Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin

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Pressurized liquid extraction (PLE) was used to extract anthocyanins from the freeze-dried skin of a highly pigmented red wine grape with six solvents at 50 °C, 10.1 MPa, and 3 × 5 min extraction cycles. Temperature (from 20 to 140 °C in 20 °C increments) effects on anthocyanin recovery by acidified water and acidified 60% methanol were also studied. Acidified methanol extracted the highest levels of total monoglucosides and total anthocyanins, whereas the solvent mixture (40:40:20:0.1 methanol/acetone/water HCl) extracted the highest levels of total phenolics and total acylated anthocyanins. Acidified water extracts obtained by PLE at 80−100 °C had the highest levels of total monoglucosides, total acylated anthocyanins, total anthocyanins, total phenolics, and ORAC values. Acidified methanol extracts obtained by PLE at 60 °C had the highest levels of total monoglucosides and total anthocyanins, whereas extracts obtained at 120 °C had the highest levels of total phenolics. High-temperature PLE (80−100 °C) using acidified water, an environmentally friendly solvent, was as effective as acidified 60% methanol in extracting anthocyanins from grape skins.

KEYWORDS: Red grape; pressurized liquid extraction; anthocyanins; phenolics; antioxidant capacity; ORAC

INTRODUCTION

Anthocyanins are natural, nontoxic, water-soluble pigments responsible for the red, purple, blue, and orange colors of fruits, vegetables, and flowers. These bright pigments benefit plants by attracting animals involved in pollination and seed dispersal, by repelling harmful insects, and by preventing damage by UV light. Anthocyanins also have many human health beneficial effects including enhancement of visual acuity (*1*), reduction of coronary heart disease (*2*, *3*), protection against age-related declines in neurological dysfunction (*4*), and maintenance of normal vascular permeability (*5*, *6*), as well as anticarcinogenic (*7*, *8*), antimutagenic (*9*), anti-inflammatory (*10−12*), and antioxidative properties (*11−13*). Grape anthocyanins play an important role in the color quality of red wines, and they have been increasingly used as food colorants and nutraceuticals (*14*, *15*).

Extraction is the first step in the commercial isolation of anthocyanins. Anthocyanins are located in cells near the plant surface (*16*) and are readily extracted from homogenized plant materials by organic solvents. Traditionally, acidified solutions of methanol, ethanol, acetone, water, and acetone/methanol/water mixtures have been used to extract anthocyanins. In a comparative study of three solvents, methanol, ethanol, and water, that were acidified with either HCl or various organic

acids, Metivier et al. (*17*) found that methanol extraction was 20% more effective than ethanol and 73% more effective than water in recovering anthocyanins from grape pomace. Most extraction procedures use acidic solvents, which denature cellular membranes and facilitate solubilization of anthocyanins (*16*). However, addition of excess acid can result in hydrolysis of labile, acyl, and sugar residues during concentration steps. Weak organic acids, such as formic acid, acetic acid, citric acid, and tartaric acid, and low concentrations of strong acids, such as trifluoroacetic acid (0.5−3.0%) and hydrochloric acid (<1.0%), are recommended for extracting anthocyanins (*16*, *18*, *19*), because addition of excess acid added to extraction solvents can lead to artifacts during anthocyanin extraction (*20*).

Elevated temperatures are reported to improve the efficiency of extraction due to enhanced diffusion rate and solubility of analytes in solvents (*21*, *22*). However, elevated extraction and concentration temperatures may simultaneously increase the rate of anthocyanin degradation. Conventional extraction and concentration of anthocyanins is typically conducted at temperatures ranging from 20 to 50 °C (*16*, *23*), because temperatures >70 °C have been shown to cause rapid anthocyanin and color degradation (*24*). The degradation rate of anthocyanins is time and temperature dependent. Therefore, high-temperature−short-time extraction conditions and processing treatments have been used successfully to retard anthocyanin degradation in fruits (*25*, *26*). Besides acid concentration and temperature, additional factors such as light, oxygen, copigments, metals, sugars, and

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their degradation products have been shown to affect the stability and antioxidant capacity of anthocyanin pigments (*16, 27*).

Pressurized liquid extraction (PLE; also known under the trade name ASE, accelerated solvent extraction) is an ideal technology for performing high-temperature−short-time extraction of phytochemicals. PLE enables rapid extraction (3−20 min) of analytes in a closed and inert environment, under high pressures (3.3−20.3 MPa) and temperatures (40−200 °C). A major advantage of PLE over conventional solvent extraction methods conducted at atmospheric pressure is that pressurized solvents remain in a liquid state well above their boiling points, allowing for high-temperature extraction. These conditions improve analyte solubilities and the desorption kinetics from the matrices (*28*). Hence, extraction solvents including water that are inefficient in extracting phytochemicals at low temperatures may be much more efficient at elevated PLE temperatures.

Recent interest in the health-beneficial properties of polyphenolics has prompted researchers to screen plants for phenolic content and antioxidant capacity. Advanced breeding lines and commercial cultivars of fruits identified to contain elevated levels of phenolics and antioxidant capacity could be used by plant breeders to genetically modify crops for improved health benefits. This research was undertaken to evaluate PLE of anthocyanins from the skin of a highly pigmented red wine grape and to investigate how different solvents and temperature conditions affect PLE extraction of anthocyanins. The total phenolic content, antioxidant capacity, and color properties of grape skin extracts obtained by each PLE extraction solvent were also determined.

MATERIALS AND METHODS

Samples and Chemicals. Grapes were obtained from a highly pigmented advanced breeding line of wine grape, Arkansas-1575 (A-1575), which was cultivated at the University of Arkansas, Agricultural Experimental Station Farm (Fayetteville, AR). The wine grape is unusual in that anthocyanins are dispersed throughout the flesh in addition to high concentrations found in the skin, although 94% of the fruits' anthocyanins are present in the skin. Harvested grapes were placed into plastic bags, flushed with nitrogen, sealed, and stored at −20 °C until analysis. Grape skin was manually separated from the flesh using a razor blade and freeze-dried. The dried skin was ground to a fine powder in a commercial coffee grinder, placed in brown vials, sealed, and stored at −20 °C.

A mixture of anthocyanin standards containing the 3-monoglucosides of delphinidin (Dpd), cyanidin (Cyd), petunidin (Ptd), pelargonidin (Pgd), peonidin (Pnd), and malvidin (Mvd) was purchased from Polyphenols Laboratories AS (Sandnes, Norway). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI) and 2,2′-azobis(2-amidinopropane) dihydrochloride from Wako Chemicals USA, Inc. (Richmond, VA). HPLC grade methanol, acetone, and ethanol and analytical grade acetic, hydrochloric, and trifluoroacetic acids were obtained from Fischer Scientific (Boston, MA).

PLE of Anthocyanins. PLE of anthocyanins from grape skin was performed using a Dionex model ASE 200 equipped with a solvent controller (Dionex Corp., Sunnyvale, CA). A portion (0.20 g) of grape skin powder was mixed with 30 g of sea sand and then placed into 22 mL extraction cells that contained a cellulose paper filter at the bottom of each cell. The PLE variables, pressure (10.1 MPa), temperature (50 °C), extraction time (3 × 5 min cycles), rinsing volume (50%), and nitrogen purge time (90 s), were used as basic conditions. After PLE, extracts were rapidly cooled to 5 °C in ice water, and their volumes were quantitatively measured.

Optimizing the Solvent System for PLE. The following solvents were tested for their ability to extract phenolics from grape skins: (1) 0.1% HCl in deionized water (acidified water) (pH 2.3); (2) 0.1% HCl

in 60% ethanol (acidified ethanol) (pH 2.2); (3) 0.1% HCl in 60% methanol (acidified methanol) (pH 2.3); (4) 0.1% HCl in 40:40:20 (methanol/acetone/water; solvent mixture) (pH 1.9); (5) 7% acetic acid in 70% methanol (pH 2.0); and (6) 0.1% trifluoroacetic acid in 70% methanol (pH 2.1). Hydrochloric acid was used as an acidulant at 0.1% because it provides a favorable medium for the formation of the stable flavylium ion and does not promote anthocyanin degradation at concentrations <1% (*16, 19*). Solvents were sparged with nitrogen for 2 h prior to PLE in order to prevent phenolic oxidation during extraction.

Optimizing PLE Extraction Temperature. To determine the effect of PLE extraction temperature on the recovery of phenolics, temperatures of 20, 40, 60, 80, 100, 120, and 140 °C were tested at basic conditions described above, using acidified water and acidified 60% methanol as solvents. After each PLE run, the extracts were rapidly cooled to 5 °C in ice water, and their volumes were quantitatively measured.

Soxhlet Extraction of Anthocyanins. Extraction of anthocyanins from grape skin was also performed in a Soxtec Avanti model 2055 extractor (Foss North America, Inc., Eden Prairie, MN). Grape skin sample (0.4 g) was placed in a 30 mm × 100 mm i.d. cellulose extraction thimble and extracted using 70 mL of water or 60% methanol using a boiling cycle for 7.5 min and a rinsing cycle for 7.5 min at 60 and 100 °C. After Soxhlet extraction, the extracts were rapidly cooled to 5 °C in ice water, and their volumes were quantitatively measured.

Determination of Total Phenolics. The amount of total phenolics in the extracts was determined using the Folin−Ciocalteu assay described by Slinkard and Singleton (*29*), using gallic acid as standard. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight.

Determination of Color Density, Polymeric Color, and Percent Polymeric Color. Indices for color density, polymeric color, and percent polymeric color in the extracts were determined using the method of Giusti and Wrolstad (*30*). PLE and Soxhlet extracts were diluted 5-fold with 0.025 M potassium chloride buffer (pH 1) until the absorbance at 520 nm was <1. Diluted samples (2.8 mL) were transferred to two cuvettess; 0.2 mL of sodium metabisulfite solution (0.90 M) was added to one cuvette, and 0.2 mL of distilled water was added to the other. After 15 min, the absorbance of both samples was measured at 420, 520, and 700 nm and compared against a blank containing distilled water. Color density of the control sample (distilled water) was calculated as

$$\text{color density} = [(A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700\,nm})] \times 5$$

Polymeric color of the bisulfite-bleached sample was calculated as

$$\text{polymeric color} = [(A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm})] \times 5$$

Percent polymeric color was calculated using the formula

$$\text{\% polymeric color} = (\text{polymeric color/color density}) \times 100$$

Determination of Antioxidant Capacity. Oxygen radical absorbance capacity (ORAC) of the extracts was measured using a modified version of the Cao et al. (*31*) procedure for use with a Perkin-Elmer HTSoft 7000 Plus Bio Assay Reader (Norwalk, CT). Concentrations of reagents were identical to those of Cao et al. (*31*) except for the working Trolox standard (Aldrich Chemical, Milwaukee, WI), which was diluted to 10 μM prior to use in the assay. For the assay, 20 μL of each sample diluted 1000-fold with phosphate buffer was mixed with 160 μL of β-phycoerythrin (3.73 mg/L, Sigma) in a clear 48-well Falcon microplate, and a baseline reading was obtained. As rapidly as possible, 20 μL of 320 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, Waco Chemicals, Richmond, VA) was added to each well using a multichannel pipet. The plate was agitated for 20 s prior to reading and for 5 s before each reading before reading at 2-min intervals for 70 min. Excitation and emission filters were 535 and 560 nm, respectively. Data were expressed as micromoles of Trolox equivalents per gram of dry weight.

HPLC Analysis of Anthocyanins. Analysis of anthocyanin glucosides was performed using a Waters Alliance model 2690 HPLC system

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Table 1. Total Phenolics, Total Anthocyanins, Antioxidant Capacity (ORAC), and Relative Amounts of the Main Groups of Anthocyanins in Grape Skin Extracts by Six Solvents Using PLE[a] at 50 °C

solvent	total phenolics[b]	total anthocyanins[c]	ORAC[d]	anthocyanin monoglucosides[e]						total acylated anthocyanins[f]	ratio of total monoglucosides to total acylated
				Dpd	Cyd	Ptd	Pnd	Mvd	total		
0.1% HCl in water	111.9a[g]	41.33a	3406a	6.86a[d]	1.34a	8.26b	3.31a	14.68a	34.46a	6.87a	5.01e
0.1% HCl in 60% ethanol	157.7b	50.34b	4137b	7.65b	1.64bc	9.38cd	4.11c	18.05b	40.82b	9.51b	4.29d
0.1% HCl in 60% methanol	163.4c	56.51e	4466cd	8.22c	1.58b	9.52d	4.94d	20.56d	44.84e	11.67c	3.84c
0.1% HCl in solvent mixture[h]	179.4d	50.48b	4508d	7.73b	1.74c	7.17a	3.35a	15.81a	35.80a	14.68d	2.44a
7% acetic acid in 70% methanol	157.5b	53.65c	4429cd	7.96bc	1.52b	9.08c	3.78b	19.20bc	41.54c	12.11c	3.43b
0.1% TFA[i] in 70% methanol	162.9c	55.07d	4369c	8.34c	1.58b	9.46d	3.91bc	19.74c	43.02d	12.05c	3.57b

[a] Pressurized liquid extraction conditions: pressure, 10.1 MPa; extraction time, 5 min for three cycle times; solvent volume, 50%; nitrogen purge time, 90 s. [b] Data expressed as milligrams of gallic acid equivalents per gram of dry weight. [c] Total anthocyanins were calculated as the sum of monoglucoside and acylated anthocyanins. [d] Data expressed as micromoles of Trolox equivalents per gram of dry weight. [e] Data expressed as milligrams per gram of dry weight of each respective compound; delphinidin 3-glucoside (Dpd), cyanidin 3-glucoside (Cyd), petunidin 3-glucoside (Ptd), peonidin 3-glucoside (Pnd), and malvidin 3-glucoside (Mvd). [f] Data expressed as milligrams of malvidin 3-glucoside equivalents per gram of dry weight. [g] Means within a column with similar letters are not significantly different ($P > 0.05$). [h] Solvent mixture contains 40:40:20:0.1 (methanol/acetone/water/HCl). [i] Trifluoroacetic acid.

(Waters Corp.,, Milford, MA) equipped with an autosampler and a Waters model 996 photodiode array detector. PLE extracts were passed through a 0.45 μM filter, and 30 μL was loaded onto a 250 × 4.6 mm Waters Symmetry C_{18} column (Alltech Associates, Inc., Deerfield, IL). Mobile phases consisted of 5% formic acid in water (A) and 100% methanol (B). The program ran under isocratic conditions for 3 min with 20% B and followed a linear gradient from 20 to 50% B in 40 min, 100% B in 45 min, and 20% B in 50 min. Peaks were detected at 510 nm at a flow rate of 0.8 mL/min and identified by comparison to the retention times of standards. Individual anthocyanin monoglucosides were quantified using authentic standards of Dpd, Cyd, Ptd, Pnd, and Mvd, and acylated anthocyanins were quantified as Mvd 3-glucoside equivalents.

To confirm that the concentration of 0.1% HCl added to extraction solvents was not great enough to catalyze hydrolysis of anthocyanin glucosides during PLE, an acidified 60% methanol extract obtained by PLE at 100 °C for 15 min was hydrolyzed in boiling 2 N HCl for 1 h and compared with a nonhydrolyzed extract. After cooling in a water bath, the hydrolysates were analyzed by HPLC as described above.

HPLC-MS Analysis of Anthocyanins. Prior to HPLC-MS analysis, anthocyanin-containing extracts were passed through a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (*18*). Anthocyanins and other phenolics were absorbed onto the C-18 cartridge, and sugars, acids and other water-soluble compounds were eluted with 2 volumes of 0.01% aqueous HCl. The non-anthocyanin phenolics were eluted from the cartridge using 2 volumes of ethyl acetate, and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotary evaporator at 35 °C, and anthocyanins were dissolved in distilled deionized water containing 0.01% HCl. An analytical Hewlett-Packard 1100 series HPLC instrument equipped with an autosampler, binary HPLC pump, and UV–vis detector was used. Reverse phase separations of anthocyanins were performed using the same HPLC conditions described above with absorption at 510 nm recorded. For HPLC-MS analysis, the HPLC apparatus was interfaced to a Bruker model Esquire LC-MS ion trap mass spectrometer. Mass spectral data were collected with the Bruker software, which also controlled the instrument and collected the signal at 510 nm. Typical conditions for mass spectral analysis in the positive ion electrospray mode included a capillary voltage of 4000 V, a nebulizing pressure of 30.0 psi, a drying gas flow of 9.0 mL/min, and a temperature of 300 °C. Data were collected using a full scan mode over a mass range of m/z 50–1000 at 1.0 s per cycle.

Statistical Analysis. Data were analyzed using ANOVA to determine differences between treatment means. Means were separated by Tukey's significant difference test ($P < 0.05$). All treatments were replicated three times.

RESULTS AND DISCUSSION

Optimizing the Solvent System for PLE. Solvent efficiency in extracting total phenolics followed the order solvent mixture > acidified methanol = 0.1% TFA in 70% methanol > acidified ethanol = 7% acetic acid in 70% methanol > acidified water (**Table 1**). ORAC values of solvent extracts generally showed the same trend as total phenolic results, and the two variables were highly correlated ($r = 0.96$) (**Table 1**).

Total anthocyanins determined by HPLC accounted for about a third of the total phenolics detected by the Folin–Ciocalteu assay. Solvent efficiency in extracting total anthocyanins followed the order acidified methanol > 0.1% TFA in 70% methanol > 7% acetic acid in 70% methanol > solvent mixture = acidified ethanol > acidified water (**Table 1**). Our results are consistent with those of Metivier et al. (*17*), who found that methanol was more effective than ethanol and water in extracting anthocyanins from grape pomace. Although the solvent mixture extracted the highest level of total phenolics, it extracted the lowest level of total anthocyanins, except for acidified water and ethanol. Presumably the lower percentage of water (20%) in the solvent mixture facilitated the extraction of procyanidins, flavonols, and phenolic acids from the grape skin, but the solvent was less effective in extracting the more polar anthocyanins than the acidified methanol solvents.

Total phenolic levels (111.9–179.4 mg/g) in grape skin extracts were 5–7-fold higher than levels found (17.7–24.9 mg/g) in seed extracts obtained from the same grape variety, whereas ORAC values (3406–4508 μmol of TE/g) of grape skin extracts were 4–5-fold higher (726–983 μmol of TE/g) than those of seed extracts (unpublished data). Anthocyanins are highly concentrated in grape skin, whereas procyanidins are found predominantly in seeds. Our results show that grape skins isolated from the highly pigmented variety A-1575 are a better source of total phenolics and antioxidant capacity than grape seeds, indicating that grape skin extracts show great promise as an antioxidant-rich nutraceutical ingredient.

Compared to other wine grapes, A-1575 is exceptionally rich in total anthocyanins. Total anthocyanin content of the six solvent extracts ranged from 17 to 23.2 mg/g of fresh weight of grape skin (58.8% moisture), which is much higher than literature values of Pinot Noir (0.75–0.80 mg/g), Cabernet Franc (0.83–1.05), Merlot (0.98–1.04), and Cabernet Sauvignon (0.79–1.14 mg/g) grape skins (*19*, *32*). These results indicate that A-1575 is a promising breeding line that may be used by plant breeders to genetically modify wine grapes for anthocyanin and color enhancement.

Figure 1 shows HPLC chromatograms of skin extracts obtained by acidified water (A), acidified methanol (B), and the solvent mixture (C). Chromatograms from the other three solvent extracts were similar to that of acidified methanol (data
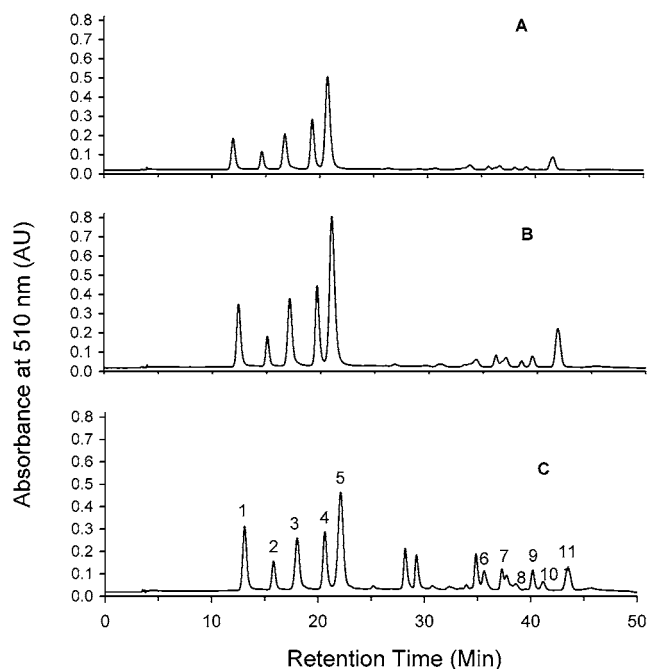
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Figure 1. HPLC chromatograms of anthocyanin extracts from grape skin using PLE extraction at 50 °C with (A) 0.1% HCl in water, (B) 0.1% HCl in 60% methanol, or (C) 0.1% HCl in 40:40:20 (methanol/acetone/water). Peaks: 1, Dpd 3-glucoside (m/z total/aglycon 465/303); 2, Cyd 3-glucoside (m/z total/aglycon 449/287); 3, Ptd 3-glucoside (m/z total/aglycon 479/317); 4, Pnd 3-glucoside (m/z total/aglycon 463/301); 5, Mvd 3-glucoside (m/z total/aglycon 493/331); 6, Mvd 3-acetylglucoside (m/z total/aglycon 535/331); 7, Dpd 3-(p-coumaroyl) glucoside (m/z total aglycon 611/303); 8, Mvd 3(p-caffeoyl)glucoside (m/z total aglycon 655/331); 9, Cyd 3-(p-coumaroyl) glucoside (m/z total aglycon 595/287); 10, Ptd 3-(p-coumaroyl) glucoside (m/z total aglycon 625/317); 11, Mvd 3-(p-coumaroyl) glucoside (m/z total aglycon 639/331).

not shown). Eleven anthocyanin peaks were identified by LC-MS according to published m/z values (*33–36*). The chromatograms clearly show that the monoglucosides of Dpd, Cyd, Ptd, Pnd, and Mvd were the predominant anthocyanins in A-1575, with minor amounts of acylated derivatives present.

Generally, acidified methanol was the most efficient solvent for extracting most anthocyanin monoglucosides except for Cyd 3-glucoside, which was best extracted by the solvent mixture (**Table 1**). Adding the contents of the five individual anthocyanin monoglucosides, solvent efficiency in extracting total anthocyanin monoglucosides followed the order acidified methanol > 0.1% TFA in 70% methanol > 7% acetic acid in 70% methanol > acidified ethanol > solvent mixture = acidified water. The total monoglucoside content of the six solvent extracts accounted for 70.9–83.3% of total anthocyanins, which compares favorably with literature values for Syrah (57.0%), Cabernet Sauvignon (65.3%), and Grenache (84.2%) grapes (*37*).

Solvent efficiency in extracting levels of total acylated anthocyanins followed the order solvent mixture > acidified methanol = 7% acetic acid in 70% methanol = 0.1% TFA in 70% methanol > acidified ethanol > acidified water (**Table 1**). Total acylated anthocyanin content of the solvent mixture extract accounted for 24.1% of total anthocyanins, which was much higher than levels found for total acylated anthocyanins (16.6–22.6%) in the other solvent extracts.

Differences in extraction efficiency for anthocyanin monoglucosides and acylated anthocyanins observed among solvents were most likely due to differences in solvent polarity, which

is reflected by the ratio of total monglucosides to total acylated anthocyanins extracted. Water and solvents containing the highest percentage of water extracted a higher ratio of monoglucosides to acylated anthocyanins, whereas the solvent mixture, which contained only 20% water, extracted a lower ratio of monoglucosides to acylated anthocyanins.

Optimizing PLE Extraction Temperature. Anthocyanins and other phenolics were extracted from grape skins by PLE at varying temperatures (20–140 °C) using acidified water and acidified methanol as solvents (pH 2.4) (**Table 2**). When acidified water was used as solvent, maximum extraction of total anthocyanins occurred at 80–100 °C, corresponding with maximum extraction of total phenolics, whereas extracts obtained at 80 °C had the highest ORAC value. Total anthocyanins were degraded at temperatures >100 °C, indicating that 100 °C is the optimum PLE temperature for isolating anthocyanins using acidified water. Soxhlet extraction with acidified water at 60 or 100 °C was much less effective than PLE in extracting phenolics and anthocyanins from grape skin. Although Soxhlet extracts obtained at 100 °C had higher levels of total phenolics and total anthocyanins than extracts obtained at 60 °C, the levels of total phenolics and anthocyanins were 2- and 3.4-fold lower than those obtained by PLE at 60 and 100 °C. Greater recovery of total phenolics with hot water extraction is consistent with results obtained by Kahkonen et al. (*38*), who reported that boiling water (refluxing) was more effective than room temperature water in extracting total phenolics in apple, cowberry, and bilberry. However, they also found that boiling water was less efficient than room temperature water in extracting anthocyanins from berries, which contrasts with our results obtained by Soxhlet and PLE extractions at 100 °C. The contrasting results obtained between the two studies may be attributed to differences in extraction time and sample matrices. Improved anthocyanin extraction from grape skin at elevated PLE temperatures was probably due to pressurized extraction conditions, including oxygen exclusion.

When acidified 60% methanol was used as PLE solvent, the highest recovery of anthocyanins occurred at 60 °C, with less observed at higher temperatures. The highest recovery of total phenolics occurred at 120 °C, whereas ORAC values were highest over the temperature range of 100–120 °C. The discrepancy between loss of anthocyanins at elevated extraction temperatures and the increased recovery of total phenolics may be explained by the greater extraction of more heat stable procyanidins and phenolic acids from grape skin (*37, 39*). Soxhlet extraction with acidified 60% methanol at 60 and 100 °C did not affect recovery of total phenolics or anthocyanins, but the extracts obtained at 100 °C had a lower ORAC value. Soxhlet extraction was less effective in extracting total phenolics compared to PLE, which is reflected by the low ORAC values obtained for Soxhlet extracts. The Soxhlet apparatus extracted a lower level of total anthocyanins at 60 °C compared to PLE at 60 °C, but it extracted a higher level of total anthocyanins at 100 °C compared to PLE at 100 °C.

Interestingly, high-temperature PLE extraction with acidified water resulted in comparable recoveries of total phenolics, total anthocyanins, and ORAC at 80 °C compared to acidified 60% methanol at 120, 60, and 120 °C, respectively. These results contrast with those obtained at 50 °C (**Table 1**), at which acidified methanol was much more effective than acidified water in extracting total phenolics and anthocyanins. Enhanced extraction of total phenolics and anthocyanins at 80–100 °C by acidified water under PLE conditions was probably related to (1) greater disruption of the grape skin matrix, which allowed

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Table 2. Total Phenolics, Total Anthocyanins, Antioxidant Capacity (ORAC), and Relative Amounts of the Main Groups of Anthocyanins in Grape Skin Extracts As Affected by PLE[a] and Soxhlet Extraction Temperature and Two Solvents

temp (°C)	total phenolics[b]	total anthocyanins[c]	ORAC[d]	anthocyanin monoglucosides[e]						total acylated anthocyanins[f]	ratio of total monoglucosides to total acylated
				Dpd	Cyd	Ptd	Pnd	Mvd	total		
						Acidified Water/PLE Extraction					
20	103.1c[g]	33.35c	2772cd	5.30c4	1.12c	5.95c	2.83c	13.68c	28.98c	4.37c	6.63g
40	106.3c	41.73e	2967d	6.52d	1.27c	7.42d	3.36d	16.76d	35.33d	6.40d	5.52f
60	128.2d	49.68f	3244e	7.65e	1.46d	8.75e	3.74d	18.89e	40.49e	9.19f	4.41d
80	179.9i	57.53i	4708j	8.49fg	1.69e	10.00f	4.27e	21.36f	45.80g	11.73g	3.90c
100	173.7hi	58.52i	4599i	9.02g	1.71e	10.20f	4.33e	21.81f	47.07h	11.45g	4.11c
120	169.3g	51.57f	4592i	8.30f	1.47d	9.42ef	3.84d	19.21e	42.24f	9.33f	4.53d
140	168.7g	36.85d	3683f	5.89cd	1.10c	6.41cd	2.71c	13.21c	29.32c	7.53e	3.89c
						Acidified Water/Soxhlet Extraction					
60	64.3a	14.46a	873a	1.93a	0.48a	2.21a	1.26a	6.44a	12.32a	2.14a	5.76f
100	75.6b	17.24b	949b	2.48b	0.54b	2.71b	1.42b	7.23b	14.38b	2.86b	5.02e
						Acidified 60% Methanol/PLE Extraction					
20	148.4e	51.64f	4139g	7.61e	1.40d	8.97e	3.70d	19.21e	40.88e	10.76g	3.80bc
40	156.7f	56.30h	4444h	8.37f	1.49d	9.85ef	3.14cd	20.51ef	44.16g	12.14h	3.64b
60	167.8g	58.04i	4406h	9.01g	1.58d	10.33f	4.12e	21.17f	46.21h	11.83gh	3.91c
80	172.9h	53.33g	4422h	8.24f	1.45d	9.47ef	3.72d	19.17e	42.05ef	11.28g	3.73bc
100	175.1i	51.21f	4590i	7.84e	1.41d	9.10e	3.68d	18.79e	40.82e	10.39fg	3.93c
120	189.5j	42.16e	4617ij	6.60d	1.07c	7.50d	2.65c	15.24d	33.06d	9.10f	3.63b
140	149.8e	34.24d	4160g	5.18c	0.95c	6.02c	2.44c	12.09c	26.68c	7.56e	3.53b
						Acidified 60% Methanol/Soxhlet Extraction					
60	154.1ef	54.22g	2876d	7.45e	1.41d	9.79ef	3.84d	20.47ef	42.96ef	11.26g	3.82bc
100	149.9e	54.56g	2646c	7.84e	1.34cd	9.05e	3.55d	19.12e	40.90e	13.66i	2.99a

[a] Pressurized liquid extraction conditions: pressure, 10.1 MPa; extraction time, 5 min for three cycle times; solvent volume, 50%; nitrogen purge time, 90 s. [b] Data expressed as milligrams of gallic acid equivalents per gram of dry weight. [c] Total anthocyanins were calculated as the sum of monoglucoside and acylated anthocyanins. [d] Data expressed as micromoles of Trolox equivalents per gram of dry weight. [e] Data expressed as milligrams per gram of dry weight of each respective compound; delphinidin 3-glucoside (Dpd), cyanidin 3-glucoside (Cyd), petunidin 3-glucoside (Ptd), peonidin 3-glucoside (Pnd), malvidin 3-glucoside (Mvd). [f] Data expressed as milligrams of malvidin 3-glucoside equivalents per gram of dry weight. [g] Means within a column with similar letters are not significantly different ($P > 0.05$).

for greater solvent contact with phenolics, (2) increased phenolic solubility, (3) faster diffusion rate, (4) increased mass transfer and extraction rate, and (5) reduced solvent viscosity and surface tension (*28*).

The highest recoveries of 3-glucosides of Dpd, Cyd, Ptd, Pnd, and Mvd and total acylated anthocyanins occurred at 80–100 °C using acidified water as PLE solvent (**Table 2**). Soxhlet extracts obtained by acidified water at 100 °C had higher levels of total monoglucosides and acylated anthocyanins than extracts obtained at 60 °C, but the levels were 3–4-fold lower than those obtained by PLE at 60 and 100 °C. The ratio of total monoglucosides to total acylated anthocyanins decreased from 20 to 100 °C, indicating that high-temperature water was more effective in extracting the more nonpolar acylated anthocyanins. The greater extraction of acylated anthocyanins by pressurized hot water may be due to a reduction in the dielectric constant of water, which decreased its polarity and facilitated extraction of more nonpolar anthocyanins (*40*). Extraction temperatures >100 °C resulted in anthocyanin degradation, which was especially marked at 140 °C. Degradation of anthocyanins at elevated PLE temperatures was confirmed by measuring the color indices of the extracts (**Table 3**). Color density and polymeric color values increased readily with increased extraction temperature, especially at temperatures >100 °C, resulting in greater contribution of polymers to color, indicating that extensive degradation of anthocyanins occurred at high extraction temperatures. The effect of heat on the stability of anthocyanins is well-known. Simpson et al. (*41*) suggested that thermal degradation of anthocyanins could occur via two mechanisms: (1) hydrolysis of the 3-glycoside linkage to form the more labile aglycon and (2) hydrolytic opening of the pyrilium ring to form a substituted chalcone, which degrades to a brown insoluble compound of a polyphenolic nature. On the basis of our HPLC profiles in which anthocyanin glucosides

Table 3. Color Indices of Grape Skin Extracts As Affected by PLE[a] and Soxhlet Extraction Temperature and Two Solvents

temp (°C)	color density index	polymeric color index	% polymeric color
	Acidified Water[b]/PLE Extraction		
20	9.6c[d]	1.3a	13.1a
40	14.9d	2.1b	13.8ab
60	19.0e	2.8c	14.8b
80	18.6e	3.1c	16.8c
100	22.7g	4.7e	20.9d
120	21.9fg	6.1f	27.7f
140	20.6f	7.6g	36.9h
	Acidified Water[b]/Soxhlet Extraction		
60	8.1a	1.2a	15.3b
100	8.3b	1.3a	15.5b
	Acidified 60% Methanol[c]/PLE Extraction		
20	20.2f	3.9d	19.2d
40	25.0h	5.0e	19.9d
60	25.2h	5.4e	21.3e
80	26.9hi	7.1g	26.2f
100	27.1hi	9.1h	33.7g
120	27.9i	9.5hi	34.1g
140	26.5hi	9.9i	37.7h
	Acidified 60% Methanol/Soxhlet Extraction		
60	35.4j	6.8g	19.2d
100	37.3k	7.4g	19.8d

[a] Pressurized liquid extraction conditions: pressure, 10.1 MPa; extraction time, 5 min for three cycle times; solvent volume, 50%; nitrogen purge time, 90 s. [b] Deionized water + 0.1% HCl. [c] 60% methanol + 0.1% HCl. [d] Means within a column with similar letters are not significantly different ($P > 0.05$).

were not converted to aglycons under high-temperature PLE conditions (**Figure 2A**) and color data showing increased amounts of polymeric color with increased extraction temperature, it appears that the latter mechanism was responsible for anthocyanin degradation.
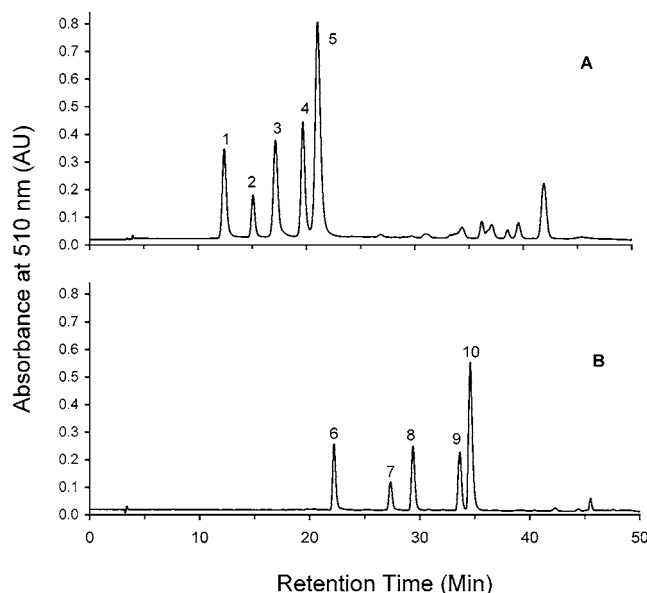
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Figure 2. HPLC chromatograms of (A) acidified 60% methanol extract obtained by PLE at 100 °C for 15 min amd (B) acidified 60% methanol extract obtained by PLE at 100 °C for 15 min and then hydrolyzed in boiling 2 N HCl for 1 h. Corresponding peaks: 1, Dpd 3-glucoside; 2, Cyd 3-glucoside; 3, Ptd 3-glucoside; 4, Pnd 3-glucoside; 5, Mvd 3-glucoside; 6, Dpd; 7, Cyd; 8, Ptd; 9, Pnd; 10, Mvd.

Using acidified 60% methanol as PLE solvent, the highest recovery of total monoglucosides occurred at 60 °C, whereas the highest recovery of total acylated anthocyanins occurred at 40−60 °C (**Table 2**). Soxhlet extracts obtained by acidified 60% methanol at 60 and 100 °C had similar levels of total monglucosides, but extracts obtained at 100 °C had higher levels of acylated anthocyanins than extracts obtained at 60 °C. The Soxhlet apparatus extracted lower levels of total monoglucosides than PLE at 60 °C but extracted higher levels of acylated anthocyanins at 100 °C compared with PLE. In contrast to the results obtained with acidified water as solvent, the ratio of total monoglucosides to total acylated anthocyanins did not change appreciably with increased extraction temperature using acidified 60% methanol as solvent, and PLE extraction temperatures >60 °C resulted in rapid anthocyanin degradation. Anthocyanin degradation in acidified methanol extracts at elevated extraction temperatures was also confirmed by color index results (**Table 3**). Although color density values did not change appreciably over the temperature range of 40−140 °C, polymeric and percent polymeric color values increased at extraction temperatures >60 °C, which paralleled anthocyanin degradation in the extracts. Color density, polymeric color, and percent polymeric color values of acidified methanol extracts were much higher than those obtained for acidified water extracts, indicating that anthocyanins were more susceptible to polymerization and degradation when extracted with methanol.

To confirm that the concentration of 0.1% HCl added to extraction solvents was not high enough to catalyze hydrolysis of anthocyanin glucosides during PLE, the anthocyanin profile of an acidified methanol extract obtained by PLE extraction at 100 °C for 15 min (**Figure 2A**) was compared to the anthocyanin profile of the same extract after boiling for 60 min in 2 N HCl (**Figure 2B**). The chromatograms show that no anthocyanidin peaks were present in the acidified methanol extract obtained by PLE extraction at 100 °C for 15 min and that each of the anthocyanidin peaks in hydrolyzed grape extract came from two sources, the corresponding anthocyanin monogluco-

sides and acylated anthocyanins. The five anthocyanidins eluted at higher retention times than those of the corresponding anthocyanins, but in the same order.

Conclusions. A-1575 is an anthocyanin-rich breeding line, with skin containing up to 56.4 mg/g (dry weight) total anthocyanins. Solvent type affected both the quantity and composition of anthocyanins extracted by PLE. Acidified 60% methanol extracted the highest levels of total monoglucosides and total anthocyanins, whereas the solvent mixture extracted the highest levels of total acylated anthocyanins and total phenolics. Optimum PLE temperatures for maximum recovery of total anthocyanins were 80−100 °C for acidified water and 60 °C for acidified 60% methanol. High-temperature PLE using acidified water, an inexpensive and environmentally friendly solvent, appears to be effective for isolating anthocyanins from grape skins.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ORAC, oxygen radical absorbing capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; PLE, pressurized liquid extraction; HCl, hydrochloric acid; TFA, trifluoroacetic acid; Dpd, delphinidin; Cyd, cyanidin; Ptd, petunidin; Pnd, peonidin; Mvd, malvidin.

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