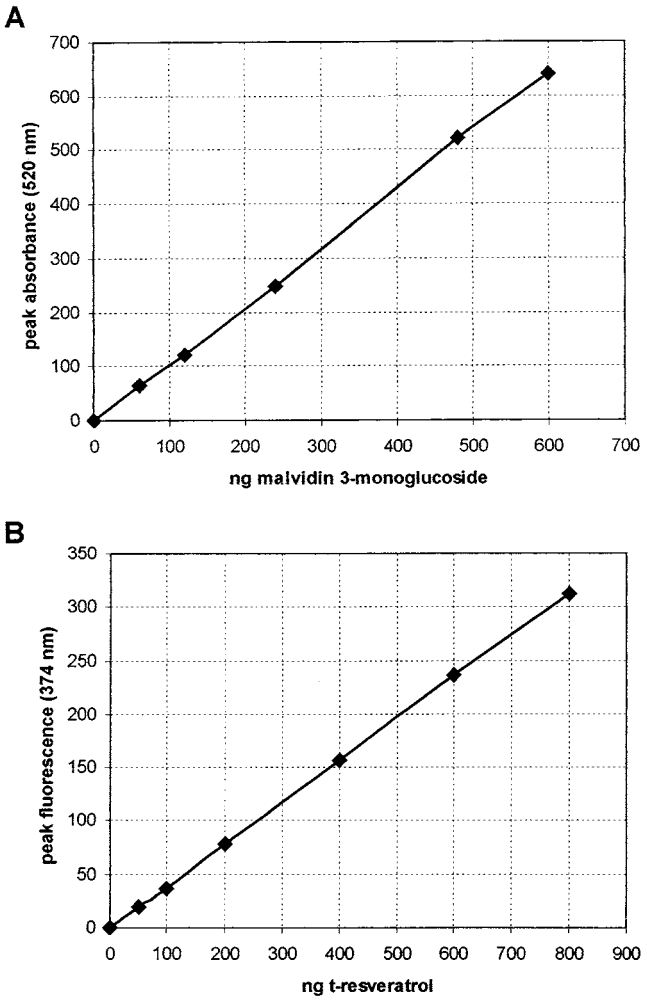

Figure 4. Dilutions of known concentrations of (**A**) malvidin 3-monoglucoside and (**B**) *trans*-resveratrol were chromatographed to verify a linear relationship between concentration and absorbance at 520 nm (anthocyanins) or fluorescence at 374 nm (stilbenes).

Calculated levels of anthocyanins and stilbenes in mature berry skins of Cabernet Franc (based on a pool of three berry skins) together with Cabernet Sauvignon, Merlot, and Pinot Noir (based on individual berry skins) are presented in **Tables 1** and **2**. Amounts of both components naturally vary from berry to berry, but the data presented in **Figure 3** and **Tables 1** and **2** are generally typical of our results. An exception is the calculated level of malvidin 3-monoglucoside in the Cabernet Sauvignon berry (22.4%), which was well below our average for Cabernet Sauvignon berries harvested from the Niagara region in 2001 (32.2%). This average percentage of malvidin 3-monoglucoside was still below the average value published by Mazza (*8*) for Cabernet Sauvignon (42.6%). In other aspects, the data match those obtained by other researchers. The lack of monoglucoside derivatives in Pinot Noir samples is characteristic of that variety. The relative levels of Merlot anthocyanins are very close to those published by Mazza (*8*).

Less information is available on stilbene levels in berries of healthy plants, as stilbenes have been analyzed primarily in infected or stressed tissues. Amounts of these compounds varied more dramatically from berry to berry than levels of anthocyanins. The average amounts calculated for *trans*-resveratrol for the Cabernet Franc and Merlot berries are close to those reported by Okuda and Yokotsuka (*20*) for unstressed grapes harvested in Japan. The *trans*-resveratrol level in the Cabernet Sauvignon berry represented in **Figure 3** (~15 times that reported by Okuda and Yokotsuka) was much higher than the average for our samples (data not shown). The greater variation in levels of stilbenes can be explained, at least partially, by differences in the control of their production relative to that of anthocyanins. The latter are developmentally induced and accumulate from veraison to maturity. Stilbenes, on the other hand, respond to environmental stimuli such as UV light, pathogenesis, injury, and salt treatment (*6*, *20*), which are highly variable throughout berry development.

In summary, the described method of separation allows for the simultaneous analysis—quantitative and qualitative—of the major classes of stilbenes and anthocyanins from grape skin, requiring <1 h per sample. Data collection requires a single extraction and one passage through HPLC. The resulting absorption peaks of anthocyanins are readily identifiable, and one quickly develops an eye for the pattern. The fluorescence patterns for stilbenes, present in much lower amounts, are more difficult to interpret in the absence of mixing experiments, in which samples are "spiked" with known standards. The measurements obtained for both anthocyanins and stilbenes are in good agreement with those produced by others, using different separation methods.

We expect this method to be of particular value in molecular analyses of the biosynthetic enzymes and putative transcriptional regulators acting on the pathways closely related to anthocyanin and stilbene production. It should be applicable as well to more applied studies of polyphenols in fruits, juices, and wines.

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