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Design and Optimization of a Semicontinuous Hot–Cold Extraction of Polyphenols from Grape Pomace

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ABSTRACT: Grape pomace contains appreciable amounts of polyphenolic compounds such as anthocyanins and procyanidins which can be recovered for use as food supplements. The extraction of these polyphenols from the pomace is usually accomplished at slightly elevated temperatures, frequently employing hydroethanolic solvents. Due to governmental regulations and the cost involved in using ethanol as a solvent, as well as the loss in polyphenolics due to thermal degradation, improved extraction techniques are required. In this study, a semicontinuous extraction apparatus employing only water was developed to maximize the recovery of anthocyanins and procyanidins from red grape pomace (*Vitis vinifera*). Water is preheated prior to its entry to the extraction cell containing the grape pomace sample, where it is allowed to then flow continuously through the unheated extraction vessel prior to its collection at ambient conditions. Extraction variables that impacted the polyphenolic recovery included pomace moisture content (crude or dried), sample mass, water flow rate, and extraction temperature. A response surface method was used to analyze the results from the extraction, and the optimal conditions were found to be 140 °C and 9 mL/min water flow rate. These conditions can produce an extract containing 130 mg/100 g DW of anthocyanins and 2077 mg/100 g DW of procyanidins. Higher yields of polyphenolics were observed using crude (wet) rather than dried pomace, hence avoiding the need to dry the pomace prior to extraction. The described semicontinuous extraction method using only water as the extraction solvent under subcritical conditions allowed the efficient extraction of polyphenols from red grape pomace without the attendant loss of polyphenolic content due to having to heat the extraction vessel prior to commencement of extraction.

KEYWORDS: *anthocyanins, grape pomace, hot–cold extraction, procyanidins, subcritical water*

INTRODUCTION

Flavonoids, such as anthocyanins and procyanidins, have been investigated for their purported health benefits and are extensively used in food, cosmetic, and nutraceutical industries.[1] Such compounds have been traditionally extracted from natural products using organic solvents such as hexane, methanol, or acetone[2] as well as with hydroalcoholic solvents.[3] There have been a number of these reported studies on extracting such polyphenolic compounds from grape pomace obtained as a residue in grape and wine industry after juice removal.[3,4]

Traditional extraction methods including liquid–liquid and solid–liquid extraction can involve long extraction times accompanied by organic solvent uptake into the remaining pomace.[5] Pressurized liquid extraction using water and ethanol or mixtures thereof have been investigated and have been shown to provide a rapid, selective, and benign method for the extraction of flavonoids from natural products.[6−9] Such studies have confirmed the efficient extraction of the targeted compounds at higher temperatures due to increased solubility of polyphenolics in the solvent and concomitant decrease in the solvent viscosity and dielectric constant.[6]

These extraction processes involve the use of solvents at temperatures above their boiling point under pressure and are called subcritical fluids.[10,11] The optimal yield of the polyphenolics is obtained by adjusting the extraction temperature, whereas pressure has a negligible effect on the extraction efficiency.[12]

Previous studies conducted in our research group used pressurized hydroethanolic solvent mixtures to extract flavonoids from grape pomace, and it was found that the maximum yield of anthocyanins and procyanidins occurred between 80 and 120 °C.[13−15] Such studies were performed by allowing solvent flow into a batch reactor containing the grape pomace followed by an increase in the temperature or pressure on the extraction vessel. However, it was observed that as the vessel was heated up to the higher extraction temperatures, the oxygen radical absorbance capacities (ORAC) were lower due to the thermal degradation of the glucoside linkages in the anthocyanins.[13] Such results were supported by other studies cited in the literature.[16−18]

One such study[19] indicated that there was significant thermal degradation of quercetin diglucosides when extracted from red onions using pressurized water at 110 °C within the first 8 min of the extraction cycle. Similarly, in another study involving the extraction of silymarins from milk thistle, it was found that the thermal degradation of these compounds occurred during extraction and can be modeled using first order kinetics.[20] With such adverse effects, it can be seen that there is still a need for an improved extraction method that can reduce degradation of

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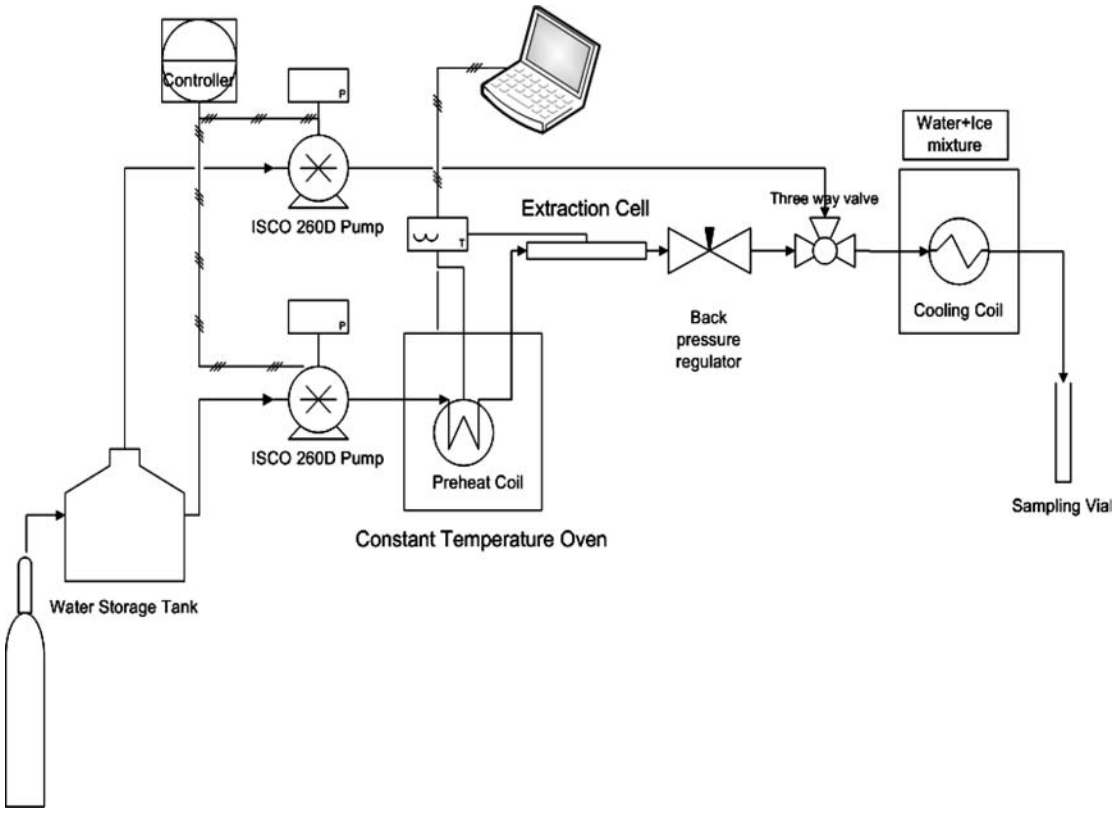

Figure 1. Process schematic of the semicontinuous hot−cold extraction apparatus used in extraction of polyphenolic compounds from grape pomace.

these thermally labile polyphenolic solutes and is amenable to being scaled up for commercial production.

Studies using a semicontinuous flow of water into a pressurized vessel placed in a constant temperature oven have been done previously to counter the disadvantages attendant with batch processing, such as long-term exposure and thermal degradation of the solute under conditions near and above the boiling point of water.[18,21,22] Although it was observed that there was a reduction in the overall extraction time, the residence time of the pomace in the extraction vessel was found to cause loss in flavonoid recovery due to their thermal degradation.[23]

In this study, the extraction solvent (water) flows through a a constant temperature oven held at a high temperature before being allowed to contact the grape pomace which is prepacked in an extraction cell placed outside the oven held at ambient conditions. Such an approach is expected to eliminate the loss of polyphenolics in the grape pomace as it occurs during the heatup of the extraction vessel. This approach to extraction is optimized for solvent flow rate, temperature, feed (or pomace) mass, oven preheat temperature, and the effect of sample moisture content (dried or crude) on the recovery of anthocyanins and procyanidins from the grape pomace matrix.

MATERIALS AND METHODS

Samples and Reagents. White zinfandel grape pomace (*Vitis vinifera;* 58% moisture content) was obtained from San Joaquin Valley Concentrates (Fresno, CA) and immediately stored at −20 °C. The pomace was then dried to 4% moisture (wet basis) using a convection oven at 40 °C to compare the extraction of polyphenols from dried versus crude pomace. Dried pomace was stored at −20 °C after coarse grinding with a coffee grinder (Black & Decker Smart Grind, Towson, MD) for 30 s. Nondried frozen pomace was ground immediately before each extraction in a commercial 6-speed blender (Sunbeam model 4181; Maitland, FL) for 30 s.

HPLC grade reagents used in analysis were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Anthocyanin standards consisting of monoglucosides of delphinidin (Dpd), cyanidin (Cyd), petunidin (Ptd), pelargonidin (Pnd), and malvidin (Mvd) were purchased from Polyphenols Laboratories AS (Sandnes, Norway). A mixture of procyanidin standards DP 1 through DP 10 previously isolated from cocoa was provided by Master Foods, Hackettstown, NJ.

Semicontinuous Extraction Using Subcritical Water. The process schematic for the semicontinuous system used for the extraction of polyphenolic compounds from grape pomace is shown in Figure 1. In this system, water (degassed using nitrogen purge) from the storage tank was pumped using an ISCO 260D constant flow syringe pump (Lincoln, NE) through a preheating coil placed in a constant temperature HP model 5890 GC oven. The oven temperature was maintained between 60 and 140 °C, which varied no more than ±1 °C at the set point temperature throughout the experiments.

The aqueous solvent contacted the grape pomace packed in an extraction cell (SS-316; 1.0 in. (i.d.) × 6.0 in. (length)) which was placed outside the constant temperature oven. An on/off switching valve (High Pressure Equipment Inc. (HIP), Erie, PA, P/N HIP15-11AF1) was placed at the outlet of the extractor cell to maintain system pressure in order to prevent flashing of water to steam due to sudden expansion as the pressure was released (i.e., this is the vapor pressure generated by hot water at the extraction temperatures). It should be noted that the tubing, extractor cell, and back-pressure regulator were insulated to prevent a rapid change in experimental temperature. The solute−solvent mixture would then contact excessive solvent at a mixing tee (HIP, Erie, PA, P/N HIP15-23AF1) placed after the extraction cell and before the back pressure regulator. The additional solvent (in this case, water) was pumped using another ISCO 260D pump at the same flow rate as the first pump. An ISCO

SFX 200 controller (Lincoln, NE) was used to control the flow rate of both the solvent pumps. The pump pressures recorded in the controller were found to be lower than 60 psi for all the experiments.

The solute−solvent mixture was then collected in a collection beaker and stored under refrigeration until needed for further analysis. The total experimental time was dependent on the chosen solvent flow rates and was determined as the time recorded from the start of the solvent flow into the collection beaker until the last visible purple drop collected in the beaker. Temperatures of the oven, preheating coil inside the oven, the coil before the extraction cell, and the temperature of the extractor cell were measured using J-type thermocouples connected to a Cole Parmer 18200-40 analog input module (Vernon Hills, IL, USA). Translated digital signals from the thermocouples were recorded using Tracer DAQ software (ver. 1.8.3; Cole Parmer Instrument Company, Vernon Hills, IL).

The experimental design for the extraction process was developed using a randomized response surface design as will be noted later in this section. The solvent flow rates were varied between 5 and 15 mL/min, and the mass of the grape pomace placed in the extraction cell varied between 5 and 25 g. The total extraction time averaged at 28, 17, and 11 min for 5, 10, and 15 mL/min flow rates, respectively. Triplicate measurements were made for the optimized conditions in this study to verify the reproducibility of the extraction process.

Conventional Extraction. The conventional extraction method for recovering anthocyanins from grape pomace was done using methanol/formic acid/water (60:37:3, v/v) while procyanidins were extracted using acetone/water/acetic acid (70:29.5:0.5, v/v) as solvents. The anthocyanin and procyanidin content extracted from grape pomace using the method of Cho et al.[24] was used as a baseline for calculating the extraction efficiencies to compare with that obtained using semicontinuous process. One gram of ground grape pomace was homogenized at ambient temperature (23.5 ± 1.5 °C) for 30 s with the above respective solvents using an Ika T18 Ultra-Turrax Tissuemizer (Wilmington, NC). The homogenized samples were filtered through Miracloth (Calbiochem, San Diego, CA) into 100 mL volumetric flasks. The extraction was repeated twice and the filtrate pooled and made up to 100 mL with deionized water. The extracts were then centrifuged for 10 min at 7012 × *g* to remove insoluble solids, and the resulting supernatants were stored in 50 mL centrifuge tubes and kept frozen before being analyzed.

Polymeric Color. The polymeric color in the grape extracts was determined using the method described in Giusti and Wrolstad.[25] Briefly, the extracts were diluted using a 0.025 M phosphate buffer until the absorbance at 520 nm was less than 1 Au units. 2.8 mL of the diluted sample was then transferred to two different cuvettes. 0.2 mL of 0.9 M sodium metabisulfite solution was added to the first cuvette, 0.2 mL of deionized water was added to the other, and their absorbances were measured at 420 nm, 520, and 700 nm after 15 min. The principle behind this spectrophotometric method was that when the sample was treated with bisulfite, the monomeric anthocyanins would lose their color unlike their polymeric constituents. The dilution of the extracts varied between 30- and 376-fold depending on the starting mass and final volume of the collected extracts.

Anthocyanin Analysis by HPLC/ESI-MS. The anthocyanins in the Zinfandel grape pomace were identified by HPLC/ESI-MS as described by Cho et al.[24] This method was discussed in detail in our previous study.[13] Briefly, the identification of the anthocyanin peaks in grape extracts was done using a Hewlett-Packard 1100 series (Agilent Technologies, Wilmington, DE) equipped with an autosampler, binary HPLC pump, and UV−vis detector. The anthocyanins were analyzed using a 250 × 4.6 mm Waters Symmetry C_{18} column (Waters Corp., Milford, MA). The solvents used were (A) 5% formic acid in water (v/v) and (B) methanol. The gradient method started with 98% A to 40% A in 60 min, followed by 98% A in 65 min, which remained isocratic until the end of the run. The HPLC system was interfaced with a Bruker Esquire LC−MS (Billerica, MA) ion trap mass spectrometer with the data acquired at 510 nm using positive ion electrospray mode with a capillary voltage of 4000 V, nebulizing pressure of 0.21 MPa, drying gas flow rate of 9 mL/min, and a temperature of 300 °C. The data were collected over the mass range of m/z 50 through 800 in full scan mode at 1.0 cycles/s.

Anthocyanin Analysis by HPLC. As described previously, the anthocyanins were separated and analyzed using the method described in Cho et al.[24] The column, solvent, and gradient method are as described in the previous section. The entire HPLC run time per sample was 90 min with a flow rate of 1 mL/min. The anthocyanin peaks were analyzed at 510 nm and compared with the retention times of a grape extract obtained using the conventional extraction method, and identified using the HPLC−MS method as described previously. The anthocyanins were expressed as milligrams per 100 g of dry weight (DW) and quantified as equivalent anthocyanin standards using external calibration curves.

Procyanidin Analysis by HPLC. The procyanidin content in the grape extracts was analyzed using the method described in Prior et al.[26] This method has been described in detail in our previous study[14] and will be briefly discussed in this manuscript. The analysis was performed using a Waters Alliance model 2690 HPLC system (Waters Corp., Milford, MA) equipped with an autosampler and a Waters model 474 fluoroscence detector. Ten milliliters of the sample was concentrated using a ThermoSavant SpeedVac concentrator (Ramsey, MN) and loaded onto a packed Sephadex column (6 cm × 1.5 cm, 3 g packed, Sigma-Aldrich, St. Louis, MO). 40 mL of 30% methanol/water (v/v) was loaded onto the Sephadex column to remove the sugars and other phenolics from the samples. The procyanidins in the grape extracts were then recovered using 80 mL of 70% acetone/water (v/v). The organic fraction was concentrated and brought back to solution using 2 mL of acetone/water/acetic acid solvent (20:29.5:0.5, v/v).

The solvent system comprised (A) dichloromethane/methanol/water/acetic acid (82:14:2:2, v/v) and (B) methanol/water/acetic acid (96:2:2, v/v). The gradient method began with 100% A at 0 min and and was brought to 88.3% A at 20 min, then to 74.4% A at 50 min, then to 12.3% A at 55 min, remaining at 12.3% A until 65 min, and then was returned to 100% A at 70 min with an additional 5 min equilibration time at 100% A. The procyanidins were analyzed using a Phenomenex 250 × 4.6 mm Luna Silica column (Torrence, CA). The total run time was 75 min with a constant flow rate of 1.0 mL/min. The procyanidins were detected at 276 nm excitation and 316 nm emission. The procyanidin polymers were quantified using external calibration curves of a polymeric fraction (DP > 10, average DP of 36.1) which were previously isolated from blueberry.[26]

Procyanidin Analysis by HPLC/ESI-MS. The procyanidins in the grape extracts were identified using HPLC/ESI-MS as described by Wang et al.[27] The procyanidins were separated with a 250 × 4.6 mm Luna Silica column (Phenomenex, Torrence, CA) attached to an Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA) with an HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The binary mobile phase used for analysis was composed of (A) dimethylchlorine/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 gradient method, running time, and solvent flow rate were as described previously. The electrospray ionization used was in negative mode using the following conditions: 50 psi nebulizer pressure, 10 L/min drying gas, and a 350 °C drying temperature. The fluorescent detection was set at 231 nm excitation and 320 nm emission. The data was collected and interpreted with Chemstation software (Version B. 01.03, Agilent Technologies, Palo Alto, CA).

Experimental Design. The variables used in the design of the semicontinuous hot−cold extraction of the grape pomace are as follows: temperature (60−140 °C), flow rate (5, 10, 15 mL/min), sample mass (5, 15, 25 g), and sample moisture content (dried or crude). Combinations of variables that were tested were predetermined by the statistical software Stat-Ease, Inc. (Minneapolis, MN) using a completely randomized response surface method. The experimental design fitted to a second degree polynomial is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot X_i + \sum_{i=1}^{k} \beta_{ii} \cdot X_{ii}^{\ 2} + \sum_{i=1} \sum_{j=i+1} \beta_{ij} \cdot X_{ii} \cdot X_{ji} \quad (1)$$

Table 1. Mean Total Anthocyanins, Total Procyanidins, and Percent Polymeric Color Extracted by Combinations of the Following Experimental Variables: Sample Moisture Content (MC), Flow Rate, Sample Mass, and Temperature

MC[a]	flow (mL/min)	mass (g)	temp (°C)	total anthocyanins[b]	total procyanidins[b]	% polymeric color[b]
dried	5	5	60	31.0	2,372.2	36.5
			140	83.6	2,059.9	37.7
		15	100	31.2	285.4	42.2
		25	60	11.2	156.4	35.9
			140	17.9	842.6	37.3
	10	5	100	39.1	148.8	
		15	60	42.8	540.7	34.2
			100	42.0	662.9	37.1
	15	5	60	11.6	359.0	
			140	57.6	1,323.0	38.6
		15	100	8.3	1,951.0	38.4
		25	60	10.2	183.8	37.3
			140	19.6	932.1	37.7
crude	5	5	140	111.9	2,240.9	39.2
		15	100	15.4	349.1	42.9
		25	60	24.2	699.6	38.0
			140	96.9	2,607.0	39.3
	10	5	100	65.4		43.0
		15	60	56.9	1,387.4	43.1
			100	80.1	1,136.0	38.4
			140	71.3	1,706.2	37.1
		25	100	34.2	1,235.2	36.3
	15	5	60	13.1	214.5	37.7
			140	119.5	939.7	38.4
		15	100	35.6	521.1	38.7
		25	140	70.6	1,078.4	35.8

[a]Sample moisture content was defined as dried (4% moisture, dry basis) or crude (58% moisture, dry basis). [b]Anthocyanins and procyanidins were quantified as mg/100 g DW and percent polymeric color quantified in perecentage points.

where Y was the dependent variable, β_0 was the central point of the system, β_i, β_{ii}, and β_{ij} corresponded respectively to the linear, quadratic, and interaction effects of the independent variables, and X_i, X_{ii}, and X_{ij} were the independent variables. A central composite design, face centered, was used. Design Expert 6.0.1 software (Stat-Ease Inc., Minneapolis, MN, USA) was used, and a 95% confidence interval was set for all the procedures. The objective of a response surface method was to evaluate the effect of different variables by creating samples that were generated at a variety of conditions and create regression equations to predict the optimal set of extraction conditions. Our goal was to maximize anthocyanin and procyanidin recovery and minimize polymeric color, or browning in the samples. The optimal conditions were repeated in triplicate to determine variability in sample replication. Data were analyzed with Stat-Ease, Inc. (Minneapolis, MN) and JMP 8 software (Cary, NC), and the significances within the model were determined at a level of $p < 0.05$.

RESULTS AND DISCUSSION

The total anthocyanin and procyanidin yield from semicontinuous hot−cold extraction of both dry (4% moisture) and crude (58% moisture) grape pomace was expressed in terms of dry weight basis calculated using eq 2.

$$\text{DW} = (\text{fresh weight} \times 100)/\text{dry weight} \quad (2)$$

The total anthocyanin and procyanidin yield from the semicontinuous hot−cold extraction of grape pomace were calculated as the sum of all the anthocyanins and procyanidins present in the grape pomace extract as characterized using the HPLC−MS. The anthocyanins and procyanidins present in the grape pomace as identified using HPLC−MS along with their respective peak numbers (in the order of their elution) has been listed and discussed in detail in our previous studies.[13,14]

The total anthocyanin and procyanidin yields from the semicontinuous hot−cold extraction of dried and crude grape pomace are given in Tables 2 and 3 as ascertained by the conventional method. For extraction of the crude pomace, they are 135.6 mg/100 g DW anthocynanins and 2,464.0 mg/100 g DW for the procyanidins. Using subcritical water alone, we were able to extract from the dried pomace 62% of the anthocyanins and 60% of the procyanidins relative to the conventional extraction method, while for the crude (wet) pomace, 96% and 84% anthocyanins and procyanidins, respectively, were extracted relative to that obtained using the conventional extraction method as described in Materials and Methods. There is no absolute quantification of the anthocyanin and procyanidin content of the pomace since it is dependent on the conventional extraction method employed. The detailed anthocyanin composition of the extracted fractions is shown in Table 2. Temperature (T_{exp}) was found to be the most and only significant variable affecting the anthocyanin recovery from the grape pomace ($p = 0.0207$), and the applicable regression equation is given by eq 3. It was found that the regression model provided an R^2 value of 0.51, indicating that as much as 51% of the variability in the results could be explained using the variables used in the study.

$$Y_1 = 34.06 + 0.35T_{exp} \quad (3)$$

where Y_1 is the predicted total anthocyanins recovered from dried pomace.

Table 2. Mean Anthocyanins[a] Extracted Using a Semicontinuous Extraction System with Combinations of the Following Variables: Sample Mositure Content (MC), Flow Rate, Mass, and Temperature[b]

				peak														
MC[c]	flow (mL/min)	mass (g)	temp (°C)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	total anthocyanins
dried	5	5	60	0.0	0.0	0.0	1.0	19.0	0.7	0.2	1.2	0.0	1.0	0.0	1.3	0.0	6.2	31.0
			140	0.1	0.0	1.1	2.8	41.0	1.5	1.2	3.6	0.2	4.0	0.3	5.3	0.0	22.0	83.6
		15	100	0.0	0.0	0.0	0.9	17.0	0.4	0.0	1.2	0.0	1.5	0.0	1.9	0.0	8.3	31.2
		25	60	0.0	0.0	0.1	0.4	5.7	0.3	0.3	0.6	0.1	0.6	0.0	0.6	0.0	2.5	11.2
			140	0.0	0.0	0.0	2.7	6.3	0.6	0.5	0.9	0.0	0.9	0.1	1.3	0.0	4.8	17.9
	10	5	100	0.0	0.0	0.0	0.9	22.0	0.0	0.0	1.3	0.0	1.8	0.0	2.0	0.0	11.0	39.1
		15	60	0.2	0.0	0.7	1.6	23.0	0.5	0.5	1.1	0.0	2.1	0.0	3.0	0.0	10.0	42.8
			100	0.1	0.0	0.6	1.4	21.0	0.6	0.6	1.8	0.1	2.1	0.2	2.7	0.0	11.0	42.0
	15	5	60	0.0	0.0	0.0	0.0	8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	11.6
			140	0.0	0.0	0.0	1.4	29.0	0.5	0.4	2.4	0.0	2.3	0.0	3.5	0.0	18.0	57.6
		15	100	0.0	0.0	0.0	0.0	6.3	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	1.8	8.3
		25	60	0.0	0.0	0.0	0.3	6.6	0.1	0.2	0.4	0.0	0.4	0.0	0.4	0.0	1.8	10.2
			140	0.0	0.0	0.0	0.6	11.0	0.2	0.1	0.9	0.0	0.9	0.0	1.2	0.0	5.2	19.6
crude	5	5	140	0.0	0.0	0.0	2.4	53.0	1.5	0.3	3.1	0.0	4.6	0.0	8.4	0.0	39.0	111.9
		15	100	0.0	0.0	0.0	0.0	11.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2	0.0	3.8	15.4
		25	60	0.0	0.0	0.0	0.8	14.0	0.1	0.0	0.8	0.0	0.9	0.0	1.4	0.0	5.8	24.2
			140	0.5	0.2	2.1	3.5	42.0	2.5	3.8	5.3	1.4	4.7	1.0	5.9	0.1	24.0	96.9
	10	5	100	0.0	0.0	0.0	0.7	40.0	0.0	0.0	1.5	0.0	1.7	0.0	3.0	0.0	18.0	65.4
		15	60	0.0	0.0	0.0	1.8	29.0	0.9	0.7	2.3	0.0	2.8	0.0	3.8	0.0	16.0	56.9
			100	0.2	0.0	0.6	2.5	40.0	1.1	1.2	3.3	0.1	3.9	0.2	5.5	0.0	21.0	80.1
			140	0.0	0.0	0.1	2.2	35.0	1.4	1.2	3.2	0.4	3.4	0.2	4.8	0.0	20.0	71.3
		25	100	0.0	0.0	0.0	1.1	20.0	0.5	0.5	1.8	0.0	1.6	0.0	1.5	0.0	7.5	34.2
	15	5	60	0.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	13.1
			140	0.0	0.0	0.0	1.5	61.0	0.0	0.5	1.3	2.1	2.0	3.6	4.8	0.0	43.0	119.5
		15	100	0.0	0.0	0.0	4.8	15.0	0.0	0.0	0.7	0.0	1.4	0.0	2.5	0.0	11.0	35.6
		25	140	0.3	0.0	1.5	2.6	35.0	1.2	1.1	2.8	0.3	3.1	0.4	4.4	0.0	18.0	70.6
conventional				0.0	0.0	0.0	2.3	43.8	2.7	1.6	1.3	3.8	5.0	0.6	12.6	0.0	61.9	135.6

[a]Anthocyanins quantified in mg/100 g DW. [b]Peak assignments, mass spectral data, and HPLC chromatogram have been presented in previous literature.[13] [c]Moisture content defined as dried (4% mositure, dry basis) or crude (58% moisture, dry basis).

Since anthocyanins are highly pigmented, these trends could also be verified and tracked visually, i.e., as the experimental temperature increased, the color of the extract changed from colorless to dark purple and the color of the pomace in the extraction cell turned brown, indicating that more anthocyanins were removed from the pomace and were solubilized in the extract. Such a relationship between temperature and the amount of total anthocyanins extracted from the grape pomace has been observed before[28−30] and has been attributed to the influence of the temperature-dependent physicochemical properties of solvent and the extracted solutes.

Mass transport studies have indicated that increased temperature impacted the extraction rate, efficiency, and selectivity of compounds due to increased vapor pressure and increased thermal desorption of polyphenolics.[28] Other studies have also attributed the greater anthocyanin yield with an increase in temperature to the increased solubility of the polyphenolic compounds in water and the enhanced dissolution power of the solvent.[31] However, this relationship between increased flavonoid yields with increasing temperature is offset due to the thermal lability of these compounds.[32] Studies have indicated that, under excessive heat, the anthocyanins had either a tendency to open its pyrilium ring, thereby forming a colorless chalcone equivalent, or cleave its sugar moiety, thus forming an anthocyanin aglycon.[29,33]

Studies done by Mishra et al.[33] indicated that mass average retention of anthocyanins heated to 126.7 °C for 25 min did not exhibit a linear degradation pattern and instead showed a sigmoidal trend. This meant that, after 25 min, the degradation curve increased substantially until it asymptoted after a certain point. Since many of our extractions were shorter than 25 min, it was possible that they were executed in the region before a sigmoidal increase in anthocyanin degradation would have occurred. Studies done by Duan et al.[20] indicated that the degradation rates for silymarins in hot water doubled when temperature increased from 100 to 120 °C and increased 8 times when temperature was increased to 160 °C. However, these degradation studies were done for a residence time of as high as 60 min, and the half-life of the silymarin compounds was found to range from 6 min at 160 °C to as high as 58 min at 100 °C. This confirms that a shorter residence time at higher temperatures might be ideal for enhancing extraction of such thermally labile compounds using water as a solvent, which is not possible using a batch process.

The regression models used in this study to predict and optimize flavonoid yield from grape pomace have been discussed on the basis of the set oven temperatures (T_{exp}). Although an optimal oven temperature of 140 °C would seem to be deleterious to the thermal degradation of polyphenolic compounds, logging of the thermocouple data described in the Materials and Methods gave us better understanding of the actual temperatures experienced by the extraction solvent continuously moved through the system. Since the water was heated solely during the time it moved through the coils within the GC oven, it is only rational that the extraction solvent might not fully reach the set point GC oven temperature value,

Table 3. Mean Procyanidins[a] Extracted Using a Semicontinuous Extraction System with Combinations of the Following Variables: Sample Moisture Content, Flow Rate, Mass, and Temperature[b]

MC[c]	flow (mL/min)	mass (g)	temp (°C)	epicatechin[a]	catechin	dimers	trimers	tetramers	pentamers	sum DP 1−5	polymers	total procyanidins
dried	5	5	60	284.6	220.7	414.8	178.8	148.3	84.4	1,331.0	1,041.0	2,372.0
			140	226.6	190.3	404.2	164.8	145.4	84.3	1,216.0	1,230.0	2,060.0
		15	100	44.1	30.2	58.6	29.1	26.1	14.0	202.1	83.3	285.4
		25	60	20.8	13.6	32.7	14.7	12.4	7.4	101.5	54.9	156.4
			140	101.5	81.5	146.6	70.7	58.8	34.4	493.5	349.1	842.6
	10	5	100	29.8	19.2	44.9	14.9	13.3	8.0	130.2	18.5	148.8
		15	60	71.0	47.4	92.3	45.1	39.7	18.2	313.8	227.0	540.7
			100	84.7	61.8	114.9	51.5	44.9	26.1	383.7	279.1	662.9
	15	5	60	46.5	34.1	56.5	30.2	24.0	15.8	207.1	151.9	359.0
			140	135.1	111.5	210.4	87.9	72.5	41.8	659.2	663.8	1,323.0
		15	100	239.1	160.9	314.2	145.0	123.0	69.9	1,052.0	899.0	1,951.0
		25	60	22.6	15.2	44.8	16.4	14.5	11.7	125.2	58.6	183.8
			140	101.1	83.7	135.7	64.6	55.7	34.3	475.1	457.0	932.1
crude	5	5	140	203.3	129.8	152.8	81.1	340.7	256.0	1,164.0	1,077.0	2,241.0
		15	100	62.7	52.1	73.7	36.8	33.0	19.6	277.9	71.2	349.1
		25	60	79.4	58.6	109.1	56.5	48.2	24.8	376.6	323.0	699.6
			140	230.7	186.8	390.2	149.0	126.2	80.7	1,163.0	1,444.0	2,607.0
	10	15	60	137.0	101.3	328.4	115.1	97.7	59.1	838.7	548.8	1,387.0
			100	141.7	102.0	198.5	89.0	78.6	51.5	661.3	474.8	1,136.0
			140	159.8	132.7	280.4	109.3	89.6	51.9	823.7	882.5	1,706.0
		25	100	142.2	102.6	206.3	94.1	82.6	51.0	678.7	556.5	1,235.0
	15	5	140	209.5	156.6	206.3	98.9	81.8	44.5	797.6	142.0	939.7
		15	100	97.0	47.9	115.2	49.8	48.2	30.0	388.1	133.0	521.1
		25	140	107.6	87.3	133.7	67.4	56.6	23.8	476.4	602.1	1,078.0
conventional				117.3	77.2	164.1	78.0	68.5	45.2	1,913.8	550.2	2,464.0

[a]Procyanidins quantified in mg/100 g DW. [b]Mass spectral data and HPLC chromatogram have been presented in previous literature[14]. [c]Moisture content was either dried (4% moisture, dry basis) or crude (58% moisture, dry basis).

especially at higher flow rates. The thermocouples placed at different points in the system indicated that when the oven temperature was set (for example) at 140 °C, the actual cell temperature was found to be 88.4 °C and the temperature of the water in the line just before it enters the cell was found to be 112 °C. Similar experiments conducted at 60 and 100 °C indicated average cell temperatures of around 35 and 50 °C respectively. However, the variation in the actual extraction cell temperatures due to the effect of mass, moisture content, and mass of pomace was found to be ±10 °C. Hence, our regression models were based on the set oven temperature as a controlling variable and the results agreed well with previous predicted data on the optimal temperatures for extraction of polyphenols from grape pomace.[13,14]

The total procyanidins extracted from grape pomace as a function of temperature, flow rate, mass of pomace, and moisture content is given in Table 1. The composition of the procyanidin in the resultant extracts is shown in Table 3. The regression model indicated that an interaction between the flow rate and mass of the pomace was found to be significant (p = 0.0269) with an R^2 value of 0.62. The regression equation for the procyanidin recovery from grape pomace is given in eq 4 as follows:

$$Y_2 = 509.16 + 6.44(\text{flow} - 10.33)(\text{mass} - 13.33) \quad (4)$$

where Y_2 is the predicted total procyanidins extracted from dried pomace. The regression model indicated that no other variables or interactions were found to significantly affect the procyanidin yield from grape pomace. Kronholm et al.[28] have noted that the extraction efficiency was dependent on the solvent flow rate and the concentration or mass charge of the sample used.

Data for percent polymeric color for the extraction of both dry and wet pomace as function of the extraction conditions are shown in Table 1. The percent polymeric color for dried pomace was found to be dependent on all variables and combinations of variables (flow rate, mass, temperature) except for a flow*flow interaction, and the model fit indicated an R^2 value of 0.69.

$$\begin{aligned} Y_3 = {} & 44.27 - 0.5T_{\text{exp}} + 0.35\,\text{flow} - 0.38\,\text{mass} \\ & - 0.004(T_{\text{exp}} - 101.33) - 0.01(T_{\text{exp}} - 101.11) \\ & (\text{flow} - 10.33) + 0.04(T_{\text{exp}} - 101.33) \\ & (\text{mass} - 13.33) - 0.04(\text{flow} - 10.33) \\ & (\text{mass} - 13.33) + 0.05(\text{mass} - 13.33) \\ & (\text{mass} - 13.33) \end{aligned} \quad (5)$$

where Y_3 is the predicted polymeric color in the grape pomace extract. The correlation of the data showed that the percent polymeric color increased with an increase in the flow rate and decreased with increasing temperature and mass. These results are in contrast to previous results that reported an increase in the polymeric color with application of heat due to an increase in the extraction temperature.[17,24] These are logical trends since the polymeric color is an indicator of anthocyanin degradation at the higher extraction temperatures. It was found that, with increasing temperature, a greater extraction of monomeric anthocyanins occurs which can account for the higher

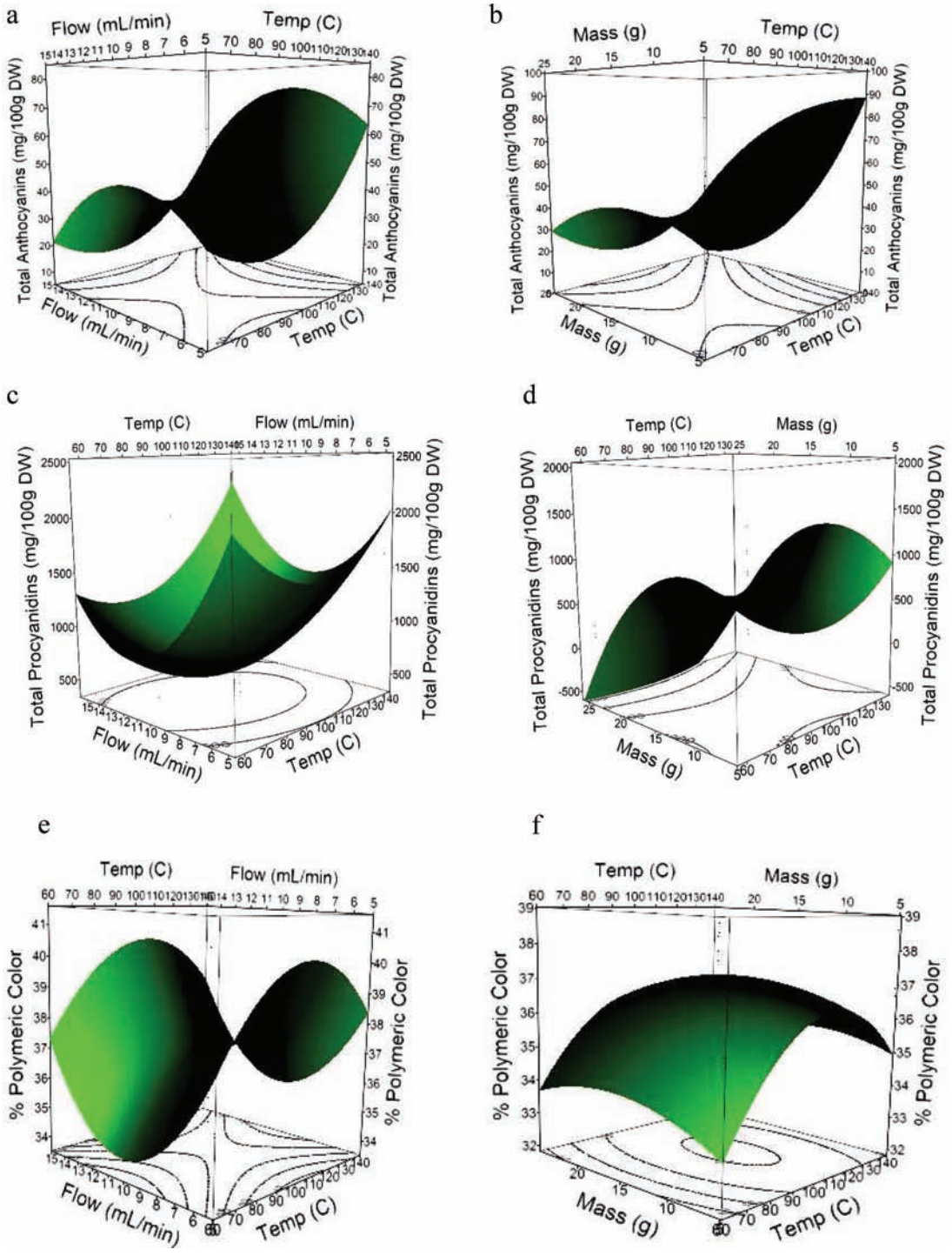

Figure 2. Response surface profile and contour graphs for extraction results obtained on the batch, semicontinuous extraction unit. Panels a, b, c, d, e, and f show the relationship between flow rate, mass, and temperature on anthocyanin recovery (mg/100 g dry weight), procyanidin recovery (mg/100 g dry weight), and percent polymeric color (%), respectively, from dried pomace.

monomeric:polymeric anthocyanin ratio with increasing temperature. It is also possible that the extraction temperatures were not sufficient for the given extraction period to promote the polymerization of the anthocyanins or the formation of anthocyanin−tannin complexes.[34]

Response surface profile graphs and contour plots for the total anthocyanins and procyanidins extracted from grape pomace are shown in Figures 2 and 3. These surface profile graphs are three-dimensional illustrations of the relationship between two independent variables and one dependent variable. Since the experimental model was second-order, the surface graph contained quadratic-type bends and twists.[35] A contour plot, which is shown at the bottom of the surface profile graph, is a two-dimensional illustration of the surface trends. Contour plots graphically intercept a surface graph starting from the optimization point and continually slice further from the optimization point. An "X-shape" is represented by a saddle point solution, a "circle" shape

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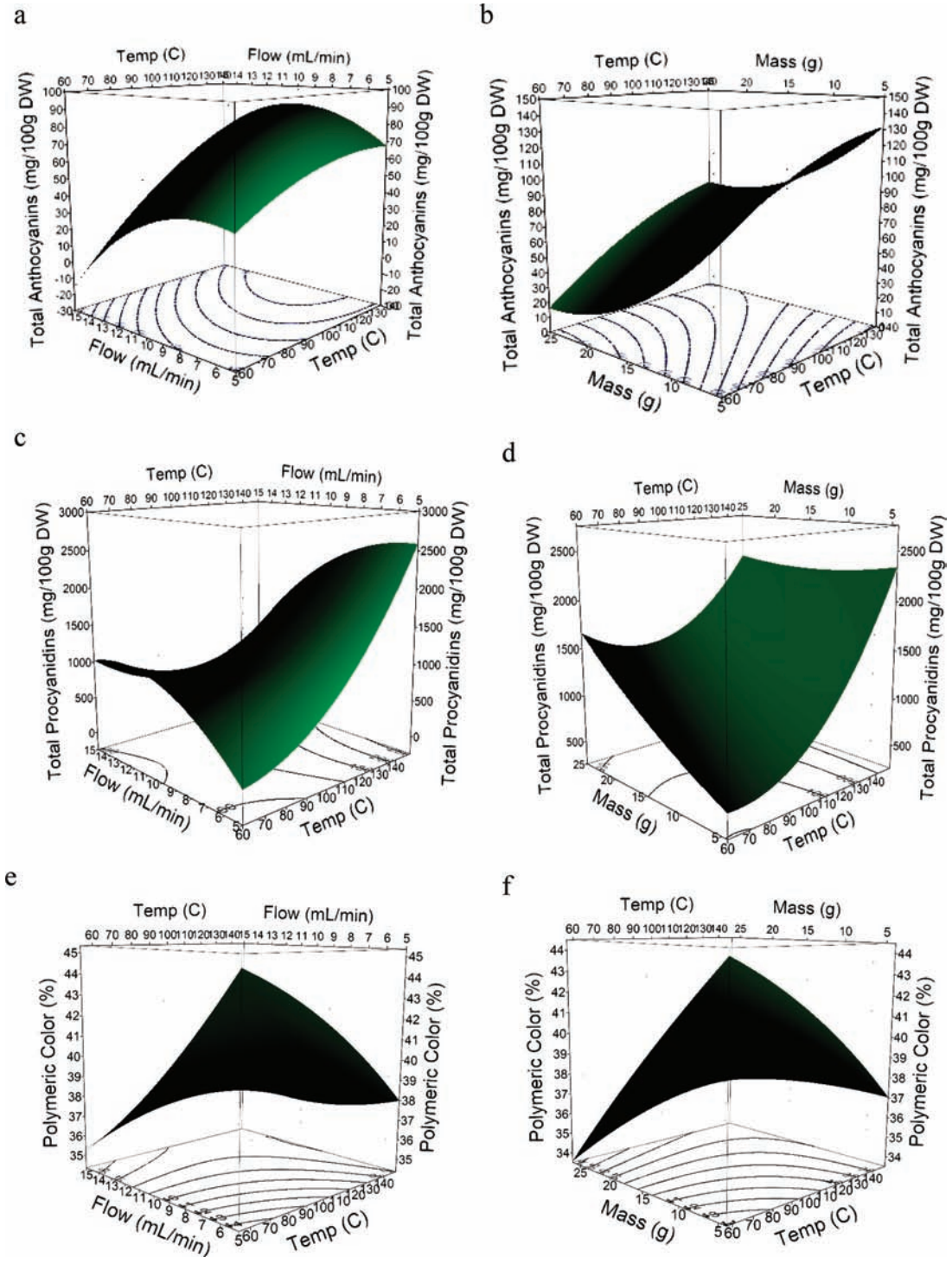

Figure 3. Response surface profile and contour graphs for the results obtained on the batch, semicontinuous extraction unit. Panels a, b, c, d, e, and f show the relationship between flow rate, mass, and temperature on anthocyanin content (mg/100 g dry weight), procyanidin content (mg/100 g dry weight), and percent polymeric color (%), respectively, from crude (wet) pomace.

represents a solution within the circle, and a "straight line" indicates a linear relationship between the variables.

In Figure 2, graphs a, b, c, d, e, and f showed images of the relationship between flow rate (mass) and temperature for anthocyanins, procyanidins, and percent polymeric color for dried pomace, respectively. The saddle point in these plots indicated the optimum conditions for the extraction of anthocyanins and procyanidins from grape pomace. At these saddle points, the predicted concentration of anthocyanins and procyanidins was found to be 43.5 and 682.8 mg in 100 g DW pomace having a polymeric color of 37.1%. However, a saddle point also means that the optimization point was a minimum point for one variable under study and a maximum point for another variable.[36] Hence, the optimal experimental conditions were determined using ANOVA, and for dried pomace, these conditions was found to be 140 °C oven temperature, flow rate of 5.9 mL/min, and a starting mass of 9.9 g. Under these conditions, 64.4 mg/100 g DW of anthocyanins (polymeric

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color of 33%) and 1,770.0 mg/100 g DW of procyanidins were extracted from grape pomace using hot water. These predicted results accounted for 48% and 71% of the respective yields obtained using a methanol- or acetone-based conventional extraction method, respectively.

These results were similar to that reported in Pinelo et al.[37] where distilled water was used to extract flavonoids from white grape pomace using a small-scale continuous extraction method. Similar to our results, Pinelo et al.[37] found that the use of smaller sample masses was preferred for two reasons. First, high sample masses promote a packing or channeling phenomenon that creates zones on the sides of the extraction cell that prevent contact between the pomace and solvent. Second, a smaller sample mass promotes a higher concentration gradient between sample and liquid, thereby enhancing the extraction rate. Although our optimal result did not require high solvent flow rate, we believe that the optimized flow rate determined in this study allowed the dried pomace to be sufficiently hydrated throughout the extraction time, thereby reducing losses due to degradation. Pinelo et al.[37] and King et al.[38] have noted that the use of less solvent produces more concentrated extracts, eliminating the need for water removal to produce a more concentrated extract for food formulation purposes. As previously discussed, Kronholm et al.[28] indicated that increase in extraction temperature was the most effective variable in improving extraction efficiencies, which is consistent with that observed for extraction of the flavonoids.

Results for crude pomace were similar to the dried pomace except there was more variability in the data. The amounts of anthocyanins and procyanidins and the percent polymeric color extracted from crude grape pomace are listed in Table 1. This variability resulted in lower R^2 values in the predicted model. One explanation for this result was that the crude sample was more heterogeneous, i.e., there were a varying proportion of skins or seeds. The results for total anthocyanins extracted from crude pomace have an R^2 value of 0.45, and only the temperature effect was significant ($p = 0.0260$). The regression model given by eq 6 is as follows:

$$Z_1 = 25.98 + 0.69T_{exp} \quad (6)$$

where Z_1 refers to the predicted total anthocyanins extracted from crude pomace. Compared to dried pomace, the intercept coefficient in crude pomace was found to be higher, indicating that more anthocyanins were extracted with crude pomace. The slope of the model was also found to be higher, which can be attributed to a greater effect of temperature on the extraction of anthocyanins from crude grape pomace as compared to dried grape pomace.

The regression model for predicting the optimized extraction of procyanidins from grape pomace (give in eq 7) was found to be similar to that for anthocyanins, with an R^2 of 0.42 and temperature as the only significant variable ($p = 0.0344$).

$$Z_2 = -626.93 + 17.87T_{exp} \quad (7)$$

where Z_2 refers to the predicted total procyanidins extracted from the crude grape pomace. The model applied to the percent polymeric color in the crude pomace extracts indicated no significant effect of the experimental variables.

Response surface profile graphs and contour plots as shown in Figure 3, panels a, b, c, d, e, and f, showed the effect of flow rate, mass, and temperature on the anthocyanin content, the procyanidin content, and the percent polymeric color of the crude pomace extracts respectively. As indicated previously, due to the limitations in the regression model, ANOVA was used to determine the optimized conditions for the extraction of polyphenolic compounds from crude grape pomace. The results indicated that there was maximum anthocyanin and procyanidin yield from crude grape pomace at an oven temperature of 140 °C, a flow rate of 8.85 mL/min, and a mass of 5 g. Under these conditions, the crude pomace extracts contained 129.8 mg/100 g DW anthocyanins, 2076.9 mg/100 g DW procyanidins, and 37.0% polymeric color, which amounted to 96% and 83.5% of that obtained using solvent extraction with methanol- or acetone-based conventional solvents, and a 10.5 unit increase in polymeric color.

The greater polyphenolic yield from crude grape pomace over dried pomace was also found in another study, where it was attributed to a quicker solvent diffusion of water through crude pomace and the attainment of kinetic equilibrium at a faster rate.[39] However, our study indicated that a slightly higher water flow rate was required for obtaining kinetic equilibrium in crude pomace when compared to dried pomace for the optimized extraction of polyphenolic compounds. For the same residence time used in the experiments, this slightly higher solvent flow rate yielded close to twice the amount of anthocyanins and 1.5 times more procyanidins from the grape pomace. The percent polymeric color for both the dried and crude pomace extracts was found to be the same.

This increased yield from the crude pomace was a significant finding since it allowed the wet pomace, as is, to be directly extracted without employing a drying step in the pomace extraction scheme. These results indicated that, even after blending crude pomace to reduce heterogeneity, the impact of enzyme-initiated degradation of anthocyanins by exposing the embedded oxidoreductases to phenolic-containing components in the pomace matrix was minimal. Rapid degradation of anthocyanins was not observed, possibly attributed to the use of frozen pomace for grinding, which decreased enzymatic activity, or because these samples had high levels of acylated malvidin. Studies have indicated that the acylated anthocyanins are more stable than nonacylated anthocyanins, and likewise, higher methoxylated anthocyanins, like malvidin, were found to be more stable than the less methoxylated, or more hydroxylated, anthocyanins.[40]

While the regression models discussed above were based on the total anthocyanins and procyanidins present in the extract at the end of the experiment (18 min), we also wanted to determine the concentration of the polyphenolic compounds in the extract as a function of the residence time. Although there have been other studies on fractioning anthocyanins by varying solvents to isolate anthocyanins or procyanidins,[41−43] there were no other studies, to our knowledge, that have fractionated them using one solvent, in this case subcritical water, using a semicontinuous extraction apparatus to determine the extraction and elution pattern of the polyphenolics.

In the time study, extracts were collected every minute for 18 min and analyzed for polyphenolic concentration. Five grams of crude pomace was used for the time study with water flow rate set at 15 mL/min. The concentration of the anthocyanins in the crude pomace extract obtained from the time-based study is presented in Figure 4. In Figure 4a, the concentration of the anthocyanins in the extracts (mg/100 g DW) was plotted as a function of time during the 18 min extraction. The cumulative anthocyanin yield (mg/100 g DW) was plotted as the secondary ordinate. In Figure 4b, respective thermocouple readings indicating the oven set point, temperature preheater

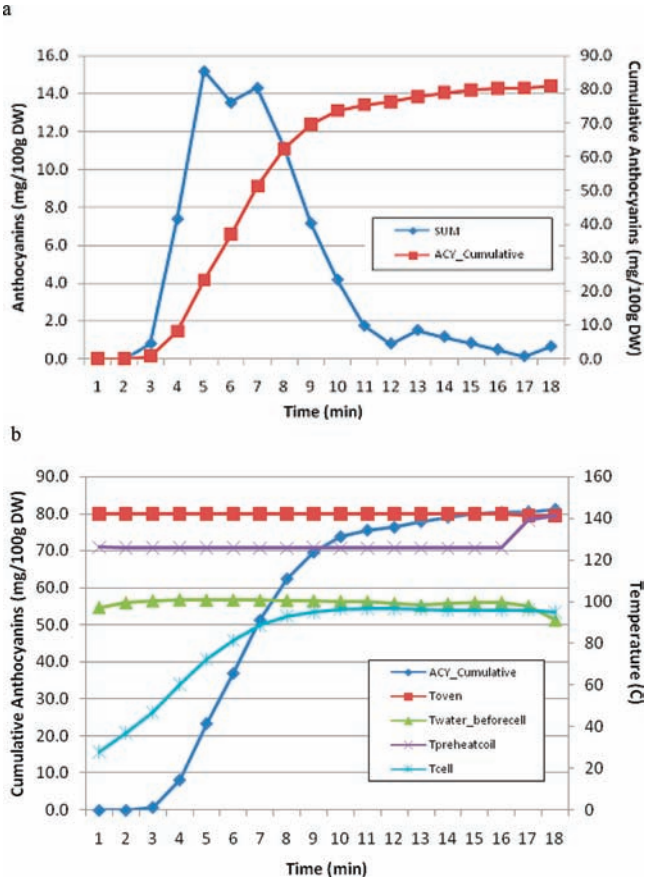

Figure 4. (a) Graph showing anthocyanins extracted (mg/100 g DW) after each minute during a batch, semicontinuous extraction using water as the solvent. The primary (Y_1) axis is the actual amount of anthocyanins extracted after each minute after commencing the extraction while the secondary ordinate shows cumulative extraction (mg/100 g DW). (b) Graph comparing cumulative extraction on the primary ordinate to temperatures (cell surface, oven, preheat coil, and cell inlet point) each minute of the extraction on the secondary ordinate.

coil placed in the oven, temperature of the water entering the extraction cell, and the extraction cell temperature are plotted as a function of the residence time.

It was found that no anthocyanins eluted until the fourth minute of the semicontinuous run, which can be attributed to the heating time (as shown in Figure 4b) required by the crude pomace to facilitate effective solvent diffusion and subsequent solubilization of the anthocyanins in the solvent. However, after the fourth minute, it was found that 85% of total anothcyanins were extracted within the next 5 min. This indicates that the majority of the anthocyanins were extracted in the first 9 min, which is similar to that reported by King et al.[39] The reduction in the anthocyanin concentration in the extracts after the first 9 min can either relate to effective extraction or onset of thermal degradation. However, evaluation of the HPLC chromatograms of the extracts did not indicate significant presence of chalcones or other byproduct that might have been obtained from the thermal degradation of the anthocyanins.

The two most prevalent anthocyanin compounds in the extracts obtained from HPLC−MS analysis were found to be malvidin-3-*O*-monoglucoside and malvidin-3-*O*-(6-*O*-*p*-coumaroyl)-monoglucoside. The malvidin monoglucosides have greater polarity compared to its acylated counterparts, which can be used to explain the bimodal extraction pattern between 5 and 7 min in Figure 4a. Based on the analyte polarity, the concentration profile maximum at 5 min can relate to malvidin monoglucoside while the maximum at 7 min can be attributed to the acylated malvidin monoglucoside. It would appear from the solubility-based fractionation pattern that the more polar anthocyanins eluted earlier from the pomace matrix during the extraction process followed by less polar anthocyanins. This extraction or elution pattern can relate to the greater polarity of water at lower temperatures and its decrease with an increase in temperature as reported previously.[22,44]

Similar results for the concentration of procyanidins in the collected extracts are shown in Figure 5a with the temperature profiles shown in Figure 5b. It was found that almost 65% of the available procyanidins were extracted from the grape pomace within the first 11 min of the extraction. These results again indicate the feasibility of a rapid extraction of polyphenolic compounds using subcritical water. Similar to the anthocyanin extraction, a bimodal pattern was also seen

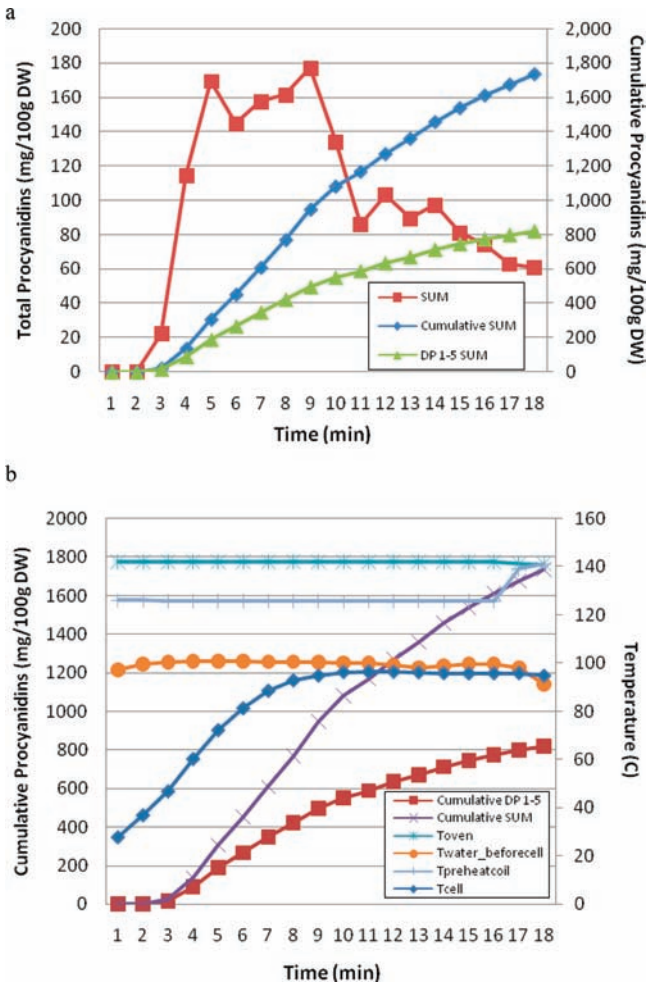

Figure 5. (a) Graph showing procyanidins extracted (mg/100 g DW) after each minute during a batch, semicontinuous extraction using water as the solvent. The primary (Y_1) axis is actual amount of procyanidins extracted after each minute after commencing the extraction while the secondary ordinate shows cumulative extraction (mg/100 g DW). (b) Graph comparing cumulative extraction on the primary ordinate to temperatures (cell surface, oven, preheat coil, and cell inlet point) each minute of the extraction on the secondary ordinate.

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here which can be attributed to the polarity of the procyanidins. The cumulative yield of the procyanidin oligomers and the total procyanidins in the extract was also obtained as a function of residence time and is given in Figures 5a and 5b. It can be seen from Figure 5a that, at the end of the 11th minute, the amount of DP 1−5 oligomeric procyanidins was almost 50% of the total procyanidins in the extracts. However, at the end of the extraction, these oligomeric procyanidins amounted to only 45% of the total procyanidins. This indicates that a shorter residence time can effectively extract the lower oligomeric antioxidants which are easily absorbed by the human body when compared to the polymeric procyanidins.[45]

The above study showed that a semicontinuous extraction system was efficient for extracting both anthocyanins and procyanidins from red grape pomace. Utilization of crude (wet) pomace gave equal or better results for the anthocyanin and procyanidin extraction recoveries compared to dehydrated or dried grape pomace. Introduction of the hot solvent into the extraction vessel held at ambient conditions also minimized the degradation of the poylphenolic compounds. Previous studies[46] have indicated the need to treat pomace by inactivating enzymes, lowering pH, removing oxygen, etc. in order to prevent degradation. However, this study showed that it was not necessary to add an additional drying and treatment step to the process to realize successful recovery of the polyphenolics from grape pomace. High extraction efficiencies were realized for both anthocyanins and procyanidins using this method, relative to that obtained using organic solvents.

The extraction pattern of the anthocyanins and procyanidins as a function of time from the grape pomace was also studied, and it was found that the anthocyanins could be efficiently extracted within 12 min. The constituent anthocyanins were found to be extracted in the order of highest to lowest polarity, while the procyanidins eluted with an increase in the degree of polymerization (DP). Such information is of value in optimizing the described process with respect to isolating specific polyphenolic fractions having variable antioxidant activity.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomas-Barberan, F. A.; Datta, N.; Singanusong, R.; Chen, S. S. Flavonoids in foods and their health benefits. *Plant Food Hum. Nutr.* **2004**, *59*, 113−122.

(2) Shrikhande, A. J. Wine by-products with health benefits. *Food Res. Int.* **2000**, *33*, 469−474.

(3) Luque-Rodríguez, J. M.; Luque de Castro, M. D.; Pérez-Juan, P. Dynamic superheated liquid extraction of anthocyanins and other phenolics from red grape skins of winemaking residues. *Bioresour. Technol.* **2007**, *98*, 2705−2713.

(4) Lapornik, B.; Prosek, M.; Wondra, A. G. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng.* **2005**, *71*, 214−222.

(5) Schwartz, S. J. *Protocols in Food Analytical Chemistry*; Wiley: New York, 2011; pp F.0.1−F.0.2.

(6) Mustafa, A.; Turner, C. Pressurized liquid extraction as a green approach in food and herbal plants: a review. *Anal. Chim. Acta* **2011**, *703*, 8−18.

(7) Carabias-Martinez, R.; Rodriguez-Gonzalo, E.; Revilla-Ruiz, R. Hernandez-Mendez. Pressurized liquid extraction in the analysis of food and biological samples. *J. Chromatogr., A* **2005**, *1089*, 1−17.

(8) Teo, C. C.; Tan, S. N.; Yong, J. W. H.; Hew, C. S.; Ong, E. S. Pressurized hot water extraction. *J. Chromatogr., A* **2010**, *1217*, 2484−2494.

(9) King, J. W.; Srinivas, K.; Zhang, D. Advances in critical fluid processing. In *Alternatives to Conventional Food Processing*; Proctor, A., Ed.; RSC Publishing: Cambridge, U.K., 2011; pp 93−144.

(10) Kammerer, D.; Claus, A.; Schieber, A.; Carle, R. A novel process for the recovery of polyphenols from grape (*Vitis vinifera* L.) pomace. *J. Food Sci.* **2005**, *70*, C157−C163.

(11) Lou, X.; Janssen, H. G.; Cramers, C. A. Parameters affecting the accelerated solvent extraction of polymeric samples. *Anal. Chem.* **1997**, *69*, 1598−1603.

(12) Sharif, A.; Saim, N.; Jasmani, H.; Ahmad, W. Y. W. Effects of solvent and temperature on the extraction of colorant from onion (Allium cepa) skin using pressurized liquid extraction. *Asian J. Appl. Sci.* **2010**, *3*, 262−268.

(13) Monrad, J. K.; Howard, L. R.; King, J. W.; Srinivas, K.; Mauromoustakos, A. Subcritical solvent extraction of anthocyanins from red grape pomace. *J. Agric. Food Chem.* **2010**, *58*, 2862−2868.

(14) Monrad, J. K.; Howard, L. R.; King, J. W.; Srinivas, K.; Mauromoustakos, A. Subcritical solvent extraction of procyanidins from red grape pomace. *J. Agric. Food Chem.* **2010**, *58*, 4014−4021.

(15) Srinivas, K.; King, J. W.; Monrad, J. K.; Howard, L. R.; Zhang, D. Pressurized solvent extraction of flavonoids from grape pomace utilizing organic acid additives. *Ital. J. Food Sci.* **2011**, *23*, 90−105.

(16) Simpson, K. L. Chemical changes in natural food pigments. In *Chemical Changes in Food During Processing*; Richardson, T., Finley, J. W., Eds.; Van Nostrand Reinhold: New York, 1985; pp 409−441.

(17) Ju, Z. Y.; Howard, L. R. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. *J. Agric. Food Chem.* **2003**, *51*, 5207−5213.

(18) Sadilova, E.; Carle, R.; Stintzing, F. C. Thermal degradation of anthocyanins and its impact on color and *in vitro* antioxidant capacity. *Mol. Nutr. Food Res.* **2007**, *51*, 1461−1471.

(19) Petersson, E. V.; Liu, J.; Sjöberg, P. J. R.; Danielsson, R.; Turner, C. Pressurized hot water extraction of anthocyanins from red onion: A study on extraction and degradation rates. *Anal. Chim. Acta* **2010**, *663*, 32.

(20) Duan, L.; Wallace, S. N.; Engelberth, A.; Lovelady, J. K.; Clausen, E. C.; King, J. W.; Carrier, D. J. Extraction of co-products from biomass: example of thermal degradation of silymarin compounds in subcritical water. *Appl. Biochem. Biotechnol.* **2009**, *158*, 362−373.

(21) Xavier, M. F.; Lopes, T. J.; Quadri, M. G. N.; Quadri, M. B. Extraction of red cabbage anthocyanins: optimization of operating conditions of the column process. *Braz. Arch. Biol. Technol.* **2008**, *51*, 143−152.

(22) Ibanez, E.; Kubatova, A.; Senorans, J. F.; Cavero, S.; Reglero, G.; Hawthorne, S. B. Subcritical water extraction of antioxidant compounds from rosemary plants. *J. Agric. Food Chem.* **2003**, *51*, 375−382.

(23) Duan, L.; Carrier, D. J.; Clausen, E. C. Silymarin extraction from milk thistle using hot water. *Appl. Biochem. Biotechnol.* **2004**, *114*, 559−568.

(24) Cho, M. J.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771−1782.

(25) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Ed.; John Wiley & Sons: New York, 2001; pp F1.2.1−F1.2.13.

(26) Prior, R. L.; Lazarus, S. A.; Cao, G.; Muccitelli, H.; Hammerstone, J. F. Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium* Spp.) using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1270−1276.
(27) Wang, W.; Bostic, T. R.; Gu, L. Antioxidant capacities, procyanidins and pigments in avocados of different strains and cultivars. *Food Chem.* **2010**, *122*, 1193−1198.
(28) Kronholm, J.; Hartonen, K.; Riekkola, M. L. Analytical extractions with water at elevated temperatures and pressures. *Trends Anal. Chem.* **2007**, *26*, 396−412.
(29) Sin, H. N.; Yusof, S.; Hamid, N. S. A.; Rahman, R. A. Optimization of hot water extraction for sapodilla juice using response surface technology. *J. Food Eng.* **2006**, *74*, 352−358.
(30) Li, Z.; Pan, Q.; Cui, X.; Duan, C. Optimization on anthocyanins extraction from wine grape skins using orthogonal test design. *Food Sci. Biotechnol.* **2010**, *19*, 1047−1053.
(31) Srinivas, K.; King, J. W.; Monrad, J. K.; Howard, L. R.; Hansen, C. M. Optimization of subcritical fluid extraction of bioactive compounds using Hansen solubility parameters. *J. Food Sci.* **2009**, *74*, E342−E354.
(32) Patras, A.; Brunton, N. P.; O'Donnell, C.; Tiwari, B. K. Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. *Trends Food Sci. Technol.* **2010**, *21*, 3−11.
(33) Mishra, D. K.; Dolan, K. D.; Yang, L. Confidence intervals for modeling anthocyanin retention in grape pomace during non-isothermal heating. *J. Food Sci.* **2008**, *73*, E9−E15.
(34) Brownmiller, C.; Howard, L. R.; Prior, R. L. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. *J. Food Sci.* **2008**, *73*, H72−H79.
(35) Fox, J. *Applied Regression Analysis and Generalized Linear Models*; Sage Publications: Thousand Oaks, CA, 2008.
(36) Liyana-Pathirana, C.; Shahidi, F. Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem.* **2005**, *93*, 47−56.
(37) Pinelo, M.; Del Fabbro, P.; Manzocco, L.; Nuñez, M. J.; Nicolib, M. C. Optimization of continuous phenol extraction from *Vitis vinifera* byproducts. *Food Chem.* **2005**, *92*, 109−117.
(38) King, J. W.; Gabriel, R. D.; Wightman, J. D. Subcritical water extraction of anthocyanins from fruit berry substrates. *Proceedings of the 6th Intl. Symposium on Supercritical Fluids Tome 1, April 28−30, 2003, Versailles, France*, pp 409−418.
(39) Sriram, G.; Surendranath, C.; Sureshkumar, G. K. Kinetics of anthocyanin extraction from fresh and dried grape waste. *Sep. Sci. Technol.* **1999**, *34*, 683−697.
(40) Yokotsuka, K.; Singleton, V. L. Disappearance of anthocyanins as grape juice is prepared and oxidized with PPO and PPO substrates. *Am. J. Enol. Vitic.* **1997**, *48*, 13−25.
(41) Vidal, S.; Hayasaka, Y.; Meudec, E.; Cheynier, V.; Skouroumounis, G. Fractionation of grape anthocyanin classes using multilayer coil countercurrent chromatography with step gradient elution. *J. Agric. Food Chem.* **2004**, *52*, 713−719.
(42) Sun, G.; Belchior, P.; Ricardo-da-Silva, J. M.; Spranger, M. I. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr., A* **1999**, *841*, 115−121.
(43) Sun, B.; Leandro, C.; Ricardo da Silva, J. M.; Spranger, I. Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1998**, *46*, 1390−1396.
(44) Herrero, M.; Cifuentes, A.; Ibanez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. *Food Chem.* **2006**, *98*, 136−148.
(45) Donovan, J. L.; Kasim-Karakas, S.; German, J. B.; Waterhouse, A. L. Urinary excretion of catechin metabolites by human subjects after red wine consumption. *Br. J. Nutr.* **2002**, *87*, 31−33.
(46) Markakis, P. Anthocyanins and their stability in foods. In *Critical Reviews in Food Technology*; Furia, T. E., Ed.; CRC Press: Cleveland, OH, 1974; pp 437−456.