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ARTICLE

Subcritical Solvent Extraction of Procyanidins from Dried Red Grape Pomace†

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Procyanidins in dried Sunbelt (*Vitis labrusca* L.) red grape pomace were extracted using accelerated solvent extraction (ASE) with pressure (6.8 MPa), one extraction cycle, and temperature (40, 60, 80, 100, 120, and 140 °C). Six ethanol/water solvents (0, 10, 30, 50, 70, and 90%, v/v) were compared to conventional extraction with acetone/water/acetic acid (70:29.5:0.5, v/v/v). Procyanidins in the extracts were identified by HPLC-ESI-MS/MS and contained degrees of polymerization (DP) of 1−5 (monomers through pentamers) and polymers (DP > 10). Generally, 50% ethanol/water (v/v) extracted more total procyanidins than other ethanol/water compositions, and contained up to 115% of total procyanidins extracted by the acetone-based conventional solvent. Additionally, 50% ethanol/water (v/v) extracted 205, 221, and 113% more epicatechin, catechin, and dimers, respectively, than conventional extraction. Results indicated greater extraction of low oligomeric procyanidins using 50% ethanol/water (v/v) solvent between 80 and 140 °C.

KEYWORDS: Extraction; grape; polyphenolic; pomace; procyanidin; subcritical

INTRODUCTION

Ten million pounds of natural grape waste, commonly called pomace, was produced in 2005 by the juice and wine industries. Pomace, which consists of skins, seeds, and stems, is often discarded as waste, used for animal feed, grape seed oil, dietary fiber, or soil fertilizer (*1*). However, even after pressing, pomace retains high levels of health-benefiting polyphenolic compounds, namely, procyanidins and anthocyanins. The health benefits associated with polyphenolics include reduction of oxidative stress, free radical scavenging capacity, reduction of cancer and disease risk, modulation of gene regulation and expression, regulation of cholesterol, and reduction of diabetes (*2*).

A major class of polyphenolics found in grapes is procyanidins. Procyanidins are known for the astringent and bitter flavors that they impart to foods and beverages (*3*). Procyanidins are condensed tannins composed of flavan-3-ol monomeric units (epicatechin and catechin) that are linked together by a C4−C8 bond in grapes. The degree of polymerization (DP) indicates the number of monomeric subunits linked together. Grape seeds contain 60% of total polyphenols in the grape and 50−70% of the procyanidins, which largely remain in the seeds even after juicing (*4*). Therefore, collection of pomace is important for procyanidin recovery.

Current trends in technology are toward sustainability-focused processing while still increasing yields and minimizing costs (*4*). To recover polyphenolics from pomace, techniques employing environmentally friendly solvents should be developed for incorporation into food, pharmaceutical, or cosmetic products.

An environmentally friendly technique commonly referred to as accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) involves the use of subcritical or superheated solvents. Subcritical water, also called hot pressurized water, is water heated above its boiling point (100 °C) but below its critical point (374 °C). The application of pressure allows it to remain in a liquid state. In comparison to ambient water, subcritical water exhibits lower polarity, viscosity, and surface tension relative to water at room temperature (*5*). Subcritical extraction conditions with ASE use increased extraction temperature and pressure to enhance the speed and efficiency of the extraction of polyphenolics from natural products (*6*). Increased temperature improves extraction yield due to increased diffusion rates, solvent penetration into the sample, mass transfer, disruption of solute−matrix interactions, and solubilization of analytes into solvents. Increased pressure allows solvents to contact the sample matrix being extracted and permits closer contact between the sample and solvent (*7*). Additional benefits of these extraction techniques include reduction in solvent cost and disposal as well as energy savings (*5*, *8*, *9*). Recently, subcritical extraction processes have been applied to extract anthocyanins from red grape

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pomace (*10*−*12*) and red cabbage (*13*) and procyanidins from red wine grape pomace (*4*), tea leaves, and grape seeds (*14*).

The objective of this study was to optimize the extraction solvent and temperature for recovering procyanidins from dried Sunbelt red grape pomace using ASE with generally recognized as safe (GRAS) solvents. Sunbelt (*Vitis labrusca* L.) was developed by the University of Arkansas and is a large blue juice grape, similar to Concord (*V. labrusca* L.), but differs in its ability to ripen more evenly in warm climates (*15*). The results from this research provide the grape juice industry with another option to extract procyanidins from grape waste for further use as nutraceuticals or nutritional supplements.

MATERIALS AND METHODS

Samples and Chemicals. Sunbelt grapes (*V. labrusca* L.) were harvested in 2006 at the University of Arkansas' Agricultural Experimental Station Farm (Fayetteville, AR). These grapes were crushed and destemmed. A 70 L Enrossi bladder press (Enoagricol Rossi s.r.l., Calzolaro, Italy) at 4 bar was used to press the must. Pomace was recovered, placed into plastic bags, sealed, and stored at −20 °C. Frozen grape pomace was freeze-dried with a VirTis Genesis freeze-dryer (Gardiner, NY). Although freeze-drying would not likely be feasible for industry application, the objective of this research was to optimize a small-scale system, which required stabilized samples by reducing water activity and slowing enzymatic degradation. The freeze-dried pomace was ground to a homogeneous fine powder by passing it through a 500 μm screen on an Udy Cyclone sample mill (Fort Collins, CO). The pomace powder was stored at −70 °C in a ThermoScientific (Waltham, MA) Ultra-Low Freezer until used for analyses.

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, dichloromethane, ethanol, acetone, and analytical grade acetic acid were purchased from EMD Chemicals Inc. (Gibbstown, NJ).

Procyanidin Extraction. Procyanidins were extracted from ground grape pomace using a Dionex model ASE 200 equipped with a solvent controller (Dionex Corp., Sunnyvale, CA). Freeze-dried grape pomace (0.5 g) was dispersed thoroughly with 25 g of sea sand (EMD Chemicals). The homogeneous sand and ground pomace mixture was loaded into a 22 mL extraction cell with a cellulose paper filter at the bottom of the cell. ASE experimental variables were pressure (6.8 MPa), one extraction cycle, flush volume (70%), nitrogen purge time (90 s), static time (0 min), and preheat time (0 min). After extraction, the volume of each collection tube was adjusted to 50 mL with deionized water. Samples were immediately centrifuged for 10 min at 7012*g* using a Beckman GS-15R centrifuge (Beckman Coulter Inc., Fullterton, CA) to remove insoluble solids. The supernatant was recovered and stored in 50 mL plastic centrifuge tubes at −20 °C.

Solvent and Temperature Optimization. Six solvents were tested for their efficacy in extracting procyanidins from ground grape pomace, including 0, 10, 30, 50, 70, and 90% ethanol/water (v/v). Six temperatures (40, 60, 80, 100, 120, and 140 °C) were used to determine effects of temperature on procyanidin extraction. Extractions at each solvent and temperature combination were performed in triplicate.

Conventional Extraction. Conventional extraction of procyanidins from ground grape pomace using acetone/water/acetic acid (70:29.5:0.5, v/v/v) as extraction solvent was considered to be the standard and used as the baseline for comparing the effectiveness of ASE extractions (*16*−*18*). Ground grape pomace (1 g) was homogenized at ambient temperature (23.5 ± 1.5 °C) for 30 s with 20 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v) using an Ika T18 Ultra-Turrax tissuemizer (Wilmington, NC). Homogenized samples were filtered through Miracloth (CalBiochem, LaJolla, CA) into 100 mL volumetric flasks. The extraction was repeated twice as described above, and filtrates were pooled and adjusted to 100 mL with deionized water. Extracts were centrifuged for 10 min at 7012*g* to remove insoluble solids, the supernatants were recovered, and extracts were stored in 50 mL plastic centrifuge tubes at −20 °C.

Procyanidin Analysis by HPLC. Procyanidins were analyzed according to the method described by Prior et al. (*19*) using a Waters Alliance model 2690 HPLC system (Waters Corp., Milford, MA) equipped with an autosampler and a Waters model 474 fluorescence detector. Ten milliliters of the ASE extract sample was concentrated to 3 mL using a ThermoSavant SpeedVac concentrator (Ramsey, MN). Concentrated extracts were loaded onto a packed column (6 cm × 1.5 cm) containing 3 g of Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO), previously hydrated for at least 3 h with deionized water. After samples equilibrated into the column, 40 mL of 30% methanol/water (v/v) was loaded onto the Sephadex column to remove sugars and other phenolics from the samples. The procyanidins were recovered from the column by eluting with 80 mL of 70% acetone/water (v/v). The acetone fraction was evaporated to dryness with the SpeedVac and reconstituted with 2 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v). These extracts were passed through 0.45 μm PTFE filters (Varian, Inc., Palo Alto, CA), and 5 μL was injected onto a 150 × 4.6 mm Luna silica column (Phenomenex, Torrence, CA). The two HPLC mobile phases consisted of (A) dichloromethane/methanol/water/acetic acid (82:14:2:2, v/v/v/v) and (B) methanol/water/acetic acid (96:2:2, v/v/v).

A normal-phase gradient started with 100% (A) and then changed to 88.3% (A) at 20 min, 74.4% (A) at 50 min, and 12.3% (A) at 55 min, remained isocratic until 65 min, and then returned to 100% (A) at 70 min, with 5 min of equilibration time at 100% (A). The entire run was 75 min with a flow rate of 1.0 mL/min. Procyanidins detected at 276 nm excitation and 316 nm emission were identified by comparison to the retention times of standards and also by HPLC-ESI-MS/MS analysis. Procyanidins were quantified using external calibration curves of a mixture of procyanidins DP1−DP10 previously isolated from cocoa, which was provided by Master Foods (Hackettstown, NJ). Polymers were quantified using external calibration curves of a polymeric fraction (DP > 10, average DP of 36.1) previously isolated from blueberry (*18*). Procyanidin results were expressed as milligrams per 100 g of dry weight (DW).

Procyanidin Analysis by HPLC-ESI-MS/MS. Procyanidins were identified by HPLC-ESI-MS/MS in Dr. Ron Prior's laboratory, Arkansas Children's Nutrition Center (Little Rock, AR). The method used was described by Khanal et al. (*20*). An Agilent 1100 HPLC was used to analyze procyanidins and was equipped with a quaternary pump, degasser, autosampler, thermostat column compartment, diode array detector, fluorescence detector, and a ChemStation (Aligent Technologies, Palo Alto, CA) data collector and analyzer. The HPLC system was interfaced with a Bruker Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) to identify molecular masses of each procyanidin peak in the chromatogram. Procyanidins were separated by DP with a 250 × 4.6 mm Luna silica column (Phenomenex) held at 37 °C. The two mobile phases consisted of (A) dichloromethane/methanol/acetic acid/water (82:14:2:2, v/v/v/v) and (B) methanol/acetic acid/water (96:2:2, v/v/v). The entire run was 70 min with a constant 0.8 mL/min flow rate and started with 0−20 min of linear 11.7% (B), then changed to 25.6% (B) at 50 min, then to 87.7% (B) at 55 min, remained at 87.7% (B) until 65 min, and changed to 0% (B) at 70 min. Ten minutes of equilibration time was allowed between runs. Fluorescence detection was monitored at 230 nm excitation and 321 nm emission, and UV detection was at 280 nm with a reference wavelength at 650 nm. The stream eluting from the HPLC at 1 mL/min entered the mass spectrometer and was ionized. Ionization was enhanced by the addition of ammonium acetate (10 mmol/L in methanol), which was pumped a flow rate of 0.06 mL/min in to the column effluent stream. The nebulizer had a nitrogen pressure of 50 psi, a flow rate of 10 L/min, and a 350 °C drying gas temperature was used. Capillary voltage was 3.5 kV, and scan range was set at m/z from 150 to 2200. Other parameters were self-adjusted by the Esquire Control software (v. 4.5). Parameters were expressed as compound stability and ion trap level and were 50 and 25% for monomers, 50 and 90% for dimers, 30 and 110% for trimers, 70 and 120% for tetramers, and 80 and 110% for pentamers, respectively. To increase sensitivity, $[M - H]^-$ ions of monomers through heptamers were used as the target masses in the ion trap. Doubly charged ions $[M - 2H]^{2-}$ were used as targets from octamers through decamers, and decamer parameters were also used for polymers. Helium was introduced at 1×10^{-6} bar as collision gas in the ion trap to help collision-induced dissociation. One hundred percent collision energy was used.

Antioxidant Capacity. Oxygen radical absorbance capacity ($ORAC_{FL}$) analyses used the method of Prior et al. (*21*). Grape pomace extracts were diluted 200-fold with phosphate buffer (pH 7). Results were determined by differences in the area under the fluorescein decay curves between blanks, samples, and standards and expressed in terms of micromoles of Trolox equivalents per gram of DW (*22*).

Experimental Design and Statistical Analysis. The experimental design was a six by six full factorial treatment completely randomized design with three replications. There were six solvents (0, 10, 30, 50, 70, and 90% ethanol/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

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk} \text{ with } i = 1,\ 2,\ ...,\ 6;$$
$$j = 1,\ 2,\ ...,\ 6;\ \text{and } k = 1,\ 2,\ 3$$

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 ($\sum_{i=1}^{7}\alpha_i = 0$), β_j is the jth temperature main effect ($\sum_{j=1}^{7}\beta_j = 0$); $(\alpha\beta)_{ij}$ is the ijth interaction effect of solvent by temperature ($(\sum_{j=1}^{7}(\alpha\beta)_{ij} = 0\ \forall i$ and $\sum_{i=1}^{7}(\alpha\beta)_{ij} = 0\ \forall j$), and $e_{ijk} \overset{iid}{\sim} N(0, \sigma^2)$ is the unobserved ijkth error random effect. The errors are assumed to be independent, identical, 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.

RESULTS AND DISCUSSION

Procyanidin Identification. Procyanidins eluted from the HPLC column in order of increasing DP and were identified relative to retention times of standards and by HPLC-ESI-MS/MS (**Figure 1**). The procyanidins quantified included two monomers (epicatechin and catechin), dimers, trimers, tetramers, pentamers, and polymers. Multiple peaks that eluted as dimers, trimers, tetramers, and pentamers were grouped together according to their DP for quantification purposes. There were no galloylated procyanidins in Sunbelt pomace, which contrasts with the highly galloylated procyanidins reported in wine (*Vitis vinifera*) grapes (*23*, *24*). Linkages in Sunbelt grape pomace were all B-type. B-type linkages have C4−C8 cross-links of catechin/epicatechin, which differ from A-type linkages that have both C4−C8 and C2−C7 cross-links of catechin/epicatechin (*25*).

Solvent and Temperature Optimization. Extraction efficiencies were calculated as a function of the extraction solvent composition and temperature according to the total and individual procyanidins in the red grape pomace. It was found that there was no ideal solvent composition due to variation in structural complexity and polarity of the compounds in the pomace. The experimental design and data of the solvent and temperature interaction are presented in **Table 1**. Total procyanidins, which was the summation of monomers, dimers, trimers, tetramers, pentamers, and polymers, had a significant solvent and temperature interaction ($P = 0.0024$) (**Figure 2**). In general, total procyanidins were optimally extracted at 80, 100, 120, or 140 °C with 50% ethanol/water (v/v) using ASE. The total procyanidin content was largely dependent on the extraction efficiency of polymers. The 50% ethanol/water (v/v) solvent extracted higher levels of polymers than the other ethanol/water solvents.

Compared to conventional extraction, extracts obtained at each combination of ethanol/water solvent and temperature contained only 26% (0% ethanol/water, 60 °C) to 115% (70% ethanol/water, 120 °C) of total procyanidins as the acetone-based

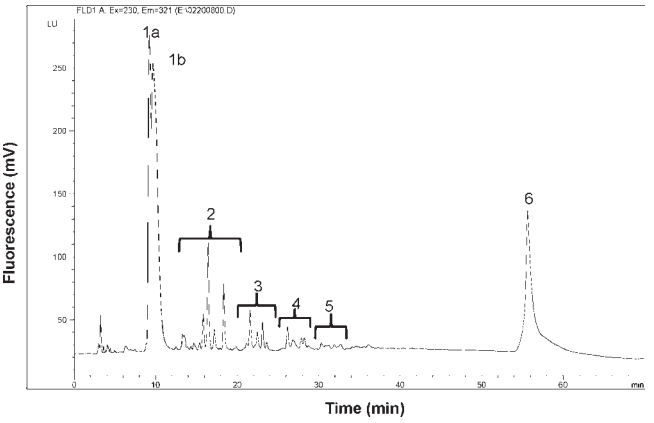

Figure 1. Representative HPLC chromatogram of procyanidins in Sunbelt grape pomace obtained by fluorescence detection (excitation, 230 nm; emission, 321 nm). Procyanidins with a degree of polymerization of 1−5 and polymers were identified by HPLC-ESI-MS/MS and quantified by external standards. Peaks: 1a, epicatechin (9.1 min, *m*/*z* 288.7); 1b, catechin (9.7 min, *m*/*z* 288.5); 2, group of dimers (15.9, 16.4, 17.2, and 18.4 min, *m*/*z* 576.7, 577.6, 576.6, and 576.8, respectively); 3, group of trimers (21.6, 22.5, 23.1, and 23.7 min, *m*/*z* 864.8, 864.8, 864.8, and 866.8, respectively); 4, group of tetramers (26.8, 27.9, and 28.2 min, *m*/*z* 1152.4, 1152.4, and 1153.6, respectively); 5, group of pentamers (30.2 and 32.6 min, *m*/*z* 1441.3 and 1439.3); 6, polymers (55.5−55.8 min, *m*/*z* undefined).

conventional solvent extracts. Although 50% ethanol/water (v/v) was found to be best for extracting total procyanidins, which agrees with research by Savova et al. (*26*), 30, 50, and 70% ethanol/water (v/v) solvent compositions tested were statistically equivalent to the conventional solvent with regard to the total amount of procyanidins extracted. Although some ethanol/water solvents were as good at extracting total procyanidins as the conventional acetone-based solvents, we observed that acetone-based solvents were a more efficient extraction solvent for procyanidin polymers than ethanol and water mixtures (**Figure 3**). This was consistent with previous studies reporting that high levels of acetone were required to extract the larger molecular weight procyanidins (*27*). To determine if acetone-based solvents could extract the polymers that ethanol-based solvents were not recovering, we collected the ASE residue and extracted with the conventional method using acetone/water/acetic acid (70:29.5:0.5, v/v/v). We did not observe additional polymer recovery from the residue using acetone-based solvents, presumably because of matrix interactions formed during the ASE that made remaining polymers unextractable. According to Hollström et al. (*28*), procyanidins can become unextractable due to strong complexation with other insoluble polymers in plants, thus rendering the procyanidin extraction imcomplete.

When grapes are pressed into juice, the procyanidins are released from the vacuole and can readily bind to cell wall polysaccharides and proteins. Procyanidins have been shown to readily associate with cell wall materials and proteins through hydrophobic interactions or hydrogen bonding, with higher DP procyanidins showing a greater propensity for binding than lower DP procyanidins (*29*). It has been estimated that between 50 and 93% of apple procyanidins could be retained in cell wall material following processing of apple juice (*30*).

Additionally, disruption of the grape tissue allows procyanidins and other polyphenols to come into contact with polyphenol oxidase, which can lead to polyphenol oxidation and polymerization. Dehydration of plant tissues can also impair procyanidin extraction, as pectins can cross-link and form

Table 1. Concentrations (Milligrams per 100 g of DW) of Procyanidin Monomers, Oligomers, and Polymers in Grape Pomace Extracts As Affected by ASE Extraction Temperature and Ethanol/Water (v/v) Solvent Mixtures[a]

solvent	temperature (°C)	epicatechin	catechin	dimers	trimers	tetramers	pentamers	total DP1−5	polymers	total
0% ethanol[b]	40	354 ± 36	514 ± 15	458 ± 42	168 ± 20	142 ± 14	76 ± 19	1712 ± 78	133 ± 23	1845 ± 94
	60	260 ± 2	348 ± 2	321 ± 11	110 ± 6	104 ± 5	53 ± 7	1195 ± 18	107 ± 20	1303 ± 24
	80	375 ± 80	586 ± 122	454 ± 98	165 ± 39	156 ± 36	78 ± 20	1815 ± 361	277 ± 134	2092 ± 461
	100	454 ± 13	609 ± 20	416 ± 6	133 ± 6	125 ± 5	68 ± 5	1806 ± 54	425 ± 68	2231 ± 120
	120	537 ± 45	743 ± 57	464 ± 45	157 ± 16	158 ± 24	97 ± 10	2156 ± 180	628 ± 167	2784 ± 334
	140	453 ± 54	786 ± 92	480 ± 38	182 ± 28	164 ± 25	120 ± 40	2184 ± 192	417 ± 14	2601 ± 178
10% ethanol	40	504 ± 12	665 ± 19	462 ± 9	133 ± 5	142 ± 2	78 ± 7	1985 ± 37	217 ± 46	2201 ± 61
	60	556 ± 15	750 ± 25	474 ± 6	143 ± 6	139 ± 6	71 ± 8	2134 ± 57	221 ± 45	2355 ± 79
	80	642 ± 54	785 ± 30	486 ± 4	161 ± 16	160 ± 19	87 ± 14	2320 ± 113	615 ± 199	2935 ± 216
	100	812 ± 28	1028 ± 78	576 ± 36	170 ± 8	170 ± 11	94 ± 7	2850 ± 156	548 ± 58	3398 ± 201
	120	852 ± 21	1097 ± 39	602 ± 24	173 ± 4	175 ± 3	91 ± 5	2990 ± 83	708 ± 60	3699 ± 135
	140	764 ± 175	1070 ± 249	541 ± 110	157 ± 41	143 ± 36	76 ± 19	2751 ± 627	803 ± 61	3554 ± 659
30% ethanol	40	565 ± 17	751 ± 23	503 ± 20	148 ± 4	152 ± 3	93 ± 4	2211 ± 56	730 ± 70	2941 ± 87
	60	513 ± 45	688 ± 51	445 ± 23	129 ± 5	126 ± 9	86 ± 1	1986 ± 130	631 ± 71	2618 ± 201
	80	630 ± 22	844 ± 31	505 ± 28	151 ± 5	144 ± 8	88 ± 5	2363 ± 92	815 ± 36	3178 ± 124
	100	875 ± 8	1143 ± 23	627 ± 13	174 ± 5	165 ± 6	82 ± 11	3066 ± 36	1562 ± 158	4627 ± 123
	120	889 ± 59	1185 ± 57	607 ± 21	177 ± 9	171 ± 9	97 ± 5	3126 ± 133	1874 ± 225	2999 ± 357
	140	902 ± 62	1246 ± 60	603 ± 49	170 ± 16	168 ± 2	95 ± 3	3185 ± 187	2272 ± 42	5456 ± 154
50% ethanol	40	522 ± 68	666 ± 90	473 ± 37	136 ± 9	154 ± 13	87 ± 5	2038 ± 218	1214 ± 116	3253 ± 146
	60	553 ± 62	753 ± 81	502 ± 31	135 ± 9	153 ± 13	97 ± 5	2193 ± 200	2346 ± 321	4539 ± 519
	80	629 ± 12	849 ± 3	534 ± 28	141 ± 5	148 ± 11	84 ± 11	2385 ± 62	2425 ± 356	4810 ± 418
	100	847 ± 125	1155 ± 109	638 ± 42	196 ± 10	191 ± 10	105 ± 4	3131 ± 297	2024 ± 300	5155 ± 597
	120	927 ± 30	1188 ± 44	601 ± 21	177 ± 6	167 ± 1	99 ± 4	3158 ± 92	1880 ± 63	5037 ± 152
	140	1050 ± 5	1403 ± 14	678 ± 8	198 ± 0	186 ± 2	111 ± 1	3626 ± 9	2083 ± 86	5708 ± 91
70% ethanol	40	420 ± 30	570 ± 38	474 ± 47	122 ± 15	119 ± 12	56 ± 4	1762 ± 144	1045 ± 301	2807 ± 271
	60	455 ± 22	636 ± 22	455 ± 20	124 ± 8	129 ± 9	81 ± 6	1881 ± 63	356 ± 44	3236 ± 105
	80	711 ± 55	962 ± 71	579 ± 52	156 ± 17	160 ± 19	100 ± 17	2669 ± 228	1942 ± 221	4611 ± 443
	100	906 ± 76	1144 ± 124	636 ± 25	184 ± 5	179 ± 6	103 ± 9	3152 ± 238	1766 ± 161	4918 ± 391
	120	1067 ± 42	1302 ± 45	677 ± 18	200 ± 8	199 ± 8	122 ± 5	3567 ± 127	2146 ± 91	5712 ± 217
	140	724 ± 270	1025 ± 374	496 ± 120	138 ± 38	125 ± 39	74 ± 25	2582 ± 866	1026 ± 317	3608 ± 1172
90% ethanol	40	279 ± 16	362 ± 14	377 ± 4	107 ± 2	103 ± 5	50 ± 7	1277 ± 15	232 ± 51	1509 ± 55
	60	387 ± 30	525 ± 34	486 ± 20	139 ± 8	150 ± 10	86 ± 15	1774 ± 112	239 ± 20	2012 ± 129
	80	491 ± 27	587 ± 100	375 ± 109	122 ± 17	102 ± 24	73 ± 9	1751 ± 267	186 ± 15	1935 ± 272
	100	824 ± 41	1123 ± 51	563 ± 18	157 ± 8	151 ± 9	88 ± 9	2906 ± 132	498 ± 47	3403 ± 158
	120	925 ± 28	1233 ± 24	653 ± 15	201 ± 9	189 ± 10	106 ± 7	3306 ± 32	670 ± 110	3966 ± 83
	140	915 ± 38	1229 ± 34	625 ± 24	197 ± 12	192 ± 9	114 ± 7	3272 ± 107	432 ± 76	3704 ± 172
conventional[c]	25	248 ± 15	313 ± 17	268 ± 8	67 ± 3	76 ± 5	72 ± 2	1044 ± 43	3912 ± 234	4955 ± 275

[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 acetone/water/acetic acid (70:29.5:0.5, v/v/v).

hydrophobic pockets that are able to encapsulate and complex the procyanidins (*3*). Also, plant cellulosic material organized in microfibrils and xyloglucans as globules allows surface adsorption and aggregation of the procyanidins (*3*). The spontaneous and rapid adsorption or precipitation of apple procyanidins to cell wall materials not only affects their selective removal from tissues but also can affect their antioxidant capacity (*31*).

Because polymeric procyanidins were a major contributor to total procyanidin content and ethanol/water solvents were less efficient at extracting polymers, we analyzed extraction efficiency of summed DP1−5 oligomeric procyanidins (**Figure 4**). When polymers were removed from the statistical analysis, ethanol/water solvents became significantly better extraction solvents than the acetone-based conventional solvent. The solvent by temperature interaction was not significant for summed DP1−5 oligomeric procyanidins ($P = 0.0517$). The solvent main effect was significant ($P < 0.0001$) and ASE extracts obtained with 50, 30, 70, 10, and 90% ethanol/water (v/v) solvents and water contained 164, 155, 149, 140, 128, and 74% more DP1−5 procyanidins, respectively, than conventional solvent extracts. This showed that ethanol/water solvents, and even water, were more efficient and selective in extracting low molecular weight procyanidins than the conventional solvent. The temperature main effect was also significant ($P < 0.0001$). ASE extracts obtained with ethanol/water solvents at 120, 140, 100, 80, 60, and 40 °C contained 192, 181, 170, 112, 78, and 72% more DP1−5 procyanidins, respectively, relative to conventional solvent extracts. This indicated that elevated temperatures were necessary to efficiently extract procyanidins from dried red grape pomace. Garcia-Marino et al. (*4*) also found that an increase in temperature was necessary to extract procyanidins having a DP of > 1.

Evaluation of monomer and dimer extraction efficiency with ethanol/water solvents was especially important because of the bioactivity of these low molecular weight procyanidins (*32*). Epicatechin, one of the two procyanidin monomer subunits, was more efficiently extracted with heated ethanol/water solvents using ASE than via conventional solvent extraction (**Figure 4**).

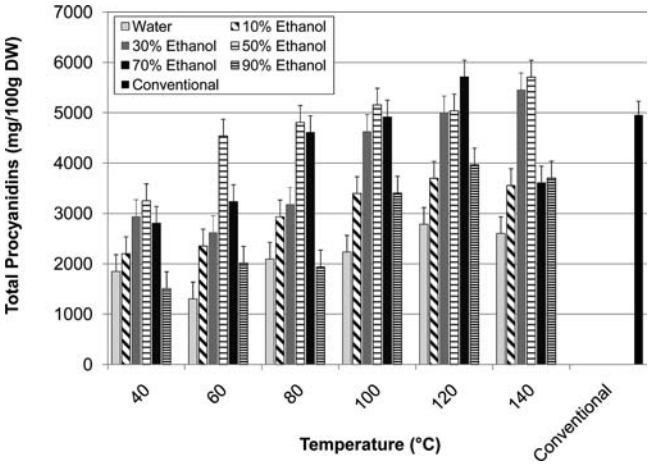

Figure 2. Concentration of total procyanidins in ASE extracts obtained with various ethanol/water (v/v) solvents and temperatures compared to conventional solvent extracts obtained with acetone/water/acetic acid (70:29.5:0.5, v/v/v) at ambient temperature. Procyanidins were quantified in milligrams per 100 g of dry weight (DW). Bars represent SEM (n = 3).

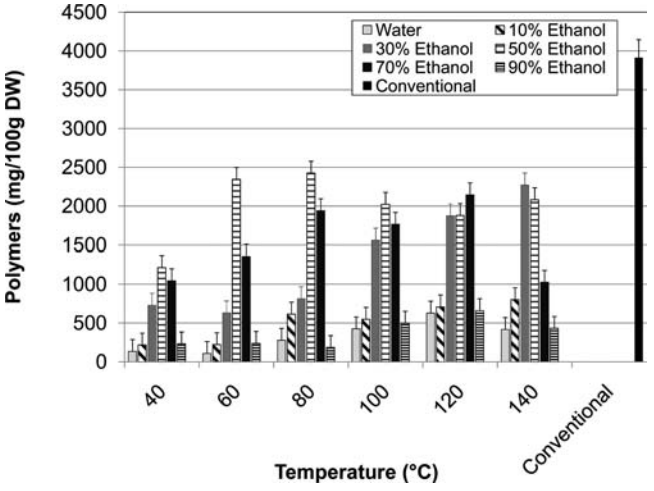

Figure 3. Concentration of procyanidin polymers in ASE extracts obtained with various ethanol/water (v/v) solvents and temperatures compared to conventional solvent extracts obtained with acetone/water/acetic acid (70:29.5:0.5, v/v/v) at ambient temperature. Procyanidins were quantified in milligrams per 100 g of dry weight (DW). Bars represent SEM (n = 3).

The solvent by temperature interaction for epicatechin was significant (P = 0.0308), and generally 30−90% ethanol/water (v/v) at temperatures above 100 °C increased epicatechin extraction. ASE extracts obtained with each ethanol/water solvent composition at each temperature condition contained between 5% (0% ethanol/water (v/v), 60 °C) and 331% (70% ethanol/water (v/v), 120 °C) more epicatechin than conventional solvent extracts. Catechin, the second procyanidin monomer subunit, was also more efficiently extracted with heated ethanol/water solvents than with conventional solvent (**Figure 4**). The solvent by temperature interaction for catechin was not significant (P = 0.0893). The solvent effect was significant (P < 0.0001) and ASE extracts obtained with 50, 30, 70, 10, and 90% ethanol/water (v/v) solvents and water contained 221, 212, 201, 188, 170, and 91% more catechin, respectively, than conventional solvent extracts. The temperature effect was also significant (P < 0.0001), and ethanol/water solvent extracts obtained at 140, 120, 100, 80, 60, and 40 °C contained 260, 260, 231, 146, 97, and 88% more catechin than conventional solvent extracts. The high yield of monomers compared to conventional solvent extraction was significant as the low molecular weight monomers are absorbed in the body to contribute to health benefits associated with procyanidins (*33*−*35*).

We believe the cause of increased monomer recovery by ethanol/water solvents is due to enhanced extraction and not due to other potential causes such as acid-catalyzed cleavage or depolymerization of larger molecular weight polymers into monomers. Acid-catalyzed cleavage was unlikely during ASE because no acid was added to the ethanol/water solvents. We cannot rule out the possibility that higher molecular weight polymers were depolymerized to lower oligomers because we did not have sufficient polymer standard to test this hypothesis. The gain of low DP procyanidins with ASE conditions was most likely due to enhanced extraction due to the preferential solubility of ethanol-based solvents for low DP procyanidins and acetone-based solvents for high DP procyanidins. We know that 50% ethanol/water (v/v) is more selective for low DP procyanidins because we used the conventional extraction method with 50% ethanol/water (v/v) instead of acetone/water/acetic acid (70:29.5:0.5, v/v/v) and found that under the exact same extraction conditions, acetone/water/acetic acid (70:29.5:0.5, v/v/v) extracted 67 and 77% less epicatechin and catechin, respectively, than 50% ethanol/water (v/v). We also know that when comparing extracts at 50% ethanol/water (v/v) at ambient temperature to extracts at 50% ethanol/water (v/v) at 120 °C, the 50% ethanol/water (v/v) extracts at ambient temperature recovered 121 and 116% less epicatechin and catechin, respectively, than extracts from 120 °C extraction. Hence, ethanol-based solvents are more selective for low-DP procyanidins, and increasing temperature improves extraction presumably by increasing mass transfer, contact between solvent and matrix, and disruption of cell wall matrices (*7*).

Extraction of dimers is also of importance due to purported bioavailability (*32*). The solvent by temperature interaction for dimers was not significant (P = 0.1055). For procyanidin dimers (**Figure 4**), the solvent effect was significant (P < 0.0001) and ASE extracts obtained with 50, 70, 30, 10, and 90% ethanol/water (v/v) solvents and water contained 113, 106, 105, 95, 91, and 61% more dimers, respectively, than conventional solvent extracts. For the significant temperature effect (P < 0.0001), extracts obtained with ethanol/water solvents heated to 120, 100, 140, 80, 40, and 60 °C contained 124, 115, 113, 82, 71, and 67% more dimers than conventional solvent extracts.

For trimers (**Figure 4**), the solvent by temperature interaction was significant (P = 0.0364) and ASE at each solvent and temperature combination contained between 59% (90% ethanol/water (v/v), 40 °C) and 198% (90% ethanol/water (v/v), 120 °C) more trimers than conventional solvent extracts. In general, any composition of ethanol/water solvent heated to temperatures 100 °C or greater increased trimer extraction. For tetramers (**Figure 4**), the solvent by temperature interaction was significant (P = 0.0246) and similar to trimers, any ethanol/water solvent heated to 100 °C or above generally improved tetramer extraction. ASE extracts obtained at each solvent and temperature combination had between 34% (90% ethanol/water (v/v), 80 °C) and 162% (70% ethanol/water (v/v), 120 °C) more tetramers, respectively, than conventional solvent extracts. For pentamers (**Figure 4**), the solvent by temperature interaction was not significant (P = 0.0751), nor was the solvent effect (P = 0.2916). The temperature effect was significant (P = 0.0005), and extracts obtained with heated ethanol/water solvents at 120, 140, 100, 80, 60, and 40 °C contained 42, 37, 25, 19, 10, and 2% more pentamers, respectively, than conventional solvent extracts.

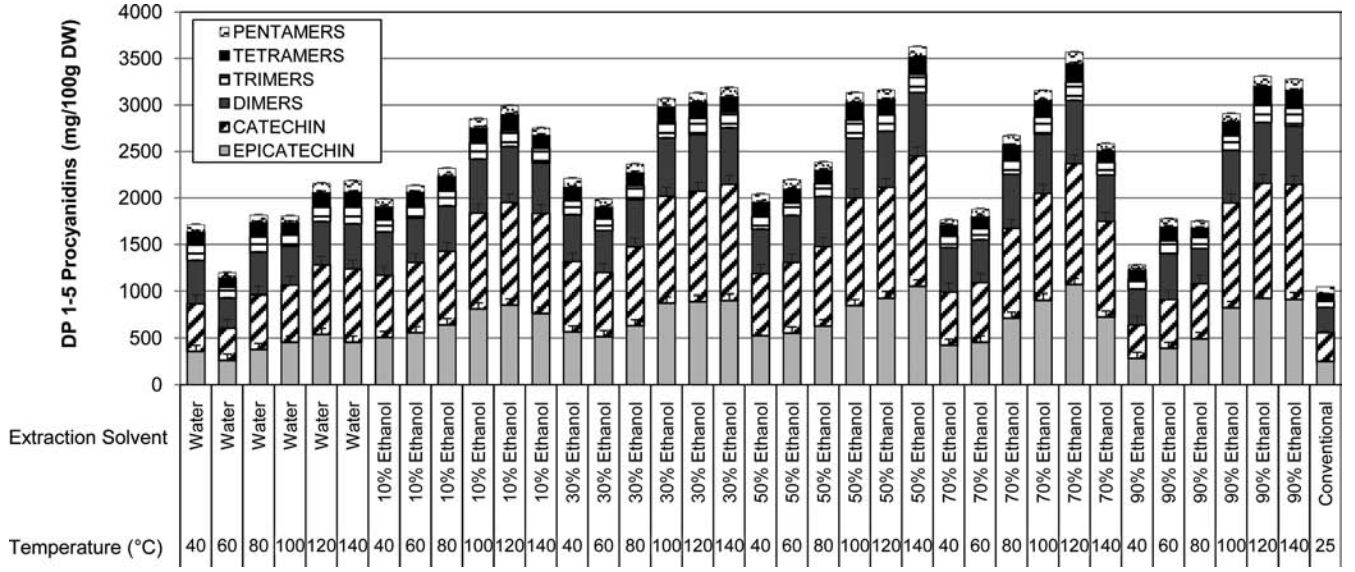

Figure 4. Concentration of DP1−5 procyanidins in ASE extracts obtained with various ethanol/water (v/v) solvents and temperatures compared to conventional solvent extracts obtained with acetone/water/acetic acid (70:29.5:0.5, v/v/v) at ambient temperature. Procyanidins were quantified in milligrams per 100 g of dry weight (DW). Bars represent SEM (n = 3).

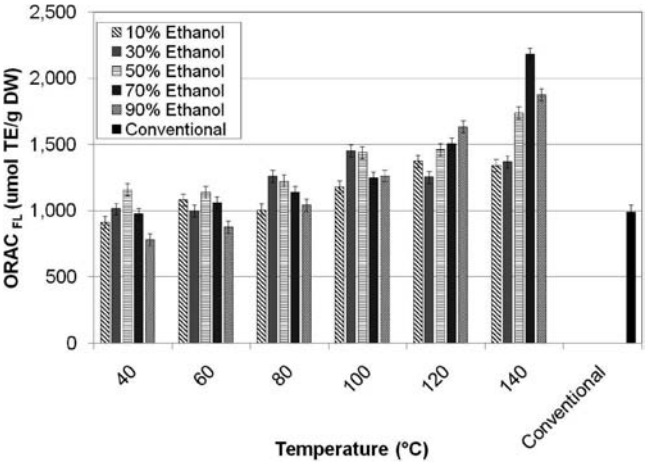

Figure 5. Antioxidant capacity ($ORAC_{FL}$) of ASE extracts obtained with various ethanol/water (v/v) solvents and temperatures compared to conventional solvent extracts obtained with acetone/water/acetic acid (70:29.5:0.5, v/v/v) at ambient temperature. Antioxidant capacity was quantified in micromoles of Trolox equivalents (TE) per gram of dry weight (DW). Bars represent SEM (n = 3).

These results showed that ethanol/water solvents became less effective in extracting procyanidins as DP increased. Also, the extraction of higher DP procyanidins was affected more by increased extraction temperature than the ethanol/water solvent used.

Antioxidant Capacity. Antioxidant capacity (**Figure 5**) was determined by the $ORAC_{FL}$ assay. Interestingly, as DP increased, the correlation between $ORAC_{FL}$ and procyanidins decreased. Correlations (r_{xy}) between $ORAC_{FL}$ and epicatechin, catechin, dimers, trimers, tetramers, pentamers, and polymers were 0.640, 0.667, 0.482, 0.508, 0.396, 0.358, and 0.271, respectively. $ORAC_{FL}$ results of ASE extracts did not correlate well (r = 0.311) with total procyanidin results of the corresponding extracts. $ORAC_{FL}$ data showed increased antioxidant capacities with increasing temperatures, whereas total procyanidin results showed lower recovery with increasing temperatures. Higher temperatures theoretically would degrade procyanidins and decrease antioxidant capacity, due to their thermal instability, instead of increasing $ORAC_{FL}$ values at higher temperatures. One possible explanation for the results is increased browning due to the formation of Maillard reaction products (MRP) at higher temperatures increased antioxidant capacity. Yilmaz and Toledo (*36*) found significant formation of MRP with potent antioxidant capacity when mixtures of an amino acid and a sugar were heated at 120 °C for 10, 20, and 30 min. It was likely that high-temperature MRPs with potent antioxidant capacity were formed when extracts were heated to 120 °C and above. Similar increases in $ORAC_{FL}$ values were observed for ethanol/water spinach extracts obtained at extraction temperatures over the range of 50−190 °C, changes that correlated well with sample browning indices (*37*). Another explanation may be that polymeric procyanidins, which were extracted in greater concentrations with higher temperatures (> 100 °C), have more potent antioxidant capabilities than monomeric or low oligomeric procyanidins (*38*, *39*).

Fifty percent ethanol/water (v/v) extracted higher levels of procyanidins from red grape pomace than other ethanol/water solvents with an optimal temperature range of 80−140 °C. Although ethanol/water solvents were less effective than conventional solvent in extracting high molecular weight polymers, they were exceedingly more effective in extracting monomers, dimers, trimers, tetramers, and pentamers. These results can be applied in the wine and juice industries to extract procyanidins from grape pomace, resulting in a more cost-effective and environmentally friendly solvent.

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NOTE ADDED AFTER ASAP PUBLICATION

Errors in calculation of polymer concentrations were discovered after the original ASAP publication of December 18, 2009. The abstract, Results and Discussion, **Figures 2−4**, and **Table 1** have been revised in the ASAP posting of January 28, 2010.

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