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LC–MS/MS and UPLC–UV Evaluation of Anthocyanins and Anthocyanidins during Rabbiteye Blueberry Juice Processing

Rebecca E. Stein-Chisholm [1], John C. Beaulieu [2,*], Casey C. Grimm [2] and Steven W. Lloyd [2]

1 Lipotec USA, Inc. 1097 Yates Street, Lewisville, TX 75057, USA; rstein07@gmail.com
2 United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124, USA; Casey.Grimm@ars.usda.gov (C.C.G.); Steven.Lloyd@ars.usda.gov (S.W.L.)
* Correspondence: John.Beaulieu@ars.usda.gov; Tel.: +1-504-286-4471

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Abstract: Blueberry juice processing includes multiple steps and each one affects the chemical composition of the berries, including thermal degradation of anthocyanins. Not-from-concentrate juice was made by heating and enzyme processing blueberries before pressing, followed by ultrafiltration and pasteurization. Using LC–MS/MS, major and minor anthocyanins were identified and semi-quantified at various steps through the process. Ten anthocyanins were identified, including 5 arabinoside and 5 pyrannoside anthocyanins. Three minor anthocyanins were also identified, which apparently have not been previously reported in rabbiteye blueberries. These were delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p-coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside). Delphinidin-3-(p-coumaroyl-glucoside) significantly increased 50% after pressing. The five known anthocyanidins—cyanidin, delphinidin, malvidin, peonidin, and petunidin—were also quantitated using UPLC–UV. Raw berries and press cake contained the highest anthocyanidin contents and contribute to the value and interest of press cake for use in other food and non-food products. Losses of 75.7% after pressing and 12% after pasteurization were determined for anthocyanidins during not-from-concentrate juice processing.

Keywords: not-from-concentrate juice; *Vaccinium ashei*; juice processing; anthocyanins; anthocyanidins

1. Introduction

Blueberries are a well-known source of health-promoting phytochemicals [1,2]. These phytochemicals can be divided into different classes based on their chemical structures. Of the major classes of phytochemicals, flavonoids are becoming popular for studies focusing on their health benefits. One of the most unique classes of flavonoids are the anthocyanins. Anthocyanins give fruits and vegetables their vibrant red, blue, and purple colors [3]. These compounds are unique because they exist in five configurations and various colors based on pH. These configurations include the blue-colored anionic quinonoidal base, the violet-colored quinonoidal base, the red-colored flavylium cation, the colorless carbinol base, and the yellow-colored (E) or (Z) chalcone [4]. At lower pH values, anthocyanins are red in color; as pH rises anthocyanins will be more blue in color but, as the pH shifts from more neutral to basic, they turn clear, then above pH 10, the alkalinity generally destroys the compound [5].

There have been over 600 naturally occurring anthocyanins reported in plants [6]. Anthocyanins are composed of an anthocyanidin backbone with varying glycosides. The five major anthocyanidin classes in blueberries are cyanidin, delphinidin, malvidin, peonidin and petunidin [7]. Cyanidin is the most common anthocyanidin found in plants and can have 76 different glycoside combinations [8]. The major anthocyanins in blueberries include 3-glycosidic derivatives of cyanidin, delphinidin, malvidin, peonidin and petunidin; with glucose, galactose and arabinose as the most abundant sugars [9]. The lesser anthocyanins consist of acetoly, malonoyl, and coumaroyl conjugated compounds [10].

Consumer demand for food and beverage products which are made from locally or regionally grown raw materials are driven by the belief that these products help local communities, provide healthier alternatives, as well as decrease carbon footprints [11]. With increased demand for natural and less processed food options, local small scale juice producers are looking at not-from-concentrate (NFC) juice products to meet demand and create niche markets [12]. In Europe, the NFC juice market segment was up 5.4% in 2016 from 2015 [13]. In the United States, NFC juices have an expected annual average growth of 5.3% through 2016 [14]. Utilizing NFC juices as a compromise between unpasteurized fresh juices and highly processed juices reconstituted from concentrates allows small-scale local producers to expand beyond the farmers markets [15,16]. Furthermore, the ability to process berries, especially locally frozen fruit into juice is an efficient way to extend their shelf life and extend the profitability of a grower's harvest season [17].

The process of making berry juice may include heating before pressing as well as enzyme treatments (creating a mash) to increase juice recovery and minimize anthocyanin loss [18]. Processing can affect the anthocyanins and other phytochemicals, as well as the macronutrients in the berries [19]. It is proposed that one of the pathways of degradation of anthocyanins is caused by native enzymes, mainly polyphenol oxidase (PPO), breaking down other polyphenols to form quinones. These in turn react with the anthocyanins, forming brown pigments [19]. Heating the berries before pressing denatures native enzymes and reduces enzymatic browning in juice [20]. The use of pectinases increases juice recovery by degrading pectin in cell walls, improving liquefaction and clarification, and aiding in filtration processes [21]. Heated mash is then pressed, removing remaining skins and seeds, resulting in an unfiltered juice. Filtration is an optional step to reduce sedimentation to clarify the juice and remove polymeric compounds which can affect overall color and turbidity [22]. Pasteurization is a safety step used to decrease spoilage and contamination. Evaluating the aforementioned processes builds information and awareness to help juice producers develop juice products that can maximize profit and quality.

The "Tifblue" variety at one point was the most widely planted rabbiteye (RAB) blueberry in the world [23]. It is still a very popular RAB berry cultivar today and is highly regarded for its appearance, productivity, harvesting and shipping qualities, as well as a standard for comparison to other selections and cultivars [24,25]. Many studies have been conducted on the processing effects on blueberry juice anthocyanins, but to our knowledge, few have been carried out on RAB (*Vaccinium ashei*) blueberries [26,27]. In this experiment, RAB blueberry anthocyanins and their anthocyanidin backbones were identified in each juice processing step and their stepwise changes were evaluated. This NFC juice evaluation contributes to the knowledge of RAB blueberry properties and pilot plant process parameters affecting polyphenolics during various juice processing steps.

2. Materials and Methods

2.1. Juice Processing

Commercial "Tifblue" RAB blueberries (*V. ashei*) were harvested (Blue River Farms, Hattiesburg, MS, USA) and commercially packaged (sorted, graded, washed, air-dried and forced-air rapid frozen at −20 °C (Nordic Cold Storage, Hattiesburg, MS, USA). Using a 37.9 L steam-jacketed kettle (Groen-A Dover Industries Co., Byram, MS, USA) 27 kg (two individual 13.5 kg boxes) of frozen berries (control)

were quickly heated to ~95 °C in roughly 14.5 min with constant stirring using a large wooden paddle. Temperature was monitored with thermal probes ("K Milkshake"; ThermoWorks, Salt Lake City, UT, USA) and the crude mash was held at 96.5 ± 1.1 °C for 3 min. The mash (sampled prior to enzyme treatment and hydraulic pressing) was then poured into a 37.9 L stainless steel vessel and allowed to cool to 55 °C for addition of pectinase enzyme. Rohapect 10 L (AB Enzymes, Darmstadt, Germany) was added at 200 mL ton^{-1} and allowed to activate with occasional stirring for 1 h. The enzyme-treated mash was pressed warm (~45 °C) in an X-1 single-layer hydraulic press (Goodnature, Orchard Park, NY, USA) at 12.4 MPa using a medium-weave polyester mesh press bag (Goodnature, #2636) for 1 min. Pressed juice from each batch was individually collected in a stainless-steel vessel and cooled overnight at 4 °C. Press cake (PRC) samples were collected and stored at −20 °C.

Half of the chilled pressed juice (PJ) was portioned off and pasteurized, delivering a pasteurized pressed unfiltered juice (PPJ) and samples were collected at this point and stored at −20 °C. The remaining pressed juice was filtered using ultrafiltration in a pilot unit (BRO/BUF, Membrane Specialists, Hamilton, OH, USA) with a 100 L hopper tank. The unit consisted of an in-line membrane filtration module (PCI B-1 Module Series, Aquious PCI Membrane, Hamilton, OH, USA) and a heat exchanger fed by a 7.5 hp screw pump. Filtration occurred with a 200,000 molecular weight cut-off (0.2 µm) XP-201 polyvinylidene fluoride (PVDF) membrane (ITT PCI Membrane Systems, Zelienople, PA, USA), with the heat exchanger run at ambient (~25 °C) attaining a product flow rate of roughly 18.9–29.9 L h^{-1}. After equilibrating the ultrafiltration unit, filtered not-from-concentrate blueberry juice was collected for sampling (UF; ultrafiltered juice), then pasteurized (UFP, ultrafiltered pasteurized).

PJ and UF samples were pasteurized using a high-temperature short-time (HTST) pasteurization unit (Electra UHT/HTST Lab-25EDH; MicroThermics, Raleigh, NC, USA) at 90 °C for 10 s, at 1.2 L min^{-1}, followed by hot-filling at 85 °C into pre-sterilized 250 mL transparent glass media bottles (Corning, Tewksbury, MA, USA) followed by inversion and ice water bath chilling. Pressed juice which was pasteurized (PPJ) and ultrafiltered pasteurized juice (UFP) were frozen at −20 °C before anthocyanins and anthocyanidins analysis.

2.2. Anthocyanin Analysis

2.2.1. Extraction

For the control and PRC samples, 5 g of raw berries were thawed and homogenized using a Tekmar Tissumizer (SDK-1810, IKA-Werke, Staufen, Germany). Using 2 g of sample, all triplicated samples were lyophilized in a VirTis Genesis 25ES freeze dryer (SP Scientific, Warminster, PA, USA). After lyophilization, 100 mg of the powder was weighed into 2 mL centrifuge tubes and 1 mL of an extraction solvent (70:30:1, $v/v/v$; methanol (MeOH): water (H_2O): trifluoroacetic acid (TFA)) was added [10]. The tubes were vortexed for 15 s and left undisturbed for 60 min. Following extraction, the tubes were sonicated for 20 min and centrifuged (IEC CL, International Equipment Company, Needham Heights, MA, USA) for 15 min at 1200 rpm. The supernatant was filtered through a 0.2 µm syringe filter into a HPLC vial then stored at −20 °C.

2.2.2. HPLC–MS/MS Chromatography

Berry and juice samples were analyzed for anthocyanins using a LC–MS/MS method. Extracted samples were analyzed on an Agilent 1200 HPLC with an Agilent Small Molecule Chip Cube interface and Agilent 6520 Q-TOF MS/MS (Agilent, Santa Clara, CA, USA). The chip contained a 40 µL enrichment column and a C18 (43 mm × 75 µL, 80 Å) column. The eluents were acidified H_2O with 0.1% formic acid (A) and 90% acetonitrile with 9.9% H_2O and 0.1% formic acid (B). The gradient was held at 2% B then raised to 20% over 10 min, and then increased to 40% B to 18 min. The MS fragmenter was set to 175 V, and the VCap at 1800 V. Capillary temperature was 300 °C with N_2 as carrier gas with a flow rate of 5 L min^{-1}. The MS scan rate was 1 scan s^{-1} (10,000 transients). Auto MS/MS had selected m/z ranges (Table 1) and the scan rate was 1 scan s^{-1}. A semi-quantified peak area

abundance was calculated for each compound using the characteristic anthocyanin parent fragment molecular weight ($[M + H]^+$) along with the fragmented MS/MS backbone anthocyanidin molecular weight (Table 1), as confirmed by the residual sugar (generally the 3 position in the C-ring or R3; the R-*O*-sugar group) moiety fragment. Averaged ion counts from these identifying fragments were utilized to measure and compare process changes in the anthocyanins in juices.

Table 1. Major and minor anthocyanin compounds and mass spectrometry variables found in blueberries.

Major Anthocyanin [z]	Sugar Moiety	Molecular Formula	$[M + H]^+$ (*m*/*z*) [y]	MS/MS (*m*/*z*) [x]	R_t (min) HPLC [w]
Delphinidin-3-arabinoside	Arabinose	$C_{20}H_{19}O_{11}$	435.0922	303.0500	10.94
Cyanidin-3-arabinoside	Arabinose	$C_{20}H_{19}O_{10}$	419.0928	287.0550	11.47
Petunidin-3-arabinoside	Arabinose	$C_{21}H_{21}O_{11}$	449.1078	317.0700	11.66
Peonidin-3-arabinoside	Arabinose	$C_{21}H_{21}O_{11}$	433.1129	301.0700	12.20
Malvidin-3-arabinoside	Arabinose	$C_{22}H_{23}O_{11}$	463.1235	331.0800	12.34
Delphinidin-3-pyranoside	Galactose/Glucose	$C_{21}H_{21}O_{12}$	465.1027	303.0500	10.38
Cyanidin-3-pyranoside	Galactose/Glucose	$C_{21}H_{21}O_{11}$	449.1078	287.0550	10.99
Petunidin-3-pyranoside	Galactose/Glucose	$C_{22}H_{23}O_{12}$	479.1184	317.0700	11.29
Peonidin-3-pyranoside	Galactose/Glucose	$C_{22}H_{23}O_{11}$	463.1235	301.0700	12.99
Malvidin-3-pyranoside	Galactose/Glucose	$C_{23}H_{25}O_{12}$	493.1340	331.0800	11.98
Minor Anthocyanin [z]	**Sugar Moiety**	**Molecular Formula**	**$[M + H]^+$ (*m*/*z*)**	**MS/MS (*m*/*z*)**	**R_t (min) HPLC [w]**
Delphinidin-3-(p-coumaroyl-glucoside)	Glucose	$C_{30}H_{27}O_{14}$	611.1395	303.0500	13.11
Cyanidin-3-(p-coumaroyl-glucoside)	Glucose	$C_{30}H_{27}O_{13}$	595.1446	287.0550	13.86
Petunidin-3-(p-coumaroyl-glucoside)	Glucose	$C_{31}H_{29}O_{14}$	625.1552	317.0700	14.02
Peonidin-3-(p-coumaroyl-glucoside)	Glucose	$C_{31}H_{29}O_{13}$	609.1603	301.0700	ND [v]
Malvidin-3-(p-coumaroyl-glucoside)	Glucose	$C_{32}H_{31}O_{14}$	639.1708	331.0800	ND
Delphinidin-3-(6″-acetyl-pyranoside)	Galactose/Glucose	$C_{23}H_{23}O_{13}$	507.1133	303.0500	ND
Cyanidin-3-(6″-acetyl-pyranoside)	Galactose/Glucose	$C_{23}H_{23}O_{12}$	491.1184	287.0550	ND
Petunidin-3-(6″-acetyl-pyranoside)	Galactose/Glucose	$C_{24}H_{25}O_{13}$	521.1289	317.0700	ND
Peonidin-3-(6″-acetyl-pyranoside)	Galactose/Glucose	$C_{24}H_{25}O_{12}$	505.1340	301.0700	ND
Malvidin-3-(6″-acetyl-pyranoside)	Galactose/Glucose	$C_{25}H_{27}O_{13}$	535.1446	331.0800	ND

[z] Compounds noted in different blueberry species, including rabbiteye, as reported in literature. [y] $[M + H]^+$ = Molecular ion weight. Values corroborated by several literature sources, listed in Table 2. [x] MS/MS = Fragmented anthocyanidin molecular weight. Subsequently, this fragment ion (the backbone anthocyanidin), is free from the sugar moiety cleavage product. [w] R_t = retention time (minutes) from the Agilent 1200 HPLC. [v] ND = Compounds not detected in experimental samples. Theoretical molecular ion weights calculated based on literature.

Confirmation of anthocyanin identification was verified by the MS/MS scan of selected parent ion fragments and the sugar moiety molecular weight denoted in Table 1, using the MassHunter Workstation 6.00 software (Agilent, Santa Clara, CA, USA) [10]. Anthocyanin standards are difficult to find and the few that are available are expensive, so an extensive literature search was utilized to determine ions (Table 2).

Table 2. Anthocyanins in blueberries from the literature.

Anthocyanin	Literature [z]
Delphinidin-3-arabinoside	[3,10,19,28–32], **[33]**, [34], **[35]**, [36], **[37]**, [38]
Cyanidin-3-arabinoside	[3,7,10,19,29–32], **[33]**, [34], **[35]**, **[37]**, [38]
Petunidin-3-arabinoside	[7,10,19,28–32], **[33]**, **[35]**, [36], **[37]**, [38]
Peonidin-3-arabinoside	[10], **[26]**, [28,29,32], **[33]**, [34], **[35]**, **[37]**, [38]
Malvidin-3-arabinoside	[7,10,19,28,29,31,32], **[33]**, [34], **[35]**, [36], **[37]**

Table 2. *Cont.*

Anthocyanin	Literature [z]
Delphinidin-3-pyranoside	[3,7,10,19], **[26]**, [28–32], **[33]**, [34], **[35]**, [36]
Cyanidin-3-pyranoside	[3,7,10,19], **[26]**, [28–32], **[33]**, [34], **[35]**, [36,38]
Petunidin-3-pyranoside	[3,7,10,19], **[26]**, [28–32], **[33]**, [34], **[35]**, [36,38]
Peonidin-3-pyranoside	[3,7,10,19], **[26]**, [28–32], **[33]**, [34], **[35]**, [36,38]
Malvidin-3-pyranoside	[3,7,10,19], **[26]**, [28–32,34], **[35]**, **[36]**, [38]
Delphinidin-3-(p-coumaroyl-glucoside) [y]	[10]
Cyanidin-3-(p-coumaroyl-glucoside)	[10]
Petunidin-3-(p-coumaroyl-glucoside)	[10]
Peonidin-3-(p-coumaroyl-glucoside)	[10]
Malvidin-3-(p-coumaroyl-glucoside)	[10]
Delphinidin-3-(6″-acetyl-pyranoside)	[7,10,28,29,31], **[36]**
Cyanidin-3-(6″-acetyl-pyranoside)	[7,10,28,31]
Petunidin-3-(6″-acetyl-pyranoside)	[7,10,28,29,31]
Peonidin-3-(6″-acetyl-pyranoside)	[7,10,28,31]
Malvidin-3-(6″-acetyl-pyranoside)	[7,10,28,29,31], **[36]**

[z] References denoted by **bold font** indicate rabbiteye blueberry samples. [y] "p" indicates *para*.

2.3. Anthocyanidin Analysis

2.3.1. Extraction

Berry samples were analyzed for anthocyanidins using a modified UPLC method [10,28], as previously modified by Beaulieu et al. (2015) [39]. For the control samples, 10 g of raw berries were thawed and homogenized using a Tekmar Tissumizer (SDK-1810, IKA-Werke, Staufen, Germany) and a 2 g sample was lyophilized (VirTis Genesis 25ES, SP Scientific, Warminster, PA, USA). Press cake (PRC) was lyophilized in 40 g samples from each batch and a 2.5 g sample was utilized for extraction. Process step samples were 2 mL of lyophilized juice. All samples were allocated and weighed before being stored at −20 °C. Extraction of the juices and control was done using the 2 g frozen lyophilized powder in 2 mL of an extraction solvent comprised of 70:30:1 MeOH:H_2O:TFA. Press cake samples were extracted with 25 mL of extraction solvent and 2.5 g lyophilized powder. Each sample was vortexed for 15 s and left undisturbed for 60 min. Following the extraction, the samples were sonicated 20 min then centrifuged (IEC CL, International Equipment Company, Needham Heights, MA, USA) for 20 min at 7000 rpm and supernatant stored at −20 °C.

2.3.2. Acid Hydrolysis

The extracted samples (2 mL) were then hydrolyzed in a 4 mL vial, in which was mixed 200 µL of 12N HCl [28]. After purging vials with nitrogen gas, the vials were sealed and vortexed for 5 s and heated at 95 °C for 20 min. Immediately after heating, the samples were stored at −20 °C. Hydrolysates were thawed and filtered using a 0.2 µm polyvinylidene difluoride (PVDF) syringe filters, into autosampler vials.

2.3.3. UPLC Chromatography

Samples were analyzed on an Acquity ultra performance liquid chromatography system equipped with an ultra violet detector (UPLC–UV) using an Acquity BEH C18 column (50 mm × 2.1 mm × 1.7 µm) (Waters Corporation, Milford, MA, USA). The flow rate was 1.0 mL min^{-1}. The eluents consisted of acidified water with 3% phosphoric acid (A) and 100% acetonitrile (ACN) (B). The gradient started at 10% B, ramped to 20% B at 2 min, increased to 100% B at 2.1 min, then held at 100% B until 2.7 min, and returned to 10% B at 2.8 min. Detection was done with single-wavelength UV at 20 points min^{-1} at 525 nm. Anthocyanidin standards cyanidin, delphinidin, malvidin, peonidin,

and petunidin (Chromadex, Santa Ana, CA, USA) were run at a concentration gradient of 0.001, 0.003, 0.010, 0.030 and 0.100 mg mL^{-1} ($r^2 \geq 0.995$) to report anthocyanidins (mg 100 g^{-1}).

2.4. Statistical Analysis

The experiment was repeated three times. Results are presented as mean ± standard deviation. Anthocyanin and anthocyanidin values obtained by HPLC–MS/MS and UPLC–UV experimentation are presented as the mean of three discreet batches, sampled three times, for a total of 9 data points for each treatment. Data were analyzed using analysis of variance (ANOVA), using the SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The means and standard deviations were compared using Tukey's studentized range method with $p \leq 0.05$.

3. Results and Discussion

3.1. Identification of Anthocyanins and Anthocyanidins

3.1.1. Anthocyanins

Anthocyanins were identified to determine the effects of processing on juice quality due to their heat sensitivity and as a marker of health benefits in blueberries [40]. Using literature to focus in and narrow down specific compounds, 10 major and 10 minor anthocyanins were evaluated, including 5 coumaroly-glucosides and 5 acetyl-pyranoside anthocyanins (Tables 1 and 2). Of these 20 compounds, 10 major and 3 minor anthocyanins were identified in the various blueberry juices and were used to visualize changes in juice quality through each processing step (Table 3).

Table 3. Average semi-quantitative LC–MS/MS anthocyanin peak area abundance in rabbiteye blueberry during various processing steps.

Treatment [z]	Del-3-ara [y]	Cya-3-ara	Pet-3-ara/Cya-3-pyr	Peo-3-ara
PRC	0.2 ± 0.05 d [x,w]	0.04 ± 0.03 d	0.2 ± 0.03 d	ND
Control	8.8 ± 1.8 a	8.4 ± 1.2 a	22.8 ± 2.9 a	5.1 ± 0.9 a
PJ	2.8 ± 0.5 b	2.4 ± 0.4 bc	7.0 ± 1.0 b	1.3 ± 0.2 bc
PPJ	2.7 ± 0.9 bc	2.6 ± 0.8 bc	7.3 ± 1.0 b	1.6 ± 0.5 ab
UF	3.6 ± 0.7 b	3.1 ± 0.6 b	8.3 ± 0.8 b	1.9 ± 0.2 b
UFP	1.7 ± 0.4 c	1.8 ± 0.4 c	4.4 ± 0.4 c	0.9 ± 0.2 c
	Mal-3-ara/Peo-3-pyr	**Del-3-pyr**	**Pet-3-pyr**	**Mal-3-pyr**
PRC	0.3 ± 0.1 d	0.5 ± 0.09 d	0.3 ± 0.03 d	0.5 ± 0.1 d
Control	49.2 ± 8.3 a	20.5 ± 4.1 a	18.8 ± 3.8 a	49.5 ± 9.7 a
PJ	16.5 ± 2.8 bc	7.1 ± 1.5 b	7.1 ± 1.6 b	24.7 ± 4.4 bc
PPJ	17.7 ± 5.6 b	6.0 ± 1.7 bc	7.0 ± 2.2 b	25.6 ± 7.9 b
UF	22.1 ± 2.3 b	8.4 ± 1.3 b	8.7 ± 1.9 b	30.5 ± 4.6 b
UFP	12.2 ± 3.6 c	4.0 ± 0.6 c	4.3 ± 0.9 c	18.2 ± 4.6 c
	Del-3-cou	**Cya-3-cou**	**Pet-3-cou**	**Total Abundance**
PRC	0.05 ± 0.01 c	ND	0.1 ± 0.05 c	2.2 ± 0.4 d
Control	0.5 ± 0.1 b	0.3 ± 0.05 b	1.4 ± 0.5 b	185.2 ± 34.0 a
PJ	1.1 ± 0.3 a	0.4 ± 0.1 a	2.1 ± 0.7 ab	72.5 ± 13.4 b
PPJ	0.9 ± 0.4 a	0.4 ± 0.1 ab	2.2 ± 1.1 a	74.0 ± 21.2 b
UF	1.1 ± 0.4 a	0.5 ± 0.2 a	2.9 ± 1.1 a	85.2 ± 21.2 b
UFP	1.0 ± 0.3 a	0.4 ± 0.08 ab	2.1 ± 0.4 ab	50.9 ± 11.5 c

[z] PRC = press cake; PJ = pressed not filtered juice; PPJ = pasteurized pressed unfiltered juice; UF = ultrafiltered juice; UFP = ultrafiltered pasteurized juice. [y] Del-3-ara = delphinidin-3-arabinoside; Cya-3-ara = cyanidin-3-arabinoside; Pet-3-ara/Cya-3-pyr = petunidin-3-arabinoside/cyanidin-3-pyranoside; Peo-3-ara = peonidin-3-arabinoside; Mal-3-ara/Peo-3-pyr = malvidin-3-arabinoside/peonidin-3-pyranoside; Del-3-pyr = delphinidin-3-pyranoside; Pet-3-pyr = petunidin-3-pyranoside; Mal-3-pyr = malvidin-3-pyranoside; Del-3-cou = delphinidin-3-(p-coumaroyl-glucoside); Cya-3-cou = cyanidin-3-(p-coumaroyl-glucoside); Pet-3-cou = petunidin-3-(p-coumaroyl-glucoside). [x] Abundance values are reported as average ion counts min^{-1} ± standard deviations, ×100,000, according to information presented per compound in Table 1. [w] Significant differences (Tukey's method with $p < 0.05\%$) per parameter are designated by letters in each column. Means not connected by the same letter are significantly different at $p < 0.05$.

Conformation of anthocyanins was determined by the anthocyanin molecular weight ion $[M + H]^+$ and the backbone anthocyanidin molecular weight ion (MS/MS), as verified in literature [10,41]. With the exception of peonidin-3-arabinoside and cyanidin-3-(p-coumaroyl-glucoside) in the press cake, all 13 major and minor anthocyanidins were identified in each processing step. This corroborates with other studies identifying anthocyanins in blueberries, including RAB berries (Table 2) [34–36,38]. Several studies on southern highbush (SHB) and northern highbush (NHB) blueberries, as well as bilberries, identified minor 5 acetyl-pyranoside anthocyanins, which were not identified in this study (Table 2). This may be due to varietal and species differences between RAB berries and other blueberry types [36,42]. A "Tifblue" RAB juice study by Srivastava et al. (2007) [26] created juice by thawing, boiling and blending the berries in a household blender before treating them with a pectinase enzyme. The berry slurry was then centrifuged and batch pasteurized to 85 °C for 2 min before bottling. Results of their bench top experiment identified 8 anthocyanins using HPLC–UV (Table 2). Previously, glucoside and galactoside anthocyanins were isolated in RAB berries [26], but they did not identify as many arabinoside compounds as our experiment. An LC–MS overlay of the major anthocyanin abundances in a raw berry sample (control) chromatogram shows how closely the compounds elute relative to each other and how MS/MS can help to differentiate some of the different compounds (Figure 1).

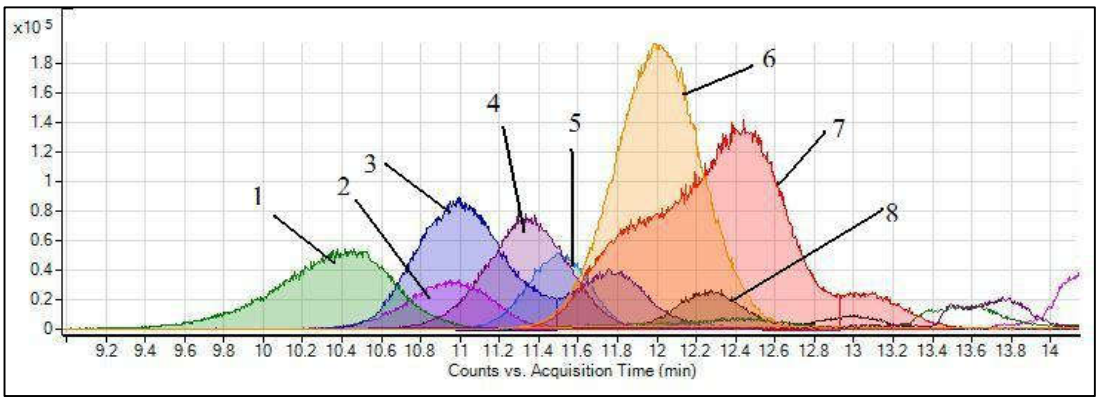

Figure 1. Overlay of $[M + H]^+$ ions of major anthocyanins in raw berry sample. (**1**) Delphinidin-3-pyrannoside (m/z 465 $[M + H]^+$); (**2**) Delphinidin-3-arabinoside (m/z 435 $[M + H]^+$); (**3**) Cyanidin-3-pyrannoside (m/z 449 $[M + H]^+$); (**4**) Petunidin-3-pyrannoside (m/z 479 $[M + H]^+$); (**5**) Cyanidin-3-arabinoside (m/z 419 $[M + H]^+$); (**6**) Malvidin-3-pyrannoside (m/z 493 $[M + H]^+$); (**7**) Malvidin-3-arabinoside/Peonidin-3-pyrannoside (m/z 463 $[M + H]^+$); (**8**) Peonidin-3-arabinoside (m/z 433 $[M + H]^+$).

In the major anthocyanins identified, separation issues were identified between the glucoside- and galactoside-containing anthocyanins. These two sugar moieties proved difficult to separate the 6-carbon structures with identical $[M + H]^+$ and MS/MS ions as well as similar elution times (Figure 1, Peaks 1, 3, 4 and 6). Peonidin-3-arabinoside was not separated from cyanidin-3-pyranoside as well as malvidin-3-arabinoside from peonidin-3-pyranoside (Figure 1, Peaks 7 and 8). These compounds' elution times, almost identical $[M + H]^+$ fragment ions MW, and difficulty to separate pyranoside compounds made separation impractical. In the past, extensive LC method development for peak separation has been reported [31]. Therefore, the unresolved co-eluding peaks were evaluated as one peak response and not individual anthocyanins (Table 3).

As part of the complexing and/or co-pigmentation of anthocyanins, they can also form esters with hydroxycinnamates and organic acids [41]. The lesser anthocyanins may consist of acetoly, malonoyl and coumaroyl compounds [10]. In this study, 5 coumaroly-glucosides and 5 acetyl-pyranoside anthocyanins were searched for and three minor coumaroyl anthocyanin

compounds were identified using MS/MS ions, as confirmed in literature (Table 1) [30,41,43]. The compounds were delphinidin-3-(p-coumaroyl-glucoside), cyanidin-3-(p-coumaroyl-glucoside), and petunidin-3-(p-coumaroyl-glucoside) which, to the best of our knowledge, have not been reported in RAB blueberries (Figure 2). These three coumaroyl anthocyanins are reported in grapes, radishes, red cabbage and black carrots [41,44]. A possibility for not identifying the other acetyl-pyranoside anthocyanins as identified by other studies in RAB berry samples may be due to the inadequate LC separation before MS detection of the compounds: including phase gradients, length of gradient, and run times. Figure 3 illustrates the MS/MS scan of the 3-coumaroyl compounds and the ion fragments of each compound. Further LC–MS/MS method development is apparently needed to better separate and further confirm identification of the minor anthocyanins in RAB berries, which could provide further tools to monitor processing variables and juice quality.

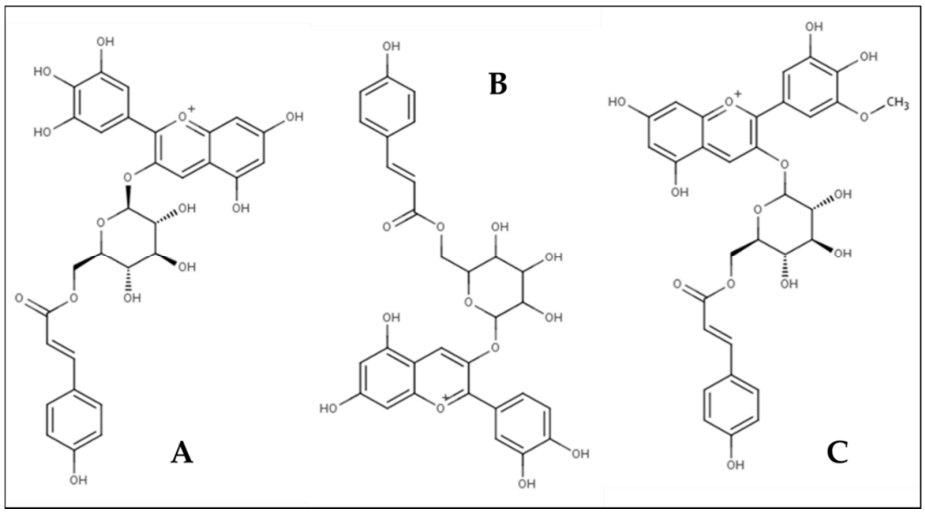

Figure 2. Chemical structure of delphinidin-3-(p-coumaroyl-glucoside) (**A**); cyanidin-3-(p-coumaroyl-glucoside) (**B**) and petunidin-3-(p-coumaroyl-glucoside (**C**). Figure adapted from (Neveu et al., 2012) [43].

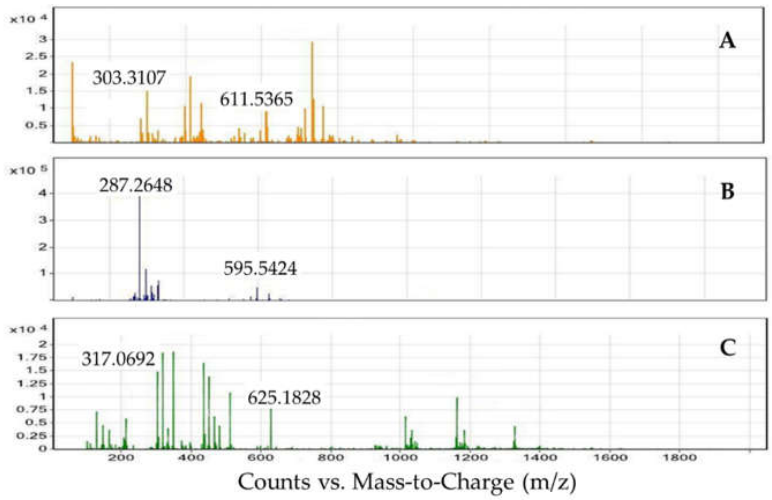

Figure 3. MS/MS spectrum scan of 3 identified minor anthocyanins in unfiltered rabbiteye blueberry juice: delphinidin-3-(p-coumaroyl-glucoside), top plate (**A**); cyanidin-3-(p-coumaroyl-glucoside), middle plate (**B**) and petunidin-3-(p-coumaroyl-glucoside) bottom plate (**C**).

3.1.2. Anthocyanidins

The five major anthocyanidins found in blueberries (cyanidin, delphinidin, malvidin, peonidin and petunidin) were all identified in every step of the juice process. A chromatogram of the anthocyanidins found in a press cake sample illustrates the elution order for the anthocyanidins in RAB blueberries (Figure 4). Acid hydrolysis of anthocyanins produces anthocyanidins which are identified with UV detectors. Sugar moieties have extremely similar molecular weights which can complicate identification [28]. By removing the glycosides from the molecule, the anthocyanidin backbones can be evaluated and precisely quantified to illustrate the classes of anthocyanin molecules present in the sample.

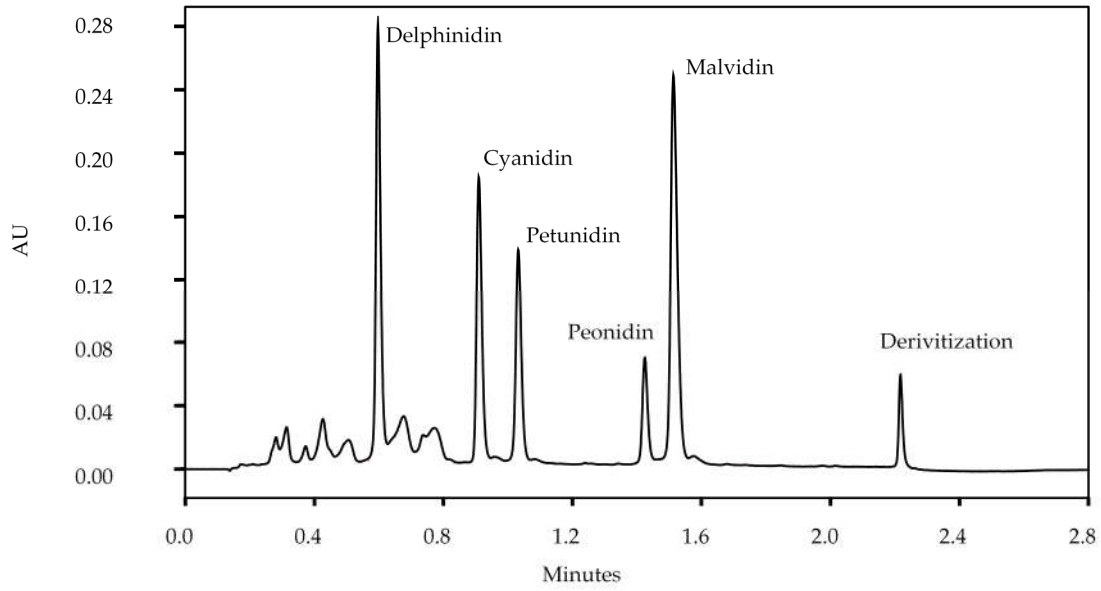

Figure 4. Anthocyanidins found in rabbiteye blueberry press cake (PRC) as detected by HPLC–UV.

3.2. Changes of Anthocyanins during Blueberry Juice Processing

Anthocyanin amounts were measured to determine the effects of processing on juice quality. Using the semi-quantitative LC–MS/MS peak area abundance data to compare anthocyanins, the most abundant major anthocyanin identified in the control rabbiteye sample was malvidin-3-pyranoside (Table 3). Malvidin-3-galactoside was the most abundant anthocyanin found in NHB blueberries [37]. Although peak resolution did not allow for the separation of the two 6 carbon sugars, herein, malvidin-3-galactoside was included in the recovery of malvidin-3-pyranoside. The least abundant major anthocyanin identified was peonidin-3-arabinoside. Comparing these results to a study by Srivastava et al. (2007) [26], malvidin-3-pyranoside was also found to be the largest anthocyanin recovered; however, they concluded that cyanidin-3-pyranoside was the least abundant anthocyanin in "Tifblue" RAB juice.

After pressing, a large percentage of anthocyanins were not transferred to the PJ from the whole berry (control). With one exception, there was an approximate 70% decrease between control and PJ in all anthocyanins (Table 3). However, malvidin-3-pyranoside (Mal-3-pyr) only had a 50% loss. Peonidin-3-arabinoside (Peo-3-ara) had the greatest loss (74%), decreasing from $5.1 \pm 0.9 \times 10^5$ to $1.3 \pm 0.2 \times 10^5$ counts. The anthocyanin decreases between the PJ and the pressed pasteurized unfiltered juice (PPJ) were minimal and not significantly different. This may be explained by the increased heat stability caused by possible polymerization and/or co-pigmentation of the anthocyanins. Co-pigmentation of anthocyanins with other anthocyanins and polyphenols increases the molecule's heat stability [45].

After the initial decrease from pressing the berries, the second greatest decrease in anthocyanins occurred when ultrafiltered juice was pasteurized (UFP). The major anthocyanins significantly decreased 40% to 53% after pasteurization in the UFP samples (Table 3). Major anthocyanins containing delphinidin had the greatest degradation, with delphinidin-3-pyranoside (Del-3-pyr) decreasing from $8.4 \pm 1.3 \times 10^5$ to $4.0 \pm 0.6 \times 10^5$ counts. Delphinidin was less stable in elevated temperatures which resulted in greater decreases in relation to the other anthocyanins due to its greater heat lability [26].

The minor anthocyanin trends after pressing were opposite compared to the major anthocyanins. Both cyanidin-3-(p-coumaroyl-glucoside) (Cya-3-cou) and petunidin-3-(p-coumaroyl-glucoside) (Pet-3-cou) increased (occasionally significantly) ~33% in PJ samples from their initial control levels. Delphinidin-3-(p-coumaroyl-glucoside) (Del-3-cou) significantly increased 50% from $0.5 \pm 0.1 \times 10^5$ to $1.1 \pm 0.3 \times 10^5$ counts after pressing (Table 3). These increases may be due to the heat degradation process by which anthocyanins break down into chalcone structures which can then undergo transformation into a coumarin glucoside [46]. One possible cause of the recoveries for the minor anthocyanins through processing involves the acylation of the anthocyanin molecule and its ability to increase stability by preventing hydration when exposed to pH changes and heat [44,47]. On the other hand, the greater anthocyanin degradation in the UFP juices as compared to the PPJ juices may be directly related to the role that co-pigmentation and anthocyanin polymers and/or polymerization play in the heat stability of the compounds themselves. Ultrafiltration removes many of the higher molecular weight co-pigments and polymers from the juice. It can be assumed that the larger the polymer or co-pigment, the greater the increase in heat stability of the compounds included in the polymer [45].

There was a significant 61% decrease in total anthocyanins between the control and PJ samples in the juice processing steps. The control berries contained $185.2 \pm 34.0 \times 10^5$ counts min^{-1}, while the PJ juice contained only $72.5 \pm 13.4 \times 10^5$ counts (Table 3). This was expected due to the large percentage of anthocyanins that remain in the press cake [48]. There was an insignificant concentration increase in the amounts of anthocyanin content of the UF samples, as compared to the PJ (Table 3). A concentrating effect regarding monomer anthocyanins has been observed in grape juice filtration using varying sizes of membranes [49]. Utilization of filtration to increase the concentration of beneficial compounds in juices is a concept that is being explored in other fruits and can increase juice nutritional value [50–53]. Polyphenolics in the retentate (recirculated to the 100 L hopper) from the filtration process were not evaluated in this experiment. However, since sugars from controls (12.7 °Brix) increased in the residual retentate (16 °Brix), the residual retentate presumably accumulates other possible value-added by-products that the producer may capitalize upon, in addition to the press cake. Removal of the anthocyanin polymers could be utilized as more stable natural food colorings [51]. Ultrafiltered juices (UFP) had significantly greater anthocyanin degradation due to pasteurization compared with the PPJ juice. A 40% decrease in total anthocyanins after pasteurization of UF juices, similar to microfiltered pomegranate juice [54], may be attributed to anthocyanin degradation from pasteurization due to the removal of the co-pigmentation of the anthocyanins by ultrafiltration [55].

Significant anthocyanin losses have also occurred in NHB blueberry juice and products [19,37,48,56,57]. There was 58.8% loss of monomeric anthocyanins in enzyme-treated, non-clarified, batch-pasteurized NHB blueberry juice prepared from frozen blanched berries, but only 27.5% loss in centrifuge-clarified juice [48]. A slightly different process using thawed, blanched NHB blueberries treated with pectinase, pressed, then clarified and followed by HTST (90 °C) pasteurization resulted in higher losses of 77.6% and 84.3% anthocyanin glycosides and anthocyanins, respectively [19]. There was a 68.0% total anthocyanin loss in juice created with individually quick frozen (IQF) NHB blueberries that were partially thawed, heated to 43 °C, treated with pectinase, pressed, then HTST pasteurized (90 °C) [37]. In puree/juice made with NHB berries that were manually crushed and machine-juiced, not filtered or clarified, then batch-pasteurized at 92 °C, there was a substantial 95.7% loss of total anthocyanins [57]. In addition, there was a 76.2% and 79.1% anthocyanin loss in Powderblue and Tifblue RAB blueberry varieties, respectively, that were thawed

12 h at 5 °C, hot water blanched, homogenized in a blender, pectinase added, centrifuged, then batch-pasteurized (85 °C) [26]. Overall, significant anthocyanin "losses" occur in processed blueberry juices and, herein, we did not prevent losses using a heated mash and UF with NFC juices.

The MS/MS PRC samples had unusually low anthocyanin peak area abundance amounts reported overall, compared to the remaining processing steps (Table 3). However, the low reports of anthocyanins in the PRC were likely due to problems with the extraction method itself (discussed below in Section 3.3.1) and did not reflect the actual amounts of anthocyanins found in the PRC. As discussed below, anthocyanidin recoveries from the PRC support the supposition that our extraction protocols for MS/MS PRC samples versus the liquid UPLC–UV samples were not comparatively effective, because the majority of anthocyanidins indeed remained in the PRC.

3.3. Changes of Anthocyanidins during Blueberry Juice Processing

3.3.1. Individual Anthocyanidins

To better define the changes caused by juice processing on anthocyanins, anthocyanidins were also evaluated. Malvidin (26.6 mg 100 g^{-1}) and petunidin (25.9 mg 100 g^{-1}) were the most abundant anthocyanidins in the control samples, while peonidin (4.1 mg 100 g^{-1}) was the least abundant. Markedly higher amounts of anthocyanidins detected in the press cake are due to the majority of anthocyanins being located in the skins of the berries [37]. Press cake samples contained 3-fold greater total anthocyanidins (265.6 mg 100 g^{-1}) than the control (85.1 mg 100 g^{-1}). Since berry skins account for 10% to 20% of the berry weight (13.0 ± 0.6% herein [58]) and as the main location for anthocyanins in the berry, the majority of anthocyanins were left behind in the press cake, as previously demonstrated [19,37,48]. Cyanidin was the most abundant anthocyanidin (77.7 mg 100 g^{-1}) recovered from PRC.

To further illustrate changes caused by juice processing, percent change was calculated for individual anthocyanidins at each step as compared against the frozen berry controls. Delphinidin retained the most from the control with 48.7% continuing into the PJ juice; however, only 22.5% of petunidin was transferred (Figure 5). After pasteurization of PJ juice, cyanidin was retained 41.8% from the initial control, while petunidin was retained only 20.3%. In UF juices, delphinidin again was retained the most in the initial control anthocyanidins (34.2%) while petunidin was retained the least (16.7%) (Figure 5). The same pattern was observed in UFP juices with delphinidin (34.9%) and petunidin (14.1%). The percent change for total anthocyanidins summarizes the data further. At pressing, 32.9% of the control total anthocyanidins were transferred to the resulting PJ juice, resulting in a 67.1% loss of total anthocyanidins (Figure 5). After pasteurization, the unfiltered pasteurized juice (PPJ) contained 29.7% of the starting control total anthocyanidins. Further processing of the pressed juice (PJ) created the UF juice which had 24.5% of the initial control berry anthocyanidins, hence increasing the total anthocyanidin loss to 75.5%. The UFP, lastly, had 21.7% of the total anthocyanins that the control berries started with, rendering losses of 78.3% (Figure 5).

3.3.2. Total Anthocyanidins

Juice producers are more concerned with total anthocyanin changes then individual compounds and the relation to how steps in the juice process affect the compounds that change clarity and cause sedimentation. Since the anthocyanins are only semi-quantified and the PRC samples were not adequately extracted for a good representation of the individual MS/MS amounts, the total anthocyanins could not be utilized to track the percent of anthocyanins that proceeded forward from the whole berry. This was, however, calculated with the UPLC quantified anthocyanidin data. To determine how much of the juice and press cake came from the raw berries for each sample, relative percentages were determined by utilizing previously collected data from pilot presses [58]. The percentage of the whole berries broken down into relative free juice and press cake amounts were 74.0 ± 1.0% and 13.0 ± 0.6%, respectively (note that through experimental batch-like transfers, UF

hopper residuals and measuring, 13 ± 0.5% juice was also lost, which was added to the value to calculate "total juice" versus cake [58]). The percent of anthocyanidins that remained in the juice and press cake after pressing, as well as percentage lost after each processing step were calculated using these estimations (Figure 6). The sequential degradation or loss/polymerization of anthocyanins begins with loss to pressing, which removed the majority of anthocyanidins. There was a 40.6% loss of anthocyanidins to the press cake and 35.1% was lost to mashing and juice loss in the press cloth and equipment. Of the total anthocyanidins in raw control berries, only 24.3% were transferred to the juice (PJ) (Figure 6). Thereafter, juice that was not filtered lost another 9.6% through pasteurization (PPJ). This translates to 29.7% of the original control amount of anthocyanidins being transferred to a non-filtered pasteurized juice product (not counting the effectually concentrated PRC loss). By filtering juice, an additional 25.4% of the anthocyanidins were removed from PJ samples before pasteurization (UF) (Figure 6). Pasteurization of ultrafiltered juice (UFP) removed an additional 12.0% of anthocyanidins in the final juice product. The UFP contained roughly 21.6% of the raw berry control anthocyanidins after processing.

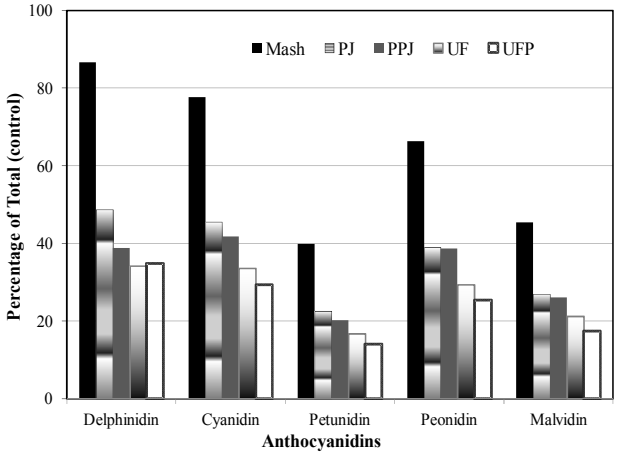

Figure 5. Percentage of control of anthocyanidins in rabbiteye blueberry juice processing steps. Mash = crude mash following the steam-jacketed kettle (prior to enzyme treatment and hydraulic pressing); PJ = pressed not filtered juice; PPJ = pasteurized pressed unfiltered juice; UF = ultrafiltration-filtered juice; UFP = filtered pasteurized juice.

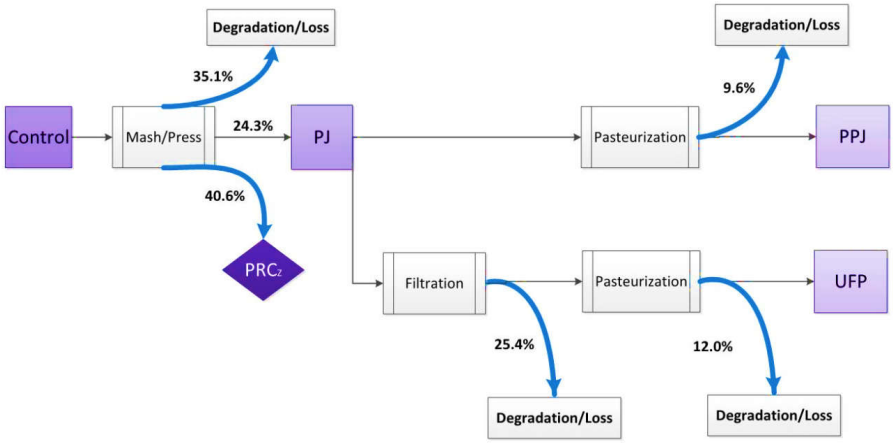

Figure 6. Percent anthocyanidin loss through unfiltered and ultrafiltered rabbiteye blueberry juice processing. PRC = press cake; PJ = pressed not filtered juice; PPJ = pasteurized pressed unfiltered juice; UFP = filtered pasteurized juice. Filtration box specifically refers to ultrafiltration (UF), as described in the M&M.

Literature on anthocyanidins in blueberries give varying results in comparison to our study. A contributing factor is the difference in calculating total anthocyanidins by summation of individual anthocyanidin amounts compared to using the pH differential method which calculates total anthocyanidins as one group of compounds based on UV spectra [59,60]. In general, blueberry results reported from a spectrophotometric method are higher in the same sample than results from methods quantifying the individual anthocyanidins [19,30,61]. Yet, the reverse situation has also been reported [62]. Although the pH differential method versus LC analyses can deliver differing results from the same sample, blueberry data tend to be closely correlated, which indicates that both approaches are reliable [60,61,63]. The loss to press cake is similar to the percentages found in the literature [19,37]. Highlighting the amount of anthocyanidins remaining in the press cake again confirms that economic opportunities abound for producers to further utilize their waste stream. Minimizing waste from the juice production scheme should be accomplished as value-added polyphenolic products could be developed from the press cake. Greener technologies, like environmentally friendly hot water extraction, is an option for removing the remaining beneficial compounds from press cake [64]. The seeds and skins which remain in the press cake can be separated and utilized for seed oil, natural coloring, and confectionary products [15], creating sustainable and unique perspectives for marketing niche products.

4. Conclusions

Ten major and three minor anthocyanins were identified in RAB blueberry juice processing steps. There were three minor anthocyanins isolated, which have not been reported previously in RAB blueberries. All five of the major anthocyanidin classes found in blueberry were identified as well. Comparing the trends of the individual anthocyanins, the major individual compounds all significantly decreased after pressing and pasteurization; the three minor anthocyanins, while increased slightly, with significant changes varying based on the process steps through juice processing. Acylation of the minor anthocyanins may increase stability when exposed to pH changes and heat, and this may explain why different trends were observed in the minor coumaroyl anthocyanins compared with the major anthocyanins. Raw control berries and press cake contained the highest anthocyanidin contents. Pressed juice only contained 24.3% of the anthocyanidins transferred from raw control berry quantities. Unfiltered juices only had 9.6% loss of anthocyanidins resulting from pasteurization, while ultrafiltered juices had 12.0% loss from pasteurization, in addition to the 25.4% anthocyanidin loss from the ultrafiltration step. Herein, we too realized that ultrafiltration concentrated monomer anthocyanins slightly and ultrafiltration should be further evaluated and optimized to increase anthocyanins in NFC juices. These findings also contribute to the value and interest of press cake for use in other food and non-food products as value-added ingredients and product quality to boost health benefits. In juice processing, the more steps in the process, the greater the loss of anthocyanins in the end product. Nonetheless, further studies evaluating the storage effects on juice quality between unfiltered and filtered NFC juices, taking into account lower molecular weight anthocyanins and polymers associated with increased health benefits, would contribute to the important role of filtration in juice processing.

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Conflicts of Interest: The authors declare no conflict of interest.

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