Extraction of Anthocyanins and Polyphenolics from Blueberry Processing Waste

J. LEE AND R.E. WROLSTAD

ABSTRACT: The effectiveness of temperature, SO_2, citric acid, and industrial juice-processing enzymes (n = 9) for producing extracts of blueberries (*Vaccinium corymbosum*, cv. Rubel) and blueberry skins that are rich in anthocyanins and polyphenolics were evaluated individually and/or in combination. Enzyme treatment had little effect on total monomeric anthocyanins and on total phenolics recovery. Various combinations of heat, SO_2, and citric acid yielded extracts with higher concentrations of ACY and TP than the control. The distribution of anthocyanins and polyphenolics in 'Rubel' was also investigated. Anthocyanins existed almost exclusively in the skins, and polyphenolics were mostly in the skins with lesser amounts in flesh and seeds. Skins were also highest in antioxidant activity. All portions contained the same individual anthocyanins but in varying amounts. Cinnamic acid derivatives and flavonol-glycosides were found in the skins and seeds, whereas the flesh contained only cinnamic acids.

Keywords: anthocyanins, polyphenolics, blueberry, skins, flesh, seeds

Introduction

The health benefits of blueberries (*Vaccinium* sp.) became widely accepted after Prior (1998) reported that blueberries had the highest antioxidant activity of 42 fruits and vegetables evaluated. This finding sparked numerous investigations into the health benefits of blueberries. Several studies have confirmed that blueberries contain high antioxidant activity compared with other fruits (Cao and others 1996; Wang and others 1996; Prior and others 1998; Vinson and others 2001). Epidemiological studies have demonstrated that a diet rich in fruits and vegetables reduces the risk of certain types of cancer, cardiovascular disease, and other chronic diseases (Steinmetz and Potter 1991; Hertog and others 1993, 1994, 1995; Criqui and Ringel 1994; AICR 1997; Ness and Powles 1997).

The Pacific Northwest is a major producer of berries, fruit juices, and fruit juice concentrates. In 2002, Oregon produced 27500000 pounds of blueberries. Of these, 40% were marketed as fresh fruit, whereas the remaining 60% were processed in some form (Coba and Goodwin 2003). A substantial amount of blueberry fruit is processed into juice and juice concentrate, which is subsequently used in beverages, syrups, and other food products. Juice processing unavoidably generates waste by-products, consisting of seeds, stems, and skins that will be found in the presscake residue. Because blueberry presscake has high amounts of anthocyanins and polyphenolics (Lee and others 2002), it is a potential source for natural colorants and nutraceuticals.

The objective of this study was to evaluate juice-processing enzymes and a number of processing parameters, for producing aqueous blueberry extract that was rich in anthocyanins and polyphenolics. The intent was to develop a process that would be suitable for manufacturing extracts from blueberry juice–processing wastes for natural colorant and nutraceutical usage. Another approach for making an anthocyanin/polyphenolic-rich extract would be to use only the skins for the starting material. For that reason, the anthocyanin and phenolic contents of the separate portions of blueberry fruit (skin, flesh, and seeds) were also determined.

MS 20040056 Submitted 1/29/04, Revised 2/23/04, Accepted 5/3/04. *Authors are with the Dept. of Food Science and Technology, Oregon State Univ., Corvallis, OR 97331. Direct inquiries to author Wrolstad (E-mail: Ron.Wrolstad@oregonstate.edu).*

Materials and Methods

Plant material

Frozen blueberries (*V. corymbosum* cv. Rubel; Grade 1) were provided by the Oregon Blueberry Commission. Blueberry skins for the extraction experiments were obtained by removing skins from partially thawed berries with the aid of a razor blade. The skins were immediately frozen in liquid nitrogen and stored at –70 °C. For the analysis of blueberry fractions, skins were removed as described previously, and seeds were manually separated from thawed flesh. Samples were frozen in liquid nitrogen and stored at –70 °C until analysis. Industrial blueberry processing waste was supplied by the J. M. Smucker Co. (Woodburn, Oreg., U.S.A.) in the forms of frozen puree waste and frozen berries described as puree stock. These materials were stored at –23 °C.

Reagents, standards, and enzymes

All solvents used in this investigation were high-performance liquid chromatography (HPLC) grade. Potassium metabisulfite, citric acid, gallic acid, catechin, epicatechin, protocatechuic acid, ellagic acid, caffeic acid, ferulic acid, *m*-coumaric acid, *o*-coumaric acid, *p*-coumaric acid, chlorogenic acid, vanillic acid, syringic acid, 2,3-dihydroxybenzoic acid, quercetin, myricetin, hydrocinnamic acid, and *P*-hydroxybenzoic acid were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and TPTZ (tripyridyltriazine) were purchased from Fluka (Buchs, Switzerland). AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] was purchased from Wako (Richmond, Va., U.S.A.).

Commercial juice-processing enzymes were provided by AB Enzymes (Columbus, Ohio, U.S.A.), DSM Food Specialties USA, Inc. (Charlotte, N.C., U.S.A.), Novozymes North America Inc. (Franklinton, N.C., U.S.A.), and Valley Research, Inc. (South Bend, Ind., U.S.A.). Substrate activities as described for the enzymes are listed in Table 1. Firms supplying the enzymes were assured that

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Table 1—Characteristics of industrial juice-processing enzymes

Enzymes	Production microorganism	Activity
E1	Information not provided	No information provided by the manufacturer, experimental stages
E2	*Aspergillus niger*	Pectinase, hemicellulase
E3	*Trichoderma reesei*	Cellulase
E4	*Aspergillus aculeatus/ Trichoderma longibrachiatum/ Aspergillus niger*	Pectinase,cellulase, cellobiase
E5	*Aspergillus aculeatus*	Pectinase
E6	Information not provided/ *Trichoderma longibrachiatum/ Aspergillus niger*	Experimental pectinase, cellulase, cellobiase
E7	Fungal cultures (did not specify)	Pectinase, cellulase
E8	*Aspergillus* species	Pectinase, hemicellulase
E9	*Aspergillus niger*	Pectinase

the identity of the individual enzymes would be kept confidential.

Enzyme evaluation

Commercial enzyme companies supplied juice-processing enzyme preparations ($n = 9$) for assessment. Frozen whole blueberries or frozen skins (250 g each) were pureed with distilled water (1:2, berries or skins:distilled water) using a Waring blender (Warings Laboratory Science, Torrington, Conn., U.S.A.) and aliquoted to Erlenmeyer flasks. The mixture was placed in a 50 °C Orbit shaker water bath (Lab-Line Instruments Inc., Melrose Park, Ill., U.S.A.) for 10 min, and then 1 mL of diluted enzyme solution (enzymes were diluted based on manufacturer recommendations) was added to the berry/skin puree-water mixture and incubated for 1 h at 50 °C. After the incubation period, samples were then placed in a boiling water bath (Precision 180 Series, Precision Scientific Inc., Chicago, Ill., U.S.A.) for 10 min to inactivate enzymes, and then immediately cooled in an ice bath. Samples were then centrifuged using an International clinical centrifuge (Intl. Equipment Co., Boston, Mass., U.S.A.) for 30 min. The supernatants were collected and kept frozen at –70 °C until analysis.

Extraction experiments

Both whole blueberries and skins were used for extraction material. Experimental conditions evaluated included temperature (50 °C and 80 °C), citric acid addition (none and 1% citric acid), and SO_2 addition (0, 50, and 100 ppm free SO_2). These processing parameters were applied individually and in combination, representing a $2 \times 3 \times 2$ randomized complete block design (RCBD). Details of the different parameters are summarized in Table 2. Blueberries were homogenized using a Polytron PT10-35 and PT-K Laboratory homogenizer (Kinematica, Luzern, Switzerland), setting 3 for 30 s, in the presence of solutions containing the appropriate concentration of acid and/or sulfur dioxide in distilled water (1:2, berries or skins:solution). Samples were then held at a constant temperature (either 50 °C or 80 °C, depending on the parameter) for 1 h in an Orbit shaker water bath (Lab-Line Instruments, Inc.). Samples were cooled in an ice bath after incubation and then centrifuged. The

Table 2—Experimental design for heat, SO_2, and citric acid combinations for extraction experiments (12 different combinations)[a]

	0 ppm of SO_2	50 ppm of SO_2	100 ppm of $S0_2$
50 °C	0% citric acid-control (H50S0C0)	0% citric acid (H50S50C0)	0% citric acid (H50S100C0)
	1% citric acid (H50S0C1)	1% citric acid (H50S50C1)	1% citric acid (H50S100C1)
80 °C	0% citric acid (H80S0C0)	0% citric acid (H80S50C0)	0% citric acid (H80S100C0)
	1% citric acid (H80S0C1)	1% citric acid (H80S50C1)	1% citric acid (H80S100C1)

[a]Codes in parentheses are abbreviations for the individual treatments.

supernatants were collected, frozen, and stored at –70 °C until analysis. All trials were duplicated.

Sample preparation

Sample materials were extracted following the procedure described by Rodriguez-Saona and Wrolstad (2001). Blueberries, blueberry fractions (skin, flesh, and seeds), and processing wastes were liquid nitrogen-powdered using a mortar and pestle. Five grams of powdered sample was sonicated with 10 mL of 100% acetone and re-extracted with 70% (70:30 acetone:water, v/v) aqueous acetone until the solution became colorless. It was then partitioned with chloroform (1:2 acetone:chloroform, v/v) to obtain the aqueous fraction. The aqueous portion was collected, and residual acetone was evaporated by a Büchi rotovapor (Westbury, N.Y., U.S.A.) at 40 °C. The aqueous extract was dissolved to a final volume of 25 mL with distilled water. Samples were then stored at –70 °C until analysis.

Determination of total anthocyanins, polymeric color percentage, and total phenolics

The pH differential method (spectrophotometric method), as described by Giusti and Wrolstad (2001), was used in determining total monomeric anthocyanins (ACY), and expressed as cyanidin-3-glucoside (molar extinction coefficient of 26900 $Lcm^{-1}mol^{-1}$ and molecular weight of 449.2 $gmol^{-1}$). Absorbance measurements were conducted at 520 and 700 nm. The unit for ACY was mg of cyanidin-3-glucoside/100 g. The percent polymeric color of samples was determined by resistance to bisulfite bleaching, as described by Giusti and Wrolstad (2001). The Folin-Ciocalteu (FC) method was used for measuring total phenolics (TP) (Waterhouse 2002) and expressed as mg of gallic acid/100 g. Absorbance measurements were conducted at 765 nm. A Shimadzu 300 UV-visible spectrophotometer (Shimadzu Inc., Kyoto, Japan) and 1-cm pathlength cells were used for both measurements. Measurements of ACY, polymeric color percentage, and TP of sample extracts were replicated 2 times. ACY and TP were determined on whole berries, berry fractions (skin, flesh, and seeds), industrial processing waste, and sample extracts from the enzyme and extraction experiments. ACY and TP were expressed as mg/100 g of berries, skin, flesh, or seeds.

Purification of anthocyanins and polyphenolics

Anthocyanin pigments and polyphenolics were isolated by solid-phase extraction as described by Rodriguez-Saona and Wrolstad (2001). The aqueous extract was applied to a C-18 Sep-Pak mini-column (Waters Associates, Milford, Mass., U.S.A.), rinsed with 10 mL water, and the water eluant discarded. After drying with a nitrogen stream, polyphenolics were eluted with 10 mL ethyl acetate. Ethyl acetate was removed from this fraction using a Büchi ro-

tovapor at 40 °C, then the residue was redissolved in 2 mL acidified water. Anthocyanins were eluted with 10 mL of acidified (0.01% HCl) methanol, and the methanol was subsequently evaporated using a Büchi rotovapor at 40 °C. The pigments were redissolved in 2 mL acidified (0.01% HCl) water. Samples were filtered through a 0.45-μm Millipore filter (type HA; Millipore Corp., Bedford, Mass., U.S.A.) before analysis by electrospray ionization mass spectrometry (ES-MS) and liquid chromatography equipped with a photodiode array detector and mass spectrometer (LC-DAD-MS).

Determination of antioxidant activity

Antioxidant activities of extracts were determined by ferric reducing antioxidant potential (FRAP) and oxygen radical absorbing capacity (ORAC) assays. FRAP assays were performed as described by Benzie and Strain (1996), using a 96-well ThermoMax microplate spectrophotometer (Molecular Devices, Foster City, Calif., U.S.A.) to measure the formation of ferrous-TPTZ complex (λ_{max} = 595 nm). FRAP measures the extract ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in a solution of TPTZ prepared in sodium acetate at pH 3.6. Absorbance was measured at 595 nm. ORAC assays followed the method described by Cao and others (1993), with the alteration of using a 96-well cytofluor 4000 microplate fluorometer (PerSeptive Biosystems, Framingham, Mass., U.S.A.), which recorded rate and duration of fluorescence. β-Phycoerythrin acted as target for the peroxyl radicals generated by AAPH (a peroxyl radical generator that destroys the fluorescence). Samples were monitored at 2-min intervals, for 2 h, at 485 nm (excitation wavelength) and 585 nm (emission wavelength). FRAP and ORAC values were expressed as μmol of Trolox (a water-soluble tocopherol analogue) equivalents/g of sample.

Anthocyanin and polyphenolic determinations by HPLC-DAD

Analytical HPLC system. Anthocyanins were separated by reversed-phase HPLC using a Hewlett-Packard 1090 (Agilent Technologies Inc, Wilmington, Del., U.S.A.) equipped with a photodiode array detector (DAD). Absorbance spectra were recorded for all peaks. Flow rate was 1 mL/min.

Anthocyanins. A Prodigy 5 μm ODS (3) 100 Å (250 × 4.6 mm) column, fitted with 4.0 × 3.0-mm inner dia guard column, from Phenomenex (Torrance, Calif., U.S.A.) was used. Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. The program used a linear gradient from 2% to 20% solvent A in 25 min; then a linear gradient of solvent A from 20% to 40% in 5 min, with simultaneous detection at 280, 320, and 520 nm (Durst and Wrolstad 2001). Injection volume was 20 μL. Column temperature was maintained at 40 °C. Samples were filtered before HPLC injection.

Polyphenolics. A Synergi 4 μm Hydro-RP 80 Å (250 × 4.6 mm) column, fitted with a 4.0 × 3.0-mm inner dia guard column, from Phenomenex was used. Solvent A was 100% acetonitrile. Solvent B was 1% (v/v) formic acid in water. All solvents used were HPLC grade. The program used a linear gradient from 5% to 25% solvent A in 50 min, then a linear gradient of solvent A from 25% to 50% in 5 min, then held for 5 min, with simultaneous detection at 260, 280, 320, and 520 nm. Injection volume was 100 μL.

Electrospray mass spectroscopy of anthocyanins

Analysis was performed using a Perkin-Elmer SCIEX API III + mass spectrometer (Toronto, Canada), equipped with an Ion Spray source and loop injection. Purified and filtered blueberry anthocyanins (5 μL), in acidified water, were injected directly into the system.

LC-DAD-MS of anthocyanins

A Hewlett-Packard 1090 HPLC (Agilent Technologies Inc.), equipped with a photodiode array detector (DAD) and mass spectrometer (MS) was used to confirm the identification of the blueberry anthocyanins. A Synergi 4 μm Hydro-RP 80 Å (250 × 2 mm) column, fitted with 4.0 × 3.0-mm inner dia guard column from Phenomenex was used. Absorbance spectra were collected for all peaks. Flow rate was 0.2 mL/min and injection volume was 20 μL. Solvent A was 5% formic acid and 80% acetonitrile (v/v), and solvent B was 5% formic acid. All solvents were HPLC grade. The initial solvent composition was 10% solvent A and 90% solvent B; then a linear gradient of 10% to 30% solvent A, and 90% to 70% solvent B in 30 min. Detection occurred simultaneously at 280, 320, and 520 nm. MS analysis was performed using a Perkin-Elmer SCIEX API III Plus triple-quadrupole mass spectrometer equipped with an ion spray source (ISV = 5500, orifice voltage = 50) and positive ion mode. LC-DAD-MS-MS was conducted on some of the peaks observed. Multiple reaction monitoring mode was used. The mass of the molecular ions (anthocyanins) of interest was scanned in 1st quadrupole (Q1) and the daughter ions (also known as fragmented ions, anthocyanidins) were monitored in 3rd quadrupole (Q3). Argon was used as the collision gas. The nebulizer gas and orifice curtain gas were nitrogen. Mass spectra data were collected with Tune 2.5 software, while Mac Spec 3.3 software was used for data processing, both provided by SCIEX (Toronto, Canada).

Statistical analyses

SPSS (Chicago, Ill., U.S.A.) version 11.0 was used for the statistical analyses. Differences among enzyme treatment means were tested using 1-way analysis of variances (ANOVA) and the Tukey Honest Significant Difference (HSD) at α = 0.05 level. ANOVA was conducted on the results for the extraction treatments and Tukey HSD was also conducted.

Results and Discussion

Anthocyanins, phenolics, and antioxidant properties of blueberries and their subfractions

The distribution of ACY, TP, FRAP, and ORAC of whole berries and in their skins, flesh, and seeds are shown in Table 3. The skins percentage by weight (19.0%) would be expected to be higher for 'Rubel' blueberries than for other commercially grown highbush cultivars because it has a smaller berry size (Ehlenfeldt and Prior 2001; Moyer and others 2002). Blueberry seeds are small and represent a relatively small portion of the berry (1.5%). In determining the weight percentage of the 3 fractions, there was a 5.1% loss from conducting the manual separating operations. The high antioxidant capacity of whole blueberries has been highly correlated to their anthocyanin and total phenolic contents (Kalt and Dufour 1997; Prior and others 1998; Kalt and others 2000). The anthocyanins exist almost exclusively in the skin, whereas phenolics and antioxidant properties are mostly in the skins. By extracting only from the skins, there is the potential for producing extracts with very high anthocyanin and polyphenolic concentrations.

Results are expressed on a per 100 g or per gram basis and also arithmetically converted to show the amounts in skin, seeds, and flesh for a 100-g berry sample. For example, the skins contained 994 mg ACY/100 g skin, which represents 188 mg in a 100-g berry sample (994 ×19%). This permits comparison with whole berries and estimation of losses. There was a 16% loss in ACY and a 43% loss in TP when comparing with values for whole berries. We suspect that the high loss of phenolics was because of enzymatic oxidation occurring during the manual separating and weighing operations. There

Table 3—Percent weight distribution, total monomeric anthocyanin (ACY), total phenolics (TP), ferric reducing antioxidant power (FRAP), and oxygen radical absorbing capacity (ORAC) of whole 'Rubel' berries and their fractions

	Weight distribution (% weight)	Berry basis				Fraction basis			
		ACY[a]	TP[b]	FRAP[c]	ORAC[d]	ACY[a]	TP[b]	FRAP[c]	ORAC[d]
Whole berry	100.0	230.0	737.5	39.9	30.7	—	—	—	—
Skins	19.0	188.5	300.4	28.7	18.0	993.8	1583.8	151.3	94.9
Flesh	74.4	5.8	119.3	7.0	6.3	7.9	160.4	9.5	8.5
Seeds	1.5	0.1	4.3	0.3	0.1	7.8	285.6	20.5	9.8
Total	-	194.5	424.1	36.1	24.5	—	—	—	—
% loss	5.1	15.5	42.5	9.7	20.4	—	—	—	—

[a]ACY was expressed as cyanidin-3-glucoside equivalents (mg/100 g of berries, 100 g of skins, 100 g of flesh, or 100 g of seeds).
[b]TP was expressed as gallic acid equivalents (mg/100 g of berries, 100 g of skins, 100 g of flesh, or 100 g of seeds).
[c]FRAP was expressed as Trolox equivalents (μmol/1 g of berries, 1 g of skins, 1 g of flesh, or 1 g of seeds).
[d]ORAC was expressed as Trolox equivalents (μmol/1 g of berries, 1 g of skins, 1 g of flesh, or 1 g of seeds).

were no reports in the literature on the composition of blueberry seeds, but the antioxidant properties were substantially lower than what has been reported for grape seeds (Pastrana-Bonilla and others 2003).

Evaluation of juice-processing enzymes

With the intent of using juice-processing enzymes to increase recovery of anthocyanins and polyphenolics from juice-processing wastes, we developed a screening assay using either whole berries or skins as substrate. Requests were made to enzyme suppliers and 9 enzyme preparations were provided for evaluation. Two of the samples were experimental samples, but the others are available commercially. The characteristics of the enzymes regarding pectinase, cellulase, cellobiase, and hemicellulase activity are shown in Table 1. The ACY, TP, and polymeric color percentage results for the enzyme-treated samples are summarized in Table 4.

The amount of anthocyanin and phenolics extracted from the whole berries by enzymes ranged from 20.5 to 28.1 mg ACY, and 82.9 to 101.0 mg TP per 100 g extract (Table 4). The control (no enzyme addition) actually gave the highest yield of anthocyanin and phenolics. Enzyme 8 (pectinase and hemicellulase activities) performed as well as the control for extracting ACY. Enzyme 6 (pectinase, cellulase, and cellobiase) extracted the least ACY and TP from the berries. Although the control yielded the highest amount of TP, enzymes 3, 4, 7, and 8 (all containing cellulase or hemicellulase activity) were equivalent in the extraction of TP. The percent polymeric color, which is an index for anthocyanin degradation, was highly variable and ranged from 40.9% (enzyme 1) to 73.4% (control). Surprisingly, the control, which had the highest anthocyanin recovery, also yielded the highest polymeric color percentage. Enzyme 1 produced the least amount of polymeric color percentage, and all enzymes, except enzyme 3 (only cellulase activity) yielded significantly less polymeric color percentage compared with the control. Depectinization with pectolytic enzymes remains an essential unit operation for producing blueberry juice and juice concentrates. So juice-processing enzymes are required even if they do not give increased recovery of anthocyanins and polyphenolics.

ACY and TP for the different enzyme treatments to skins ranged from 100.4 to 137.9 mg of ACY, and 232.2 to 278.5 mg of TP per 100 g extract. There were also differences between berries and skins when making comparisons to the control. Enzyme 7 (pectinase and cellulase activities) had the highest ACY, TP, and polymeric color percentage values. Enzymes 1, 6, 9, and control were equivalent to enzyme 7 in their ability to extract ACY. Enzymes 1, 2, 4, 6, 9, and control were equivalent to enzyme 7 in extracting TP from skins. The percent polymeric color yield ranged from 28.5% to 36.3%,

Table 4—Results from the industry-recommended enzymes (n = 9) in extracting total anthocyanins (ACY), total phenolics (TP), and polymeric color percentage (%PC) from whole berries and skins (100 g of extract)[a]

	Whole berries			Skins		
Enzymes	ACY[b]	TP[c]	%PC	ACY[b]	TP[c]	%PC
E1	27.1ab	88.7abc	40.9a	128.2bc	264.8abc	28.5a
E2	24.9ab	87.6ab	41.8ab	109.0ab	251.9abc	32.2ab
E3	27.2ab	101.0cd	67.6cd	101.4a	235.6ab	35.8ab
E4	23.7ab	90.4abcd	45.0ab	109.9ab	242.9abc	33.7ab
E5	23.5ab	87.9ab	45.5ab	100.4a	232.2a	32.5ab
E6	20.5a	82.9a	49.9b	127.1bc	268.3abc	31.6ab
E7	26.7ab	91.7abcd	43.9ab	137.9c	278.5c	36.3b
E8	28.1b	98.3bcd	61.6c	106.5ab	234.5a	35.2ab
E9	23.2ab	85.9ab	44.9ab	136.7c	276.1bc	35.7ab
Control	28.3b	103.3d	73.4d	113.3abc	248.6abc	32.3ab

[a]Values in each column sharing the same superscript are not significantly different from each other (Tukey's HSD, $P \leq 0.05$).
[b]Total anthocyanin was expressed as cyanidin-3-glucoside equivalents.
[c]Total phenolics was expressed as gallic acid equivalents.

which was not as variable as polymeric percentage values obtained from whole berries. Enzyme 1 (unknown experimental enzyme) produced the least amount of polymeric color percentage. Lower amounts of polymeric color percentage from skins processed with extracts might be explained by the absence of blueberry pulp (source of endogenous enzymes and chlorogenic acid). Skrede and others (2000) demonstrated that adding unblanched blueberry pulp to pasteurized juice increased anthocyanin degradation significantly. Blueberry anthocyanins have been found to degrade rapidly in the presence of chlorogenic acid and polyphenoloxidase (PPO) but not by PPO alone (Kader and others 1997, 1998).

Delphinidin-glycosides (based on its structure, delphinidin is the most labile) were degraded the most, and malvidin-glycosides appear to have degraded the least by processing enzyme treatments (Figure 1c), especially when the starting material was whole berries, which implied that the native enzymes present in the blueberry destroyed anthocyanin (Skrede and others 2000).

Landbo and Meyers (2001) tested 5 different processing enzymes (the enzymes had pectinase, macerase, or protease activity) with varying degrees of extraction efficiency from black currant presscake residue. Enzyme-assisted extractions yielded more polyphenolics, but were not found to increase anthocyanins when compared with a control (no addition of enzyme). Particle size has shown to influence the amount of phytochemicals extractable from black currant pomace, where a reduction in particle size increased

Table 5—Total anthocyanin content, total phenolic content, and pH of the extracts obtained from whole berries and skins from the individual and combination of the heat, SO_2, and citric acid treatments[a]

	Treatment combinations			Whole berries[b]			Skins[b]		
Trt nr[c]	Heat levels (°C)	SO_2 levels (ppm)	Citric acid levels (%)	ACY	TP	pH	ACY	TP	pH
1	50	0	0	27.5	55.9	3.9	102.7	146.9	3.6
2	50	0	1	42.6	62.6	2.8	156.8	186.2	2.7
3	80	0	0	50.2	71.4	3.9	151.7	260.4	3.7
4	80	0	1	62.4	88.8	2.8	220.2	312.2	2.7
5	50	50	0	92.2	307.3	3.9	462.9	745.2	3.7
6	50	50	1	96.5	316.4	3.3	469.9	748.5	3.0
7	80	50	0	97.5	420.6	3.9	401.0	701.9	3.5
8	80	50	1	102.2	413.8	3.2	373.0	659.3	2.8
9	50	100	0	90.6	386.0	3.9	494.5	906.9	3.5
10	50	100	1	82.2	337.5	3.4	504.5	924.1	3.2
11	80	100	0	92.1	641.0	3.8	409.8	938.8	3.2
12	80	100	1	91.4	684.3	3.3	446.1	978.3	2.9

[a]Total anthocyanin content (ACY) expressed as cyanidin-3-glucoside equivalents), total phenolic content (TP) expressed as gallic acid equivalents
[b]100 g of extract basis.
[c]Trt nr = treatment number.

the amount extracted. Also, Stanley and Miller (2001) showed fine milling was more influential than the use of commercial enzymes or heating in extraction of phytochemicals from blueberry juice–processing waste. Meyer and others (1998) tested pectinase and cellulase in phenolic extraction of grape pomace and found that pectinase had positive effects, but that cellulase had a negative effect in the extraction of polyphenolics. The benefit of pectinase was correlated with plant cell wall breakdown from the enzymes (Meyer and others 1998). Some commercial juice-processing enzymes have shown negative effects on the anthocyanin content (color loss) of cranberry juice because of β-galactosidase activity (Wrolstad and others 1994; Wightman and Wrolstad 1995). In this study, the enzymes used did not exhibit side activities, such as β-galactosidase activity and/or β-glucosidase activity, because the enzyme side activities are specific (Wrolstad and others 1994). There was no clear evidence of decrease in β-galactosides and β-glucosides from the proportions of individual anthocyanin profiles (Figure 1).

Table 6—The results from ANOVA tables for the extraction experiments[a]

		Whole berries		Skins	
Effects	d.f.[b]	ACY	TP	ACY	TP
Blocks	1	0.001	0.005	0.001	0.001
Heat levels (H)	1	0.001	0.001	0.001	0.006
SO_2 levels (S)	2	0.001	0.001	0.001	0.001
Citrate levels (C)	1	0.001	0.515	0.001	0.109
H × S	2	0.001	0.001	0.001	0.001
S × C	2	0.001	0.530	0.001	0.056
C × H	1	0.443	0.012	0.889	0.869
H × S × C	2	0.145	0.001	0.154	0.402

[a]Total anthocyanin content (ACY), expressed as cyanidin-3-glucoside equivalents) and total phenolic content (TP), expressed as gallic acid equivalents)
[b]d.f. = degrees of freedom

Extraction experiments

In the United States, the legal definitions of fruit juices and fruit juice concentrates require that juice be extracted by physical methods and/or aqueous extraction (Title 21, U.S. Code of Federal Regulations 73). Thus, anthocyanin-based colorants under the classification of fruit and vegetable juices cannot be extracted with organic solvents. For this reason, the use of ethanol (or other organic solvents) was not included in this investigation. Previously, we showed that high-temperature-short-time treatment and SO_2 could increase anthocyanin recovery when blueberries were processed into juice and concentrate (Lee and others 2002). Also, anthocyanins were more stable in acidic conditions (Wrolstad 2000), and Kalt and others (2000) showed that blueberry juice contained more anthocyanins, phenolics, and antioxidant activity at lower pH, so addition of citric acid might increase pigment recovery. In this experiment, the addition of 50 and 100 ppm SO_2, the addition of 1% citric acid, and extraction temperatures of 50 °C and 80 °C were tested individually and in combination (Table 5). ACY and TP concentrations for the different treatments are shown in Table 5, and statistical results are shown in Table 6 and 7. Abbreviations of the treatments are provided (Table 1).

ACY extracted from whole berries ranged from 27.5 mg/100 g to 102.2 mg/100 g and TP from 55.9 mg/100 g to 684.3 mg/100 g extract (Table 5). The pH of the extracts ranged from 2.8 to 3.9. Addition of citric acid lowered the pH of the extract by 0.5 to 1.1 units. The addition of SO_2 did not alter the pH of the extracts significantly. Extraction of both ACY and TP contents of samples cannot be explained by main effects alone, namely, heat (H), SO_2 (S), or citric acid levels (C). There was no significant evidence for H × S × C effect (3-way interaction), or C × H effect (2-way interaction) for ACY content (Table 6). The effect of heat changed with a change in SO_2 level. Higher levels of both heat and SO_2 increased the extraction of ACY from whole berries. As an increase in extraction temperature increases the rate of diffusion (Cacace and Mazza 2002), in this case it increased the extraction of anthocyanins. Elevated extraction temperature also aids in the breakdown of blueberry tissue, opening up cells, further increasing the yield of anthocyanin within the extract. Cacace and Mazza (2002) speculated that one explanation for increased anthocyanin extraction by the addition of SO_2 is that the added SO_2 reduced the dielectric constant of water and increased the solubility of phenolics, but the extact mechanism is unknown. Native enzymes present in the blueberry, which degrade phenolics, are inhibited by increased extraction temperature (Skrede and others 2000) and SO_2 addition (Sapers 1993). Also, the effect of SO_2 addition changes with the level of citric acid. Addition of citric acid aided in extracting ACY with 0 and 50 ppm SO_2 but decreased the extraction at 100 ppm SO_2. The highest ACY extracts were with treatments H50S50, H80S50, and H80S100, also from

Table 7—The results of the effect of heat, SO_2, and citric acid from 2-way or 3-way Tukey HDS[a]

		Whole berries		Skins	
	Levels	ACY	TP	ACY	TP
H × S effect	H50S0	35.0a	—	129.8a	166.6a
	H80S0	56.3b	—	185.9a	286.3b
	H50S50	93.3cd	—	466.4cd	746.9c
	H80S50	99.9d	—	387.0b	680.6c
	H50S100	86.4c	—	499.5d	915.6d
	H80S100	91.7cd	—	428.0bc	958.6d
S × C effect	S0C0	38.9a	—	127.2a	*
	S0C1	52.5b	—	188.5a	*
	S50C0	94.8cd	—	432.0b	*
	S50C1	99.4d	—	421.4b	*
	S100C0	94.8cd	—	452.1b	*
	S100C1	86.8c	—	475.3b	*
H × S × C effect	H50S0C0	*	55.9a	*	*
	H50S0C1	*	62.6a	*	*
	H80S0C0	*	71.4a	*	*
	H80S0C1	*	88.8a	*	*
	H50S50C0	*	307.3b	*	*
	H50S50C1	*	316.4b	*	*
	H80S50C0	*	420.6c	*	*
	H80S50C1	*	413.8c	*	*
	H50S100C0	*	386.0c	*	*
	H50S100C1	*	337.5b	*	*
	H80S100C0	*	641.0d	*	*
	H80S100C1	*	684.3d	*	*

[a]Values in each column sharing the same letter are not significantly different from each other (Tukey's HSD, $P < 0.05$). * = not significantly different; — = not applicable. ACY = total anthocyanin content; TP = total phenolic content.

S50C0, S50C1, and S100C0. For TP, there was a significant 3-way interaction among the factors. In general, an increase in SO_2 addition, and heat levels, yielded higher amounts of TP. A temperature of 80 °C and 100 ppm of free SO_2 (H50S100C0 and H50S100C1) extracted the most TP. The addition of 1% citric acid aided in the extraction of TP at low temperature and when SO_2 was not added. The control had the lowest amount of ACY and TP (Table 7).

ACY extracted from the skins ranged from 102.7 mg/100 g to 504.5 mg/100 g, and TP from 146.9 mg/100 g to 978.3 mg/100 g extract (Table 5). The pH of the extracts ranged from 2.7 to 3.7. Addition of citric acid lowered the extract pH values in all cases. Again, the extraction of ACY and TP from skins cannot be explained by the main effects alone (Table 6) because there were interactions that were significantly different. There were no significantly different 3-way interactions and C × H effect for ACY content. From the H × S effect, combination of heat and addition of SO_2 assisted in the extraction of ACY from the skins when compared with low heat and no SO_2 addition. Low heat (50 °C) and addition of 50 ppm or 100 ppm of SO_2 (H50S50C0, H50S50C1, H50S100C0, and H50S100C1) yielded the highest amount of ACY. Use of high heat aided in extracting TP, when no SO_2 and citric acid was used. From the S × C effect, the use of SO_2 increased the extraction of ACY when used in combination with citric acid addition. Using 100 ppm of SO_2 and 1% citric acid yielded the highest ACY content from the skins. From the TP results, there was a significant interaction between heat and SO_2. TP values of the skins treated with the 2 different levels of citric acid were not significantly different. Higher heat aided the extraction of TP at 0 ppm of SO_2, but not when 50 ppm or 100 ppm of SO_2 was added. High heat (80 °C) and addition of citric acid (H80S0C1) was effective in extracting ACY when no SO_2 was added to the skins. High-sulfured water (H50S100 and H80S100) extracted the highest amount of TP from the skins. Control had the lowest amount of ACY and TP.

Although whole berries and blueberry skins were used in these experiments, the extraction conditions should also apply to juice-processing waste products such as presscake residue. Processing waste obtained by a local processor was analyzed for ACY and TP content. Puree waste was high in ACY (96.9 mg/100 g) and TP (540.7 mg/100 g). Puree stock contained 47.2 mg of ACY/100 g of stock, and 223.1 mg of TP/100 g. Puree waste appeared to be a good source for further extraction.

During the early 1960s, a combination of heat treatment (62 °C) and pectinase before pressing had been reported to be effective in blueberry juice processing for improving yield, color, and soluble solids (Fuleki and Hope 1964). Whole berries and skins were extracted a single time in this study. Because the residual appears to still retain pigment, multiple extractions of the residual might increase the final ACY and TP yield. Bakker and others (1998) reported an increase in anthocyanin extraction with the increase in total SO_2 addition (0, 75, and 150 ppm) to must. They also reported no noticeable effect on the aroma and taste of the final wine produced. Bocevska and Stevcevska (1997) have demonstrated boosting SO_2

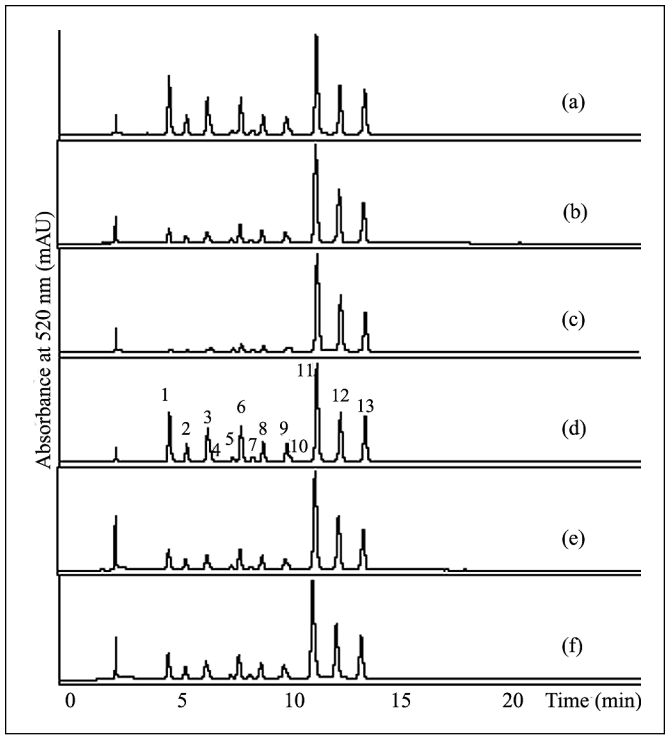

Figure 1—Liquid chromatography equipped with a photodiode array detector (LC-DAD) profiles of anthocyanin: whole berry (a), whole berry processed as control (b), whole berry processed with enzyme 6 (c), skin (d), skin processed with control (e), and skin processed with enzyme 6 (f). Corresponding anthocyanin peak assignments and peak area percentages (values in parentheses) for chromatogram (d). Corresponding anthocyanin peak assignments are as follows: 1 = delphinidin-3-galactoside (12.8), 2 = delphinidin-3-glucoside (4.4), 3 = delphinidin-3-arabinoside (8.4), 4 = cyanidin-3-galactoside (2.0), 5 = cyanidin-3-glucoside (0.9), 6 = petunidin-3-galactoside (5.3), 7 = cyanidin-3-arabinoside (1.1), 8 = petunidin-3-glucoside (4.8), 9 = petunidin-3-arabinoside (4.6), 10 = peonidin-3-galactoside (1.0), 11 = malvidin-3-galactoside (27.8), 12 = malvidin-3-glucoside (13.9), and 13 = malvidin-3-arabinoside (13.0).

addition (500 to 5000 ppm of total SO_2) increased the extract purity obtained from wine grape pomace. Cacace and Mazza (2002, 2003) have conducted an intensive study of extracting phytochemicals, such as anthocyanins and polyphenolics, from black currants using sulfured water (28 to 1372 ppm of SO_2), aqueous ethanol (39% to 95% ethanol in water), and heat (6 °C to 74 °C). They recommended that 19 L of ethanol per kg milled frozen berries, 1000 to 1200 ppm of SO_2 (they did not indicate whether these values were total or free SO_2), and an extraction temperature of 30 °C to 35 °C. Sixty percent aqueous ethanol yielded the most total phenolics but had little effect on the anthocyanin content of the extract.

Free SO_2 concentrations of 0, 50, and100 ppm were selected for this study because previous work (Lee and others 2002) showed that after pasteurization, the amount of total SO_2 left in the product was less than 10 ppm. The minimum level of total SO_2 for labeling is 10 ppm (Title 21, U.S. Code of Federal Regulations 101.100). Recently, researchers have demonstrated that steam-blanching blueberries for 3 min increased the recovery of anthocyanins and cinnamic acids (Rossi and others 2003). From this study, a combination of heating and SO_2 will increase the yield of phytochemicals from whole blueberry fruit and from their process by-products.

Anthocyanin profile of 'Rubel' skins and seeds

The anthocyanin pigment profile of 'Rubel' blueberry skins obtained from the LC-DAD is shown in Figure 1d. Thirteen peaks were identified. They contained the glycosides of malvidin (54.7%), delphinidin (25.7%), petunidin (14.7%), cyanidin (3.9%), and peonidin (1.0%), based on peak area percentage. The major anthocyanin present in 'Rubel' was malvidin-galactoside (27.8%).

Peak assignments were based on their spectra and retention time. Peaks that were present in low quantities were difficult to identify based on their spectra alone. Using tools such as ES-MS and LC-DAD-MS helped in identification of peak assignments. Anthocyanins (mostly in the oxonium form at low pH) generate a positive ion that can be detected by MS. An anthocyanin's single positive charge allows the mass-to-charge ratio (m/z) to correspond directly to the molecular weight of the anthocyanin. Based on the ES-MS results alone, it was difficult to rule out any of the 15 possible combinations of glycosides of delphinidin, cyanidin, peonidin, petunidin, and malvidin. The 13 peaks identified by HPLC can be assigned to the 8 ES-MS peaks (Figure 2). Anthocyanins with the same m/z were not differentiated by ES-MS. Malvidin-3-galactoside and malvidin-3-glucoside (both have the same m/z) had the largest peak in the ES-MS data (Figure 2, peak 8) and were also the largest peaks in HPLC (Figure 1d, peaks 12 and 13).

Results for LC-DAD-MS analysis are shown in Figure 3. Resolution was not as good with the LC-DAD-MS system, probably because the mobile phase contained formic acid, as phosphate buffer needed to be avoided. Despite this, LC-DAD-MS provided more information than LC-DAD alone. Blueberry anthocyanin peaks that co-eluted in the new LC system still differed in m/z, so the individual anthocyanins could be distinguished. For example, malvidin-3-glactoside (Figure 3a, peak 12) and peonidin-3-glucoside (Figure

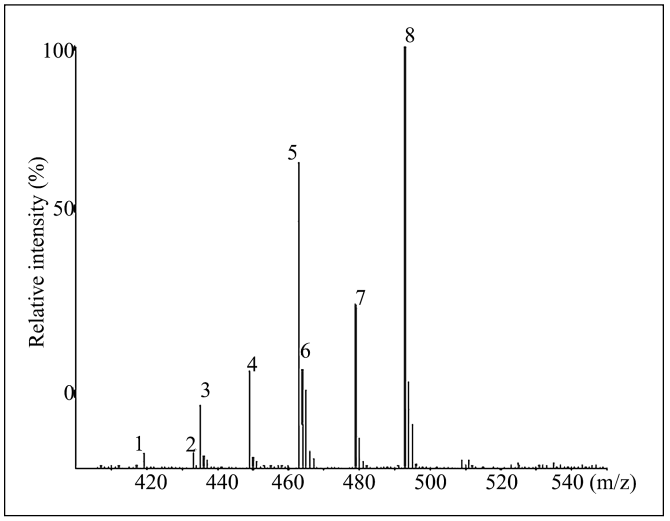

Figure 2—Electrospray mass spectroscopy (ES-MS) separation of 'Rubel' blueberry skin anthocyanin. Corresponding presumptive anthocyanin peak assignments and mass-to-charge ratio (m/z) in parenthesis: 1 = cyanidin-3-arabinoside (419), 2 = peonidin-3-arabinoside (433), 3 = delphinidin-3-arabinoside (435), 4 = petunidin-3-arabinoside, cyanidin-3-galactoside, and cyanidin-3-glucoside (449), 5 = peonidin-3-galactoside, peonidin-3-glucoside, and malvidin-3-arabinoside (463), 6 = delphinidin-3-galactoside and delphinidin-3-glucoside (465), 7 = petunidin-3-galactoside and petunidin-3-glucoside (479), and 8 = malvidin-3-galactoside and malvidin-3-glucoside (493).

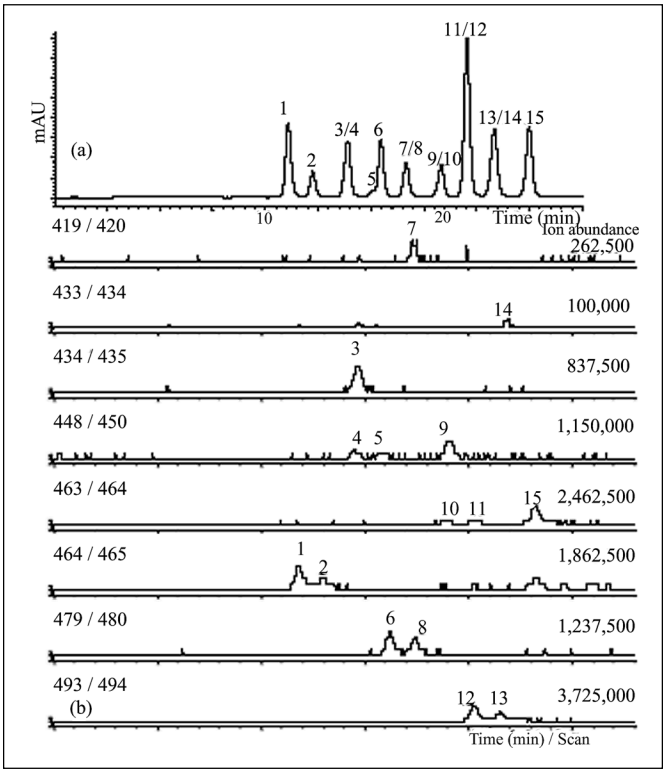

Figure 3—Liquid chromatography equipped with a photodiode array detector mass spectroscopy (LC-DAD-MS) results of 'Rubel' skin anthocyanin. LC-DAD profile (a) and extracted ion chromatograms (b). Corresponding anthocyanin peak assignments are as follows: 1 = delphinidin-3-galactoside (465.2), 2 = delphinidin-3-glucoside (465.2), 3 = delphinidin-3-arabinoside (435.0), 4 = cyanidin-3-galactoside (449.2), 5 = cyanidin-3-glucoside (449.2), 6 = petunidin-3-galactoside (479.2), 7 = cyanidin-3-arabinoside (419.0), 8 = petunidin-3-glucoside (479.2), 9 = petunidin-3-arabinoside (449.0), 10 = peonidin-3-galactoside (463.2), 11 = peonidin-3-glucoside (463.2), 12 = malvidin-3-galactoside (493.2), 13 = malvidin-3-glucoside (493.2), 14 = peonidin-3-arabinoside (433.0), and 15 = malvidin-3-arabinoside (463.0).

3a, peak 11) co-eluted. The 2 compounds could be detected, however, with MS. From the LC-DAD-MS results there were a total of 15 peaks in 'Rubel' skins. There was a low concentration of peonidin-3-arabinoside in 'Rubel' skins, which made identification based on retention time and spectra difficult. LC-DAD-MS permitted positive identification of 'Rubel' skin anthocyanins. By using a combination of retention time, peak spectra, and m/z, identification of anthocyanins was simplified. LC-DAD-MS-MS was also performed on some peaks that co-eluted and were present in low concentrations on the LC-DAD-MS system (peaks 3/4 and 11/12 in Figure 3). The m/z of the corresponding molecular ion ▶ fragmented ion were 435.0 ▶ 303.0 (delphinidin-arabinoside, peak 3 in Figure 3), 449.2 ▶ 287.0 (cyanidin-galactoside, peak 4 in Figure 3), 463.2 ▶ 301.0 (peonidin-glucoside, peak 11 in Figure 3), and 493.2 ▶ 331.0 (malvidin-galactoside, peak 12 in Figure 3).

The anthocyanin profile for whole berries was the same for skin alone (Figure 1a and 1d), which was expected because the anthocyanins exist almost exclusively in the skins. The extracts of flesh and seeds were slightly different, having proportionately less delphinidin-glycosides. We believe this difference can be attributed to differences in seed anthocyanins because pigments in the flesh probably diffused from the skin.

Blueberry puree–waste extracts had a similar anthocyanin HPLC profile as the stock, but the anthocyanin profiles for the industrial juice–processing wastes differed slightly in that the proportions of delphinidin, petunidin, and malvidin glucosides decreased when compared with the stock, which might be because of processing enzyme side activity (such as glucosidase activity).

Polyphenolics profile

The HPLC separation for blueberry polyphenolics is shown in Figure 4, 5, and 6. Figure 4 represents a chromatogram of compounds that have been reported in blueberries. Most phenolics in the berry exist as glycosides or esters. In this study, hydrolysis was not conducted on the phenolic fractions, so only the native form was observed. Chlorogenic acid (5-caffeoylquinic acid), 58% of the total peak measured at 320 nm, was the major polyphenolic present in 'Rubel' (Figure 5), confirming previous reports (Lee and others 2002).

The skin, flesh, and seeds exhibited different polyphenolic profiles (Figure 6). The skins mainly consisted of cinnamic acids and flavonol-glycosides, with some minor peaks such as gallic acid and syringic acid. The flesh consisted of only cinnamic acids. Chlorogenic acid, 60% of the total peak area of 320 nm absorbers, was the main phenolic compound present in the flesh. The seed fraction also had cinnamic acids and flavonol-glycosides, with numerous unidentified peaks. The LC conditions used in this study were not ideal for analysis of procyanidins, but procyanidins in blueberry seeds have been reported (Prior and others 2001; Gu and others 2002). Prior and others (2001) reported 6 μg of total procyanidin/g of dry 'Rubel.' They also identified B-type procyanidin monomers through octamers by normal-phase HPLC-ESIMS. Gu and others (2002) reported lowbush blueberries to contain 20 mg of procyanidin/g of freeze-dried blueberries. Despite the efforts to identify polyphenolics based on their retention time and UV spectra, there were still numerous peaks unidentified that did not correspond to available standards (Figure 4).

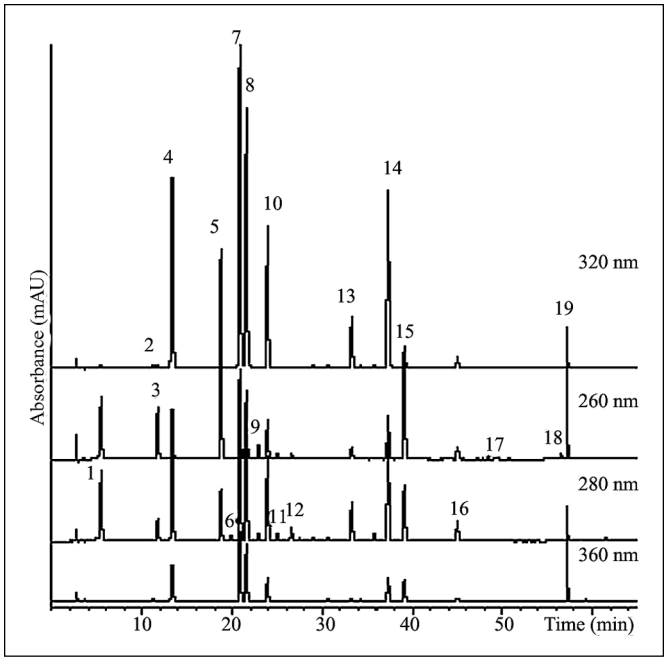

Figure 4—Polyphenolics separation of 20 standards. The peak assignments are as follows: 1 = gallic acid, 2 = 1-O-caffeoylquinic acid, 3 = protocatechuic acid, 4 = 3-O-caffeoylquinic acid (neochlorogenic acid), 5 = *P*-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid, 6 = catechin, 7 = 5-O-caffeoylquinic acid (chlorogenic acid), 8 = 4-O-caffeoylquinic acid (cryptochlorogenic acid), 9 = vanillic acid, 10 = caffeic acid, 11 = syringic acid, 12 = epicatechin, 13 = *P*-coumaric acid, 14 = hydroxycinnamic acid, 15 = *m*-coumaric acid and ferulic acid coeluting, 16 = *o*-coumaric acid, 17 = myricetin, 18 = ellagic acid, and 19 = quercetin. Peak numbers are on the maximum detected absorbance.

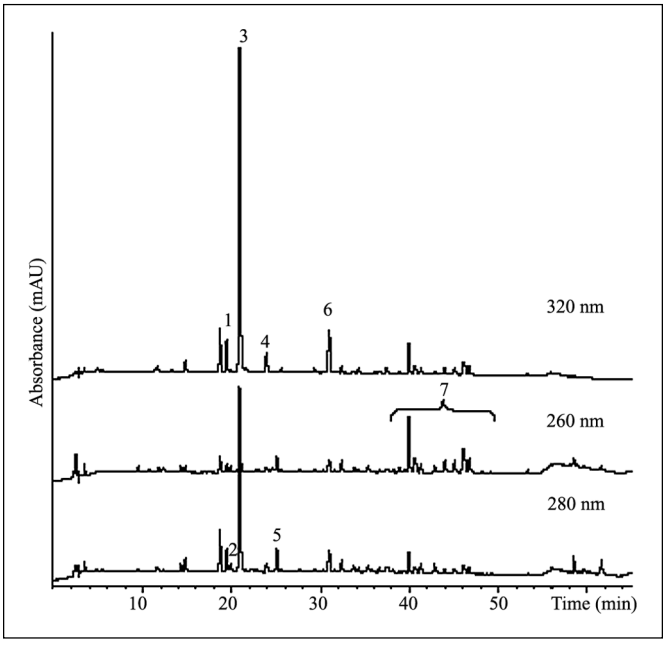

Figure 5—High-performance liquid chromatography (HPLC) separation (detection at 320, 260, and 280 nm) of 'Rubel' whole berry polyphenolic fraction. Corresponding phenolics peak assignments: 1 = cinnamic acid derivative, 2 = catechin, 3 = chlorogenic acid, 4 = caffeic acid, 5 = syringic acid, 6 = cinnamic acid derivative, and 7 = flavonol-glycosides.

The puree waste polyphenolic profile for the industrial blueberry juice–processing wastes showed the puree stock to have higher amounts of cinnamic acids and flavonol-glycosides than the puree waste. Enzymatic destruction of polyphenolics in the puree is a possible explanation.

Conclusions

A combination of heat and SO_2 increased the extraction of anthocyanins and polyphenolics from whole berries and skins. Processing enzymes produced an extract from whole berries lower in polymeric color percentage but were not effective in extracting ACY and TP. However, enzymes were helpful in extraction of ACY and TP from skins. Blueberry skins were the highest in ACY, TP, and antioxidant activity compared with flesh and seed fractions. Blueberry processing waste is high in ACY and TP and has the potential to be a good source of natural colorants and nutraceuticals. The findings will be useful to processors who would like to increase their recovery of anthocyanin- and polyphenolic-rich extracts, reduce byproduct disposal, and enhance the value of their fruit commodities through more than 1 avenue.

Acknowledgments

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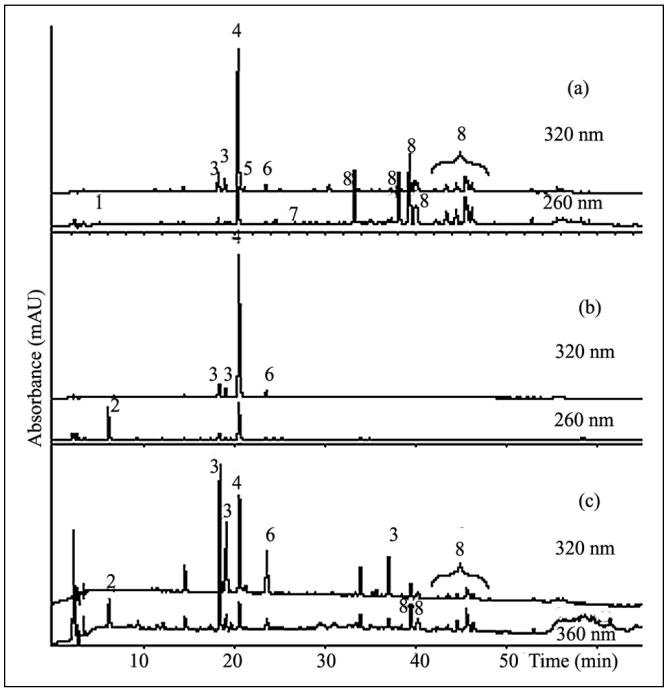

Figure 6—Polyphenolic profile of the 3 blueberry fractions (skins [a], flesh [b], and seeds [c]) separated by liquid chromatography (LC) (detection at 320 and 260 nm shown). Corresponding tentative peak assignments are as follows: 1 = gallic acid, 2 = residual acetone left from extraction, 3 = cinnamic acid derivatives, 4 = chlorogenic acid (5-caffeoylquinic acid), 5 = 4-caffeoylquinic acid (chlorogenic isomer), 6 = caffeic acid, 7 = syringic acid, and 8 = flavonol-glycosides.

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