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ARTICLE

Subcritical Solvent Extraction of Anthocyanins from Dried Red Grape Pomace

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Accelerated solvent extraction (ASE) was used to optimize and determine the effectiveness of an alternative, environmentally friendly extraction procedure using subcritical solvents to recover anthocyanins from freeze-dried, ground Sunbelt red grape pomace. Anthocyanins were extracted from pomace using the following ASE variables: pressure (6.8 MPa), one extraction cycle, and temperature (40, 60, 80, 100, 120, and 140 °C). Conventional solvent extraction with methanol/water/formic acid (60:37:3 v/v/v) was compared to four hydroethanolic solvents (10, 30, 50, and 70% ethanol in water, v/v). Anthocyanins in the extracts were identified and quantified by HPLC-MS and HPLC. There was an insignificant interaction between solvent and temperature (p = 0.0663). Solvents containing 70 and 50% ethanol in water extracted more total anthocyanins (463 and 455 mg/100 g of DW, respectively) than other solvents. The total amounts of anthocyanins extracted at 100 °C (450 mg/100 g of DW), 80 °C (436 mg/100 g of DW), and 120 °C (411 mg/100 g of DW) were higher than at the other temperatures. Solvents containing 70 and 50% ethanol in water extracted similar amounts of anthocyanins as conventional extraction solvent.

KEYWORDS: Anthocyanin; extraction; grape; polyphenolic; pomace; subcritical fluid

INTRODUCTION

Grape pomace consists of the skin, stems, and seeds of grapes that remain after processing in the wine and juice industry. Ten million tons of grape pomace was produced in 2005 from 66 million tons of harvested grapes (*Vitis vinifera* L.) (*1*). Much of this pomace was discarded as natural waste, used as a residual sugar source for secondary fermentation to ethanol, or utilized as animal feed or compost (*2*). Grape pomace typically retains polyphenolics after juicing, with as much as 20−30% of the total phenolics in the skins and 60−70% of phenolics found in the seeds (*3*). Interest in extracting anthocyanins from grape pomace has arisen due to their numerous health-benefiting properties (oxidative stress reduction, free radical scavenging properties, assisting in cancer and disease risk reduction, as well as cholesterol regulation) (*4*). In addition, anthocyanin-containing extracts have potential as natural colorants.

Anthocyanins are naturally occurring phenolic compounds called flavonoids, which consist of three phenolic rings with glycoside substitutions in the 3- and 5-positions of the flavan structure (**Figure 1**) (*5*). Anthocyanins are well-known for the red, blue, purple, and violet pigments they impart to fruits and vegetables (*6*). Anthocyanins have been extracted from grape pomace using a combination of acids, methanol, acetone, and chloroform (*6*, *7*), some of which are toxic, expensive, and environmentally hazardous. In addition, the extracted anthocyanins must undergo detoxification before incorporation into food products by filtering, desulfurizing, and concentrating the extracts by vacuum evaporation (*2*).

Extraction processes using generally recognized as safe (GRAS) solvents (i.e., water and ethanol) have been investigated for their effectiveness in comparison to extractions using acids, methanol, acetone, and chloroform. Previous studies have used ethanol and water mixtures to extract anthocyanins from wine grapes employing various concentrations above 50% ethanol in water (v/v) (*8*−*11*); however, no optimal ethanol concentration has been reported for extracting anthocyanins from table grapes, which vary significantly from wine grapes in anthocyanin composition (*12*). Other techniques for extracting anthocyanins from grape pomace include ultrasonication, application of high hydrostatic pressure, pulsed electric fields (*13*), and accelerated solvent extraction (ASE).

ASE is also known as pressurized liquid extraction (PLE), and both use solvents at increased temperature and pressure to increase the speed and efficiency of the extraction. Increasing temperature improves anthocyanin extraction by increasing the solute diffusion rate, accelerating mass transfer, solubilizing anthocyanins into the solvents, and reducing solute−matrix interactions. Also, increasing extraction pressure improves contact between the sample and extraction solvent, thereby facilitating solvent penetration into matrices such as grape pomace (*14*).

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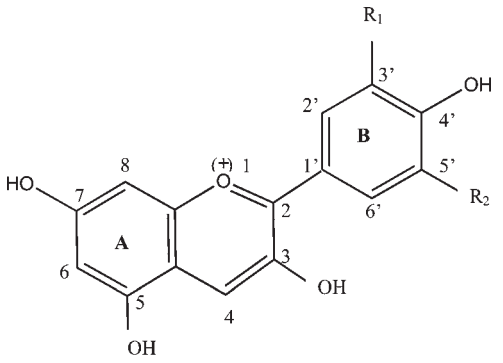

Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1. Structures of six naturally occurring anthocyanidins (no sugar attached at the 3-position) with A and B aromatic rings and R_1 and R_2 substitution sites.

ASE technology under subcritical conditions therefore can improve extraction efficiency of anthocyanins from grape pomace. Subcritical water, also called pressurized low-polarity water, is water heated above its boiling point (100 °C), but below its critical point (374 °C). These conditions allow water to remain in a liquid state due to the applied pressure. In comparison to ambient water, subcritical water acts similarly to organic solvents because of its decreased polarity, surface tension, and disassociation constant (*15*). Benefits of this "green" extraction technology include decreased energy costs and increased speed of extraction (*15*−*17*). Recently, subcritical water extraction has effectively been used to recover anthocyanins from red grape pomace (*18*−*20*) and red cabbage (*21*).

Although many novel environmentally benign extraction technologies have been reported using superheated solvents with high pressure and GRAS solvents, no study has determined an optimal GRAS solvent and temperature combination to extract anthocyanins from table grape pomace. The objective of this study was to optimize the selection of solvent composition and temperature conditions for extracting anthocyanins from Sunbelt (*Vitis labrusca* L.) red grape pomace using subcritical solvents and an ASE system. Sunbelt grapes were developed by the University of Arkansas and are a large blue table (juice) grape similar to Concord (*Vitis labrusca* L.) but developed to withstand and ripen evenly in warmer southern climates (*22*).

MATERIALS AND METHODS

Samples and Chemicals. Sunbelt grapes (*V. labrusca* L.) (*22*) were harvested, crushed, and destemmed at the University of Arkansas' Agricultural Experimental Station Farm (Fayetteville, AR) in 2006. The must was then pressed in a 70 L Enrossi bladder press (Enoagricol Rossi s. r.l., Calzolaro, Italy) at 4 bar and cooled immediately. The pomace (seeds and skins) was recovered, placed into plastic freezer bags, sealed, and stored at −20 °C. We used whole pomace in the experiment and did not separate seeds and skins because we wanted to simulate commercial conditions. The frozen grape pomace was removed from storage bags and freeze-dried with a VirTis Genesis freeze-drier (Gardiner, NY). Freeze-dried pomace was then ground to a homogeneous fine powder (500-μm) using an Udy Cyclone Sample Mill (Fort Collins, CO). The pomace powder was stored at −70 °C in a ThermoScientific Ultra-Low Freezer (Waltham, MA) until used for extraction and analyses.

Anthocyanin standards of the 3-monoglucosides of delphinidin (Dpd), cyanidin (Cyd), petunidin (Ptd), pelargonidin (Pgd), peonidin (Pnd), and malvidin (Mvd) were 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 (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). HPLC-grade methanol, ethanol, and acetone and analytical-grade formic and acetic acids were acquired from EMD Chemicals Inc. (Gibbstown, NJ).

Anthocyanin Extraction. A Dionex model ASE 200 equipped with a solvent controller (Dionex Corp., Sunnyvale, CA) was used to extract anthocyanins from ground grape pomace. A 0.50 g sample of grape pomace was loaded into a 22 mL extraction cell with an inserted cellulose paper filter at the bottom of the cell. The ASE experimental variables were 6.8 MPa pressure, one extraction cycle, 70% flush volume, 90 s nitrogen purge time, 0 min static time, and 0 min preheat time. After extraction, the final sample volume was adjusted to 50 mL with deionized water. A Beckman GS-15R centrifuge (Beckman Coulter Inc., Fullerton, CA) was used to immediately centrifuge samples for 10 min at 7012*g* to remove insoluble solids in the samples extracted by the ASE. The supernatant was recovered and stored at −20 °C.

Solvent and Temperature Optimization. Four hydroethanolic solvents (10, 30, 50, and 70% ethanol in water, v/v) and six temperatures (40, 60, 80, 100, 120, and 140 °C) were used on the ASE to optimize the extraction of anthocyanins from ground grape pomace. Each extraction was performed in triplicate.

Conventional Extraction. Conventional extraction of anthocyanins from ground grape pomace was used for comparison as a standard to determine the efficiency of the ASE extractions. The method of Hager et al. (*23*) was used for this purpose. Briefly, 2 g of ground grape pomace plus 20 mL of methanol/water/formic acid (60:37:3, v/v/v) was homogenized at ambient temperature (23.5 ± 1.5 °C) for 30 s with an Ika T18 Ultra-Turrax tissuemizer (Wilmington, NC). The homogenate was filtered through Miracloth (Calbiochem, San Diego, CA), and the filtrate was collected. The residue was isolated, and the extraction was repeated twice with 20 mL of extraction solvent. The filtrates were pooled and adjusted to 100 mL with the extraction solvent. The extract was immediately centrifuged similarly to the ASE-derived extracts for 10 min at 7012*g* to remove insoluble solids, and the supernatant was collected for analysis and stored at −20 °C. These conventional extractions were performed in triplicate.

Anthocyanin Analysis by HPLC. Anthocyanins were analyzed according to a modified method of Cho et al. (*24*) using a Waters Alliance model 2690 HPLC system (Waters Corp., Milford, MA) equipped with an autosampler and a Waters model 996 photodiode array detector. Unconcentrated ASE extracts were passed through a 0.45 μm PTFE filter (Varian, Inc., Palo Alto, CA), and 50 μL was injected onto a 250 × 4.6 mm Waters Symmetry C_{18} column (Waters Corp., Milford, MA). The two mobile phases forming the mobile phase gradient consisted of (A) 5% formic acid/water and (B) 100% methanol. The gradient system started with 98% A, was changed to 40% A at 60 min, and then switched back to 98% A at 65 min, at which it remained isocratic until the run ended. The entire HPLC run time was 90 min with a flow rate of 1.0 mL/min. Anthocyanin peaks were detected at 510 nm and were identified by comparison with the retention times of a standard grape pomace extract analyzed using HPLC-MS. Individual anthocyanin derivatives were quantified as Dpd, Cyd, Ptd, Pnd, and Mvd glucoside equivalents, using external calibration curves of each respective anthocyanin standard. Results were expressed as milligrams per 100 g of dry weight (DW).

Anthocyanin Analysis by HPLC-MS. HPLC-MS was used to identify each anthocyanin peak in HPLC chromatograms as described by Cho et al. (*24*). Anthocyanin samples were prepared in the same way as for HPLC analysis. A Hewlett-Packard 1100 series HPLC (Agilent Technologies, Wilmington, DE) equipped with an autosampler, binary HPLC pump, and UV−vis detector was used in the HPLC analysis. The same gradient system was used as stated above for the HPLC analysis of the anthocyanins with UV detection at 510 nm. The HPLC system was interfaced with a Bruker Esquire LC-MS (Billerica, MA) ion trap mass spectrometer, and data were collected at 510 nm with the accompanying LC-MS software, using positive ion electrospray mode with a capillary voltage of 4000 V, a nebulizing pressure of 0.21 MPa, a drying gas flow rate of 9.0 mL/min, and a temperature of 300 °C. Data were collected over the mass range of m/z 50 through 800 in full scan mode at 1.0 s/cycle (*24*).

Antioxidant Capacity. Oxygen radical absorbance capacity ($ORAC_{FL}$) analysis of the extracts followed the method of Prior et al. (*25*) using fluorescein as fluorescent probe. The grape pomace extracts were diluted 200-fold with a phosphate buffer (pH 7) prior to the $ORAC_{FL}$ analysis. Results were expressed as micromoles of Trolox equivalents per gram of dry weight (*24*).

Experimental Design. The experimental design was a four by six full factorial treatment completely randomized design with three replications. There were four solvents (10, 30, 50, and 70% ethanol in water, v/v) and six temperatures (40, 60, 80, 100, 120, and 140 °C) with every sample tested at every level of the variables. The linear statistical model used for the analysis was

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

$$\text{with } i = 1,\ 2,\ ...,\ 4;\ j = 1,\ 2,\ ...,\ 6;\ \text{and } k = 1,\ 2,\ 3$$

where Y_{ijk} is the observed measured response of the kth replication of the ith solvent on the jth temperature, μ is the overall population average response, α_i is the ith solvent main effect effect ($\sum_{i=1}^{4} \alpha_i = 0$), β_j is the jth temperature main effect ($\sum_{j=1}^{6} \beta_j = 0$), $(\alpha\beta)_{ij}$ is the ijth interaction effect of solvent by temperature [$\sum_{j=1}^{6} (\alpha\beta)_{ij} = 0\ \forall i$ and $\sum_{i=1}^{6} (\alpha\beta)_{ij} = 0\ \forall j$], and $e_{ijk} \overset{iid}{\sim} N(0,\sigma^2)$ is the unobserved ijkth error random effect. The errors are assumed to be independent, identically, and normally distributed with mean zero and common variance σ^2. The general linear model for this two-way ANOVA with interaction factorial experiment was fitted for each response with JMP 8 software (Cary, NC). Significance is reported when model effects p values are smaller than the 5% significance level. Significant differences, between treatment means, interaction effects, and main effects are reported and examined using the LSMeans of the fitted model.

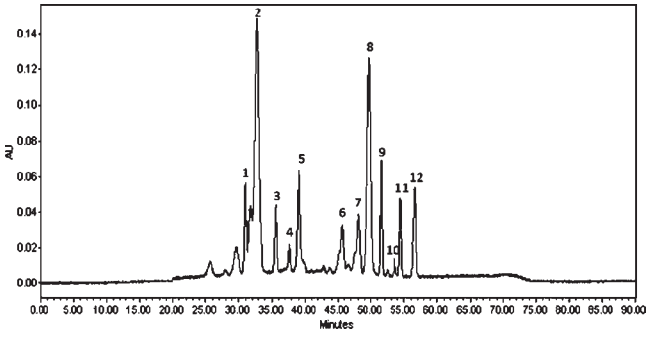

Figure 2. Representative HPLC chromatogram of Sunbelt red grape pomace anthocyanins extracted by ASE using 50% ethanol in water (v/v) at 80 °C. Twelve peaks were identified by HPLC-MS (**Table 1**).

Because the two factors in this research, solvent and temperature, were quantitative with levels to address the overall form of the relationship of each factor and their interactions on each response, we also fitted a second-order response surface regression model that approximated well enough the two-way ANOVA model described above. This approach allows us to better describe, understand, and display visually the form of each factor effects with the aid of JMP's prediction profiler. JMP profiler output helps visualize the predicted values of each response at the optimal setting that happens to maximize all responses simultaneously with the highest desirability.

RESULTS AND DISCUSSION

Anthocyanin Identification. Anthocyanins eluted from the HPLC C_{18} column in order of decreasing polarity (**Figure 2**). Twelve individual anthocyanin peaks were tentatively identified in the Sunbelt grape pomace by HPLC-MS (**Table 1**). The two largest peaks of the HPLC-MS chromatogram were peaks 2 and 8, or Mvd-3,5-*O*-diglucoside and Pnd-3-(6-*O*-coumaroyl)-5-*O*-diglucoside, which coeluted with Mvd-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside, respectively. Malvidin diglucosides were the most prominent in the red grape pomace samples. Of the limited literature on Sunbelt grapes, no other studies have looked at the composition of anthocyanins by HPLC, and therefore there are no data with which to compare our results (*26*, *27*). For table grapes, previous studies identified anthocyanin compositions and found mainly 3-monoglucoside derivatives (*28*, *29*), whereas we found many diglucosides in the Sunbelt grape pomace. Similarly, wine grapes contain mainly 3-monoglucosides (*30*). Previous studies indicated table grape anthocyanins were acylated with coumaric, acetic, or caffeic acids (*28*, *29*); similarly, Sunbelt grapes were acylated with coumaric and acetic acids.

In contrast to previously characterized table and wine grapes, Sunbelt red grape pomace contained acylated diglucosides, which are known to be more stable than the more commonly found mono- and diglucosides (*31*). Because Sunbelt is a hybrid of Concord and an unknown father (pollen) and was bred to be more stable in hotter climates, it is possible the high levels of diglucosides came from muscadine or another cultivar with higher levels of diglucosides. Anthocyanin composition in grapes is mainly influenced by genetics, but anthocyanin content can be influenced by maturation and by different seasonal, environmental, and soil conditions (*28*).

Solvent and Temperature Optimization. When the solvent and temperature extraction efficiencies for individual anthocyanins were analyzed, there was not one ideal solvent or temperature due to the structural complexity of each anthocyanin compound. Overall, determining an optimal set of conditions for all compounds

Table 1. Peak Assignments, Retention Times (RT), and Mass Spectral Data of Anthocyanins Detected in Extracts of Sunbelt Red Grape Pomace

			(*m*/*z*) values	
peak	HPLC RT (min)	anthocyanin identification	M^+	fragments
1	29.2	peonidin-3,5-*O*-diglucoside	625	463, 301
2	31.2	malvidin-3,5-*O*-diglucoside	655	493, 331
3	33.8	petunidin-3-*O*-monoglucoside	479	317
4	35.8	peonidin-3-*O*-monglucoside +	463	301
		petunidin-3-*O*-(6-acetyl)-5-*O*-diglucoside	683	317
5	37.4	malvidin-3-*O*-(3-acetyl)-5-*O*-diglucoside	697	535, 493, 331
6	44.3	delphinidin-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	773	611, 465, 303
7	46.8	petunidin-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	787	625, 479, 317
8	48.4	peonidin-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside +	771	609, 463, 301
		malvidin-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside	801	639, 493, 331
9	50.2	delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside	611	303
10	52.1	cyanidin-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside	595	287
11	53.3	petunidin-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside	625	317
12	55.5	malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside	639	331

Table 2. Concentrations (Milligrams per 100 g of DW) of Anthocyanins in Grape Pomace Extracts As Affected by ASE Extraction Temperature and Ethanol/Water (v/v) Solvent Mixtures[a]

		peak												
solvent[b]	temp (°C)	1	2	3	4	5	6	7	8	9	10	11	12	total
10% ethanol	40	ND	211.04 ± 7.58	ND	ND	11.65 ± 0.86	5.90 ± 2.44	ND	93.03 ± 2.84	ND	ND	ND	ND	312.91 ± 6.96
	60	ND	191.35 ± 28.32	ND	ND	11.39 ± 4.47	10.14 ± 3.67	ND	88.83 ± 15.63	0.02 ± 3.05	ND	ND	3.09 ± 3.09	304.82 ± 57.91
	80	ND	230.34 ± 34.70	ND	ND	16.72 ± 5.22	23.63 ± 1.97	ND	118.04 ± 17.63	11.18 ± 3.07	ND	ND	12.48 ± 3.58	412.39 ± 64.78
	100	ND	217.77 ± 7.72	ND	ND	21.22 ± 2.25	19.77 ± 3.31	ND	109.65 ± 7.36	9.68 ± 1.41	ND	ND	8.97 ± 1.90	387.05 ± 20.94
	120	ND	179.61 ± 31.72	ND	ND	6.97 ± 4.78	9.01 ± 4.87	ND	84.53 ± 19.41	4.95 ± 5.59	ND	ND	3.64 ± 3.64	288.71 ± 69.65
	140	ND	124.09 ± 0.80	ND	ND	5.52 ± 0.51	5.52 ± 0.51	ND	64.74 ± 0.84	0.58 ± 1.40	ND	ND	ND	195.04 ± 2.67
30% ethanol	40	0.07 ± 0.07	234.33 ± 9.33	ND	ND	16.75 ± 1.56	19.10 ± 2.61	ND	120.95 ± 6.01	21.56 ± 4.21	ND	ND	14.59 ± 4.03	427.45 ± 24.99
	60	0.08 ± 0.08	200.01 ± 22.48	ND	ND	11.74 ± 5.06	15.72 ± 2.99	ND	102.17 ± 12.47	16.72 ± 4.13	ND	ND	7.31 ± 4.03	353.74 ± 50.05
	80	5.39 ± 2.46	205.75 ± 18.59	ND	ND	17.78 ± 4.08	16.46 ± 3.88	ND	98.17 ± 10.10	17.45 ± 4.01	ND	ND	7.99 ± 3.50	369.00 ± 46.35
	100	7.38 ± 0.90	238.50 ± 0.57	ND	ND	27.89 ± 1.69	24.90 ± 1.69	ND	116.65 ± 0.76	26.27 ± 0.91	ND	ND	15.18 ± 0.88	456.76 ± 0.10
	120	3.99 ± 2.02	228.93 ± 2.70	ND	ND	29.17 ± 2.94	21.61 ± 2.61	ND	113.48 ± 0.25	23.07 ± 1.08	ND	ND	13.98 ± 1.27	434.24 ± 3.71
	140	1.24 ± 1.24	191.73 ± 26.13	ND	ND	22.40 ± 5.66	17.30 ± 5.49	ND	94.83 ± 14.18	17.55 ± 6.41	ND	ND	6.85 ± 4.49	351.89 ± 62.85
50% ethanol	40	4.11 ± 2.30	198.65 ± 14.05	ND	ND	23.83 ± 3.69	28.45 ± 3.62	ND	111.55 ± 8.89	26.43 ± 3.36	ND	ND	10.70 ± 2.62	403.72 ± 32.72
	60	1.08 ± 0.59	214.39 ± 6.67	ND	ND	25.45 ± 1.99	21.75 ± 1.83	ND	120.43 ± 4.53	29.36 ± 1.93	ND	ND	13.00 ± 1.74	425.45 ± 19.10
	80	4.40 ± 1.17	239.32 ± 2.66	ND	ND	32.26 ± 0.43	24.82± 1.01	ND	133.57 ± 1.57	33.32 ± 0.81	ND	ND	15.91 ± 0.51	483.60 ± 3.77
	100	3.51 ± 0.98	249.08 ± 3.56	ND	ND	35.18 ± 1.74	24.68 ± 0.65	0.23 ± 0.16	131.94 ± 3.70	34.82 ± 1.75	ND	ND	17.99 ± 1.27	497.43 ± 13.54
	120	1.32 ± 0.75	232.21 ± 12.62	ND	ND	29.14 ± 3.99	21.80± 3.31	0.72 ± 0.72	126.64 ± 8.35	31.58 ± 3.22	ND	ND	16.97 ± 2.83	460.37 ± 34.86
	140	0.39 ± 0.33	233.21 ± 3.58	ND	ND	29.11 ± 1.10	20.69± 1.91	0.39 ± 0.39	128.66 ± 1.89	29.24 ± 0.95	ND	ND	15.67 ± 0.96	457.36 ± 9.73
70% ethanol	40	4.36 ± 2.08	210.59 ± 14.70	ND	ND	26.42 ± 1.79	30.05 ± 1.6	1.74 ± 1.74	127.66 ± 5.69	30.05 ± 1.68	ND	ND	14.08 ± 2.16	437.06 ± 25.31
	60	5.26 ± 1.61	217.64 ± 8.36	ND	ND	27.74 ± 1.78	24.80 ± 1.22	ND	133.60 ± 1.71	33.71 ± 0.33	ND	ND	15.33 ± 0.79	458.06 ± 9.65
	80	7.60 ± 1.71	230.72 ± 9.56	ND	ND	31.12 ± 0.99	25.06 ± 1.20	ND	135.59 ± 2.01	34.24 ± 0.82	ND	ND	16.18 ± 1.25	480.51 ± 8.78
	100	14.53 ± 1.53	199.95 ± 2.45	ND	ND	27.86 ± 1.58	24.44 ± 0.17	1.70 ± 1.70	137.14 ± 2.26	34.86 ± 1.07	ND	ND	17.43 ± 0.45	457.91 ± 8.62
	120	8.12 ± 4.48	212.82 ± 16.62	ND	ND	28.51 ± 2.40	24.64 ± 1.69	01.6 ± 016	136.68 ± 1.53	33.92 ± 0.96	ND	ND	16.00 ± 0.55	460.83 ± 19.33
	140	13.22 ± 1.01	222.23 ± 15.36	ND	ND	33.67 ± 1.22	25.69 ± 1.00	0.32 ± 0.32	136.92 ± 3.42	33.33 ± 0.92	ND	ND	17.35 ± 0.64	482.73 ± 21.36
conventional[c]	25	12.30 ± 0.38	133.63 ± 5.32	14.19 ± 0.14	7.18 ± 0.203	31.49 ± 0.54	28.49 ± 0.68	30.87 ± 1.49	96.06 ± 3.66	34.54 11.37	2.9 ± 0.31	22.39 ± 0.94	28.83 ± 0.93	442.88 ± 15.29

[a] Mean values ± standard error (n = 3). [b] Ethanolic-based solvents were prepared in various concentrations of ethanol/water (v/v). [c] Conventional solvent was methanol:water:formic acid (60:37:3, v/v/v).

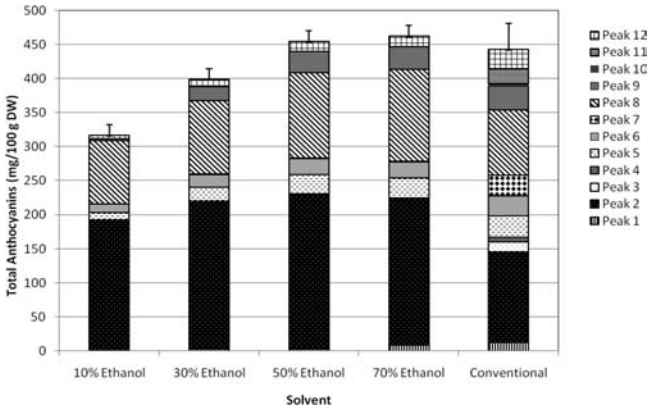

Figure 3. Comparison of anthocyanins extracted from red grape pomace with four hydroethanolic solvents and a conventional solvent. Data were averaged for all temperatures tested (40−140 °C). Results are presented in mg/100 g of dry weight (DW). Bars represent SEM (n = 3).

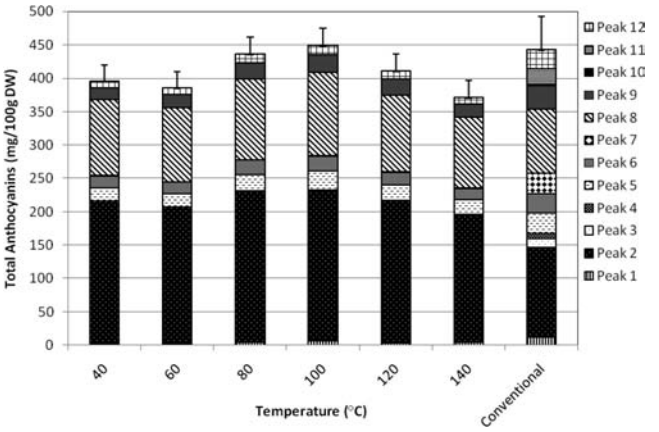

Figure 4. Comparison of anthocyanins extracted from red grape pomace as a function of extraction temperatures. Data were averaged for all solvents evaluated (10, 30, 50, and 70% ethanol in water). Results are presented in mg/100 g of dry weight (DW). Bars represent SEM (n = 3).

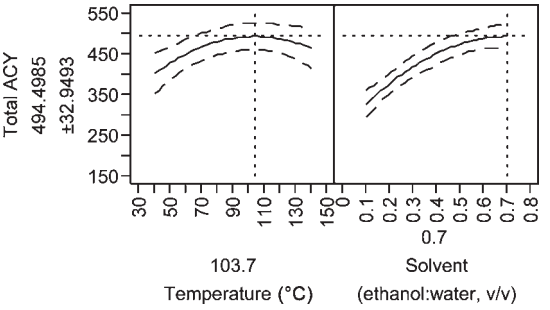

Figure 5. Optimal extraction conditions for total anthocyanins (mg/100 g of DW) shown by a response surface regression method.

in a sample is practically impossible due to the various polarities and thermal stabilities of these compounds. Therefore, the results of these studies are based on total anthocyanins from the summation of all 12 peaks in the HPLC-MS chromatogram, not the optimal conditions for each compound present in the grape extract. Experimental design data including mean values for all of the individual anthocyanins present in the extracts are presented in **Table 2**.

The solvent and temperature interaction of the extraction of total anthocyanins from ground red grape pomace using the ASE system was insignificant (p = 0.0663), but the main effect of solvent composition was significant (p < 0.0001) (**Figure 3**). The efficacy of the hydroethanolic solvents in terms of their ability to extract anthocyanins followed the order 50% = 70% > 30% > 10% ethanol in water (v/v), indicating higher levels of ethanol (50−70%) were needed to extract the maximum amount of anthocyanins from the pomace under subcritical conditions. These results were similar to previous studies that used 50−95% ethanol in water solvents to extract polyphenolics from wine grapes (*8*−*11*, *32*, *33*). We did not test the extraction efficiency of ethanol/water concentrations >70% because we found in previous studies that there was insufficient water present to hydrate the dried sample and facilitate anthocyanin extraction, thus leading to very poor anthocyanin recovery with >70% ethanol in water solvents.

The effect of temperature on the extraction of anthocyanins from Sunbelt grape pomace was also significant (p = 0.0131) (**Figure 4**). More anthocyanins were extracted at 80, 100, and 120 °C, whereas fewer anthocyanins were extracted at 40, 60, and 140 °C. This optimal temperature range (80−120 °C) to extract anthocyanins is most likely due to two factors. First, adding ethanol to water lowers the boiling point of the solution below 100 °C, and, second, anthocyanins are thermally labile, and lower temperatures minimized their thermal degradation. However, lower temperatures (40−60 °C) yielded a lower amount of anthocyanins because there was probably not enough heat solubility of anthocyanins into the extraction solvent. Of course, thermal degradation of the anthocyanins was also minimized at these lower temperatures.

ASE-derived extraction data were compared to the conventional solvent extraction method with methanol/water/formic acid (60:37:3, v/v/v) to determine the efficacy of ASE extractions. Compared to the conventional method, 70, 50, 30, and 10% ethanol in water extracts contained 105, 103, 90, and 72% of anthocyanins, respectively. The 30, 50, and 70% hydroethanolic extracts contained comparable amounts of total anthocyanins relative to that obtained with the conventional method. Compared to the conventional extraction method, extracts obtained at 100, 80, 120, 40, 60, and 140 °C contained 102, 99, 93, 89, 87, and 84% of anthocyanins, respectively. All ASE extracts collected from 40 to 140 °C contained comparable amounts of total anthocyanins as the conventional extract. These results demonstrate that hot pressurized GRAS solvents were equally as effective as conventional extraction techniques in extracting anthocyanins from grape pomace.

Although the solvent−temperature interaction was insignificant (p = 0.0663), a general trend showed increased extraction of anthocyanins using 50 or 70% ethanol in a temperature range of 80−120 °C. According to the response surface method using regression as described in the experimental design, the optimal extraction condition is 70% ethanol at 103.7 °C (**Figure 5**).

Although total anthocyanin levels extracted with hot, pressurized hydroethanolic solvents were similar in quantity to conventional extraction solvents, the methanol-based non-GRAS conventional solvent recovered a greater diversity of anthocyanins than the heated ethanol-based GRAS solvents (**Figures 3** and **4**). Specifically, methanol-based conventional solvents extracted Ptd-3-*O*-monoglucoside (peak 3), Ptd-3-*O*-monoglucoside co-eluting with Ptd-3-*O*-(6-acetyl)-5-*O*-diglucoside (peak 4), Ptd-3-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside (peak **7**), Cyn-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside (peak 10), and Ptd-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside (peak 11), which were either undetected in the ethanol-based ASE extracts or in very low levels. Because the methanol-based conventional solvent exclusively extracted these anthocyanins, one hypothesis was that methanol

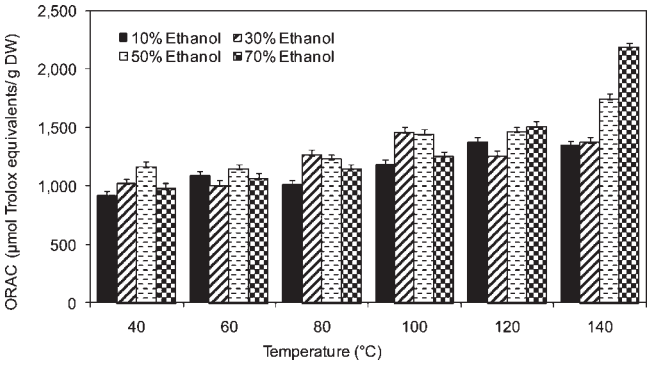

Figure 6. Antioxidant capacity ($ORAC_{FL}$) of red grape pomace extracts obtained by four hydroethanolic solvents at six different extraction temperatures. Results are presented in micromoles of Trolox equivalents (TE) per gram of dry weight (DW). Bars represent SEM ($n = 3$).

was much more specific for solubilizing these anthocyanins (*34*). Another possible explanation is that these compounds are more tightly bound to the cell wall or located in harder to reach vacuolar or cytoplasmic regions and are extracted only with a high-speed homogenization method and not a high-pressure and high-temperature method. To test these hypotheses, we used the conventional extraction method with two solvents, (1) methanol/water/formic (60:37:3, v/v/v) and (2) ethanol/water (50:50, v/v). We found no significant differences in composition or concentrations of anthocyanins in extracts obtained with the two solvents. This suggests the difference in anthocyanins extracted between the ASE and conventional method is due to the extraction technique and not the solvent selectivity for certain anthocyanin compounds. To confirm these results, we ran the ASE method with the conventional solvent, methanol/water/formic acid (60:37:3, v/v/v), and found no differences in anthocyanin composition as when running the ASE with 50% ethanol in water. These results indicate certain bound anthocyanin moieties are released only when using a blending technique, which was employed in conventional extraction that homogenizes the pomace with solvent at high speeds (*34*). ASE conditions may also promote binding of these specific compounds to proteins or other cell wall materials and prevent extraction of anthocyanins using hydroethanolic solvents (*34*).

Antioxidant Capacity. The $ORAC_{FL}$ assay determined the antioxidant capacity (**Figure 6**) of the grape pomace extracts. There was a significant solvent−temperature interaction for $ORAC_{FL}$ ($p < 0.0001$). Generally, $ORAC_{FL}$ values increased with extraction temperatures and ethanol concentration. Anthocyanins extracted from the pomace were most likely the major contributor to the antioxidant capacity of the samples as they are present in abundant quantity and are known as potent antioxidants (*35*, *36*). However, it is possible that other phenolics such as procyanidins, flavonols, and phenolic acids not measured in the study also contributed to antioxidant capacity. Because anthocyanins are potent antioxidants, we anticipated that extracts containing the highest amounts of anthocyanins would have the highest antioxidant capacity. However, the $ORAC_{FL}$ results did not correlate well ($r = 0.2762$) with the optimal solvent and temperature ranges for extracting anthocyanins from red grape pomace. The $ORAC_{FL}$ data showed increased antioxidant capacities with increased temperatures and ethanol concentrations. Theoretically, increasing extraction temperatures could degrade anthocyanins and reduce the antioxidant capacity of the resultant extract. Simpson (*37*) suggested that anthocyanin thermal degradation occurred either by hydrolyzing the 3-glycoside to form an unstable aglycone or by opening the pyrilium ring to form a chalcone. Because our samples browned with increased temperatures, presumably thermal degradation of anthocyanins caused formation of a chalcone, which is known to degrade into a brown insoluble compound (*38*). However, as remarked previously, increasing extraction temperatures yielded extracts with increased antioxidant capacity. One possible explanation for the results is the formation of Maillard reaction products (MRPs) at higher temperatures, which contain potent antioxidant capacity and presumably increased the antioxidant capacity of those extracts obtained at higher temperatures (140 °C). Yilmaz and Toledo (*39*) demonstrated that mixtures of amino acids and a sugar that were heated at 120 °C for 10, 20, and 30 min formed MRPs exhibiting high antioxidant capacity, which parallels the results found in our extraction experiments. Our results are also consistent with a previous study on spinach in which extracts obtained with hydroethanolic solvents at temperatures from 50 to 190 °C had increased ORAC values, which correlated with the induction of sample browning (*40*).

The results from this study indicate that ethanol levels of 50−70% (v/v) are needed to extract the optimal level of anthocyanins from red grape pomace. However, the larger the water percent in the extraction solvent, the more environmentally friendly and inexpensive will be the extraction medium. The use of even lower concentrations of ethanol in the hydrodroethanolic solvents, although reducing the yield, also lowers solvent cost and storage. The results in this study showed that temperatures of 80, 100, or 120 °C extracted more anthocyanins than obtained at the lower and higher temperatures of 40, 60, or 140 °C.

These results can be applied in the juice industries to extract anthocyanins from table grape pomace using a more cost-effective and environmentally friendly solvent. Hence, if the juice industries adopt such a process that extracts anthocyanins using 50% ethanol in water (v/v) solvent between 80 and 120 °C, an economic credit should be realized from what traditionally has been viewed as a waste stream.

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