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Proanthocyanidin A2 purification and quantification of American cranberry (*Vaccinium macrocarpon* Ait.) products

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ABSTRACT

In this study, five common proanthocyanidin purification techniques were evaluated prior to phloroglucinolysis, followed by HPLC analysis. An optimized purification method was then used to identify and quantify the proanthocyanidins (extension and terminal units of epigallocatechin, catechin, epicatechin, A type trimer, and A type dimer) of commercially available cranberry products (juices, concentrates, tablets, and capsules; $n = 17$). Two size exclusion beads (Toyopearl 4 TSK HW-40C and Sephadex LH-20) were found suitable for proanthocyanidin purification, though proanthocyanidin extension and terminal unit composition was contingent upon the cleanup procedure utilized. These data illustrate that purification methods require consideration prior to conducting any cranberry proanthocyanidin analyses, and have to be accounted for when comparing values between studies. Total proanthocyanidin levels ranged from 11.7 (juice) to 436.4 (tablet) mg/100 mL or 100 g values obtained from Sephadex LH-20 purification, while total anthocyanin levels ranged from 0.54 (juice) to 98.00 (tablet) mg/100 mL or 100 g.

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1. Introduction

Cranberries' phenolic compounds determine their color (due to anthocyanin) development (Francis, 1995), astringency (Peleg & Noble, 1999), food antibacterial effects (Cote, Caillet, Doyon, Sylvain, & Lacroix, 2011), fungal resistance (Vvedenskaya & Vorsa, 2004), storability (Ozgen, Palta, & Smith, 2002; Wang & Stretch, 2001), and ultimately cultivar selection (Vorsa, Polashcok, Cunningham, & Roderick, 2003; Vorsa & Polashock, 2005). The same anthocyanin (phenolics) that color cranberries are also the determining factor for harvest dates and crop values; with some grower bonuses based on the amount of cranberry fruit color (personal communication, B. Strik).

Proanthocyanidin A type phenolics are found in *Vaccinium* berries (Gu et al., 2003, 2004; Hellstrom, Torronen, & Mattila, 2009; Koerner, Hsu, Lee, & Kennedy, 2009; Kylli et al., 2011; Maatta-Riihinen, Kahkonen, Torronen, & Heinonen, 2005; Tarascou et al., 2011), plum (Gu et al., 2003, 2004; Hellstrom et al., 2009), peanuts (Gu et al., 2003, 2004; Hellstrom et al., 2009; Monagas et al., 2009), cinnamon (Gu et al., 2003, 2004), avocados (Gu et al., 2003), curry powder (Gu et al., 2003), and other sources listed in Xie and Dixon (2005). But, Cranberries contain proanthocyanidin types A and B (Tarascou et al., 2011), which are useful as quality indicators (e.g., flavor, mouthfeel, etc., Peleg & Noble, 1999) for cultivar selection, food processing efficiency measurements, and storage assessments.

Since American cranberries (*Vaccinium macrocarpon* Ait.), and other *Vaccinium* berries (e.g., lingonberry, blueberry, European cranberry, etc.) or their products (juices, concentrates, and dietary supplements) contain proanthocyanidins, anthocyanins, ascorbic acid, fructose, and other compounds; although there have been mixed results in the literature on its effectiveness, regular consumption are thought to prevent urinary tract infections (UTIs) and offer other potential health benefits (Carlsson, Wiklund, Engrstrand, Weitzberg, &

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Lundberg, 2001; Foo, Lu, Howell, & Vorsa, 2000; Howell, 2002; Zafriri, Ofek, Adar, Pocinio, & Sharon, 1989). Problems with experimental differences regarding the ingested form (extract, juice, juice cocktail, concentrate, capsule, tablet, etc.), dosage, duration, and frequency, or the age and other demographics of subjects, or relevant infective organism monitored in the clinical trial have frequently been cited (Barbosa-Cesnik et al., 2011; Jepson & Craig, 2007, 2008; McMurdo, Argo, Phillips, Daly, & Davey, 2009) and were well summarized by Raz, Chazan, and Dan (2004) and Jepson and Craig (2008). While further study is still needed, these products may offer anti-bacterial traits without the problems associated with prophylactic use of antibiotics, but since the prevalent research has focused on prevention of UTIs, the possible treatment of UTIs by cranberry product also needs to be studied; well reviewed by Jepson and Craig (2008, references therein) and Guay (2009, references therein).

Some of the variable results seen in cranberry-prevention UTI clinical trials might be due to variations in the concentration of proanthocyanidin (and other compounds) that naturally fluctuate in the fruit, and its fruit products. In the current literature, purification and analysis methods widen the range of target compound concentrations reported (Cunningham, Vannozzi, O'Shea, & Turk, 2002; Hammerstone, Lazarus, & Schmitz, 2000; Maatta-Riihinen et al., 2005; Prior et al., 2010; Wallace & Giusti, 2010) and need to be evaluated and uniformed for accurate comparison of results. The first objective of this study was to optimize proanthocyanidin purification by utilizing five commonly used proanthocyanidin purification methods. The second objective was to utilize the selected purification procedure and determine the proanthocyanidin A2 level in easily attainable market place cranberry products in juice, concentrate, capsule, and tablet forms. Anthocyanin levels of these samples were also determined.

2. Materials and methods

2.1. Sample material

All (n = 17) readily available cranberry juices (code Z), concentrates (code Y), capsules (code X), and tablets (code W) were purchased from local marketplaces in Boise and Nampa, ID, USA. Samples represented eleven companies (actual manufacturer, not distributer; Table 1). All samples were coded and listed in Table 1. Only capsules and tablets that had cranberry as the main ingredient (label information provided as cranberry fruit or cranberry concentrate) was used in this study.

2.2. Reagents, chemicals, and standards

All chemicals, reagents, and standards used in this study were analytical or HPLC grade from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), unless indicated otherwise.

2.3. Extraction

Cranberry capsules and tablets were extracted as described in Lee and Finn (2007). Gelatin or cellulose capsule portions were removed and only the powder in the capsules was retained for extraction. Tablets were ground to a fine powder using a coffee bean grinder (model KSM2; Braun GmbH, Kronberg, Germany). Powders were extracted once with acetone, then two additional times with 70% aqueous acetone, before the acetone was evaporated (Labconco Rotary Evaporator; Labconco corp., Kansas city, MO, USA) and re-dissolved in water (Lee & Finn, 2012) prior to purification (described in section below). All samples were stored in −80°C until extraction, phloroglucinolysis (for proanthocyanidins), and HPLC analyses.

Table 1 – Summary of purchased samples from local stores. All available juices, concentrates, capsules, and tablets were purchased.

Sample form and type	Number of samples obtained	Codes given
Juice	7	Z1–Z7
Concentrate	2	Y1, Y2
Capsule	6	X1–X6
Tablet	2	W1, W2
Total	17	

2.4. Proanthocyanidin purification

One liquid sample (Z1) was used to evaluate the different sample preparation methods to determine which was most suitable for proanthocyanidin composition analysis. Five sample preparation methods were evaluated: (1) no-purification, (2) liquid–liquid extraction (2:1 = sample:ethylacetate, v/v) with both fractions collected, (3) C18 cartridge (Water Corp., Milford, MA, USA) column purified with ethylacetate and methanol fractions collected (Lee & Finn, 2007), (4) Toyopearl TSK HW-40C (will refer to as Toyopearl 40C; Tosoh Bioscience LLC, Montgomeryville, PA, USA) purified (described below), and 5) Sephadex LH-20 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) purified (described below).

The size exclusion resins were prepared as following: A 5 mL Fisher Scientific PreSep-R column (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) was packed with size exclusion resins per the column and resin manufacturer's guidelines. The packed column bed was rinsed with 15 mL of water, then 5 mL of sample was applied, rinsed with 15 mL double-distilled water (all water in this study was double-distilled), 15 mL of 25% aqueous methanol, and 15 mL of 70% aqueous acetone (proanthocyanidin collected at this step). Elution was aided by pulling a vacuum with a KNF lab filtration pump (KNF Neuberger, Inc., Trenton, NJ, USA). These elution steps were modified from Koerner et al. (2009) and Hammerstone et al. (2000). The no-purification sample and a portion of the collected eluents were evaporated using nitrogen (N) gas via a N-Evap 112 N evaporator (Organomation Associates Inc., Berlin, MA, USA) with the water bath set to 40°C. These dried samples then underwent phloroglucinolysis (Koerner et al., 2009) and analysis by HPLC, as previously described (Lee, Kennedy, Devlin, Redhead, & Rennaker, 2008).

After selection of optimum purification method (Fig. 1; both size exclusion resins, 100 mg/L phloroglucinol, and 20 and 135 min incubations), this procedure was then used for

the remaining commercial samples (all commercial liquid samples and aqueous extracts of capsules and tablets). All purified samples underwent phloroglucinolysis, with two incubation periods (20 and 135 min) followed by HPLC (with monomer correction) with conditions as described in Lee et al. (2008), Lee (2010), and Koerner et al. (2009). The 20 min incubation period originated from analysis methods developed to determine grape proanthocyanidins (Lee et al., 2008; Lee, 2010), while the 135 min was determined to be optimal for cranberry proanthocyanidins (Koerner et al., 2009). Proanthocyanidin levels were expressed as epicatechin (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). Purification was conducted in triplicates and all commercial products were analyzed in two replicates.

2.5. HPLC conditions for proanthocyanidin analysis

Briefly, an Agilent HPLC 1100 (Agilent Technologies Inc., Palo Alto, CA, USA) was used for this investigation. Proanthocyanidins were analyzed before and after phloroglucinolysis by HPLC/DAD (for quantification) and HPLC/DAD/ESI-MS/MS (for aiding in identification). Peaks were monitored at 280, 320, 370, and 520 nm and all peak spectra were collected. Peaks were identified based on retention time, UV–VIS spectra, external standards (when available), and previously published research (Koerner et al., 2009; Lee et al., 2008). A graduated micro-splitter valve (P-470, Upchurch Scientific, Oak Harbor, WA, USA) reduced the flow from the column into the ion trap MS. MS condition was as described in Lee and Rennaker (2007).

2.6. Anthocyanin analysis by HPLC

Cranberry anthocyanins were separated by previously published HPLC method (Lee & Finn, 2007, 2012). All samples were expressed as cyanidin-3-glucoside purchased from Polyphenol Laboratories AS (Sandnes, Norway). Peaks were identified based on retention time, UV–VIS spectra, and previously published papers (Brown, Murch, & Shipley, 2012; Grace, Massey, Mbeunkui, Yousef, & Lila, 2012; Hale, Francis, & Fagerson, 1986; Hong & Wrolstad, 1990a, 1990b; Sapers & Hargrave, 1987; Viskelis et al., 2009; Vorsa & Polashock, 2005; Vorsa et al., 2003; Vvedenskaya & Vorsa, 2004; White, Howard, & Prior, 2010, 2011). All peaks were monitored at 520 nm and Agilent HPLC 1100 (Agilent Technologies Inc.) was used. Detailed guard and analytical columns information,

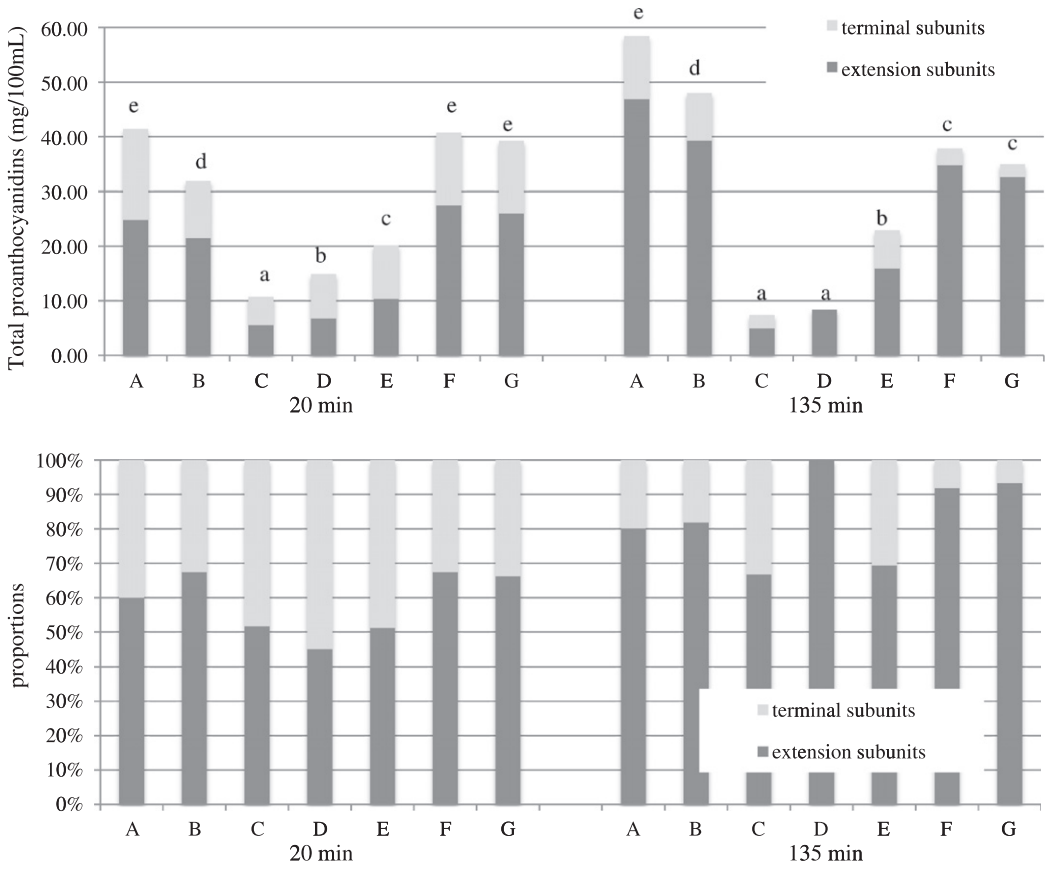

Fig. 1 – Total proanthocyanidin levels from the different sample preparation procedures ($n = 3$). Both incubation periods (20 and 135 min) are presented in concentrations (top) and percent proportions (bottom). Different lower case letters within each incubation time indicate significant difference ($p \leqslant 0.05$). Individual subunit values (with standard errors) are presented in Table 2. Presented as sums or proportions of extension and terminal subunits. The corresponding samples preparation code is as follows: A – no purification, B – liquid–liquid water fraction, C – liquid–liquid ethylacetate fraction, D – C_{18} ethylacetate fraction, E – C_{18} methanol fraction, F – Toyopearl 40C, and G – Sephadex LH-20.

mobile phase composition, gradient program, etc. are in Lee and Finn (2007).

2.7. Statistical analysis

Statistical analysis was conducted using Statistica for Windows version 7.2 (StatSoft Inc., Tulsa, OK, USA). Differences among the different sample preparation methods and samples were resolved using one-way analysis of variance (ANOVA) and t-test or Tukey Honest Significant Difference (HSD; $\alpha = 0.05$).

3. Results and discussion

3.1. Cranberry proanthocyanidin

Proanthocyanidins identified after phloroglucinolysis had the extension units of epigallocatechin, catechin, epicatechin, proanthocyanidin A2 dimer, and proanthocyanidin A type trimer, and the terminal units of catechin, epicatechin, proanthocyanidin A2 dimer, and proanthocyanidin A type trimer. A representative chromatogram of cranberry proanthocyanidin separation can be found in Koerner et al. (2009). Proanthocyanidin A2 is the focus of this paper, since potential health benefits consuming cranberry products have, so far, been linked to proanthocyanidin A2 (Foo et al., 2000; Howell et al., 2005). Total proanthocyanidin values are shown in Table 2. Proanthocyanidin A type trimer extension and terminal units were tentatively identified (**mother** ions, in bold, and their *fragments*, in italic types) of **988**, *861, 709, 575, 449, 411,* and *287* for the extension subunit and **864** (as reported by Foo et al., 2000), *711, 573, 451,* and *289* for the terminal unit.

3.2. Evaluation of the purification methods

Purified samples from size exclusion resins (both Toyopearl 40C and Sephadex LH-20) provided proanthocyanidin levels equivalent to no-purification samples (Fig. 1; not significantly different when the total proanthocyanidin A2 values were compared), but as the chromatograms from the no-purification samples were muddled with interfering compounds, a purification method was required for analyses of commercial samples.

It is unclear why the high and low levels between the proanthocyanidin A2 extension and terminal subunits were interchanged when the no-purification samples were contrasted to both of the size exclusion resin purified samples. This exchange in concentration might be due to the presence of interfering compounds in the no-purification samples that were eliminated for the purified samples, which possibly provided access for the extension units to be cleaved. Liquid–liquid (water and ethylacetate fractions) extraction and C18 (ethylacetate and methanol fractions) purified samples provided significantly lower total proanthocyanidin levels compared to the size exclusion resins (Toyopearl 40C and Sephadex LH-20) purified samples (data not shown). As purification by Toyopearl 40C and Sephadex LH-20 appeared to better purify proanthocyanidins without significantly altering the levels when compared to non-purified samples, the remaining commercial cranberry products were purified using these two size exclusion media (Table 2).

A2 extension subunit values were higher after the 135 min incubation period then the shorter 20 min incubation period, but the opposite trend was observed for A2 terminal unit values (Fig. 1). This is different from what was previously reported by Koerner et al. (2009) where 135 min incubation period was optimal for both extension and terminal units of A2. This difference might be due to the slight dissimilarity in sample form. Our samples contained other proanthocyanidin units whereas Koerner et al. (2009) evaluated a pre-purified form of A2 only. This ratio alteration should be further investigated since despite this difference in the ratio of A2 extension and terminal units changing with incubation duration, the total proanthocyanidin A2 was not significantly different from the no-purification sample (Fig. 1; Table 2). There was no significant difference between using 50 mg versus 100 mg of phloroglucinol/L, which was also unlike Koerner et al. (2009) data.

3.3. Proanthocyanidin A2 levels in purchased samples

To compare our values to Koerner (2010), regarding proanthocyanidin A2 values in commercial cranberry products, 100 mg of phloroglucinol/L with 135 min incubation period was used for our commercially purchased cranberry products. Overall, the trends obtained from Toyopearl 40C and Sephadex LH-20 purified proanthocyanidin A2 values were similar (Table 3). Cranberry juice proanthocyanidin A2 ranged from 3.0 (Z2) to 15.1 (Z7) mg/100 mL ($n = 7$), which was similar to Koerner (2010) reported range of 5.9 to 19.1 mg/100 mL ($n = 5$). Only one (Y2) out of the two cranberry concentrates was higher (Table 3) in total proanthocyanidin A2 compared to a juice sample (Z7) even on a mg/100 mL basis. Capsules and tablets ($n = 8$) total A2 (Table 3) ranged from 7.3 (X6) to 136.1 (W2) mg/100 g (18.6-fold difference between low and high) from Toyopearl 40C purified samples. Vinson, Bose, Proch, Kharrat, and Samman (2008) reported there was a wide range of total phenolic concentration (by Folin–Ciocalteu method) in the different cranberry products from frozen berries (high) to jellied sauce (low) on a per serving basis.

It is still unknown whether ingesting dry or liquid forms are more effective for potential health beneficial properties. If the assumption that one capsule or tablet weighs approximately 1 g, total proanthocyanidin range wound be reduced to 0.23 to 3.54 mg/g (purified by Toyopearl 40 C) which is at the lower end of the liquid sample range. Additional studies are needed to clarify what concentrations and forms of proanthocyanidin A2 are most effective for achieving health benefits, since most published work was conducted using cranberry juice there are no scientific data regarding dosage for efficient utilization of a cranberry product (Guay, 2009; Jepson & Craig, 2008). Future studies examining UTI prevention with cranberry juice, or any other cranberry product, should incorporate cranberry compositional results; so individual compounds and their concentrations can be linked to the outcomes from health benefit investigations.

According to Foo et al. (2000), A2 trimers exhibited the most anti-adhesion activity by *p*-fimbriated *Escherichia coli* in *in vitro* comparisons against other A2 dimers reported in

Table 2 – Summary of the proanthocyanidin concentration of individual extension and terminal subunits, in mg/100 mL, obtained from the five purification methods. Values in parenthesis are standard errors. Results from both incubation periods are presented. Different lower case letters within the incubation time indicate significant difference ($p \leqslant 0.05$). Abbreviation of the subunits after phloroglucinolysis are as follows: extension units of epigallocatechin (EGC-P), catechin (C-P), epicatechin (EC-P), proanthocyanidin A2 dimer (A2-P), and proanthocyanidin A type trimer (A type trimer-P), and the terminal units of catechin (C), epicatechin (EC), proanthocyanidin A2 dimer (A2), and proanthocyanidin A type trimer (A type trimer).

	Extension units (-P)					Terminal units				Total
	EGC-P	C-P	EC-P	A2-P	A type trimer-P	C	EC	A2	A type trimer	
20 min incubation										
No purification	6.4 (0.2)	0.9 (0)	14.8 (0.1)	2.2 (0.1)	0.6 (0)	1.4 (0.1)	3.4 (0.1)	11.4 (0.8)	0.4 (0)	41.4 e (0.9)
Liquid–liquid H_2O	5.8 (0.1)	0.9 (0)	12.5 (0.2)	1.8 (0.1)	0.5 (0)	0.8 (0)	2.5 (0)	6.8 (0.5)	0.3 (0)	31.9 d (0.1)
Liquid–liquid ethylacetate	0.7 (0)	0.5 (0)	3.0 (0.1)	1.0 (0)	0.3 (0)	0.8 (0)	1.2 (0.1)	3.0 (0.1)	0.2 (0)	10.8 a (0.1)
C_{18} ethylacetate	1.0 (0)	0.1 (0)	3.9 (0.1)	1.4 (0)	0.3 (0)	0.2 (0)	1.1 (0.1)	6.8 (0.1)	–	14.9 b (0.4)
C_{18} MeOH	2.0 (0.1)	0.5 (0)	5.8 (0.2)	1.5 (0.1)	0.6 (0.1)	0.7 (0)	2.3 (0.2)	6.5 (0.3)	0.3 (0)	20.2 c (0.6)
Toyopearl 40C	3.3 (0)	0.5 (0)	16.2 (0.2)	5.7 (0.2)	1.8 (0)	1.1 (0.1)	3.9 (0.2)	7.4 (0.4)	0.9 (0)	40.8 e (0.3)
Sephadex LH-20	3.2 (0.1)	0.3 (0)	15.6 (0.3)	5.1 (0.1)	1.8 (0.1)	0.7 (0.1)	3.2 (0.1)	8.5 (0.3)	0.9 (0)	39.3 e (0.6)
135 min incubation										
No purification	14.4 (0.5)	1.4 (0.1)	21.1 (0.2)	9.4 (0.1)	0.6 (0)	1.8 (0.1)	4.5 (0.1)	5.2 (0.8)	0.1 (0)	58.5 e (0.5)
Liquid–liquid H_2O	12.3 (0.4)	1.3 (0.1)	17.4 (0.1)	7.8 (0.2)	0.5 (0)	0.7 (0.1)	3.6 (0.1)	4.3 (0.4)	0.1 (0)	48.0 d (0.8)
Liquid–liquid ethylacetate	1.0 (0.2)	0.2 (0)	2.6 (0.1)	0.8 (0)	0.4 (0)	0.2 (0)	0.9 (0.1)	1.2 (0.1)	0.2 (0)	7.4 a (0.4)
C_{18} ethylacetate	1.4 (0)	0.2 (0)	4.2 (0.3)	2.6 (0.1)	–	–	–	–	–	8.4 a (0.5)
C_{18} MeOH	2.4 (0.1)	0.5 (0)	8.7 (0.1)	4.0 (0.2)	0.3 (0)	0.6 (0)	2.9 (0.2)	3.6 (0.9)	–	23.0 b (1.4)
Toyopearl 40C	4.0 (0.4)	0.7 (0.1)	18.7 (0.2)	11.0 (0.3)	0.4 (0)	0.3 (0)	0.4 (0.1)	2.3 (0.5)	–	37.8 c (0.9)
Sephadex LH-20	3.0 (0)	0.5 (0)	18.1 (0.2)	10.5 (0.1)	0.4 (0)	0.2 (0)	0.3 (0.1)	1.8 (0.1)	–	35.0 c (0.4)

–: indicates not detected.

cranberry. A2 trimers separated out (Table 3) in these samples ranged from 1.6 (Z2) to 25.6 (W2) mg/100 mL or 100 g purified via Toyopearl 40C and 2.5 (Z2) to 143.8 (W2) mg/100 mL or 100 g purified via Sephadex LH-20.

3.4. *Cranberry products anthocyanin composition*

The focus of this paper was proanthocyanidin composition and content of cranberry products, but anthocyanins (a more commonly measured phenolic quality indicator) were determined and presented in Table 4. The established cranberry anthocyanin profile is as follows (in the order of elution): cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, and peonidin-3-arabinoside (listed in Table 4 and Fig. 2- Z3). Anthocyanin concentration ranged from 0.54 (Z2) to 98.00 (W2) mg/100 mL or 100 g by HPLC. All samples' cyanidin to peonidin based anthocyanin ratios ranged from 0.70 to 0.99 (Table 5) within Vorsa et al. (2003) findings of 0.5–3.6. Z2 contained an additional peak 7 (Fig. 2). After observing this unknown peak, two additional cranberry juice cocktails (one labeled as the identical cranberry juice cocktail and the other labeled as a diet cranberry juice cocktail) manufactured by the same company were purchased and analyzed, and peak 7 was prominently present in those bottles as well. As indicated by Lee and Wrolstad (2004) and Lee, Durst, and Wrolstad (2002), the final processed product will contain the same anthocyanin profiles as the starting material, but the individual anthocyanin proportion will alter due to the differences in compound extractability and stability during processing and storage. It is unusual to have a 7th prominent additional peak (0.9 mg/100 mL) that was higher in concentration than three naturally occurring anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, and peonidin-3-glucoside; Table 4) also found in that product.

Literature analyzing American cranberry (*V. macrocarpon* Ait.) fruit, pomace, and a reputable commercial juice source contained six anthocyanins not deviating from the following: the galactosides, glucosides, and arabinosides of cyanidin and peonidin (Hale et al., 1986; Hong & Wrolstad, 1990b; Viskelis et al., 2009; Vorsa et al., 2003; Vvedenskaya & Vorsa, 2004; White et al., 2010, 2011). Although Brown et al. (2012) did not report peonidin-3-glucoside in their samples, which might be due to a lower detection limit from the method used. Some minor variations in the proportion of the individual anthocyanin were seen due to ripeness and cultivar differences (Brown et al., 2012; Sapers & Hargrave, 1987; Vvedenskaya & Vorsa, 2004).

Again, it is unusual to have this 7th peak (beyond the six mentioned above) in cranberry products. There were past reports of some cranberry juice processors adulterating their products by adding enocyanin (grape colorant made from presscake/pomace) in the late 1980s to early 1990s (Francis, 1985; Hong & Wrolstad, 1986, 1990a; Lee & Hong, 1992) that can be distinguished by the presence of glucosides of delphinidin, petunidin, and malvidin. In a more recent case, *Vaccinium* berry (bilberry; *V. myrtillus*) powder purchased from China was adulterated with Amaranth (E123; food dye; Penman et al., 2006). Manufacturers purchasing cranberry concentrates or powders to create products should request an ingredient anthocyanin profile from their component supplier to ensure it is pure American cranberry and not adulterated with other colorants. For authentication of the berry anthocyanin profile, the correctly classified fruit (as *V. macrocarpon* and not other

Table 3 – Proanthocyanidin (PROACD) composition and concentrations of the commercially available cranberry products (n = 17). All units were expressed in mg epicatechin/100 mL (Z1–Y2) or 100 g (X1–W2). Results from 100 g of phloroglucinol/L and 20 min incubation period. Different lower case letters within the column per each size exclusion media indicate significant difference ($p \leqslant 0.05$). Summary of sample codes given in Table 1.

Sample codes	Extension subunits (−P)					Terminal subunits				Total PROACD	Sum of A2	Sum of A type trimer
	EGC-P	C-P	EC-P	A2-P	A type trimer-P	C	EC	A2	A type trimer			
Toyopearl 40C												
Z1	3.3	0.5	16.2	5.7	1.8	1.1	3.9	7.4	0.9	40.8 (0.3) bc	13.1 (0.2) d	2.7 (0) abc
Z2	1.7	0.5	4.1	1.4	1.0	0.6	1.3	1.6	0.7	12.8 (0.3) a	3.0 (0.1) ab	1.6 (0) ab
Z3	1.2	0.5	5.3	1.7	1.0	0.6	1.4	2.4	0.8	15.0 (0.1) a	4.1 (0) abc	1.8 (0) ab
Z4	1.1	0.7	7.3	2.8	1.1	0.7	2.3	5.5	0.8	22.5 (0.1) a	8.3 (0.2) bc	2.0 (0.1) ab
Z5	1.2	0.7	6.6	2.4	1.1	0.7	2.1	2.9	0.7	18.3 (0.1) a	5.3 (0.2) abc	1.8 (0.1) ab
Z6	2.4	0.7	10.4	3.0	1.6	0.8	2.8	3.5	1.0	26.3 (0.2) abc	6.6 (0) abc	2.6 (0) abc
Z7	4.4	0.8	23.6	6.8	3.7	1.0	4.7	8.3	1.9	55.3 (0.2) cde	15.1 (0.3) d	5.6 (0.1) cde
Y1	2.3	0.6	14.8	5.2	2.4	1.0	5.5	8.9	1.7	42.3 (0.8) bcd	14.0 (0.6) d	4.0 (0.1) bcd
Y2	17.2	2.4	62.4	17.7	8.4	2.8	18.4	32.9	4.3	166.5 (10.2) h	50.5 (2.5) hi	12.8 (0.7) g
X1	7.7	2.3	21.4	7.1	3.4	2.2	7.6	14.5	0.7	66.9 (0.1) def	21.6 (0.3) e	4.1 (0) bcd
X2	9.9	3.5	56.8	17.7	9.3	3.7	19.1	45.4	0.4	165.9 (0.9) h	63.1 (1.0) j	9.7 (0.2) f
X3	38.6	3.2	84.2	22.8	15.8	3.5	12.2	21.7	0.2	202.3 (0.2) i	44.5 (1.3) gh	16.1 (0) h
X4	7.2	2.2	20.9	8.2	3.2	2.5	7.4	24.3	0.7	76.7 (1.7) ef	32.5 (0.9) f	4.0 (0) bc
X5	21.0	2.8	45.8	14.8	5.9	3.4	16.9	38.0	0.4	149.0 (3.6) g	52.7 (0.7) i	6.3 (0.2) de
X6	2.3	1.4	5.1	2.5	1.8	0.0	3.0	4.8	2.2	23.1 (0.9) a	7.3 (0.5) bc	4.0 (0.2) bc
W1	8.5	2.7	48.2	12.8	6.0	4.0	17.4	35.1	0.6	135.4 (1.2) g	47.9 (0.6) gh	6.6 (0) de
W2	15.7	6.2	137.6	46.0	25.4	4.7	28.7	90.1	0.2	354.6 (6.2) j	136.1 (0.5) k	25.6 (0.9) i
Sephadex LH-20												
Z1	3.2	0.3	15.6	5.1	0.4	0.7	3.2	8.5	0.0	37.0 (0.5) bc	13.5 (0.2) cdefg	0.4 (0) a
Z2	1.5	0.5	3.9	1.2	0.9	0.5	1.1	1.2	0.7	11.7 (0.8) ab	2.5 (0.2) abc	1.6 (0.1) b
Z3	1.2	0.6	5.2	1.5	1.0	0.6	1.3	2.7	0.8	14.8 (0.6) ab	4.1 (0.2) abcd	1.8 (0.1) b
Z4	1.0	0.7	7.0	2.4	1.1	0.7	2.0	6.0	0.9	21.7 (0.2) abc	8.3 (0) abcde	2.0 (0) b
Z5	1.1	0.6	6.5	2.1	1.1	0.8	1.9	4.9	0.8	19.8 (0.4) ab	6.9 (0.1) abcd	1.8 (0) b
Z6	2.1	0.6	10.0	2.9	1.5	0.7	2.4	3.4	0.9	24.5 (0.4) abc	6.3 (0.2) abcd	2.4 (0.1) b
Z7	4.2	0.9	23.9	7.1	3.7	0.7	4.3	7.7	1.7	54.3 (1.6) d	14.8 (0.4) defg	5.4 (0.2) efg
Y1	3.3	0.6	19.4	6.6	3.0	1.1	5.7	11.5	1.8	53.0 (0.7) d	18.1 (0.4) efg	4.7 (0.2) df
Y2	21.4	2.3	81.3	21.1	10.6	3.3	18.0	40.7	5.1	203.8 (0.4) g	61.8 (1.0) j	15.7 (0) i
X1	8.4	1.9	21.6	6.6	3.3	1.9	5.9	11.9	0.7	62.2 (2.7) d	18.5 (0.4) efg	3.9 (0) cde
X2	9.2	2.5	56.3	17.9	8.7	2.6	15.4	45.5	0.4	158.5 (1.7) f	63.4 (0.2) j	9.1 (0) hi
X3	72.7	3.5	136.9	32.0	23.8	2.8	15.7	26.3	0.2	313.9 (9.6) h	58.4 (2.5) j	24.0 (0.7) j
X4	7.6	1.9	20.6	7.1	3.2	1.9	6.5	19.7	0.7	69.2 (1.0) d	26.8 (1.8) h	3.8 (0) cde
X5	21.6	2.3	45.5	13.2	5.8	3.1	13.3	31.9	0.3	136.9 (0.4) e	45.1 (1.2) i	6.1 (0) fg
X6	2.1	1.4	5.4	2.5	1.6	0.0	3.1	7.7	3.6	27.5 (0.9) abc	10.2 (0.6) bcdef	5.2 (0.1) efg
W1	8.4	2.4	49.6	12.9	7.8	2.4	14.3	35.8	0.5	134.0 (6.3) e	48.7 (2.9) i	8.3 (0.3) h
W2	27.4	4.5	195.5	53.3	34.2	2.8	27.9	90.6	0.2	436.4 (3.8) j	143.8 (1.0) k	34.4 (0.2) k

Vaccinium berries) should be obtained, extracted, analyzed, and compared for determining adulteration, since a processed product might contain pigment from sources other than American cranberry (Hong & Wrolstad, 1986).

This discrepancy in anthocyanin profile might be caused by miss-identification of *V. macrocarpon* (American cranberry) and *V. oxycoccus* (small cranberry; wild cranberry; European cranberry) berries, but based on the proportion of the glycosylation it is highly unlikely that this occurred (Andersen, 1989; Obon, Diaz-Garcia, & Castellar, 2011). The two species of cranberries have distinct anthocyanin profiles (see Obon et al., 2011 and Andersen, 1989 for example chromatograms of *V. oxycoccus*). In Obon et al. (2011), *V. oxycoccus* berries contained mainly cyanidin-galactoside with minor levels of delphinidin-galactoside, delphinidin-glucoside, cyanidin-glucoside, and cyanidin-arabinoside. In *V. oxycoccus* berries analyzed by Andersen (1989), cyanidin-glucoside and peonidin-glucoside were the main pigment with minor levels of delphinidin-glucoside, cyanidin-galactoside, cyanidin-arabinoside, petunidin-glucoside, peonidin-galactoside, malvidin-glucoside, and peonidin-arabinoside. Again, *V. macrocarpon* contains mainly galactosides and arabinosides of cyanidin and peonidin, with minor levels of glucosides of cyanidin and peonidin (Hong & Wrolstad, 1990b; Vorsa & Polashock, 2005; Vorsa et al., 2003).

All samples except Z2 and Z6 evaluated in this study had the anthocyanin glycosylation abundance pattern of galactoside > arabinoside > glucoside (high to low); that is the typical pattern for *V. macrocarpon* fruit (Table 5; Vorsa & Polashock, 2005; Vorsa et al., 2003). Z2 and Z6 anthocyanin patterns were arabinoside > galactoside > glucoside (high to low). Z2 and Z6 were from the same company, and more arabinoside than galactoside might be an indication of a degraded product or harsh processing treatment, as galactoside containing anthocyanins are more stable than arabinoside containing anthocyanins (Francis, 1989), and another indication that this company's products are unusual.

Table 4 – Individual anthocyanin by HPLC results. All units were expressed in mg of cyanidin-3-glucoside/100 mL (Z1–Y2) or 100 g (X1–W2). Z2 contained 0.09 mg/100 mL of peak 7 (Fig. 2) that is not accounted for in the total. Summary of sample codes given in Table 1.

Samples	Anthocyanins determined by HPLC (listed in the order of elution)						
	1	2	3	4	5	6	Total
	Cyanidin-3-galactoside	Cyanidin-3-glucoside	Cyanidin-3-arabinoside	Peonidin-3-galactoside	Peonidin-3-glucoside	Peonidin-3-arabinoside	
Z1	2.52	0.15	1.70	4.02	0.59	1.60	10.57
Z2	0.08	0.04	0.13	0.13	0.04	0.11	0.54
Z3	0.19	0.02	0.13	0.28	0.05	0.12	0.79
Z4	2.47	0.13	1.86	3.39	0.43	1.41	9.68
Z5	1.51	0.13	1.35	2.09	0.29	1.01	6.38
Z6	0.86	0.14	1.90	1.32	0.34	1.45	6.01
Z7	1.06	0.12	1.74	1.75	0.36	1.47	6.51
Y1	0.54	0.07	0.69	0.90	0.19	0.58	2.98
Y2	16.98	0.77	13.17	27.20	4.61	12.53	75.26
X1	5.54	0.39	3.48	7.53	0.79	2.70	20.43
X2	9.83	0.58	7.73	11.98	1.43	5.16	36.71
X3	4.50	0.45	5.75	10.21	1.48	6.76	29.16
X4	12.74	0.62	7.88	16.39	1.61	5.71	44.95
X5	6.41	0.42	4.23	8.20	1.09	2.96	23.30
X6	0.68	0.24	0.56	0.80	0.29	0.41	2.99
W1	3.83	0.49	3.51	5.43	1.10	2.76	17.12
W2	24.70	1.47	18.68	34.72	3.69	14.74	98.00

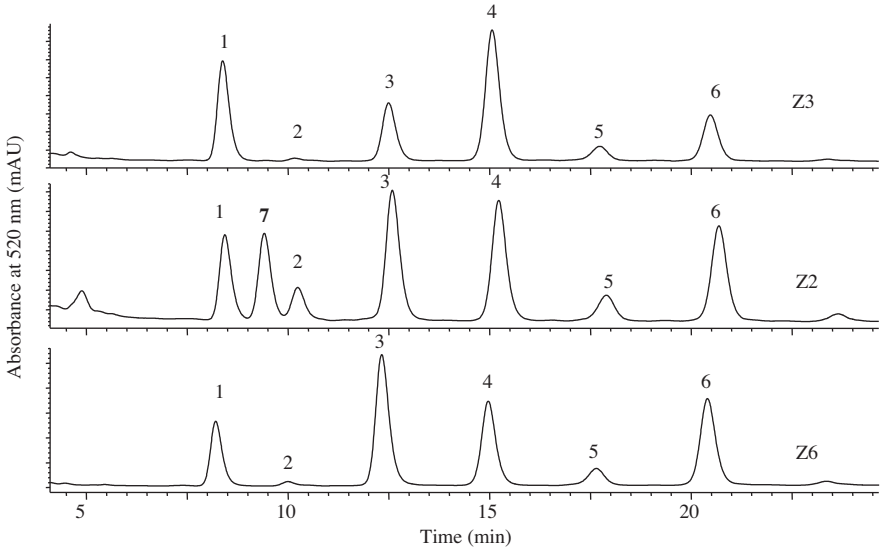

Fig. 2 – Anthocyanin profiles of three cranberry juice products: Z3, Z2, and Z6. Z3 profile similar to what was found in American cranberry (*Vaccinium macrocarpon* Ait.) fruit. Z2 had an unusual peak 7th that is not found in American cranberry fruit. Z6 was from the same manufacturer and had a different proportion of individual anthocyanins compared to Z3, but lacked peak 7. Z2 had an extra ingredient of "natural flavors" listed compared to Z6, which might account for the difference in profile. Authenticity of Z2-cranberry juice cocktail is questioned. Z6 still has a slightly unusual anthocyanin profile as well, when compared to Z3, where arabinosides containing anthocyanins (peaks 3 and 6; see Table 5) are elevated. Peaks 1 through 6 identifications are listed in Table 4.

Z6 did not have this 7th peak (Fig. 2) found in Z2. Peak 7 is clearly a contributor to the product red color, hence its detection at 520 nm. One of the extra ingredients in Z2 was "natural flavors" that could be a source (21CFR101.22) of this unknown peak. "Natural flavors" were also an ingredient in the two additional samples purchased from this manufacturer. Although this analysis of Z2 juice appears to have red colorant added, technically it may not be adulterated since its label declared "natural flavors"; though it is miss-leading, particularly to consumers assuming that the product's bioactive components are solely from cranberry. Based on the alteration in glycosylation, an extra peak in the anthocyanin profile, and having the lowest anthocyanin and proanthocyanidin concentrations Z2 was considered

Table 5 – Individual anthocyanins summarized based on the concentration and proportion of anthocyanidins or sugar substitution. Ratio between cyanidin and peonidin also calculated. Summary of sample codes given in Table 1.

Samples	Cyanidin based (mg/100 mL or 100 g)	Peonidin based (mg/100 mL or 100 g)	% cyanidin based	% peonidin based	Ratio cyanidin/peonidin	% galactoside	% glucoside	% arabinoside
Z1	4.36	6.21	41	59	0.70	62	7	31
Z2	0.26	0.28	48	52	0.91	40	15	45
Z3	0.34	0.45	43	57	0.75	60	9	31
Z4	4.46	5.22	46	54	0.85	60	6	34
Z5	2.99	3.39	47	53	0.88	56	7	37
Z6	2.90	3.11	48	52	0.93	36	8	56
Z7	2.92	3.58	45	55	0.82	43	7	49
Y1	1.30	1.67	44	56	0.78	48	9	43
Y2	30.93	44.33	41	59	0.70	59	7	34
X1	9.41	11.02	46	54	0.85	64	6	30
X2	18.14	18.57	49	51	0.98	59	5	35
X3	10.71	18.45	37	63	0.58	50	7	43
X4	21.24	23.72	47	53	0.90	65	5	30
X5	11.05	12.25	47	53	0.90	63	6	31
X6	1.49	1.50	50	50	0.99	50	18	33
W1	7.83	9.28	46	54	0.84	54	9	37
W2	44.85	53.15	46	54	0.84	61	5	34

the lowest quality (also the cheapest; $0.04/oz) product evaluated in this study.

Wu and Prior (2005) reported 13 anthocyanins in their purchased cranberry juice sample and we suspect that it might have been an adulterated sample. Presence of additional peaks that deviate from the established cranberry fruit anthocyanin profile indicate other colorant sources (Castaneda-Ovando, Pacheco-Hernandez, Paez-Hernandez, Rodriguex, & Galan-Vidal, 2009; Hong & Wrolstad, 1990a; Obon et al., 2011). Proanthocyanidin profile utilization to determine cranberry proanthocyanidin adulteration with blueberry has been suggested by Prior et al. (2010), but using anthocyanin profiles would be easier to implement since blueberries have a distinct anthocyanin profiles (Lee, Finn, & Wrolstad, 2004) that are unlike American cranberry.

Cost was not linearly related to total proanthocyanidin or anthocyanin levels in the products here (i.e., no guarantee it had more cranberry phenolics because it cost more). For example, X1 cost $0.11/capsule, and X4 and X6 cost $0.10/capsule, but their total proanthocyanidin levels were 66.9, 76.7, and 23.1 (Toyopearl 40C purified) mg/100 g and total anthocyanin levels of 20.43, 44.95, and 2.99 mg/100 g, respectively. Although, determination of total anthocyanin levels to extrapolate whether or not cranberry product contains a high proanthocyanidin level might be useful, additional work is needed to confirm this. In this study, high anthocyanin containing products contained high levels of proanthocyanidin (both media) with r value of 0.87.

There are four NIST (National Institute of Standards and Technology) certified reference standards available for cranberry: cranberry, low calorie cranberry juice cocktail, cranberry extract, and cranberry containing solid oral dosage form (Standard Reference Material, SRM, numbers from 3281 to 3284, respectively). Additional analysis of amino acids, dietary fiber, elements, solids, sugars, vitamins, etc. (Wood et al., 2011) have been determined for SRM 3281 (cranberry-fruit) and SRM 3282 (low calorie cranberry juice cocktail), but a need for standardized cranberry phenolic reference material remains.

4. Conclusion

This work displayed the range in proanthocyanidin values from different clean up procedures, which emphasize the importance of selecting the right sample preparation method prior to detailed phenolic analysis. A larger pool of commercial samples should be analyzed in the future. Whether the media used to purify the proanthocyanidin fraction, the phloroglucinolysis conditions, or any other preparation step, there remains a need to uniform proanthocyanidin purification prior to HPLC analysis for comparison of results (Krenn, Steitz, Schlicht, Kurth, & Gaedcke, 2007).

Cranberry product manufacturers should obtain an anthocyanin HPLC profile of their ingredients from suppliers before formulating their intended products. Phenolic profiles by HPLC have been previously been used for chemotaxonomy and as a tool for detecting adulteration, and should see greater use from food and dietary supplement manufacturers to prevent the creation of adulterated American cranberry products. Some commercial sources of cranberry phytochemicals were higher quality than others.

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