Characterization of Polyphenolics in Cranberry Juice and Co-Products

Brittany Lynn White
University of Arkansas

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CHARACTERIZATION OF POLYPHENOLICS IN CRANBERRY JUICE AND CO-PRODUCTS
CHARACTERIZATION OF POLYPHENOLICS IN CRANBERRY JUICE AND CO-PRODUCTS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science

By

Brittany L. White
University of Arkansas
Bachelor of Science in Food Science, 2007

May 2011
University of Arkansas
ABSTRACT

Historically, cranberry juice has been consumed to prevent urinary tract infections. These and other health benefits, including reduced risks of cancer and cardiovascular disease, are believed to be due to the presence of polyphenolics, specifically flavonoids. Cranberry pomace is the by-product of cranberry processing which consists of seeds and skins. This research focuses primarily on the effects of processing on the flavonoid composition of cranberries and cranberry pomace. First, the concentration of flavonoids during juice processing was evaluated. It was determined that anthocyanins were degraded readily compared to other flavonoids, and a significant amount of flavonoids were retained in the pomace. Additionally, hydrolysis of flavonol glycosides to aglycones occurred due to heat treatment. Next, dried cranberry pomace from industry was characterized based on its proximate and polyphenolic content. The pomace consisted primarily of insoluble dietary fiber and had high concentration of anthocyanins (111.5 mg/100 g DW), flavonols (358.4 mg/100 g DW), and procyanidins of DP 1-6 (167.3 mg/100 g DW). Next, extrusion processing was used to alter the flavonoid composition of cranberry pomace. Extrusion processing increased the amounts of DP1 and DP2 procyanidins and decreased the amounts of procyanidins with DP≥4. Total flavonols and antioxidant capacity also increased after extrusion. Alkaline hydrolysis was also used to extract procyanidins from cranberry pomace and alter their composition. When compared to conventional extraction, alkaline hydrolysis increased procyanidin oligomer extraction with the greatest increase being DP1 (14.9x) and A-type DP2 (8.4x) procyanidins. Treatment of the residue remaining after conventional extraction using alkaline hydrolysis resulted in further procyanidin extraction, indicating
that procyanidins are not fully extracted by conventional methods. This was confirmed by staining samples of cranberry pomace and residues remaining after conventional extraction and alkaline hydrolysis with dimethylaminocinnamaldehyde to visualize procyanidins. Alkaline hydrolysis was also used on other fruit processing by-products, and it was revealed that these by-products also contained bound procyanidins. This research indicates that cranberry pomace is a rich source of polyphenolics, which could be extracted for nutraceutical purposes. Additionally, the composition of procyanidins in the pomace could be altered by extrusion or alkaline hydrolysis to increase their bioavailability and health-promoting properties.
This dissertation is approved for
Recommendation to the
Graduate Council

Dissertation Director:

____________________________________
Dr. Luke Howard

Dissertation Committee:

____________________________________
Dr. Ron Buescher

____________________________________
Dr. Ya-Jane Wang

____________________________________
Dr. Ronald Prior

____________________________________
Dr. Edward Gbur
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Chapter 4


Chapter 5


Chapter 6

I. INTRODUCTION

North American cranberry (Vaccinium macrocarpon) production in the United States in 2009 was over 690 million pounds (1). Of this, less than 5 percent was sold as fresh fruit with the remainder being further processed into juices, sauces, or sweetened dried cranberries. In turn, cranberry processing produces millions of pounds of waste material, known as cranberry pomace, each year. Cranberry pomace is the solid matter left after pressing of the fruit for juice or sauce production, and it is composed primarily of seeds and skins of the fruit. It has limited applications in animal feeds due to its low protein content and presents environmental problems when it is disposed of in the soil or landfills due to its low pH (2). Therefore, it is of utmost importance to the cranberry processing industry to explore alternate uses of cranberry pomace.

Berries and other fruits are frequently touted for their health benefits. Historically, cranberry juice has been consumed to prevent urinary tract infections. These and other health benefits, including reduced risks of cancer and cardiovascular disease, are believed to be due to the presence of various polyphenolic compounds, including flavonoids and phenolic acids, in the plants (3, 4). The seeds and skins of fruits are known to contain high levels of polyphenolic compounds, particularly anthocyanins, flavonols, and procyanidins, which provide protection to plants from parasites and UV radiation (5). Therefore, cranberry pomace, which is primarily seeds and skins, should be explored as a rich source of these compounds.

Procyanidins are a class of polymeric flavonoids composed of flavan-3-ol monomeric units. They are named as such because they are readily converted to cyanidin when heated under acidic conditions (6). The most common linkages between the monomeric units is the $4\beta \rightarrow 8$ or B-type linkage. The A-type linkage is less common
and contains both a $4\beta \rightarrow 8$ and $2\beta \rightarrow O \rightarrow 7$ linkage (7). Cranberries contain particularly high levels of procyanidins compared to other fruits, and they are unique in that several of the procyanidins contain at least one A-type linkage. The health benefits associated with procyanidins are numerous and can be attributed to various properties such as their reducing capacity, their ability to bind proteins and metal ions, and cell signaling capabilities (6). Procyanidins from cranberry have been recognized for their anticancer properties and most notably for their ability to prevent urinary tract infections by inhibiting bacterial adhesion to the urinary tract (8-10). The antiadhesion property of cranberry procyanidins has been attributed to the presence of A-type linkages (11).

A majority of the procyanidins in cranberry pomace are polymeric (12). Donavan et al. (13) and Gonthier et al. (14) have shown that although polymeric procyanidins are good antioxidants, they are poorly absorbed compared to their monomeric subunits. Although typically present in smaller quantities, monomers, dimers, and to some extent trimers are better absorbed and maintain similar health benefits (15, 16). Additionally, there has been recent evidence that many procyanidins in plant foods resist extraction because they are tightly bound to cell wall material, and reported procyanidin contents in the literature have likely been underestimated (17).

Extrusion is a common processing method in the food industry used to produce various cereals and snack foods. Extrusion may involve several different operations; however the simplest definition is the forcing of a dough-like material through a restriction or die. Other functions of an extruder can include, but are not limited to homogenization, shearing, thermal cooking, gelatinization, and texture alteration (18). Extrusion can alter both physical and chemical properties of substrates. A decrease in
polymeric procyanidins (DP $\geq 6$) as well an increase in oligomers (DP $\leq 4$) was observed following the extrusion of sorghum grain (19). This suggests either cleavage of high molecular weight procyanidins to form smaller molecules or enhanced extraction of low molecular weight procyanidins in response to heat and shear. Therefore, extrusion processing may potentially enhance the bioavailability of cranberry pomace polyphenolics by converting polymeric procyanidins to oligomers and monomers. Enhanced bioavailability could translate to improved functionality of the compounds in biological systems.

Similarly, alkaline treatments are commonly used to extract bound phenolic acids and other phenolic compounds from grains such as rice, wheat, and corn (20). It is known that phenolic compounds, namely ferulic acid, are insoluble and bound to cell wall materials. There is limited information, however, on the effectiveness of alkaline treatment to release bound phenolic compounds in fruits possibly because many phenolic compounds in fruits, including anthocyanins are known to be unstable under alkaline conditions. Furthermore, there is even less research regarding the possible release of procyanidins from fruit and vegetables by alkaline treatment. Researchers have shown that strong alkaline conditions can result in cleavage of the C-C interflavan bond connecting the monomeric units of procyanidins (21). Hence, alkaline hydrolysis also has the potential to improve the functionality of procyanidins from cranberry pomace by enhanced extraction of monomers and oligomers through depolymerization of polymeric compounds.

The first objective of this experiment is to follow the polyphenolic composition of cranberries throughout juice processing. The next goal is to fully characterize the
proximate and polyphenolic composition of cranberry pomace. Then, processing technologies such as extrusion and alkaline hydrolysis will be evaluated on their ability to release bound procyanidins and depolymerize polymeric procyanidins into lower molecular weight compounds. Extraction of procyanidins by alkaline hydrolysis will also be evaluated using other fruit processing by-products. Applications of this research could provide a use for the waste product of cranberry processing which currently has little functionality. Enhanced extraction and molecular weight modulation of the procyanidins in cranberry pomace could lead to production of functional extracts that could be used as nutraceuticals or natural additives to juices and other beverages to add the aforementioned health benefits to these products.
LITERATURE CITED

1. National Agricultural Statistics Service (NASS)


II. LITERATURE REVIEW

A. FLAVONOIDS

Flavonoids are a group of naturally occurring plant compounds that contain a basic C6-C3-C6 flavan ring (Figure 1). Various groups may be substituted at specific positions on the rings giving rise to different classes of flavonoids. Depending on the substituting groups, flavonoids may have different chemical and biochemical properties. Three of the major classes of flavonoids present in foods include anthocyanins, flavonols, and flavanols (1).

Synthesis of flavonoids (Figure 2) occurs via the phenylproponoid pathway beginning with the conversion of phenylalanine to cinnamate by phenylalanine ammonia lyase (PAL). Flavonoid biosynthesis begins with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA which gives naringenin chalcone. The chalcone is then isomerized to a flavanone by chalcone flavanone isomerase (CHI). At this point the pathway divides into branches resulting in different flavonoid classes. Flavanone 3-hydroxylase (F3H) converts flavanones to dihydroflavonols which are then reduced by dihydroflavonol reductase (DFR) to form flavan-3,4-diols. Flavan-3,4-diols may be converted to anthocyanidins by anthocyanidin synthase (ANS). Anthocyanins are then formed through glycosylation of anthocyanidins by UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT) (2).

Functions of flavonoids in plants vary depending on their chemical structure. One of the most evident is the impartation of color to the plant, while others include protection from UV rays and parasites. Flavonoids may also act in enzyme regulation and cell signaling. The astringency of many plant-derived foods is attributed to the ability of flavonoids to precipitate proteins, which forms insoluble substances in the mouth (1).
**Anthocyanins.** Anthocyanins are pigments that impart a pink, red, blue, or purple color to fruits and vegetables and are primarily present in epidermal tissues (3). They may be present in different forms based on pH, which, in turn, determines whether they are colored or uncolored. The aglycone forms of anthocyanins are known as anthocyanidins and are very unstable. There are six major anthocyanidins (Figure 3) which differ in the number and location of hydroxyl and methoxyl groups on the flavan ring.

As they are naturally present, anthocyanins are typically glycosylated at position 3 on the C ring with 1 to 3 monosaccharide units, although some may contain more. Sugars are typically attached to the flavan nucleus as well as to other sugars, if present, through ester linkages (4). Anthocyanins may also be acylated with organic acids such as malic, tartaric, malonic, or acetic acid or phenolic acids such as coumaric, caffeic, gallic, or ferulic acid (4). Both glycosylation and acylation increase anthocyanin stability which, in turn, allows them to be more resistant to light, pH, and oxidation. Additionally, they may be attached to other flavonoids which may further increase stabilization (3).

Cranberry anthocyanins are primarily cyanidin and peonidin glycosides. The sugar moieties are strictly glucose, galactose, or arabinose, with no acylated anthocyanins. The four major anthocyanins that have been identified in the cranberry fruit include cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside. Cyanidin-3-glucoside and peonidin-3-glucoside are also present in smaller quantities, and trace amounts of other various anthocyanins have been identified (5). A previous study found that fresh cranberries contained 26.2 mg total anthocyanins per g of dry matter (5).
Flavonols. Flavonols are a class of flavonoids that are characterized by a hydroxyl group at position 3, a carbonyl group at position 4, as well as a double bond between carbon 2 and 3 on the C ring (Figure 4). Like anthocyanins, flavonols are typically glycosylated with glucose, galactose, rhamnose, or arabinose. Mono- and diglycosides are common, but some may be present as triglycosides. The sugars may also be in either the furanose or pyranose conformation which may result in bioavailability differences in vivo. Flavonols may also be acylated with phenolic acids (1). Various flavonols present in plant foods impart bitter and astringent flavors. Flavonols do not typically contribute to the color of fruits, but they absorb light strongly in the UV range and appear yellow at high concentrations. Upon oxidation, however, they may contribute to fruit discoloration (1).

Cranberries contain particularly high levels of quercetin derivatives compared to other fruits. The major flavonol in both freeze dried cranberry powder and fresh fruit was determined to be quercetin-3-galactoside. This was followed by quercetin-3-rhamnopyranoside and quercetin-3-arabinofuranoside in the freeze dried powder and fresh fruit, respectively. (6, 7). Fresh cranberries were found to contain 112-250 mg/kg quercetin, 11-24 mg/kg myricetin, and 0-3 mg/kg kaempferol in their glycosidic forms (8).

Flavan-3-ols and Procyanidins. Flavanols consist of monomeric catechins (flavan-3-ols) as well as polymers of catechins which are called procyanidins. While catechin and epicatechin are the most common flavanols in fruit, galallocatechin, epigallocatechin, are also found (Figure 5) (3). Procyanidins get their name from their ability to be converted to cyanidin upon heating under acidified conditions. Procyanidins
are categorized by and distinguished from other proanthocyanidins by their 3, 5, 7, 3', and 4' hydroxylation pattern. Grape seed and green tea procyanidins may be esterified at the 3-hydroxyl group with gallic acid (9). Epicatechin is the major free flavan-3-ol in cranberries, but catechin is also present (10). The primary function of flavan-3-ols and proanthocyanidins in plants is to provide protection against predators (11). One of the major protective properties of proanthocyanidins is their ability to bind to and subsequently precipitate proteins, which gives them astringency and makes them unpalatable to potential predators. Proanthocyanidins can also form complexes with metals such as zinc and iron, which is believed to be partially responsible for their antibacterial activities (12). These properties, while beneficial to plants, can cause proanthocyanidins to be anti-nutritional by reducing the bioavailability of nutrients, such as minerals and proteins.

The epicatechin monomeric units may be linked in one of two ways to form procyanidins. The most common linkage between the flavan rings is a 4β → 8, which is known as a B-type linkage. The A-type linkage is much less common and consists of both 4β → 8 and 2β → O → 7 linkages (Figure 6). The presence of A-type linkages provides additional structural stability to procyanidin molecules because it is more rigid than the B-type linkage. Additionally, A-type linkages are more resistant to cleavage under harsh conditions such as heating at extreme pH values. Procyanidins with A-type linkages are common in cranberry and have also been found in peanut skin, plum, avocado, and curry (13). Degree of polymerization (DP) in reference to procyanidins is the number of monomeric units linked together through either an A-type or B-type linkage to form an oligomeric or polymeric procyanidin. The DP of procyanidins can
range from two (dimer) to 200. The major procyanidin in cranberries is an A-type dimer, but they also contain significant levels of polymeric procyanidins (DP > 10) (14).

Thiolytic degradation was performed on the procyanidins from freeze dried cranberry to determine average DP. Excluding the monomeric flavan-3-ols, the average DP was 8.5, while including the monomers, the DP was found to be 8.3. Using LC-MS/MS, the researchers were also able to identify several procyanidin oligomers in cranberry containing A-type linkages as well as the location of the linkage (15).

**Development and Maturation.** Changes in the composition of flavonoids during the maturation of fruits depend primarily on the type of fruit as well as the specific variety and have implications in fruit quality. Changes are reflected in the fruits’ color, astringency, and texture. This phenomenon has been studied in two varieties of the American cranberry, ‘Ben Lear’ and ‘Stevens.’ The concentrations of both procyanidins and total flavonols decreased during fruit growth with the highest level found in the earliest stage. The decline of procyanidins occurs more rapidly than that of total flavonols; however both exhibit a slight increase during ripening. The synthesis of anthocyanins begins once the fruit has stopped growing and continues rapidly throughout ripening (16).

**Processing and Stability.** In 2009, 690 million pounds of cranberries were harvested in the United States. Of this, 95% were further processed into products such as juice, dried cranberry, and cranberry sauce, with the remaining 5% being sold as fresh fruit (17). Cranberry processing involves coarsely chopping the berry and passing it through a screen to produce a pulp, which is then treated with pectolytic enzymes and pressed. The by-product of cranberry pressing is known as pomace and includes the
seeds and skins. Much of the fiber as well as many vitamins and minerals in cranberries are not expressed in the juice, but rather remain in the pomace. A majority of the flavonoids, including anthocyanins, procyanidins, and flavonols also remain in the pomace.

There has been considerable evidence suggesting that flavonoids are easily degraded during processing. The major factors that affect flavonoid stability are temperature, light, pH, and the presence of endogenous enzymes such as polyphenol oxidase or glycosidases. High temperatures can accelerate degradation by the other factors and may cause hydrolysis of flavonoid glycosides. Flavonoids are generally more stable at a lower pH. Anthocyanins in particular are sensitive to pH changes because they are converted from the stable flavilium cation to less stable carbinol pseudobases or quinodal bases which are more easily oxidized. Polyphenol oxidase, if not inactivated can react with simple phenolics to form quinones which in turn can degrade other flavonoids. Additionally, glycosidases may be naturally present in plant cells and can cleave the sugar from many flavonoids, thus decreasing their stability.

Many studies concerning cranberry juice processing have focused on anthocyanin retention because of their contribution to the visual quality of juices and overall susceptibility to degradation by heat, light, and enzymatic activity. Anthocyanin retention in cranberry juice is generally less than 50% (18). Several studies have reported that anthocyanin stability is dependent upon the type of sugar attached and not the aglycone, and glucosides are more stable than galactosides which are in turn more stable than arabinosides (19, 20).
Although flavonol retention during cranberry juice processing has never been evaluated, flavonols are generally well retained compared to anthocyanins. In general flavanol stability appears to be dependent primarily on the sugar attached rather than the aglycone with arabinosides being considerably less stable than other glycosides (21). Additionally, significant quantities of flavonol aglycones have been reported in processed cranberry products, but they are not prominent in fresh cranberries (6, 22). Likewise, procyanidin retention during cranberry juice processing has not been assessed, however commercial cranberry juice contains a higher proportion of monomers, dimers, and trimers and fewer higher oligomers compared to fresh berries (5).

**Antioxidant Activity.** Flavonoids and other phenolic compounds have been of particular interest in disease prevention research due to their antioxidant capacity or ability to scavenge free radicals. There are many types of free radicals or oxidants that may be generated through various physiological reactions as well as exposure to foreign substances such as chemicals, radiation, drugs, smog, or ozone, to name a few. Examples of such radicals include O$_2^-$, $^1$O$_2$, ·HO, HO$_2^-$ NO·, ONOO′, HOCl, RO(O)·, and LO(O)· (23). Formation and propagation of radicals is thought to be a major contributor to aging and the onset and proliferation of many diseases such as coronary heart disease, cancer, cataracts, and diabetes. Antioxidants act by quenching free radicals to decrease their reactivity. Some antioxidants may be particularly effective at quenching one type of radical, while not as affective against another (24).

Cranberries are known to have one of the highest antioxidant capacities among all fruits. This has been attributed to the presence of flavonoids (anthocyanins, flavonols, and procyanidins) and phenolic acids. Extracts from cranberries have been reported to
inhibit LDL oxidation (7) and oxidative and inflammatory damage to the vascular endothelium (25). ORAC values for whole cranberry and a cranberry extract were determined to be 275 and 106 µmol TE/g dry matter (5).

B. CRANBERRIES AND HEALTH

Urinary Tract Infections (UTIs). For years, cranberry juice has been consumed for the prevention of urinary tract infections, and this has been repeatedly documented. Urinary tract infections are responsible for 7 million doctor visits, 1 million emergency room visits, and 100,000 hospitalizations each year, resulting in a total treatment cost of $1.6 billion (27). It was previously believed that the high acid content of cranberries caused acidification of urine which prevented UTIs. However, a study in 1984 evaluated the ability of a cranberry juice cocktail, cranberry concentrate, and fresh cranberry juice to inhibit the adherence of E. coli isolated from patients with bacteruria. The undiluted fresh juice, which contained no additives, showed the greatest anti-adherence properties (28). In the same study, mice were given cranberry juice cocktail in lieu of their normal water supply for 2 weeks. The urine of the mice that drank the cranberry juice inhibited bacterial adherence significantly more than did the urine of the control mice given water (28). This was the first study that demonstrated the anti-adhesion mechanism, and it is now widely accepted that the mechanism of action is cranberries’ ability to inhibit the adherence of bacteria to uroepithelial cells. A later study confirmed that cranberry juice is more effective at inhibiting bacterial adherence than displacing bacteria that have already adhered suggesting its use as a preventative rather than a curative measure (29). Clinical studies have clearly demonstrated the ability of cranberry to prevent urinary tract infections. In one study, 538 nursing home residents were given either 110 mL of
cranberry juice or a cranberry extract in capsules. A significant decrease in the number of urinary tract infections was observed with a reduction from 27 infections per month to 20 per month (30). Several other clinical trials have shown that UTI occurrence can be reduced by the consumption of cranberry juices, extracts, and powders daily for several weeks (31, 32).

Recent studies have focused on the components of cranberry that impart anti-adhesive properties. A 1998 study determined that procyanidins isolated from cranberry inhibited P-fimbriated E. coli adherence to uroepithelial cell surfaces, leading researchers to believe that these compounds may be responsible for the activity of cranberry (33). A later in vivo study found that when mice were given cranberry procyanidins in their drinking water, their urine exhibited anti-adherence activity (34). It is believed to be, specifically, the presence of A-type linkages in cranberry procyanidins that are responsible for anti-adhesive properties. Procyanidin trimers containing one A-type linkage inhibited adhesion, but an A-type dimer was less effective (35). Procyanidins containing B-type linkages did not exhibit anti-adhesion activity (36).

It has been hypothesized that the mechanism of anti-adhesion is the ability of procyanidins to interact with proteins on the surface of bacterial cells, which are required for adhesion (37). A recent multi-centric, randomized, double blind study on the dosage effect of anti-adhesion activity in urine following consumption of a cranberry powder standardized by procyanidin content determined that 36 mg per day can offer UTI protection, but 72 mg per day might offer 24 h protection (38). Discrepancies between in vitro/ex vivo findings and the bioavailability of cranberry procyanidins, however, have restricted elucidation of a single mechanism of action. Although procyanidins prevent
adhesion in vitro, they have never been isolated from the urine after cranberry consumption (39).

**Stomach Ulcers.** Increasing evidence suggests that cranberries can also prevent the adhesion of *Helicobacter pylori*, a pathogen that can survive in the extremely acidic conditions of the stomach and cause ulcers (40). Like the bacteria that cause UTIs, *H. pylori*, adheres to the gastric mucous by means of adhesive proteins on the surface of the bacteria (41). Burger et al. (41) first demonstrated that a non-dialyzable material (NDM) from cranberry prevented adhesion of *H. pylori* to gastric mucosal cells. Cranberry NDM has also been found to inhibit significantly more bacterial strains than the antibiotic metronidazole, and a combination of the two treatments was more effective than either treatment alone (42).

Clinical evidence has supported the ability of cranberry to prevent stomach ulcers *in vivo*. In one study, 189 adults who were infected with *H. pylori* consumed 500 mL cranberry juice cocktail daily. After 35 days, there was a significant decrease in number of infections compared to a placebo (43). This indicates that cranberry juice might be able to treat an already present bacterial infection in the stomach as opposed to just preventing one.

**Oral Health.** Consistent with cranberries’ ability to prevent UTIs and stomach ulcers, they may also be affective against oral bacterial infections. In the mouth, bacteria can bind to teeth and gums and eventually form biofilms. The bacteria prompt an immune response in the mouth and produce acids that in turn cause dental caries. The major pathogens of concern in oral health are *Streptococcus mutans*, *Streptococcus sobrinus*, and *Porphyromonas gingivalis* (18). Both cranberry juice and cranberry NDM
have been shown to prevent and reverse bacterial co-aggregation *in vitro* and in a saliva matrix (44, 45). Procyanidins and to a lesser extent flavonols isolated from cranberries suppressed biofilm formation and acid production of *S. mutans*, but anthocyanins were ineffective (46). A subsequent study confirmed that A-type procyanidin oligomers from cranberries reduce biofilm formation in vitro. Additionally, the study demonstrated that A-type procyanidins prevent the development of dental caries in vivo when 1.5 mg/mL procyanidins are applied twice daily for 60 seconds (47).

**Cognitive Function.** Degeneration of cognitive function has been linked to an increase in oxidative stress in brain tissue which results in accumulation of free radicals. Additionally, there are relatively low levels of antioxidants in the brain, which leads to an imbalance of defense mechanisms and free radicals (48). Hence, the ability of flavonoids to preserve cognitive function is generally attributed to their ability to scavenge free radicals. However, it is unclear whether these compounds can pass the blood-brain barrier in their active forms or accumulate in the brain in significant quantities to provide protection against oxidative stress. Therefore, it is possible that other mechanisms, such as modulation of various cellular and molecular processes, are responsible for the protective effect observed by flavonoids (48).

A few in vitro and in vivo studies have evaluated the ability of cranberries to protect cognitive function. Joseph et al. (49) demonstrated that extracts of several fruits including cranberries, protected against losses of Ca$^{2+}$ and preserved cell viability in COS-7 cells that were treated with dopamine or amyloid-β. An animal study performed by the same researchers found that aged rats that were given freeze-dried cranberries in their diet (2% w/w) for 8 weeks, showed more strength and balance than control rats.
Additionally, the cranberry diet enhanced neuronal signal transduction and lessened decreases in motor function (50). Evidence that these effects can translate to prevention or amelioration of neural degeneration or Alzheimer’s disease in humans, however, is limited, and more research is needed to identify any beneficial effects (48).

**Coronary Heart Disease (CHD).** Coronary heart disease is caused by atherosclerosis, which is the deposition of cholesterol and lipids on the walls of arteries. This begins with the uptake of oxidized LDL by endothelial cells of the arteries. Therefore, the ability of flavonoids to prevent coronary heart disease focuses primarily on their ability to inhibit LDL oxidation and platelet aggregation and deposition. Research also focuses on their ability to inhibit enzymes involved in lipoprotein synthesis and induce endothelium-dependent vassorelaxation (51).

A cranberry extract has been shown to significantly reduce LDL oxidation as well as induce expression of hepatic LDL receptors which increased intracellular uptake of cholesterol. This increased uptake of cholesterol by liver cells decreases the amount of plasma cholesterol in circulation (52). Another study has shown that a number of fruits, including cranberry, exhibit the ability to bind bile acids in vitro with the potential to lower LDL and cholesterol levels (53).

**Cancer.** One of the earliest studies involving the potential anticancer activity of cranberries in vitro came in 1996. The study showed that extracts of cranberry as well as other berries from *Vaccinium* species inhibited ornithin decarboxylase (ODC) expression and induced quinine reductase, a detoxification enzyme (54). ODC is involved in the biosynthesis of polyamines which play a role in cellular proliferation (55). Another study also showed that whole cranberry extract inhibited OCD in mouse epithelial cells (56). A
commercial cranberry powder extract consisting of water soluble phenolics inhibited the propagation of several human tumor cell lines including 2 oral cancer cell lines, 4 colon cancer cell lines, and 3 prostate cancer cell lines (57). The total extract was generally more effective than anthocyanin and proanthocyanidin fractions, suggesting a synergistic effect.

It has only recently been suggested that procyanidins from cranberry may contribute to its anticancer activities. Procyanidins from whole cranberry fruit were isolated and shown to inhibit the growth of human lung, colon, and leukemia cancer cell lines (55). New tumor colony formation was decreased by treatment with a procyanidin rich fragment as well as a whole-cranberry extract from Early Black cranberries (55). The study showed that in one cell line the procyanidin fraction was more effective than the whole cranberry extract.

Quercetin is one of the most abundant flavonols in fruits and vegetables, including cranberries. Therefore, much work has been done to determine its anticancer activities. It has been shown to inhibit growth of many human cancer cell lines including breast and pancreas (58).

**Metabolism and Bioavailability.** For many of the health benefits which have been demonstrated in vitro to translate to the same benefits in vivo, it is necessary for the active molecules to be absorbed and directed to the proper tissue in their bioactive forms. Therefore, it is important to understand what happens to these molecules once they are consumed including what form the molecules are in when and if they are absorbed. This is an area in which much research needs to be conducted as little is known.
Flavonoid absorption and metabolism is affected by structural differences in the molecules. Anthocyanins form cation structures at low pH which may contribute to their reported low absorption rates. It is suggested that they may be absorbed in their native form or as modified metabolites. Gut microflora may degrade the flavonoids in the intestinal tract before they are absorbed or excreted. Once absorbed, the molecules either enter the bloodstream or travel to the liver and kidney where they are further metabolized. Anthocyanin glycosides consumed have been observed in the plasma suggesting that no modifications were needed for absorption (59). Wu and Prior (60) evaluated the presence of anthocyanins in pig urine following the consumption of black raspberries. They found metabolites to dominate in the urine including glucuronidated and methylated anthocyanins. Some anthocyanins were deglycosylated as well, preventing them from being glucuronidated or methylated. As quercetin glycosides are metabolized in vivo, they are usually converted to sulfates and glucuronides as well (55).

Flavan-3-ol and procyanidin absorption is largely dependent on the size of the molecule. One study showed that, in a simulated gastric environment, procyanidins were hydrolyzed to smaller molecular weight monomers and dimers (61). A later study, however, evaluated procyanidin stability in 6 healthy adults after the consumption of a cocoa beverage and found that the procyanidins were indeed stable during gastric transit as they reached the small intestine unchanged (62). Once the flavanols are in the small intestine, they may be absorbed in the jejunum to the epithelial lining, where they may be methylated and glucuronidated. These compounds then proceed to the liver where they may be further methylated and glucuronidated as well as sulfated (63). Procyanidins larger than trimers, however, have shown to be poorly absorbed due to their size (64).
Holt and others (65) detected catechin and epicatechin as well as a procyanidin dimer in the plasma of human subjects as early as 30 min after they consumed a cocoa beverage. The same study which determined that cocoa procyanidins decreased diabetes induced cataracts also found that levels of epicatechin and its metabolites reached its highest levels in the plasma between 1 – 2 hours, while the B2 dimer was poorly absorbed (66). Although the large molecular weight molecules may not be absorbed, they may still protect against certain diseases of the intestine, such as cancer, by inhibiting oxidation in an area that is not typically rich in dietary antioxidants (3). Additionally, the compounds may be fermented by gut microflora to produce lower molecular weight phenolic acids which may be subsequently absorbed (67).

C. BOUND PROCYANIDINS

Polyphenolic compounds, including procyanidins, are usually stored in the vacuoles of plant cells and are separated from other cellular components. However, some can also be associated with other cellular components, including proteins and carbohydrates because of the natural affinity of procyanidins to bind with these components. Additionally, complexation can be perpetuated after cell injury when vacuoles may rupture and its contents are released. As phenolic compounds are released, they may then associate with cell wall polysaccharides and proteins. Complexes generally form through hydrogen bonding and hydrophobic interactions (68). Procyanidins in particular have a strong affinity for cell wall proteins and carbohydrates (69), and polymeric procyanidins have a greater binding affinity than monomers and other oligomers. In light of this, the presence of “unextractable” procyanidins has emerged, and it is believed that procyanidin contents in plant materials has been
underestimated due to the presence of procyanidins bound so tightly to cell wall material that they are not released by normal extraction methods (70, 71).

There have been a number of studies conducted to study procyanidin – cell wall interactions (72-74). It was determined that isolated procyanidins bind readily to cell wall carbohydrates, particularly pectin, and binding affinity increased with increasing DP of the procyanidin. Pinelo et al. (68) proposed that interactions between polyphenolics and cell wall material may be hydrophobic interactions resulting in phenols residing in hydrophobic pockets of carbohydrates or hydrogen bonding between hydroxyl groups on phenolics and cell wall carbohydrates. It is also possible that procyanidins may be covalently linked to cell wall components during flavonoid biosynthesis or under various oxidative conditions (75). Hydrogen bonding and hydrophobic interactions can be considered reversible binding, whereas, covalent linkages between procyanidins and cell wall material is analogous to lignin binding, and is deemed irreversible (75).

Hellström and Matilla (76) have developed a method to determine unextractable procyanidins in plant materials by acid-catalyzed depolymerization of the compounds into flavan-3-ols and benzylthioethers using thioacidolysis. They have used this method to determine the amount of unextractable procyanidins in several plant materials including cranberries (70). They found that a significant amount of procyanidins in many plant materials were “unextractable.” Other researchers have used butanol:HCl with heat to release bound procyanidins (71, 77). This method is based on the principle that under heat and acid and in the presence of oxygen, procyanidins are converted to cyanidin which can be measured spectrophotometrically (Figure 7). Researchers found
that apples, peaches, and nectarines contain higher levels of non-extractable procyanidins than extractable procyanidins (71).

D. EXTRUSION

Extrusion cooking has become a popular food processing technique especially in the snack food and cereal industry. It is considered a continuous, high-temperature-short-time (HTST) processing method and is capable of preserving desirable food components and destroying microorganisms (78). The end products typically have a low moisture content (< 15%) which allows them to be shelf-stable (79). Extrusion introduces a number of operations to a food matrix during processing including, but not limited to, mixing, heating, shearing, shaping, and puffing (80).

All extruders consist of primarily the same components. These include a feeding area where the raw material is introduced, a place for liquids or steam to be injected, a barrel segment consisting of screws and kneading devices, and a die from which the material is forced out (Figure 8). Some extrusion systems may include a holding bin for raw materials, an automatic feeder, and a cutting device to cut the product after it has been forced through the die (81). Extrusion systems vary in the type and number of screws present in the barrel. Twin-screw extruders contain two screws side by side which can be co-rotating or counter-rotating as well as intermeshing or non-intermeshing allowing for a variety of configurations. The configuration of the screws largely determines the amount of shear placed on the food matrix (81).

As a mixture proceeds through the extrusion system, it is subjected to various processes. In the feeding zone, the material begins to be conveyed towards the end of the extruder by the screws as it is compressed and excess air is expelled. In the kneading
zone, compression continues and the matrix becomes less granular and denser. In the final cooking zone, shear rate is highest and the matrix continues to be compressed. Enough pressure builds up in this region to expel the product through the die (81).

**Extrusion and Phenolic Compounds.** Extrusion presents mechanical stress in the form of heat and shear to the substrates, which may alter physical as well as chemical characteristics of the product. Since extrusion is a common food processing practice, many studies have focused on its effects on the nutritional aspects of food including phenolic compounds, which are known to be heat sensitive; these effects may be beneficial or detrimental.

A significant decrease in anthocyanins (> 90%) was seen in extruded corn breakfast cereals containing blueberry, cranberry, raspberry, and grape powders (82). The fruit powder containing breakfast cereals retained some antioxidant activity with the highest retention being in the cranberry cereal. Anthocyanin losses (65-68%) were also observed in extruded blueberry – corn cereals (83). In contrast, no significant loss of total phenolics was observed in an extruded snack food, however the total antioxidant capacity was negatively affected (84). The antioxidant activity of dark buckwheat extrudates was not significantly different than the raw dark buckwheat, however the HPLC profile of phenolics was altered, suggesting a change in the composition of the phenolic compounds (85). A loss and redistribution of isoflavone content was observed in extruded wheat flour with wet okara. Increases in genistin and daidzin were observed which is believed to be the result of cleavage of the malonyl group from malonyl daidzin and malonyl genistin (86). This suggests that extrusion cooking is capable of breaking covalent bonds of molecules in the food that is being processed.
The procyanidin distribution of sorghum was also altered by extrusion. A decrease in high DP procyanidins and an increase in low DP procyanidins was observed likely as a result of cleavage of the larger molecules into smaller ones (87). Similarly, increases in procyanidin monomer, dimer, and trimer contents were observed when blueberry pomace, grape pomace, and grape seeds were extruded with white sorghum (88, 89). This implies that extrusion may improve the functionality of procyanidin containing foods since it has been suggested that lower molecular weight compounds are better absorbed than larger ones (90). This implication is supported by a study that found that the bioavailability of catechin in weanling pigs fed extruded sorghum was improved compared to those that were fed non-extruded sorghum (91).

E. ALKALINE HYDROLYSIS

Alkaline treatments are commonly used to extract bound phenolic acids and other phenolic compounds from grains such as rice, wheat, and corn. It is known that phenolic compounds, namely ferulic acid, are insoluble and bound to cell wall materials. Treatment with different concentrations of sodium hydroxide for varying lengths of time has proven to be effective in releasing these bound phenolic compounds (92, 93). There is limited information, however, on using alkaline conditions to release bound phenolic compounds in fruits. This is likely due to the fact that many phenolic compounds in fruits, especially anthocyanins are known to be unstable under alkaline conditions. Furthermore, there is even less research regarding the possible release of procyanidins from fruit and vegetables by alkaline treatment. Researchers have shown that, like acid, alkali conditions can result in cleavage of the C-C interflavan bond which connects the monomeric units to form procyanidins; however, prolonged treatment can cause further
degradation by opening of the A-ring of the flavan-3-ol. Research regarding the effect of alkaline conditions on proanthocyanidins has been limited to purified compounds (94, 95).

F. ANALYSIS OF FLAVONOIDS

Oxygen Radical Absorbance Capacity (ORAC). ORAC is a common assay used to screen extracts and individual compounds for antioxidant activity. It measures the antioxidant activity of molecules against peroxyl radicals (ROO·). This reaction is biologically significant in that it mimics reactions of antioxidants with lipids both in food systems and in the body. In the assay, the peroxyl radical, generated by 2,2'-azobis(2-amidino-propane dihydrochloride (AAPH) and Cu²⁺-H₂O₂, reacts with the chemical probe, fluorescein, causing a loss in fluorescence as follows:

\[ \text{ROO}^- + \text{fluorescent probe} \rightarrow \text{ROOH} + \text{oxidized probe} \]

In the presence of an antioxidant, the peroxyl radical reacts with the antioxidant, thus sparing fluorescein from oxidation as follows:

\[ \text{ROO}^- + \text{AH} \rightarrow \text{ROOH} + \text{A}^- \]

ORAC values are then determined by differences in the area under the curves between antioxidant samples, blanks, and Trolox standards, which is a water soluble vitamin E derivative. The values are reported as Trolox equivalents (TE) (24). The method was originally designed to measure only hydrophilic antioxidant capacity, but has been modified to also measure lipophilic antioxidants, which are biologically significant as well (26).

HPLC-MS. High-performance liquid chromatography coupled with mass spectrometry is the most common method of detection, identification, and quantification of a variety of flavonoids in fruit and vegetable extracts. Several methods have been
developed and optimized to separate several classes of compounds; however, not one method can effectively separate all flavonoids at once due to significant structural diversity. Reverse phase HPLC with a column containing C18 packing material is most commonly used to separate anthocyanins and flavonols. The mobile phases generally contain acidified- aqueous mixtures of methanol or acetonitrile (96, 97). A solvent gradient with decreasing polarity is applied to the column; hence more polar compounds are eluted first and more non-polar compounds last. In contrast, normal phase HPLC uses a column with polar packing material, such as silica, and, a gradient of increasing polarity is applied. Consequently, compounds are then eluted according to increasing polarity.

Detection of compounds upon elution from the column is usually done using various spectral properties of the compounds including their ability to fluoresce or absorb at a specific UV-Vis wavelength. Fluorescence detectors monitor compounds at specific excitation and emission wavelengths, and these are commonly used to detect procyanidins. Photodiode array detectors scan a wide range of wavelengths and are commonly used to detect compounds that absorb in the UV-Vis range. Anthocyanins and flavonols are detected by absorption at 520 and 360 nm, respectively (4, 96). The signal produced by the detector is proportional to the concentration of the compound detected, and quantification is done using external calibration curves of purified standards.

Mass spectroscopy (MS) is used in tandem with HPLC to identify compounds based on characteristic mass to charge ratios. The major elements of an MS instrument include an ionizer, an analyzer, and a detector. In the ionizer, the compounds are vaporized and ionized into electrically charged particles. In the analyzer, ions are
separated using electric and magnetic fields which results in ions travelling at different speeds based on their mass to charge ratio. The detector records the relative quantities of each ion. Each flavonoid has a characteristic fragmentation pattern, allowing for identification of the parent compound (98).

**MALDI-TOF-MS.** Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a type of direct mass spectrometry used to analyze compounds with large masses that are generally not suitable for conventional mass spectrometric techniques (99). MALDI involves mixing of the compounds of interest, which are not readily ionized alone, with an easily ionizable matrix. Common matrices are simple phenolic acids, such as dihydroxybenzoic acid, which have a low molecular weight and do not interfere with detection of the compounds of interest. As the name suggests, the matrix assists in the ionization of the target compounds upon being bombarded with an N₂ laser. TOF refers to the mass analyzer, in which compounds are facilitated through according to their relative size. The mass of the compound is determined by the amount of time it takes it to travel through the analyzer. Little to no ionization occurs using MALDI-TOF-MS, so masses reported by the detector represent parent compound masses. MALDI-TOF-MS has been used to identify large phenolic compounds including procyanidin oligomers and polymers and well as anthocyanin tannin complexes (100).
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H. FIGURE CAPTIONS

**Figure 1.** Basic flavan 3 ring structure.

**Figure 2.** Diagram of the major pathway of flavonoid biosynthesis. Adapted from Jaakola (2).

**Figure 3.** Structure and substitution patterns of common anthocyanidins present in fruits and vegetables.

**Figure 4.** Structure and substitution patterns of common flavonols present in fruits and vegetables.

**Figure 5.** Structures of common flavan-3-ols; epicatechin (a); catechin (b); epicatechin gallate (c).

**Figure 6.** Examples of B-type (a) and A-type (b) procyanidin linkages.

**Figure 7.** Mechanism of procyanidin depolymerization in the presence of heat and acid.

**Figure 8.** Basic diagram of a twin screw extruder.
Figure 1
Phenylalanine

\[ \text{PAL} \]

\[ \text{C4H, 4CL} \rightarrow \text{Hydroxycinnamic acid conjugates} \]

4-coumaroyl-CoA

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| Aurones      | CHI  |                   |
|--------------|-----|                   |
| Flavanones   |     |                   |

| Flavones     | F3H  |                   |
|--------------|-----|                   |
| Isoflavonoids|     |                   |
| Dihydroflavonols | | |

| Flavonols    | DFR  |                   |
|--------------|-----|                   |
| Leucoanthocyanins | | |

| Proanthocyanidins | ANS |                   |
| (condensed tannins) |     |                   |
|                   |     |                   |
|                   |     |                   |
|                   |     |                   |
|                   |     |                   |

| Anthocyanidins   | UFGT |
|                 |     |                   |
|                 |     |                   |
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Figure 2
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Figure 3
Flavonol $R_1$ $R_2$

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<tr>
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<td>OCH$_3$</td>
<td>H</td>
</tr>
</tbody>
</table>

Figure 4
Figure 5
Figure 6
Figure 7
III. THE IMPACT OF PROCESSING ON THE ANTHOCYANIN, FLAVONOL, AND PROCYANIDIN CONTENT OF CRANBERRY JUICE

A. ABSTRACT

Cranberry juice is the most common form of cranberry consumption; however, there is limited information on the changes of polyphenolic content of the berries during processing. In this study, we evaluated the effects of three different pretreatments (grinding + blanching; only grinding; only blanching) for cranberry juice processing on the concentrations of anthocyanins, flavonols, and procyanidins throughout processing. Flavonols and procyanidins were retained to a greater extent than anthocyanins, and pressing resulted in the most significant losses in polyphenolics due to removal of the seeds and skins. Flavonol aglycones increased during processing as a result of heat treatment. Drying of cranberry pomace resulted in increased extraction of flavonols and procyanidin oligomers but lower extraction of polymeric procyanidins. Our results indicate that cranberry polyphenolics are relatively stable during processing compared to other berries; however more work is needed to determine their fate during storage of juices.

KEYWORDS: Anthocyanins; cranberries; flavonols; juicing; processing; procyanidins
B. INTRODUCTION

For centuries, the American cranberry (*Vaccinium macrocarpon*) has been recognized for its health benefiting and medicinal properties. Early American colonists were introduced to the berry by the Native Americans, who used cranberries as a food, a meat preservative, and treatment for infections and various maladies (1). Cranberries soon became a common ingredient in sauces, jellies, and most notably, juices. According to the USDA-NASS, less than 5% of cranberries are sold fresh with the remaining 95% processed into juice, sauce, or sweetened dried cranberries (2).

Berries are widely recognized for their various health-promoting properties including the reduced risks of cancer, cardiovascular disease, and other chronic diseases, which have been attributed to polyphenolic compounds, including anthocyanins, flavonols, and procyanidins (3, 4). The polyphenolic composition of cranberries has been evaluated extensively. There are six major anthocyanins in cranberries, including the arabinosides, glucosides, and galactosides of cyanidin and peonidin. The major flavonols in cranberries are glycosides of quercetin and myricetin with quercetin-3-galactoside predominating. Cranberries contain procyanidins with varying degrees of polymerization and are unique in that a majority of the procyanidins contain at least one A-type linkage, which consists of both $\beta_4 \rightarrow 8$ and $\beta_2 \rightarrow O \rightarrow 7$ bonds between two monomeric units. Most procyanidin containing species have only B-type linkages, which are single $\beta_4 \rightarrow 8$ linkages between monomeric units.

Cranberries are perhaps most recognized for their role in urinary health, specifically their ability to prevent recurrent urinary tract infections (5). The exact mechanism for cranberries role in urinary tract prevention has yet to be elucidated,
however recent research has attributed the property to the presence of procyanidins containing A-type linkages. These compounds are believed to prevent uropathogenic bacteria from adhering to the epithelial lining of the urinary tract, thus preventing the onset of an infection (6).

There has been considerable evidence suggesting that polyphenolic compounds in berries are readily degraded during juice processing. Many studies regarding cranberry juice processing have focused on retention of anthocyanins due to their contribution to the visual quality of juices and overall susceptibility to degradation by heat, light, and enzymatic activity. Anthocyanin retention in cranberry juice is generally less than 50% (7). Although flavonol retention during cranberry juice processing has never been evaluated, flavonols are generally well retained compared to anthocyanins. However significant quantities of flavonol aglycones have been reported in processed cranberry products but not in fresh cranberries (8, 9). Likewise, procyanidin retention during cranberry juice processing has not been assessed, however commercial cranberry juice contains a higher proportion of monomers, dimers, and trimers and fewer higher oligomers compared to fresh berries (10).

Various factors including temperature, duration, and pre-treatment of the fruit can affect the retention of polyphenolic compounds in juice. It is important to understand how processing alters the polyphenolic compounds in cranberries so that efforts may be made to retain them and maximize the health benefits of cranberry juice. Therefore, the aim of this study was to evaluate the effect of pre-treatments such as grinding and blanching on the polyphenolic content of cranberry juice.
C. MATERIALS AND METHODS

Frozen Berries. Frozen cranberries were obtained from Decas Cranberry Company (Carver, MA) and stored at -20°C until processing.

Juice processing. A juice processing schematic is shown in Figure 1, with sampling points indicated by an asterisk. Frozen berries were assigned to one of three processing methods, which included (A) grinding + blanching; (B) grinding + no blanching; (C) no grinding + blanching. Grinding was performed using an industrial food processor (model RSI6V; Robot Coupe USA. Inc, Jackson, MS). Berries were blanched by heating the mash to 95°C and holding for 3 min. Depectinization was achieved by adding 0.12% w/v of a commercial pectinase enzyme tailored for cranberries obtained from Decas Cranberry Company and holding at 45°C for 1 h or until the mash produced a negative pectin precipitation test. Following depectinization, the mash was pressed in a 25 L Enrossi bladder press (Enoagricol Rossi s.r.l., Calzolaro, Italy) to separate the juice from the pomace. Juices were allowed to settle overnight and were then clarified by centrifugation for 10 min at 6000 x g in an Allegra™ X-22R Centrifuge (Beckman Coulter, Brea, CA). The clarified juice was then filled into 5-mL screw top glass tubes and pasteurized by heating to 90°C and holding for 10 min.

Drying of Pomace. Pomace resulting from pressing of juices was dried by freeze drying using a VirTis Genesis freeze dryer (Gardiner, NY) or oven dried using a forced air oven. Oven drying was performed at three different temperatures (40, 60, or 80 °C), and samples were allowed to dry until the pomace reached a moisture content of 4 – 5%.

Extraction of polyphenolics. Fresh cranberries and samples from each step along the processing method were homogenized with 20 mL of acetone:water:acetic acid
(70:29:5:0.5, v/v/v) using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp., Mason, OH) and filtered through Miracloth (Calbiochem, LaJolla, CA). The residue was collected, the extraction repeated two more times, and the volume of the extracts was adjusted to 100 mL with extraction solvent. Juice samples were analyzed directly and required no extraction.

**HPLC analysis of anthocyanins.** Extracts (8 mL) were dried using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY) and re-suspended in 1 mL of 3% formic acid in water. Individual anthocyanins were separated by reverse phase HPLC according to the method described by Cho et al. (11). Anthocyanin glycosides were quantified as corresponding anthocyanin glucosides using external calibration curves of a mixture of anthocyanin glucoside standards purchased from Polyphenols Laboratories (Sandnes, Norway).

**HPLC analysis of flavonols.** Extracts (8 mL) were dried using a SpeedVac® concentrator and re-suspended in 1 mL of 50% methanol in water. Individual flavonols were separated by reverse phase HPLC according to the method described by Schieber et al. (12). Flavonol glycosides were quantified as rutin equivalents and aglycones as either quercetin or myricetin using external calibration curves.

**HPLC analysis of procyanidins.** Acetone was evaporated from extracts (20 mL), and the resulting aqueous fraction was subjected to solid-phase extraction according to the method described by Gu et al. (13) to remove interfering sugars, and other phenolic compounds. Resulting extracts were evaporated to dryness using a SpeedVac® concentrator, re-suspended in 2 mL of extraction solvent, and filtered through 0.45 µm filters. Procyanidins were separated by the method of Hammerstone et al. and quantified.
using external calibration curves of a mixture of procyanidin standards (DP1 – DP6) isolated from cocoa and obtained from Masterfoods (Hacketstown, NJ). A-type procyanidins were quantified as B-type equivalents. Polymeric procyanidins (DP > 10) were quantified by extrapolation of a slope vs. degree of polymerization curve.

**Calculations.** To account for dilution and concentration effects, anthocyanin, flavonol, and procyanidin concentrations were converted to original berry weight so that all products could be compared on an equivalent basis. This was done using the following equation:

\[ C_{\text{berry}} = C_{\text{product}} \times R \]

where \( C_{\text{berry}} \) = concentration on original berry weight basis, \( R \) = mass of the product divided by mass of original berry, and \( C_{\text{product}} \) = concentration in the product.

**Statistical analysis.** Three samples were taken at each sampling point along the juicing process. Data are expressed as means ± standard error. The effects of processing on anthocyanin, flavonol, and procyanidin concentrations were determined by one way analysis of variance (ANOVA) using JMP® 8.0 (Cary, NC). Differences between means were determined using the Student’s \( t \)-test (\( \alpha = 0.05 \)).

**D. RESULTS AND DISCUSSION**

**Processing Effects on Anthocyanins.** Changes in six anthocyanin glycosides were evaluated throughout juice processing, and the results are presented in Figure 2. Blanching resulted in significant losses of total anthocyanins, but this was unaffected by grinding. It appeared that the type of sugar attached affected the stability of the anthocyanin during blanching; the most stable were the glucosides of cyanidin and peonidin with 100% retention after blanching, while their respective galactosides were
the least stable, with only 76 – 77% retention after blanching. Several studies have reported that anthocyanin stability is dependent upon the type of sugar attached and not the aglycone, and glucosides are more stable than galactosides which are in turn more stable than arabinosides (14, 15). However, we found that during blanching, the arabinosides were more stable than the galactosides. No further losses of total anthocyanins were observed during depectinization.

The greatest losses in total anthocyanins were observed during juicing, presumably due to the exclusion of seeds and skins in the pomace, which retained 16 – 20% of the total anthocyanins present before pressing. Interestingly, a greater amount of anthocyanins were expressed in the unclarified juice from the berries that were ground but not blanched (Process B). Immediate clarification by centrifugation did not result in anthocyanin loss due to sedimentation. Slight losses were observed due to pasteurization only in berries pretreated using Process B. In contrast to blanching, but consistent with previous studies, the order of anthocyanin stability during pasteurization was (from most to least stable) glucosides > galactosides > arabinosides. Total recovery of anthocyanins in pasteurized juices ranged from 39 – 53%, which is consistent with a previous study on cranberries (16).

These findings suggest that grinding of the fruit prior to processing enhances expression of anthocyanins into the juice, and blanching of the berries to inactivate endogenous enzymes, such as polyphenol oxidase and glycosidases, does not necessarily provide additional protection against anthocyanin degradation. In this case, blanching reduced expression of anthocyanins into the juice, likely because it caused moisture loss in the berries, which resulted in decreased mass transfer of anthocyanins into the juice.
This is in stark contrast to studies involving blueberry processing that found blanching to significantly improve anthocyanin retention in processed products (17), (18). Anthocyanin retention in red raspberry juice, however, was unaffected by blanching (17). This is likely due to the difference in activities of endogenous enzymes in different berries. Polyphenol oxidase in blueberries is responsible for significant losses in anthocyanins during processing and must be inactivated by blanching to prevent these losses. This enzyme is apparently less active in cranberries either due to their naturally low pH or insufficient quantities of enzyme or its substrate, simple phenols.

**Processing Effects on Flavonols.** Changes in flavonols were followed throughout processing, and the results are presented in Figure 3. Blanching resulted in a significant increase in total flavonols, and no change was observed after blanched berries were depectinized. Process B berries, which were not blanched, also contained higher levels of total flavonols after depectinization, but not as high as berries that were blanched, indicating that flavonols are relatively heat stable compared to anthocyanins, and blanching and grinding facilitated extraction of flavonols by membrane disruption. Pressing resulted in significant losses of flavonols due to the exclusion of seeds and skins in the pomace, which retained approximately 25% of the total flavonols present before pressing. Clarification and pasteurization had no effect on total flavonol concentration of the juices. Individual flavonol retention appeared to be dependent upon the type of sugar attached rather than the aglycone. Myricetin arabinoside and quercetin arabinofuranoside were considerably less stable than the other glycosides with only 22 and 24% retained in pasteurized juices, respectively. This is consistent with a study that found quercetin arabinoside to be the least stable flavonol glycoside during storage of apple juice (19).
Interestingly, quercetin arabinopyranoside was also present in the fresh cranberries and showed remarkably greater stability (55%) than the furanoside.

Changes in the composition of flavonols were also observed during processing. Flavonol aglycones, myricetin and quercetin, were observed after blanching, but not in fresh berries, and their quantities increased as processing progressed. This was coupled with slight decreases in the amount of flavonol glycosides, indicating deglycosylation of the glycosides into aglycones as a result of processing. In pasteurized juices, flavonol aglycones comprised 24 – 25% of the total flavonols. Additionally, the pomace contained a higher percentage of aglycones (26 – 43%) than did the juices, indicating that aglycones are not as readily expressed in juices because they are less polar than their glycosidic counterparts. Other researchers have also noted significant quantities of flavonol aglycones in processed cranberry products but not in fresh berries (8). It was previously hypothesized that the presence of flavonol aglycones in cranberry pomace was the result of deglycosylating side activities of pectinase enzymes used for juice processing (20); however, this was unlikely the case since the aglycones were found immediately after blanching before addition of the enzyme. Therefore, since cranberries have a very low pH (2.6) compared to other berries, we believe that it is a combination of heat and low pH that causes deglycosylation of flavonol glycosides. To test this hypothesis, we treated solutions of quercetin-3-glucoside under conditions that mimicked blanching and found that in the solution that was adjusted to pH 2.6 and blanched, quercetin levels increased by 15%. However, in the solution that was blanched at their original pH (4.9), quercetin levels decreased by 6%. This is the first time that it has been demonstrated that a combination of heat and low pH in cranberries can result in the
formation of significant quantities of flavonol aglycones. Increases in quercetin have also been observed in cranberry jam, however, not to the extent that we found in this study (9).

**Processing Effects on Procyanidins.** Changes in procyanidin content and composition were also evaluated throughout processing and the results are presented in Figure 4 and Table 1. Blanching resulted in an increase in both total procyanidin oligomers (DP1 – DP6) and polymers (DP>10). No changes in procyanidin concentrations were observed as a result of depectinization. Berries pretreated by process B retained higher levels of polymeric procyanidins after depectinization than those that were blanched, indicating that blanching caused a decrease in polymeric procyanidins. This could be the result of depolymerization of polymeric compounds to smaller ones or binding of polymers to the cell wall material which may render them unextractable. Polymeric procyanidins have been shown to readily bind to the cell wall (21). Pressing resulted in significant losses of procyanidins as a result of seed and skin exclusion in the pomace, which retained 19 – 27% of the oligomers and 40 – 76% of the polymeric procyanidins. Interestingly, juices resulting from berries that were pretreated by Process B (grinding, no blanching) had higher levels of procyanidin oligomers than did the other juices. Clarification and pasteurization had no effect on the concentration of procyanidin oligomers in the juices. The pomaces contained a higher percentage of their procyanidins as polymeric compounds than did the other products, which suggests that the lower oligomers are more readily expressed in the juice than polymeric procyanidins.

**Effect of Drying on Polyphenolics in Pomace.** Polyphenolics were extracted from pomaces that were either freeze dried or oven dried (40, 60, or 80 °C), and these
results are presented in Table 2. Drying had no effect on total anthocyanins in the pomace. This is consistent with a study on grape pomace, in which no losses in total extractable polyphenols or antioxidant activity were observed at temperatures less than 100 °C compared to freeze dried pomace (22). Freeze drying resulted in increased extraction of flavonol glycosides, but they were unaffected by oven drying. Oven drying at 60 and 80 °C, however, did increase the amount of flavonol aglycones in the pomace. This was also observed during juice processing and is likely due to heat induced deglycosylation of flavonol glycosides at the naturally low pH of cranberries. Drying also increased the amount of procyanidin oligomers extracted from the pomace, with the freeze dried pomace containing the highest amount. In contrast, polymeric procyanidins were lower in dried pomace when compared to fresh. This could be due to depolymerization of polymeric procyanidins or increased binding of the polymeric compounds to the cell wall matrix during drying. The increase in flavonols and procyanidin oligomers after drying indicates that these compounds were ineffectively extracted from the fresh pomace, and drying aided in extraction. Drying has been shown to decrease cell wall porosity, resulting in collapse of the cell wall structure, which decreases the affinity between cell wall polysaccharides and polyphenolics (23). In this study, polymeric procyanidins were not impacted by the decrease in porosity, as their binding seemed to increase upon drying. Polymers have a higher affinity for cellular material than do lower molecular weight compounds due to the presence of multiple binding sites on large molecules (21, 23).

In summary, flavonols and procyanidins were generally more stable than anthocyanins during cranberry juice processing. The most significant losses were
observed during pressing as a result of exclusion of the seeds and skins. Blanching of the fruit prior to processing did not improve polyphenolic retention; however, grinding of the fruit appeared to facilitate expression of polyphenolics into the juice. Increases in flavonol aglycones were observed during processing as a result of deglycosylation of flavonol glycosides by heating. Drying of cranberry pomace did not affect anthocyanin concentrations; however, extraction of flavonols and procyanidin oligomers was improved by drying, likely as a result of a decrease in porosity of the cell wall. Our results indicate that cranberry polyphenolics are relatively stable during juice processing compared to other berries; however more work is needed to determine their fate during storage.

E. ACKNOWLEDGMENT

We thank Decas Cranberry Company, Inc. for providing the fresh cranberries and pectinase used in this study.
F. LITERATURE CITED


2. National Agricultural Statistics Service (NASS)


G. FIGURE CAPTIONS

**Figure 1.** Flow chart of cranberry juice processing with sampling points indicated by asterisks.

**Figure 2.** Concentration of anthocyanins throughout cranberry juice processing. Error bars represent standard errors of total anthocyanins (n=3).

**Figure 3.** Concentration of flavonols throughout cranberry juice processing. Error bars represent standard errors of total flavonols (n=3).

**Figure 4.** Concentration of procyanidin oligomers throughout cranberry juice processing. Error bars represent standard errors of total procyanidins (n=3).
<table>
<thead>
<tr>
<th>Processing Step</th>
<th>Pretreatment</th>
<th>Polymer Concentration (mg/100 g Fresh Berry)</th>
<th>% Polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>–</td>
<td>206.2 ± 9.3c</td>
<td>81.7</td>
</tr>
<tr>
<td>Blanched</td>
<td>A</td>
<td>251.8 ± 8.4b</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>245.3 ± 18.5b</td>
<td>82.6</td>
</tr>
<tr>
<td>Enzyme Treated Mash</td>
<td>A</td>
<td>261.4 ± 20.9b</td>
<td>81.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>319.6 ± 11.5a</td>
<td>85.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>172.7 ± 8.6d</td>
<td>76.1</td>
</tr>
<tr>
<td>Unclarified Juice</td>
<td>A</td>
<td>104.2 ± 17.7e-h</td>
<td>82.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>103.5 ± 8.2e-i</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>100.7 ± 4.3f-j</td>
<td>78.5</td>
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<tr>
<td>Clarified Juice</td>
<td>A</td>
<td>107.7 ± 3.6e-g</td>
<td>80.4</td>
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<td></td>
<td>B</td>
<td>74.0 ± 10.9jk</td>
<td>67.5</td>
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<tr>
<td></td>
<td>C</td>
<td>86.2 ± 4.5g-k</td>
<td>78.0</td>
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<tr>
<td>Pasteurized Juice</td>
<td>A</td>
<td>76.1 ± 4.0h-k</td>
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</tr>
<tr>
<td></td>
<td>B</td>
<td>69.4 ± 2.7k</td>
<td>68.0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>75.3 ± 7.2i-k</td>
<td>73.6</td>
</tr>
<tr>
<td>Pomace</td>
<td>A</td>
<td>109.7 ± 1.7e-g</td>
<td>88.9</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>127.7 ± 3.9ef</td>
<td>92.5</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>130.4 ± 0.5e</td>
<td>90.0</td>
</tr>
</tbody>
</table>
Table 2. Concentration of Polyphenolics in Fresh, Freeze Dried and Oven Dried Cranberry Pomace

<table>
<thead>
<tr>
<th>Pomace Type</th>
<th>Anthocyanins</th>
<th>Glycosides</th>
<th>Aglycones</th>
<th>Oligomers</th>
<th>Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>362.5 ± 36.6a</td>
<td>160.0 ± 20.2b</td>
<td>119.0 ± 10.1b</td>
<td>332.7 ± 18.9d</td>
<td>2629.1 ± 85.0a</td>
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<tr>
<td>Freeze Dried</td>
<td>366.1 ± 8.6a</td>
<td>191.1 ± 1.6a</td>
<td>121.4 ± 0.7b</td>
<td>556.5 ± 12.6a</td>
<td>1860.5 ± 59.5bc</td>
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<tr>
<td>Dried (40°C)</td>
<td>380.7 ± 18.1a</td>
<td>152.2 ± 7.0b</td>
<td>137.0 ± 9.8b</td>
<td>498.1 ± 0.8b</td>
<td>1954.4 ± 36.2b</td>
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<tr>
<td>Dried (60°C)</td>
<td>342.9 ± 7.8a</td>
<td>137.9 ± 2.4b</td>
<td>163.6 ± 3.4a</td>
<td>462.9 ± 8.0c</td>
<td>1771.8 ± 32.0c</td>
</tr>
<tr>
<td>Dried (80°C)</td>
<td>365.9 ± 11.5a</td>
<td>149.7 ± 0.8b</td>
<td>161.4 ± 6.5a</td>
<td>503.0 ± 4.8b</td>
<td>1939.8 ± 44.1bc</td>
</tr>
</tbody>
</table>
Figure 1

Figure 2
Figure 3
Figure 4
IV. PROXIMATE AND POLYPHENOLIC CHARACTERIZATION OF CRANBERRY POMACE

A. ABSTRACT

The proximate composition and identification and quantification of polyphenolic compounds in dried cranberry pomace were determined. Proximate analysis was conducted based on AOAC methods for moisture, protein, fat, dietary fiber, and ash. Other carbohydrates were determined by the difference method. Polyphenolic compounds were identified and quantified by HPLC-ESI-MS. The composition of dried cranberry pomace was 4.5% moisture, 2.2% protein, 12.0% fat, 65.5% insoluble fiber, 5.7% soluble fiber, 8.4% other carbohydrates, 1.1% ash, and 0.6% total phenolics. It contained six anthocyanins (111.5 mg/100 g DW) including derivatives of cyanidin and peonidin. Thirteen flavonols were identified (358.4 mg/100 g DW), and the aglycones myricetin (55.6 mg/100 g DW) and quercetin (146.2 mg/100 g DW) were the most prominent. Procyanidins with degrees of polymerization (DP) of 1 – 6 were identified (167.3 mg/100 g DW), the most abundant being an A-type of DP2 (82.6 mg/100 g DW).

Keywords: Anthocyanins; cranberry; flavonoids; flavonols; polyphenolics; pomace; procyanidins
B. INTRODUCTION

Cranberry pomace is the main by-product of the cranberry processing industry. It is composed primarily of skin, seeds, and stems left over after pressing the fruit for juice or preparing it for canning. Cranberry pomace has limited applications in animal feeds because of its low protein content, and it presents environmental problems when it is disposed of in the soil or landfills because of its low pH \((1)\). It is important to the cranberry processing industry that alternate uses of cranberry waste material be evaluated.

Fruits, including cranberries, are often touted for their health benefits \((2)\). Historically, cranberry juice has been consumed to prevent urinary tract infections. These and other health benefits, including reduced risks of cancer and cardiovascular disease, are believed to be due to the presence of various polyphenolic compounds, including anthocyanins, flavonols, and procyanidins \((3, 4)\). Given that the seeds and skins of fruits are known to contain significant levels of polyphenolics, cranberry pomace, which includes seeds and skins, should be explored as a source of these compounds.

Anthocyanins are pink, blue, red, or purple pigments that are present primarily in epidermal tissues of fruits and vegetables \((5)\). They may be present in different forms based on pH, which, in turn, determines whether they are colored or uncolored. They are generally glycosylated with various sugars that increase their stability. The aglycone forms of anthocyanins, known as anthocyanidins, are very unstable. There are six major anthocyanidins naturally present, and the two commonly found in cranberries, cyanidin and peonidin are presented in Figure 1a and 1b.
Flavonols are a class of flavonoids that are characterized by a hydroxyl group at position three, a carbonyl group at position four, and a double bond between carbons two and three on the C ring of the flavan structure. Quercetin and myricetin are the two flavonols commonly found in cranberries (Figure 1c and 1d). Like anthocyanins, flavonols are typically glycosylated. They may also be acylated with phenolic acids (6). Unlike anthocyanins, flavonols are typically colorless, but they absorb light strongly in the UV range and appear yellow at high concentrations. Cranberries contain particularly high levels of derivatives of the flavonol quercetin compared to other fruits.

Procyanidins are a class of polymeric flavonoids composed of flavan-3-ol units, primarily epicatechin, which may be converted to cyanidin upon heating with acid (7). Epicatechin monomers, in turn, may be linked together by one of two options. The most common linkages between the flavan rings are the $4\beta \rightarrow 8$ or $4\beta \rightarrow 6$ which are B-type linkages (Figure 1e). The A-type linkage is less common and consists of both $4\beta \rightarrow 8$ and $2\beta \rightarrow O \rightarrow 7$ linkages (Figure 1f) (8). Degree of polymerization (DP) in reference to procyanidins is the number of monomers linked together through either A- or B-type linkages to form oligomers or polymers.

Cranberries contain high levels of procyanidins, which have been shown to have antioxidant properties due to their reducing ability (7). They are also able to bind proteins and metal ions, which may further contribute to their health benefits (7). Procyanidins from cranberry have been recognized for anticancer properties and inhibition of bacterial adhesion to the urinary tract and human gastric mucus (9, 10).

Although much work has been done to determine polyphenolic compounds and their quantities present in fresh cranberry fruit, there is little qualitative or quantitative
information on the compounds that remain in the pomace. Identification and subsequent quantification of the polyphenolics present in cranberry pomace could lead to further studies on extraction of the compounds to be used as nutraceuticals or as natural additives to juices and other beverages, therefore adding the aforementioned health benefits to these products. This paper outlines the proximate composition as well as the identification and quantification of the major polyphenolic compounds present in cranberry pomace.

C. MATERIALS AND METHODS

Chemicals. HPLC-grade acetone, methanol, acetonitrile, acetic acid, and formic acid were obtained from EMD Biosciences (Madison, WI). Sephadex LH-20 was purchased from Sigma Chemical Co. (St. Louis, MO).

Reference compounds. Anthocyanin standards of cyanidin-3-glucoside and peonidin-3-glucoside were purchased from Polyphenols Laboratories (Sandnes, Norway). A standard consisting of procyanidin oligomers purified from cocoa was obtained from Masterfoods (Hacketstown, NJ). Quercetin and myricetin standards were purchased from Sigma Aldrich (St. Louis, MO).

Sample. Dried cranberry pomace was obtained from Decas Cranberry Company (Carver, MA, USA). The pomace was a blend of several cranberry varieties. It was ground to a powder by passing through a 1000 μm sieve screen using a Cyclone Sample Mill (Udy, Fort Collins, CO) and stored at -70 C until analysis.

Proximate analysis. Ground cranberry pomace was analyzed for proximate composition by AOAC methods for moisture (925.10), crude protein (960.52), crude fat (920.39), ash (900.02), and dietary fiber (985.29). Other carbohydrates were determined
according to the difference method by subtracting the other components from 100. All analyses were performed in triplicate and values averaged.

**Extraction of polyphenolics.** Polyphenolics from cranberry pomace were extracted with acetone:water:acetic acid (70:29.5:0.5 v/v/v) using a Euro Turrax Tissuemizer (Tekmar-Dohrmans Corp., Mason, OH). Samples (1 g) were added to 20 mL of solvent, homogenized for 1 min, and filtered through Miracloth (Calbiochem, LaJolla, CA). The residue was collected and two more extractions were performed. The filtrates were pooled, and the volume of the extract was adjusted to 100 mL with extraction solvent. Extracts were stored at -70 C until analysis. Extractions were performed in triplicate.

**Sephadex LH-20 isolation of procyanidins.** A cleanup step was conducted according to the methods of Gu et al. (11) to eliminate interfering compounds for analysis of procyanidins. Three g of Sephadex LH-20 was hydrated for 4 h and packed into a 6-x 1.5-cm column. The column was attached to a Sep-Pak vacuum manifold (Waters Corp., Milford, MA) connected to a vacuum pump. Polyphenolic extract (10 mL) was concentrated using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY) to remove the acetone from the solvent mixture prior to cleanup. Concentrated extracts were loaded onto the column and eluted with 40 mL of aqueous methanol (30% v/v) to remove sugars, phenolic acids, anthocyanins, and flavonols; this fraction was discarded. The columns were then eluted with 80 mL aqueous acetone (70% v/v); this fraction, containing procyanidins, was collected.

**Reverse-phase high-performance liquid chromatography (HPLC) analysis of anthocyanins.** Anthocyanins were analyzed with a Waters Alliance model Delta 600 HPLC (Waters Corp., Milford, MA), equipped with a model 600 pump, model 717 Plus
auto sampler, and a model 2996 photodiode array detector. Polyphenolic extract (8 mL) was concentrated, re-suspended in 1 mL of 3% formic acid, and filtered through a 0.45 µm filter prior to HPLC analysis. Compounds were separated on a 4.6- x 250-mm Symmetry® C18 column (Waters Corp., Milford, MA) using the reverse-phase HPLC method described by Cho et al. (12). The mobile phase consisted of a linear gradient of 5% formic acid (A) and methanol (B) from 2% to 60% B for 60 min with a flow rate of 1 mL/min. Anthocyanins were detected at 520 nm and were quantified using external calibration curves of a mixture of anthocyanin glucoside standards (Polyphenols Laboratories, Sandnes, Norway).

Reverse-phase analysis of flavonols. Flavonols were analyzed with a Waters HPLC system. Polyphenolic extract (8 mL) were concentrated, re-suspended in 1 mL of 50% methanol, and filtered through a 0.45-µm filter prior to HPLC analysis. Compounds were separated using an Aqua® 5-µm, 250- x 4.6-mm C18 column (Phenomenex, Torrence, CA) using reverse-phase HPLC. A binary gradient consisted of 2% acetic acid (A) and 0.5% acetic acid in water:acetonitrile (1:1 v/v) (B). The linear gradient consisted of 0 to 50 min, 10 to 55% B; 50 to 60 min, 55 to100% B; 60-65 min, 100 to 10% B; 65 to 70 min, 10% B isocratic. The peaks were monitored at 360 nm, and compounds were quantified as quercetin or myricetin equivalents using external calibration curves of authentic standards.

HPLC analysis of procyanidins. Procyanidins were analyzed with a Waters Alliance 2690 HPLC (Waters Corp., Milford, MA) equipped with a Waters Model 474 fluorescence detector according to the method of Hammerstone et al. (13) with slight modifications. The eluent from the isolation of procyanidins was collected, concentrated,
and re-suspended in 2 mL of acetone, water, and acetic acid (70:29.5:0.5 v/v/v) and then filtered through a 0.45 µm nylon filter prior to HPLC analysis. Compounds were separated using a 5-µm, 250- x 4.6-mm Luna silica column (Phenomenex, Torrence, CA, USA). The binary gradient consisted of dichloromethane, methanol, water, and acetic acid ((A) 82:14:2:2; v/v/v/v) and methanol, water, and acetic acid ((B) 96:2:2; v/v/v). The gradient was as follows: 0 to 20 min, 0 to 11.7% B; 20 to 50 min, 11.7 to 25.6% B; 50 to 55 min, 25.6 to 87.7% B; 55 to 65 min, 87.7% B isocratic; 65 to 70 min, 87.7 to 0% B. This was followed by a 5 min equilibration period. The peaks were monitored by fluorescence detection with excitation at 276 nm and emission at 316 nm, and compounds were quantified based on an external calibration curve of a mixture of standard procyanidins purified from cocoa, which ranged from monomers to decamers (14).

**High-performance liquid chromatography – electrospray ionization – mass spectrometry (HPLC-ESI-MS) of polyphenols.** Individual anthocyanins and flavonols were identified by HPLC-ESI-MS using an HP 1000 series HPLC connected to a Bruker Esquire 2000 quadrapole ion trap mass spectrometer (Bruker Daltronics, Billerica, MA). HPLC separations of the compounds were conducted as previously described. Data were collected in the positive ion electrospray mode for anthocyanin analysis and in the negative ion electrospray mode for flavonols. Conditions of the mass spectrometer were as described by Cho et al. (12).

Procyanidins were also identified by HPLC-ESI-MS using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a UV-vis detector. HPLC separations of the compounds were conducted as previously described. The HPLC was
connected to a Bruker Esquire ion trap mass spectrometer (Bruker Daltronics). Conditions of the mass spectrometer were as described by Gu et al. (8).

Matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry (MALDI-TOF-MS) of procyanidins. The procyanidin extract resulting from Sephadex LH-20 solid phase extraction was mixed in a 1:1 ratio with 1 M dihydroxybenzoic acid (DHB) in 90% methanol. The sample (2 µL) was spotted onto a ground stainless steal MALDI target using the dry droplet method. Analysis was conducted using a Bruker Reflex III MALDI-TOF-MS. The system was equipped with a 337 nm N₂ laser, and data were obtained in the positive ion reflectron mode.

D. RESULTS AND DISCUSSION

Proximate analysis. Cranberry pomace was analyzed for moisture, crude protein, crude fat, and ash. Total carbohydrates were determined by the difference method. The results of proximate analysis are presented in Table 1. The pomace was composed mainly of insoluble dietary fiber (65.5%) with smaller portions of fat, soluble fiber, protein, and ash. This was consistent with studies performed on cranberry cell wall material, which found that the majority of the cell wall consisted of cellulose, pectin, and hemicellulose with minor levels of protein, fat, starch, and ash (14). The relatively high fat content of cranberry pomace (12.0%) can be attributed to oil from the cranberry seeds and wax present on the skins (15).

Anthocyanins. Six anthocyanin glycosides were identified by HPLC-ESI-MS in cranberry pomace. The HPLC chromatogram (Figure 4) indicated the presence of six peaks with baseline separation. Mass spectral data used for peak identification and quantification of individual anthocyanins are presented in Table 2. The anthocyanins
identified were the galactosides, glucosides, and arabinosides of cyanidin and peonidin. Cyanidin 3-arabinoside was the most prominent anthocyanin, followed by peonidin 3-arabinoside, peonidin 3-galacoside, and cyanidin 3-galactoside. Minor amounts of peonidin 3-glucoside and cyanidin 3-glucoside were also observed. This was consistent with anthocyanins identified in fresh cranberries by Wu and Prior (16).

Peaks with the same mass spectral fragmentation properties were identified based on HPLC elution order. On the C18 column used for anthocyanin separation, compounds eluted in order of decreasing polarity; therefore, the galactoside of cyanidin eluted first, followed by its glucoside then arabinoside. This preceded the galactoside, glucoside, and arabinoside of peonidin. The pomace contained a total of 121.4 ± 5.9 mg/100 g DW anthocyanins. We analyzed fresh cranberries of the variety either Howe or Early Black and found that they contained 562 mg/100 g DW of total anthocyanins. Values in the pomace, however, were comparable to a spray-dried cranberry extract, which contained 160 mg/100g DW (16).

Some anthocyanins that are present in fresh cranberries, but do not remain in the cranberry pomace, are expressed in the cranberry juice; however, others may be degraded by heat or enzymes during the mashing step. Anthocyanins are very heat labile (17) and may have been degraded due to the heat applied during juice processing. Fresh fruits contain polyphenol oxidase, an enzyme that is responsible for tissue browning by conversion of phenolic substances to quinones which polymerize to give brown pigments. Upon crushing of fruit during juicing, polyphenol oxidase comes into contact with phenolics, which are released from the vacuoles, and causes degradation. Anthocyanins can also be degraded by pectinase enzymes, which are used in the juicing industry to
improve juice extraction. Wightman and Wrolstad (18) found that enzyme preparations used in cranberry juicing contain glycosidase activity, specifically β-galactosidase activity, capable of destabilizing the anthocyanins by producing aglycones.

**Flavonols.** Cranberries have one of the highest flavonol contents of all fruits and berries (19). The major flavonol in both freeze-dried cranberry powder and fresh fruit is quercetin-3-galactoside, followed by quercetin-3-rhamnopyranoside and quercetin-3-arabinofuranoside in freeze-dried powder and fresh fruit, respectively (20, 21).

Thirteen flavonols were identified in cranberry pomace by HPLC-ESI-MS (Table 3 and Figure 5). Most of the flavonols identified were glycosides of myricetin and quercetin; however, high amounts of myricetin and quercetin as aglycones were identified (55.6 and 146.2 mg/100 g DW, respectively). Fresh fruit analyzed in our lab (Early Black or Howe) contained only 3.71 and 3.65 mg/100 g DW of myricetin and quercetin, respectively. Since these compounds were not present in high levels in the fresh fruit, they must be a result of processing. As previously mentioned, pectinase enzymes are often used during juice processing to improve juice yield. These pectinase enzyme mixtures have glycosidase activity capable of cleaving the sugar from the flavonol glycoside, leaving the aglycone (18). Unlike anthocyanin aglycones, flavonol aglycones are stable enough to be observed by HPLC. Aglycones of myricetin and quercetin were also found in a processed cranberry powder (20).

Additionally, two unique flavonols, quercetin coumaryl galactoside and quercetin benzoyl galactoside, both acylated flavonols, were found in cranberry pomace. These flavonols are not typically found in fresh cranberries, and are also likely formed as a result of processing. Quercetin coumaryl galactoside was found in *Ledum palustre* L.
(22), whereas quercetin benzoyl galactoside has been reported in a processed cranberry powder (20) and only recently for the first time in fresh cranberries (24). Mass spectral data indicate two flavonols with fragmentation patterns corresponding to quercetin 3-arabinoside in cranberry pomace, indicating different conformations of the same flavonol. Vvedenskaya et al. (20) identified quercetin 3-arabinoside in both the pyranose and furanose forms in cranberry powder using nuclear magnetic resonance.

The pomace contained 358.4 ± 16.3 mg/100 g DW total flavonols. To compare this to the fresh fruit, fresh cranberries (Early Black or Howe) were analyzed in our lab for flavonols and found to contain 651.5 mg/100 g DW. This suggests that more than half of the flavonols from fresh cranberries were not expressed in the juice; that is, they were retained in the pomace, and are stable during processing.

**Procyanidins.** Cranberry pomace was analyzed for the type and size of procyanidins present. The HPLC profile with fluorescence detection of procyanidins in cranberry pomace is presented in Figure 6, and data on the identification and quantification of procyanidins can be found in Table 4. The HPLC chromatogram indicates the presence of a small amount of monomeric (-) – epicatechin (DP1), which was confirmed using an external standard. The mass spectral data (Figure 7) indicate the presence of both A-type and B-type linkages in the procyanidins. An A-type dimer was the primary procyanidin, with minor levels of A-type trimers, tetramers, and higher oligomers. These findings were consistent with Gu et al. (8) who used LC-MS/MS on an extract from whole cranberries to identify several procyanidin oligomers in cranberry containing A-type linkages and the location of the linkage. The researchers also performed thiolytic degradation of procyanidins in the cranberries to determine their DP.
Excluding the monomeric flavan-3-ols, the average DP was 8.5, and including the monomers, the DP was 8.3. Using LC-MS/MS, the researchers were also able to identify several procyanidin oligomers in cranberry containing A-type linkages as well as the location of the linkage.

MALDI-TOF-MS was also used to identify procyanidins in cranberry pomace (Figure 8). This allowed for identification of high molecular weight procyanidins present in the pomace, (DP4 – DP13), which could not be identified by ESI-MS due to their size. Each of the peaks represent sodium adducts of procyanidin oligomers containing varying amounts of A-type linkages. The compound with a molecular weight of 1173.3 represents a sodium adduct of a DP4 procyanidin containing two A-type linkages, whereas the compound with molecular weight 1175.3 represents a sodium adduct of a DP4 procyanidin containing only one A-type linkage. A compound containing an A-type linkage is 2 mass units smaller than the same DP procyanidin containing only B-type linkages, and each additional A-type linkage would result in a loss of 2 more mass units. MALDI-TOF-MS also revealed the presence of DP5 through DP12 procyanidins containing two A-type linkages. Additionally, the compound with the molecular weight 3768.5 represents the sodium adduct of a DP13 procyanidin containing no A-type linkages. Reed et al. (25) also identified procyanidins in a cranberry presscake that were fractionated using Sephadex LH-20. They identified procyanidins containing DP5 – DP23 in the aqueous acetone fraction, whereas we identified procyanidins with DP4 – DP13 in the aqueous acetone fraction. They also identified procyanidins in the aqueous methanol fraction; however, we discarded this fraction. They also identified compounds that were 16 mass units greater than predicted
procyanidins indicating the possibility of oligomers containing one epigallocatechin unit. However, our MALDI-TOF MS data did not indicate the presence of any such compounds. Our MALDI-TOF spectrum more closely resembles the one presented by Neto et al. (26).

The pomace contained 167.3 mg/100 g of procyanidins DP1 – DP6 on a dry pomace weight basis. These data may not be representative of all of the procyanidins present in cranberry pomace. Recent studies have indicated that many of the procyanidins are highly bound to plant cell wall material, and, therefore, unextractable (23, 24). Since cranberry pomace is composed primarily of dietary fiber (cell wall material), it is possible that many of the polyphenolic compounds, including procyanidins may be enclosed within this portion of the pomace. Therefore, many of the polyphenolics may be destined for the gastrointestinal tract. Some studies have attempted to measure the amount of unextractable procyanidins in plant products using acid catalyzed degradation by butanol:HCl or thiolysis and have found that a significant amount of procyanidins in many plant materials are indeed unextractable under normal extraction conditions (29, 30, 31). Therefore, additional extraction procedures need to be developed that enhance the extraction of procyanidins.

E. ACKNOWLEDGEMENTS

We thank Decas Cranberry Company, Inc. for providing the cranberry pomace used in this study. We also thank Rohana Liyanage and the University of Arkansas Statewide Mass Spectrometry Facility for assistance and use of the MALDI-TOF instrument.
F. LITERATURE CITED


2. Kahlon, T.S.; Smith, G.E. In vitro binding of bile acids by blueberries (Vaccinium spp.), plums (Prunus spp.), prunes (Prunus spp.), strawberries (Fragaria X ananassa), cherries (Malpighia punicifolia), cranberries (Vaccinium macrocarpon) and apples (Malus sylvestris). Food Chemistry, 2007, 100, 1182-1187.


G. FIGURE CAPTIONS

**Figure 1.** Structures of common polyphenolics found in cranberries. a) cyanidin b) Peonidin c) quercetin d) myricetin e) procyanidin dimer exhibiting a B-type linkage f) procyanidin dimer exhibiting an A-type linkage.

**Figure 2.** HPLC chromatogram of anthocyanins in cranberry pomace identified by HPLC-ESI-MS and detected at 520 nm

**Figure 3.** HPLC chromatogram of flavonols in cranberry pomace identified by HPLC-ESI-MS and detected at 360 nm

**Figure 4.** HPLC chromatogram of procyanidins in cranberry pomace identified by HPLC-ESI-MS and detected by fluorescence with ex. 276 and em. 316.

**Figure 5.** HPLC-ESI-MS spectra of procyanidins in cranberry pomace (DP2 – DP6) obtained in the negative ion mode.

**Figure 6.** MALDI-TOF-MS spectrum of procyanidins in cranberry pomace.
Table 1. Proximate Composition of Cranberry Pomace

<table>
<thead>
<tr>
<th>Component</th>
<th>Crude Content (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>4.5</td>
</tr>
<tr>
<td>Protein</td>
<td>2.2</td>
</tr>
<tr>
<td>Fat</td>
<td>12.0</td>
</tr>
<tr>
<td>Ash</td>
<td>1.1</td>
</tr>
<tr>
<td>Insoluble Fiber</td>
<td>65.5</td>
</tr>
<tr>
<td>Soluble Fiber</td>
<td>5.7</td>
</tr>
<tr>
<td>Other carbohydrates&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.4</td>
</tr>
<tr>
<td>Total Phenolics</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Carbohydrate content was determined by the difference method.
Table 2. Peak Identification, Retention Times, Mass Spectral Data, and Quantification of Anthocyanin Glycosides<sup>a</sup> Detected in Cranberry Pomace Using HPLC-ESI-MS

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>Anthocyanin</th>
<th>Mass to charge ratios (m/z)</th>
<th>Concentration (mg/100g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.6</td>
<td>Cyanidin-3-galactoside</td>
<td>449 [M+H]&lt;sup&gt;+&lt;/sup&gt; 287 [M – galactose]</td>
<td>13.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>32.2</td>
<td>Cyanidin-3-glucoside</td>
<td>449 287 [M – glucose]</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>33.6</td>
<td>Cyanidin-3-arabinoside</td>
<td>419 287 [M – arabinose]</td>
<td>49.6 ± 6.8</td>
</tr>
<tr>
<td>4</td>
<td>35.2</td>
<td>Peonidin-3-galactoside</td>
<td>463 301 [M – galactose]</td>
<td>20.1 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>36.7</td>
<td>Peonidin-3-glucoside</td>
<td>463 301 [M – glucose]</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>38.1</td>
<td>Peonidin-3-arabinoside</td>
<td>433 301 [M – arabinose]</td>
<td>26.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>121.4 ± 5.9</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data expressed as equivalents of cyanidin or peonidin glucosides.

<sup>b</sup> Mean ± standard deviation (n = 3).
### Table 3. Peak Identification, Retention Times, Mass Spectral Data, and Quantification of Flavonols
detected in Cranberry Pomace Using HPLC-ESI-MS

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>Flavonol</th>
<th>Mass to charge ratios (m/z)</th>
<th>Concentration (mg/100g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.4</td>
<td>Myricetin 3-xyloside</td>
<td>449 316 [M – xylose]</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>37.3</td>
<td>Myricetin 3-arabinoside</td>
<td>449 316 [M – arabinose]</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>38.3</td>
<td>Quercetin 3-galactoside</td>
<td>463 300 [M – galactose]</td>
<td>12.8 ± 3.6</td>
</tr>
<tr>
<td>4</td>
<td>41.0</td>
<td>Quercetin 3-xyloside</td>
<td>433 300 [M – xylose]</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>42.0</td>
<td>Quercetin 3-arabinopyranoside</td>
<td>433 300 [M – arabinose]</td>
<td>15.2 ± 3.6</td>
</tr>
<tr>
<td>6</td>
<td>43.4</td>
<td>Quercetin 3-arabinofuranoside</td>
<td>433 300 [M – arabinose]</td>
<td>16.7 ± 3.5</td>
</tr>
<tr>
<td>7</td>
<td>44.2</td>
<td>Quercetin 3-rhamnoside</td>
<td>447 300 [M – rhamnose]</td>
<td>18.5 ± 3.4</td>
</tr>
<tr>
<td>8</td>
<td>48.0</td>
<td>Myricetin</td>
<td>317 315 [M – xylose]</td>
<td>55.6 ± 2.6</td>
</tr>
<tr>
<td>9</td>
<td>49.0</td>
<td>Methoxyquercetin 3-xyloside</td>
<td>447 300 [M – xylose + methoxy]</td>
<td>11.4 ± 3.7</td>
</tr>
<tr>
<td>10</td>
<td>55.1</td>
<td>Quercetin 3-coumaroyl galactoside</td>
<td>609 463 [M - galactose]</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>11</td>
<td>55.7</td>
<td>Unidentified</td>
<td>583 316</td>
<td>12.1 ± 3.5</td>
</tr>
<tr>
<td>12</td>
<td>58.1</td>
<td>Quercetin</td>
<td>300 (quercetin)</td>
<td>146.2 ± 22.7</td>
</tr>
<tr>
<td>13</td>
<td>58.7</td>
<td>Quercetin 3-benzoyl galactoside</td>
<td>567 300</td>
<td>27.5 ± 3.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>358.4 ± 16.3</strong></td>
</tr>
</tbody>
</table>

\(^a\) Flavonols are expressed as quercetin or myricetin equivalents.  
\(^b\) Mean ± standard deviation (n = 3).
<table>
<thead>
<tr>
<th>Peak</th>
<th>Procyanidin</th>
<th>Mass to Charge ratio (m/z)</th>
<th>Concentration (mg/100g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>Monomers (catechin/epicatechin)</td>
<td>289</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>DP2 (A)</td>
<td>A-type dimer</td>
<td>575</td>
<td>82.6 ± 2.3</td>
</tr>
<tr>
<td>DP2 (B)</td>
<td>B-type dimer</td>
<td>577</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>DP3 (A)</td>
<td>A-type trimer</td>
<td>863</td>
<td>30.8 ± 2.5</td>
</tr>
<tr>
<td>DP3 (B)</td>
<td>B-type trimer</td>
<td>865</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>DP4 (B)</td>
<td>B-type tetramer</td>
<td>1153</td>
<td>22.9 ± 3.6</td>
</tr>
<tr>
<td>DP5 (A)</td>
<td>A-type pentamer</td>
<td>1439</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>DP6 (A)</td>
<td>A-type Hexamer</td>
<td>1721</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td><strong>Total Oligomers</strong></td>
<td></td>
<td><strong>167.3 ± 5.9</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Means ± standard deviation (n=3)*
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
V. POLYPHENOLIC COMPOSITION AND ANTIOXIDANT CAPACITY OF EXTRUDED CRANBERRY POMACE

A. ABSTRACT

Cranberry pomace was mixed with corn starch in various ratios (30/70, 40/60, 50/50 pomace/corn starch DW) and extruded using a twin-screw extruder at three temperatures (150, 170, 190 °C) and two screw speeds (150, 200 rpm). Changes in the anthocyanin, flavonol, and procyanidin contents due to extrusion were determined by HPLC. Antioxidant capacity of the extrudates was determined using Oxygen Radical Absorbance Capacity (ORAC). Anthocyanin retention was dependent upon barrel temperature and percent pomace. The highest retention was observed at 150 °C and 30% pomace. Flavonols increased by 30 to 34% upon extrusion compared to an un-extruded control. ORAC values increased upon extrusion at 170 and 190 °C. An increase in DP1 and DP2 procyanidins was also observed; however, a decrease was observed in DP4 through DP9 oligomers. These data suggest that extrusion alters the polyphenolic distribution of cranberry pomace and has application in the nutraceutical industry as a means of improving the functionality of this co-product.

Keywords: Anthocyanins; cranberry; extrusion; flavonols; pomace; procyanidins
B. INTRODUCTION

Cranberry pomace is the by-product of the cranberry processing industry and is composed of skin, seeds, and stems, which remain after the fruit has been pressed for juice or prepared for canning. Applications for cranberry pomace are limited. Its low protein content makes it unsuitable for animal feeds, and its low pH present problems when it is disposed of in the soil (1).

Plant foods have been recognized for their health benefits including reduced risk of cancer and cardiovascular disease. Cranberries in particular are recognized for their ability to prevent urinary tract infections (2). These benefits have been attributed to the presence of polyphenolic compounds such as anthocyanins, flavonols, and procyanidins (3, 4). These compounds are found primarily in the seeds and skins of the fruit; thus, many are retained in the pomace. Cranberry pomace, therefore, should be explored as a source of polyphenolic compounds.

Anthocyanins are pigmented compounds present in the epidermal tissues of fruits and vegetables (5). There are six major anthocyanins found in cranberries, and they include the glucosides, galactosides, and arabinosides of cyanidin and peonidin (6). Minor anthocyanins that might be present in some cranberry varieties include glycosides of malvidin, petunidin, delphinidin, and pelargonidin. Flavonols are another class of polyphenolic compounds present in cranberries. Flavonols impart bitter and astringent flavors to fruits and vegetables (7). Cranberries contain relatively high levels of glycosides and other derivatives of the flavonol quercetin, compared to other fruits. As many as 22 different flavonol glycosides have been identified in cranberries including primarily derivatives of quercetin and myricetin, and two kaempferol derivatives (8).
Procyanidins are a class of polymeric compounds composed of flavan-3-ol monomeric units. These monomeric units may be linked in two ways. The most common linkages are called B-type and are $\beta_4 \rightarrow 6$ or $\beta_4 \rightarrow 8$. The less common A-type linkage contains both a $\beta_4 \rightarrow 8$ and a $\beta_2 \rightarrow O \rightarrow 7$ linkage. Cranberries are unique in that they contain many procyanidins with A-type linkages (9). It is the A-type linkages in cranberry procyanidins that are believed to be responsible for cranberries’ ability to prevent urinary tract infections by inhibiting bacteria from adhering to the epithelial lining of the urinary tract (10).

A majority of the procyanidins found in cranberries have a high degree of polymerization (DP) (11). Donovan and others (12) and Gonthier and others (13) have shown that although high DP procyanidins are good antioxidants, they are poorly absorbed relative to their monomeric subunits. Although typically present in smaller quantities, monomers, and even dimers and trimers are better absorbed and maintain similar health benefits (14, 15).

Extrusion has become a popular food processing technique especially in the cereal and snack food industry. It is considered a high-temperature-short-time (HTST) processing method and is capable of preserving desirable food components and destroying microorganisms. The end products are typically low moisture, which allows them to be shelf-stable (16). Extrusion may include several different operations; however, the simplest definition is the forcing of a dough-like material through a restriction or die. Other functions of an extruder can include, but are not limited to homogenization, shearing, thermal cooking, gelatinization, and texture alteration (17). Extrusion presents mechanical stress in the form of heat and shear to the substrates,
which may alter physical as well as chemical characteristics of the product. Since extrusion is a common food processing practice, many studies have been done to determine its effects on the nutritional aspects of food including flavonoids, which are known to be heat sensitive; these effects may be beneficial or detrimental.

The objectives of this research were to determine the effect of extrusion processing on the anthocyanin, flavonol, and procyanidin composition of cranberry pomace mixed with corn starch. Additionally, the effect of extrusion on the antioxidant capacity of cranberry pomace was evaluated. This research aims to investigate an alternative use of cranberry pomace by improving its functionality.

C. MATERIALS AND METHODS

**Sample Preparation.** Dried cranberry pomace (Decas Cranberry Company, Carver, MA) was stored at -20 °C prior to extrusion. For extrusion, the pomace was mixed with corn starch (National Starch, Bridgewater, NJ) in ratios of 30/70, 40/60, and 50/50 cranberry pomace/corn starch on a dry weight basis and mixed using an industrial kitchen mixer (Hobart, Troy, OH). Corn starch served as facilitator for extrusion and allowed extrudates to expand upon exiting the die. Water was added to the mixtures to bring the moisture content to 30%, and they were mixed again. Mixtures were stored at 4 °C overnight prior to extrusion to allow for moisture equilibration.

**Extrusion.** Cranberry pomace – corn starch mixtures were extruded using a PolyLab-scale Rheomix twin-screw extruder (Thermo Haake, Karlsruhe, Germany) equipped with a 6-mm capillary rod die. The barrel temperature of zone one was set at 90 °C for all extrusion runs to prevent moisture loss upon introducing the mixture to the extruder. The mixtures were continuously fed manually into the extruder. Extrudates
were collected with extruder barrel and die temperatures of 150, 170, and 190 °C and screw speeds of 150 and 200 rpm. Extrudates were allowed to cool, placed in sealed bags, and stored at -20 °C until analysis. Moisture content of the extrudates was determined by the AOAC oven method.

**Extraction of polyphenonics from extrudates.** Extrudates were ground using a commercial coffee grinder prior to extraction of polyphenolics. Ground Extrudates (1 g) were mixed with 20 mL of acetone:water:acetic acid (70:29.5:0.5 v/v/v), homogenized with a T18 Basic Ultra-Turrax® homogenizer (IKA WORKS, Wilmington, NC, USA), and filtered through Miracloth. Two more extractions were performed, the extracts were pooled, and the volume adjusted to 100 mL with extraction solvent.

**Sephadex LH-20 isolation of procyanidins.** Procyanidins were isolated from the extracts according to the method described by Gu et al. (18). This was done to prevent the interference of other compounds, such as anthocyanins, sugars, and flavonols, during HPLC analysis. Briefly, 10 mL of extract were concentrated using a SpeedVac® vacuum concentrator (ThermoSavant, Holbrook, NY) to remove the acetone. The remaining aqueous extract (3 mL) was loaded onto a manually packed 6- by 1.5-cm column containing 3 g of Sephadex LH-20 (hydrated for at least 4 h). The column was attached to a Sep-Pak vacuum manifold (Waters Corp., Milford, MA) and vacuum pump. The column was flushed with 40 mL of 30% methanol, and this fraction was discarded. The procyanidins were then eluted from the column with 80 mL of 70% acetone. This fraction was collected for HPLC analysis.

**HPLC analysis of procyanidins.** The aqueous acetone fractions resulting from the Sephadex LH-20 isolation of procyanidins were evaporated to dryness using a
SpeedVac® vacuum concentrator, re-suspended in 2mL acetone:water:acetic acid (70:29.5:0.5), and filtered through 0.45 µm filters prior to HPLC analysis. Procyanidins were separated according to the method described by Kelm et. al. (19) with slight modifications. A 5-µm, 250- by 4.6 mm Develosil diol 100A column (Phenomenex, Torrence, CA) was attached to a Waters Alliance 2690 HPLC (Waters Corp., Milford, MA) equipped with a model 474 scanning fluorescence detector. The mobile phase consisted of a binary gradient of 98:2 v/v acetonitrile:acetic acid (A) and 95:3:2 v/v/v methanol:water:acetic acid (B). The flow rate was set at 0.8 mL/min, and the gradient proceeded as follows: 0 to 35 min, 0 to 40% B; 35 to 49 min, 40% B isocratic; 49 to 50 min, 40 to 100% B; 50 to 57, 100% B isocratic; 57 to 60 min, 100 to 7% B; 60 to 70 min, 7% B isocratic. Procyanidins were monitored using fluorescence detection (ex 276 nm, em 316 nm), and monomers through nonamers were quantified based on an external calibration curve consisting of a mixture of procyanidins standards (DP1 - DP9) previously purified from cocoa (19). Results were expressed on a DW basis.

**HPLC analysis of anthocyanins.** Eight mL of polyphenolic extracts were evaporated to dryness using a SpeedVac® vacuum concentrator, re-suspended in 1 mL 3% formic acid and filtered through 0.45 µm filters prior to HPLC analysis. A 4.6- by 250-mm Symmetry® C18 column (Waters Corp., Milford, MA) was attached to a Waters Alliance Delta 600 HPLC (Waters Corp., Milford, MA) equipped with a model 2996 photodiode array detector. Separations were conducted based on the method described by Cho et al (20). The mobile phase consisted of a linear gradient of 5% formic acid (A) and methanol (B). The flow rate was set at 1.0 mL/min of 2% to 60% B for 60 min. Anthocyanins were detected at 520 nm and quantified using external calibration curves of
antocyanin glucoside standards obtained from Polyphenols (Sandnes, Norway). Results were expressed on DW basis.

**HPLC analysis of flavonols.** Eight mL of polyphenolic extracts were evaporated to dryness using a SpeedVac® vacuum concentrator, re-suspended in 1 mL 50% methanol and filtered through 0.45 μm filters prior to HPLC analysis. A 5-μm, 4.6- by 250-mm Aqua® C18 column (Phenomenex, Torrence, CA) was attached to a Waters HPLC (Waters Corp., Milford, MA) equipped with a model 996 photodiode array detector. The mobile phase consisted of a gradient of 2% acetic acid (A) and 0.5% acetic acid in 1:1 v/v water:acetonitrile (B). The flow rate was set at 1.0 mL/min and the gradient proceeded as follows: 0 to 50 min, 10 to 55% B; 50 to 60 min, 55 to 100% B; 60 to 65 min, 100 to 10% B; 65 to 70 min, 10% B isocratic. Flavonols were detected at 360 nm and quantified using external calibration curves of a quercetin or myricetin standards (Sigma Chemical Co., St. Louis, MO). Results were expressed on DW basis.

**Oxygen Radical Absorbance Capacity (ORAC).** The antioxidant capacities of cranberry pomace and extrudates were evaluated using the method described by Prior and others (21). The method was carried out using a FLUOstar Optima microplate reader (BMG Labtechnologies, Durham, NC, USA). Extracts were diluted 100-fold with phosphate buffer (7mM, pH 7) prior to analysis. Clear 48-well (590 µL each) Falcon plates (VWR, St Louis, MO, USA) were used. 40 µL of diluted sample, Trolox standards (6.25, 12.5, 25, 50 μM), and a blank solution (phosphate buffer) were added to each well. The instrument automatically injected 400 µL of fluorescein (0.108 μM) followed by 150 μL of 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH) (31.6 mM) to each well. Fluorescence was detected at 485 nm (excitation) and 520 nm (emission) after the
addition of fluorescein, AAPH, and every 192 s after for 112 min to allow for a 95% loss of fluorescence. Results were calculated based on differences between the blank, sample, and standard trolox curves. A standard curve was generated by plotting the concentrations of trolox against the area under each curve. ORAC values were calculated using the regression equation obtained and expressed as μmol trolox equivalents / g DW.

**Experimental design and statistical analysis.** Treatments were applied in a split-split plot randomized block design with extruder barrel temperature as the whole plot, percent pomace as the split-plot, and screw speed as the split-split-plot. As previously mentioned, there were three levels of extruder barrel temperature, three levels of percent pomace, and two levels of screw speed, resulting in 18 conditions. Each condition was run in triplicate to give a total of 54 extrusion runs. Levels of each factor within each plot were randomized.

Analysis of variance and separation of the means were carried out using the PROC MIXED procedure of SAS (SAS V.9.1, SAS Institute, Cary, NC). Where effects were significant, comparisons were performed using protected LSD to determine differences among treatments. Additionally, to determine if a treatment was different than the control, confidence intervals (α=0.05) of the least square means were used. If the confidence interval for the means included 1, the treatment was considered not different from the control.

**D. RESULTS AND DISCUSSION**

**Anthocyanins.** Consistent with previous reports on fresh cranberries, six anthocyanins were identified in cranberry pomace, including the glucosides, galactosides, and arabinosides of cyanidin and peonidin (6). The anthocyanin composition of the
cranberry pomace is presented in Table 1. Significant losses of total anthocyanins were observed for all extrusion conditions, and anthocyanin loss was dependent upon extruder barrel temperature and percent pomace. Differences in screw speed were not significant, and there were no significant interactions among the factors. Figure 1 shows the change in anthocyanin content for each barrel temperature. At 150 °C, 46% of anthocyanins were lost. Losses were greater with higher barrel temperatures of 170 °C and 190 °C, with only 61% and 64% losses, respectively. Degradation was observed for all individual anthocyanins; however cyanidin-3-arabinoside and peonidin-3-arabinoside were the most affected. The anthocyanin retention observed at 150 °C was much higher than anthocyanin retentions reported for extruded corn breakfast cereals containing blueberry, cranberry, raspberry, and grape powders (10%) (22), and in extruded blueberry – corn cereals (32%) (23). This discrepancy was likely due to differences in moisture content, amount of anthocyanin containing material, and extrusion conditions.

Anthocyanin losses were also dependent upon the level of pomace in the extruded mixture (Figure 2). The least loss in anthocyanins was observed in the mixture containing only 30% pomace, with only 50% of anthocyanins lost. Extrudates containing 50% pomace lost the greatest amount of anthocyanins, with only 35% retention. This suggests possible protection of anthocyanins by the starch present in the extrudate mixture.

Flavonols. The flavonol composition of the cranberry pomace is presented in Table 1. Thirteen flavonols were identified in the pomace including glycosides of myricetin and quercetin as well as the aglycones. Figure 3 shows the change in total flavonol content for each barrel temperature. An increase in total flavonols was observed
upon extrusion at all conditions when compared to an un-extruded control, but there were no significant differences among the conditions and no interactions among the factors. The extrudates contained 30 to 34% more total flavonols than the control. The greatest increase was observed in quercetin-3-rhamnoside; however, all others showed a similar increase overall. Although there has been limited research regarding the stability of flavonols, this suggests that flavonols are heat stable compared to the anthocyanins. The heat stability of quercetin in onions was demonstrated by baking and sautéing as researchers observed 7 to 25% increases in the quercetin content (24). Researchers attributed the increases to a concentration effect upon cooking. However, in this study, moisture losses were accounted for. Therefore, the increase in total flavonols may be explained otherwise. There is evidence that many flavonols may be bound to cell wall components especially after injury to the cells (25). Therefore, the apparent increase in total flavonols that we observed in this study may be explained by enhanced extraction of the compounds due to disruption of the pomace matrix upon extrusion.

**Procyanidins.** Cranberry pomace contains procyanidins with DP1 through DP9 (Table 1). Changes in procyanidin distribution due to extrusion were observed and varied according to the DP of the procyanidin. No significant differences were observed among the treatments, however, when treatments were compared to the control, differences did exist. Additionally, there were no significant interactions among the factors. Changes in content of each procyanidin oligomer at different temperatures are displayed in Figure 4. Increases in DP1 and DP2 procyanidins were observed as a result of extrusion. DP1 procyanidins increased 61 to 157%, while DP2 procyanidins increased 49 to 164%. A decrease in DP3 procyanidins was observed at 150 °C, but there were no
significant changes under any of the other extrusion conditions. A significant reduction in procyanidins with DP ≥ 4 was observed in the extrudates for all conditions. Procyanidin losses were apparent as procyanidin DP increased, with 23 to 28% losses of DP4 procyanidins and 68 to 77% losses of DP9 procyanidins.

These results are similar to those observed in extruded blueberry pomace and white sorghum mixtures, in which extrusion increased the monomer, dimer, and trimer contents of the blueberry pomace (26). Similar results were also observed in extruded sorghum grain. Procyanidins with DP ≤ 4 increased upon extrusion, whereas above DP5, a reduction in procyanidins was observed (27). A loss and redistribution of isoflavone content was observed when wheat flour was extruded with wet okara. Increases in genistin and daidzin were observed which is believed to be the result of cleavage of the malonyl group from malonyl daidzin and malonyl genistin (28). Additionally, when dark buckwheat was extruded, the HPLC profile of phenolics was altered, therefore leading researchers to believe there was change in the composition of the phenolic compounds (29). This suggests that extrusion cooking is capable of breaking covalent bonds in phenolic compounds.

Very little is known about the interaction of polyphenolics, particularly procyanidins, with other plant components. The ability of procyanidins to bind proteins is what gives many plant foods their astringency. As a fruit matures, the level of extractable procyanidin oligomers decreases significantly (30, 31). These compounds are either further metabolized or bound so strongly to other components of the plant, such as the cell wall, that they are no longer easily extracted. The latter theory is supported by research performed on procyanidins and apple cell wall material, where procyanidins
were shown to readily bind to a suspension of cell wall material, with degree of binding increasing as the degree of polymerization of the procyanidin oligomer increased (32). A method has been developed to determine the amount of un-extractable procyanidins in plant materials (33). The proportion of un-extractable procyanidins of total procyanidin content determined using this method varied among plant materials with a table grape containing 63% and a cultivar of apple containing 4.1% un-extractable procyanidins. Based on this theory of the presence of un-extractable procyanidins, it is possible that the increases we observed in low molecular weight procyanidins is due to alteration of the plant cell wall material present in the pomace, which, in turn, facilitated an increase in extraction of the compounds in extruded material. However, we cannot rule out the possibility that large molecular weight procyanidins were depolymerized to monomers and dimers in response to the heat and shear incurred during extrusion.

The increase in low molecular weight procyanidins observed in this study is important because procyanidin absorption is largely dependent on the size of the molecule. Procyanidins larger than trimers have been shown to be poorly absorbed due to their size (12). Holt and others (34) detected catechin and epicatechin as well as a procyanidin dimer in the plasma of human subjects as early as 30 min after they consumed a cocoa beverage. One study determined that cacao procyanidins decreased diabetes induced cataracts and found that levels of epicatechin and its metabolites reached its highest levels in the plasma between 1 – 2 hours, but the B2 dimer was poorly absorbed (35). However, even if large molecular weight molecules are not absorbed, they may still protect against certain diseases of the intestine, such as cancer, by inhibiting oxidation in an area that is not typically rich in dietary antioxidants (5).
Additionally, these compounds may be converted to smaller molecules such as phenolic acids by colonic microflora, which could subsequently be absorbed to provide additional health benefits (36).

**Antioxidant Capacity.** Antioxidant capacity of the extrudates and an unextruded control were determined using the ORAC assay. Cranberries are known to have a high antioxidant capacity due to the presence of flavonoids (anthocyanins, flavonols, and flavanols) and phenolic acids. Extracts from cranberries have been reported to inhibit LDL oxidation (37) and oxidative and inflammatory damage to the vascular endothelium (38). ORAC values for whole cranberry and a cranberry extract were determined to be 275 and 106 µmol TE /g dry matter, respectively (6). The ORAC value for unextruded cranberry pomace was determined to be 281.3 ± 25.8 µmol TE /g dry matter.

ORAC values were found to be dependent upon the barrel temperature of the extruder (Figure 5). ORAC values increased with increasing temperature, and extrudates produced at 170 °C and 190 °C had ORAC values of 16 and 30% higher than the control values, respectively. Similarly, the antioxidant activity of dark buckwheat extrudates was not significantly different than the raw dark buckwheat (29). However, the antioxidant capacity of an extruded snack food was negatively affected (39). The higher ORAC values of extrudates obtained at elevated barrel temperatures of 170 and 190 °C were most likely due to the formation of Maillard reaction products, which possess reducing capacity (40). The extrudates obtained at 190 °C were visibly darker than the other samples, indicating Maillard browning.
Extrusion of cranberry pomace resulted in significant losses of total anthocyanins. However, increases in flavonols and low DP (1-2) procyanidins and a decrease in high DP (4-9) procyanidins were observed. The antioxidant capacity of the extrudates increased at higher temperatures. Applications of this research could provide a use for the waste product of cranberry juicing which currently has little functionality due to its low protein content and low pH. Furthermore, it could lead to the improved functionality of polyphenolic compounds, particularly procyanidins and flavonols, of cranberry pomace. However, means of curbing the loss of anthocyanins due to heat need to be evaluated. The resulting product could be incorporated into a dietary supplement or explored as a functional snack food.

E. ACKNOWLEDGMENTS

We thank Decas Cranberry Company, Inc. for providing the cranberry pomace and National Starch for providing the corn starch used in this study.
F. LITERATURE CITED


G. FIGURE CAPTIONS

Figure 1. Change in total anthocyanin content of cranberry pomace extruded at different temperatures. Values with different letters are significantly different (p<0.05). * Indicates values that are significantly different (p<0.05) than an unextruded control.

Figure 2. Change in total anthocyanin content of cranberry pomace extruded at different percentages of pomace. Values with different letters are significantly different (p<0.05). * Indicates values that are significantly different (p<0.05) than an unextruded control.

Figure 3. Change in total flavonol content of cranberry pomace extruded at different temperatures. * Indicates values that are significantly different (p<0.05) than an unextruded control.

Figure 4. Change in procyanidin oligomer content of cranberry pomace extruded at different temperatures. * Indicates values that are significantly different (p<0.05) than an unextruded control. DP1 = monomer, DP2 = dimer, DP3 = trimer, etc.

Figure 5. Change in antioxidant capacity of cranberry pomace extruded at different temperatures. Values with different letters are significantly different (p<0.05). * Indicates values that are significantly different (p<0.05) than an unextruded control.
Table 1. Polyphenolic Content and Composition of Cranberry Pomace

<table>
<thead>
<tr>
<th>Polyphenolic Compound</th>
<th>Concentration (mg/100 g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
</tr>
<tr>
<td>Cyanidin 3-galactoside</td>
<td>13.2 ± 0.2</td>
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<tr>
<td>Cyanidin 3-glucoside</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Cyanidin 3-arabinoside</td>
<td>49.6 ± 6.8</td>
</tr>
<tr>
<td>Peonidin 3-galactoside</td>
<td>20.1 ± 0.5</td>
</tr>
<tr>
<td>Peonidin 3-glucoside</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>Peonidin 3-arabinoside</td>
<td>26.6 ± 0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>121.4 ± 5.9</strong></td>
</tr>
<tr>
<td><strong>Flavonols</strong></td>
<td></td>
</tr>
<tr>
<td>Myricetin 3-xyloside</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Myricetin 3-arabinoside</td>
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</tr>
<tr>
<td>Quercetin 3-galactoside</td>
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</tr>
<tr>
<td>Quercetin 3-xyloside</td>
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</tr>
<tr>
<td>Quercetin 3-arabinopyranoside</td>
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<tr>
<td>Quercetin 3-arabinofuranoside</td>
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</tr>
<tr>
<td>Quercetin 3-rhamnoside</td>
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<tr>
<td>Myricetin</td>
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</tr>
<tr>
<td>Methoxyquercetin 3-xyloside</td>
<td>11.4 ± 3.7</td>
</tr>
<tr>
<td>Quercetin 3-coumaroyl galactoside</td>
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</tr>
<tr>
<td>Unidentified</td>
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<tr>
<td>Quercetin</td>
<td>146.2 ± 22.7</td>
</tr>
<tr>
<td>Quercetin 3-benzoyl galactoside</td>
<td>27.5 ± 3.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>358.4 ± 16.3</strong></td>
</tr>
<tr>
<td><strong>Procyanidins</strong></td>
<td></td>
</tr>
<tr>
<td>Monomer (DP1)</td>
<td>5.12 ± 0.0</td>
</tr>
<tr>
<td>Dimer (DP2)</td>
<td>52.7 ± 1.7</td>
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<tr>
<td>Trimer (DP3)</td>
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<tr>
<td>Tetramer (DP4)</td>
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<td>Pentamer (DP5)</td>
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<tr>
<td>Octamer (DP8)</td>
<td>16.1 ± 2.9</td>
</tr>
<tr>
<td>Nonomer (DP9)</td>
<td>13.2 ± 1.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>186.5 ± 8.8</strong></td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
VI. RELEASE OF BOUND PROCYANIDINS FROM CRANBERRY POMACE BY ALKALINE HYDROLYSIS

A. ABSTRACT

Procyanidins in plant products are present as extractable or unextractable/bound forms. We optimized alkaline hydrolysis conditions to liberate procyanidins from dried cranberry pomace. Five mL of sodium hydroxide (2, 4, or 6N) was added to 0.5 g of cranberry pomace in screw top glass tubes, and the tubes were flushed with nitrogen. The tubes were then placed into a water bath set at 25, 40, or 60 °C with shaking for varying amounts of time, which depended on the temperature. Sodium hydroxide was then neutralized to pH 6 – 7 with concentrated HCl. Procyanidins were extracted with ethyl acetate and analyzed using normal phase high performance liquid chromatography (HPLC). Alkaline hydrolysis resulted in an increase in low molecular weight procyanidins, and the increase was greater at higher temperature, short time combinations. The most procyanidins (DP1 – DP3) were extracted at 60 °C for 15 m with each concentration of NaOH. When compared to conventional extraction using homogenization with solvent, treatment with NaOH increased procyanidin oligomer extraction by 3.8 to 14.9-fold, with the greatest increase being DP1 (14.9x) and A-type DP2 (8.4x) procyanidins. Treatment of the residue remaining after conventional extraction with NaOH resulted in further procyanidin extraction, indicating that procyanidins are not fully extracted by conventional extraction methods.

Keywords: Alkaline; cranberry; hydrolysis; pomace; procyanidins; unextractable
B. INTRODUCTION

Cranberries (*Vaccinium macrocarpon*) are growing in popularity due to the increasing information regarding their health benefits. Cranberry juice has long been recognized for its ability to prevent urinary tract infections; however there are several other health benefits associated with cranberries which include antioxidant, antitumor, antiulcer, anti-inflammatory, and antiatherosclerotic activities (1-4). Cranberry pomace is composed primarily of seeds, skins, and stems, which are leftover from the juicing and canning processes of the cranberry processing industry (5). The seeds and skins of berries are a rich source of polyphenolic compounds, which have shown to be responsible for the numerous health benefits associated with the berries.

Procyanidins are a class of polyphenolic compounds that impart astringency and bitterness to many plant products. In plants, they are believed to serve as a defense mechanism against potential predators because their bitterness and astringency is undesirable to animals, insects, and microbes (6). Procyanidins are formed via the condensation of the flavan-3-ols catechin and epicatechin and consist of 2 to several monomeric units (6). Structurally, the monomeric units may be linked in one of three ways. The ‘B’-type linkage is the most common and consists of 4β→8 linkage between units. Units connected by both a 2β→O-7 and a 4β→8 are more rigid than ‘B’-type linkages and are denoted as ‘A’-type. The final type of linkage is the ‘C’-type linkage, which consists of a C-4→C-6 linkage (6). Recently, the ability of cranberries to prevent urinary tract infections has been attributed to the presence of procyanidins containing ‘A’-type linkages (7). The bioavailability of procyanidins is dependent upon the size of the molecule with monomers and dimers being absorbed and present in blood at
relatively low levels, but those larger than trimers are not absorbed (8, 9). Whether absorption is required for procyanidins to impart their health benefits is still unknown.

Polyphenolic compounds, including procyanidins, are commonly perceived to be found mainly in the vacuoles of plants where they are separated from other cellular components. However, many may also be associated with cellular components, such as the cell wall, especially after cell injury when vacuoles may rupture. This results in the release of phenolic compounds which may then associate with cell wall polysaccharides through hydrogen bonding and hydrophobic interactions (10). Procyanidins in particular have a strong affinity for cell wall material (11), with higher molecular weight compounds having a greater affinity for binding than smaller compounds. The idea of “unextractable” procyanidins has been of great interest recently because it is believed that the procyanidin contents in plant materials has been underestimated due to the presence of procyanidins bound so tightly to cell wall material that they are not released by normal extraction methods (12-16).

Alkaline treatments are commonly used to extract bound phenolic acids and other phenolic compounds from grains such as rice, wheat, and corn. It is known that phenolic compounds, namely ferulic acid, are insoluble and bound to cell wall materials. Treatment with different concentrations of sodium hydroxide for varying lengths of time has proven to be effective in releasing these bound phenolic compounds (17, 18). There is limited information, however, on the effectiveness of alkaline treatment to release bound phenolic compounds in fruits possibly because many phenolic compounds in fruits, including anthocyanins are known to be unstable under alkaline conditions. Furthermore, there is even less research regarding the possible release of procyanidins
from fruit and vegetables by alkaline treatment. Researchers have shown that strong alkaline conditions can result in cleavage of the C-C interflavan bond connecting the monomeric units of procyanidins; however, prolonged treatment can cause further degradation by opening of the A-ring of the flavan-3-ol. Research regarding the effect of alkaline conditions on procyanidins has been limited to purified compounds (19, 20). Additionally, the effects of alkaline conditions on A-type linkages common in cranberries have yet to be detailed. We have recently reported that cranberry pomace contains significant levels of procyanidins with primarily A-type linkages. In this paper we report on the efficacy of sodium hydroxide treatment in releasing bound procyanidins from cranberry pomace in the form of low molecular weight monomers and oligomers, thus providing a valuable source of procyanidins with potential biological activity.

C. MATERIALS AND METHODS

**Chemicals and standards.** HPLC-grade acetone, methanol, acetonitrile, ethyl acetate, acetic acid, and formic acid were obtained from EMD Biosciences (Madison, WI). Sephadex LH-20 was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from Fisher Scientific (Pittsburg, PA). Procyanidin standards derived from cocoa (DP1-DP9) were obtained from Masterfoods Inc., (Hackettstown, NJ).

**Sample.** Dried cranberry pomace was obtained from Decas Cranberry Company (Carver, MA) and stored at -20 °C until use. The pomace was ground to pass through a 1000 µm sieve screen using an Udy Cyclone Sample Mill (Fort Collins, CO) and stored at -70 °C.
**Alkaline treatment of cranberry pomace.** Ground cranberry pomace (0.5 g) was weighed and placed into glass, screw-top tubes. Five mL of 2N, 4N, or 6N NaOH was added to the tubes, and the tubes were then flushed with nitrogen for 30 s, capped, and vortexed. Tubes were then placed in a shaking water bath (200 rpm) set at 25, 40, or 60 °C for 5 m to 24 h depending on the temperature. After tubes were removed from the water bath, they were placed in an ice bath, and their pH was adjusted to 6 to 7 using 4N HCl.

**Alkaline treatment of residue following conventional solvent extraction.** Dried cranberry pomace (0.5 g) was extracted using the homogenization method described below. After three extractions, the residue was collected, and the excess acetone was removed using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY). The residue was then alkaline treated using 5 mL of 2N NaOH for 15 min at 60 °C. The pH was adjusted to 6 to 7 using 4N HCl, and procyanidins were extracted as described below.

**Extraction of procyanidins.** Neutralized samples from the alkaline treatment of pomace were transferred to 250 mL plastic bottles. Lipids were extracted by shaking with hexane (40 mL) and centrifuging for 10 m at 10,864 x g; the lipid fraction was discarded. Procyanidin monomers, dimers, and trimers were extracted with ethyl acetate (40 mL) and centrifuged for 10 m at 10,864 x g. Ethyl acetate extraction was repeated, and the extracts were pooled.

A separate extraction method was used to extract procyanidin monomers through polymers. This was performed using a T18 Basic Ultra-Turrax® homogenizer (IKA WORKS, Wilmington, NC, USA). Neutralized samples were mixed with 20 mL of
acetone/water/acetic acid (AWA, 70:29.5:0.5 v/v/v), homogenized for 1 m, and filtered through Miracloth. The extraction was repeated two more times, the extracts pooled, and volume adjusted to 100 mL with extraction solvent.

Although extraction with ethyl acetate only allowed quantification of monomers, dimers, and trimers, it provided a quick means to screen extraction conditions because it required no further clean-up step. Once an ideal condition was determined, higher oligomers were extracted using homogenization with AWA which extracts procyanidin monomers through polymers (DP ≥ 10).

**Sephadex LH-20 isolation of procyanidins.** Procyanidins extracted by homogenization with AWA were isolated from sugars and other phenolic compounds by solid phase extraction using Sephadex LH-20 according to the method described by Gu et al. (21).

**Purification of polymeric procyanidins from cranberry pomace.** Twenty grams of cranberry pomace were extracted with 500 mL acetone/water/acetic acid (70:29.5:0.5 v/v/v) by homogenization. The extract was divided into 25 mL aliquots, and acetone was evaporated from them using a vacuum concentrator. The extracts were then manually loaded onto a column containing 3 g of hydrated Sephadex LH-20. Sugars and other phenolics were eluted from the column with 30% aqueous methanol, and monomers and procyanidin oligomers were eluted with 100% methanol. Polymeric procyanidins were then eluted with 70% aqueous acetone, and this fraction was collected. Acetone was removed from the polymer fraction using a vacuum concentrator, and the remaining aqueous portion was freeze dried to obtain a light pink powder.
Alkaline treatment of purified polymeric procyanidins. Polymeric procyanidins (10 mg) were alkaline treated for 15 min at 60 °C using 1 mL of 2N NaOH. The pH was adjusted using 4N HCl, and the total volume was adjusted to 5 mL with acetone/water/acetic acid solvent. Procyanidins were then directly analyzed by HPLC.

HPLC analysis of procyanidins. Ethyl acetate extracts and extracts resulting from LH-20 isolation were evaporated to dryness using a SpeedVac® concentrator, resuspended in 2 mL AWA and filtered through 0.45 µm filters for HPLC analysis. Procyanidins were separated using the method of Hammerstone et al. (22) with modifications as described previously (23). Procyanidins were quantified using a mixture of standards (DP1 – DP10) isolated from cocoa (24). A-type procyanidins were quantified as B-type equivalents. Identification of the procyanidins in cranberry pomace by LC-MS and MALDI-TOF-MS was previously reported (23).

Experimental design and analysis. A split-plot randomized block design was used for treatment application with water bath temperature as the whole plot and sodium hydroxide concentration as the split plot. Time was nested within temperature, which is denoted as time [temperature]. A single treatment consisted of one temperature, one time, and one sodium hydroxide concentration. Three water bath temperatures (25, 40, 60 °C) and three sodium hydroxide concentrations (2, 4, and 6N) were evaluated. Time of treatment varied depending on temperature. There were a total of 39 treatments (Table 1), and levels of each factor were randomized within each plot.

Analysis of variance and mean separations were determined by the PROC MIXED procedure using SAS (SAS .9.1, SAS Institute, Cary, NC). Differences between means were determined using the protected LSD (α=0.05).
D. RESULTS

Alkaline treatment of cranberry pomace. Cranberry pomace was treated with varying concentrations of sodium hydroxide at different temperatures for different amounts of time. Monomeric (DP1), dimeric (DP2), and trimeric (DP3) procyanidins were extracted from the alkaline treated pomace. The overall analysis of variance is presented in Table 2. When averaged over all normalities and times the effect of temperature was significant for DP1 – DP3 procyanidins, and these results are presented in Table 3. Monomers were extracted better at 40 °C and 60 °C than at 25 °C. Dimers and trimers were extracted best at 60 °C, followed by 40 °C and 25 °C. Generally, higher temperatures resulted in increased extraction of procyanidins.

The highest order reaction which showed significance for DP1 – DP3 procyanidins was normality x time [temperature]. The significance of this interaction indicates that release of procyanidins at certain time/temperature combinations is different depending on the normality of sodium hydroxide used. Comparisons were made among all time – temperature – normality combinations. The effects of temperature and normality over time for DP1 release is presented in Figure 1. Several conditions yielded the highest amount of monomers (DP1), and all of the conditions were at either 40 or 60 °C. The effects of temperature and normality over time for DP2 release is presented in Figure 2. Overall, dimer (DP2) release was greatest at 60 °C for 15 m using all three concentrations of NaOH. The effects of temperature and normality over time for DP3 release is presented in Figure 3. When comparing all conditions, trimer (DP3) release was greatest at 60 °C for 15 m using 2 and 4N NaOH. Since cranberry pomace contains more dimers than other procyanidin oligomers, the ideal extraction
condition was chosen based on dimer extraction. Therefore, 60 °C for 15 min was chosen as the “best” condition to release procyanidins. Since, at this temperature, there were no differences in NaOH concentrations, the lowest concentration (2N) was chosen, and this condition was used for further experiments.

Procyanidins extracted from cranberry pomace treated with NaOH were compared to those extracted using conventional extraction by homogenization, and these results are presented in Figure 4. Higher amounts of procyanidins were extracted from the pomace following NaOH treatment compared to conventional extraction. HPLC chromatograms of procyanidins in cranberry pomace before and after treatment with NaOH are presented in Figure 5. The procyanidins in the pomace were previously identified by LC-MS and MALDI-TOF-MS (23). Procyanidin monomers (DP1) and oligomers (DP2 – DP6) were extracted at higher levels after treatment with NaOH. The increases were most evident in DP1 (14.9-fold) and DP2 (11.4-fold) procyanidins. In total, treatment with NaOH resulted in a 9.4-fold increase in procyanidins of DP1 – DP6. Homogenization with AWA also allowed for extraction of polymeric procyanidins (DP ≥ 10). There was a reduction in polymeric procyanidins in pomace treated with NaOH (518.9 mg/100 g DW) compared to conventional extraction (1188.6 mg/100 g). Including polymeric procyanidins, alkaline hydrolysis resulted in a 30% increase in total procyanidins compared to conventional extraction with 1685 mg/100 g DW and 1292 mg/100 g DW extracted, respectively. MALDI-TOF-MS was used to confirm the presence of DP2 – DP6 procyanidins in the alkali treated pomace using previously described conditions (21). The ethyl acetate fraction was found to contain dimers (m/z
and trimers ($m/z$ 887), while the AWA extract contained dimers, trimers, tetramers ($m/z$ 1173), pentamers ($m/z$ 1461), and hexamers ($m/z$ 1749).

**Alkaline treatment of residue following conventional solvent extraction.** To estimate the amount of bound procyanidins in cranberry pomace, anthocyanins, flavonols, and “free” procyanidins were extracted from cranberry pomace by homogenization with AWA, and the resulting residue was collected and treated with 2N NaOH at 60 °C for 15 m. We have previously identified and quantified the anthocyanins, flavonols, and procyanidins obtained by conventional extraction of the pomace (23). The amount of procyanidin oligomers further extracted from the residue after NaOH treatment is shown in Figure 4. Treatment of the residue with NaOH resulted in further extraction of procyanidins that were not released by conventional extraction. In total, 716.4 mg/100 g DW procyanidins with DP1 – DP6 were released from the residue compared to 165.7 mg/100 g DW that were extracted by the conventional method. The release procyanidins were primarily in the form of monomers, dimers, and other lower oligomers.

**Alkaline treatment of purified polymeric procyanidins.** Polymeric procyanidins isolated from cranberry pomace were treated with sodium hydroxide under the optimized conditions to estimate the contribution of depolymerization in our observed increase in low molecular weight procyanidins. Figure 6 shows the HPLC chromatograms of the purified polymer before and after alkaline treatment. It is clear that the polymer was depolymerized to primarily monomers and dimers. On a weight basis, however, approximately 5% of the polymer was converted to dimer and less than
1% was converted to monomers. The identity of the dimer was identified as an A-type by MALDI-TOF-MS \((m/z \ 599)\).

**E. DISCUSSION**

**Alkaline treatment of cranberry pomace.** Treatment of cranberry pomace with NaOH effectively enhanced the extraction of procyanidin monomers and oligomers. This was coupled with a significant loss in polymeric procyanidins. An increase in reaction temperature allowed for enhanced extraction of DP1 – DP3 procyanidins. Additionally, the time needed for procyanidins to be released was much lower at 60 °C (15 m) than at 25 °C (>24 h). Under harsher conditions (e.g. longer treatment times, higher temperatures), procyanidin yields were lower, indicating degradation. It appears that, in this experiment, release of procyanidins was accompanied by procyanidin degradation; however, at the optimum condition identified in this study, we observed a significant increase in procyanidin extraction unlike any other that has previously been reported. The mechanism by which alkaline conditions resulted in such a significant increase in low molecular weight procyanidins is not fully understood, but it is likely a combination of depolymerization of polymeric procyanidins through cleavage of the C-C interflavan bond and enhanced extraction of bound procyanidins.

**Depolymerization of Polymeric Procyanidins.** We isolated polymeric procyanidins from cranberry pomace and treated them with sodium hydroxide under the optimized conditions. In doing so, we observed that depolymerization of the polymer to lower molecular weight procyanidins occurred in isolated compounds. However, only a small percentage of the polymer was actually converted, while the remainder was likely degraded. It has been previously demonstrated that alkaline conditions are capable of
breaking the C-C interflavan bonds of procyanidins from pine bark similar to the way in which they are cleaved under acidic conditions (19). However, these studies have only been conducted on isolated compounds, and hydrolysis is generally performed in the presence of various nucleophiles such as bezylmercaptan or phloroglucinol, which quench the carbocation formed as each extension unit is released from the polymer, and this results in the formation of flavan-3-ols with nucleophilic adducts (20). When the pomace and residue were treated with alkali, it is likely that high pH in combination with elevated temperatures resulted in depolymerization of polymeric procyanidins to monomers and other oligomers. Alkaline conditions can also cause degradation of flavan-3-ols and procyanidins by opening of the A-ring leading to the formation of various side products such as catechinic acid; however, depolymerization occurs prior to opening of the A-ring, and this likely requires oxygen (19). In preliminary experiments, we observed that tubes flushed with nitrogen yielded much higher procyanidin levels than those that were not, further validating the idea that degradation beyond depolymerization requires oxygen. Although it is likely that some further degradation is occurring at the conditions that we are using, we have optimized the conditions to produce the highest possible yields of procyanidin oligomers. Exclusion of oxygen allowed us to achieve depolymerization without further degradation.

Other researchers have used the depolymerization principle to synthesize dimeric procyanidins (25). They isolated polymeric procyanidins from chokeberry and subjected them to acid catalyzed depolymerization in the presence of the monomeric flavan-3-ols catechin and epicatechin. They were able to synthesize significant levels of procyanidin dimers based on the fact that upon depolymerization, a procyanidin extension unit
becomes a positively charged carbocation that is capable of reacting with catechin or epicatechin, rather than the typical nucleophiles mentioned above, to form a stable dimer. Given the high levels of dimeric procyanidins observed in this study, it is possible that the carbocation intermediates that are formed are reacting with catechin or epicatechin that is already present in the pomace or released as a terminal unit to form a dimer. Additionally, it has been demonstrated that A-type linkages are resistant to acid catalyzed cleavage, and therefore might also be resistant to base catalyzed cleavage (21). This could also explain the high levels of A-type dimers that we observed following alkaline treatment.

**Enhanced Extraction of Procyanidins.** The increase in total procyanidins after alkaline hydrolysis and the further release of procyanidins from the treated residue indicate that there are bound procyanidins present in cranberry pomace that are released by treatment with alkali. There has been significant interest recently in the presence of bound or unextractable procyanidins in plant materials. There is evidence that many procyanidins are not able to be extracted by conventional methods of extraction, but the means by which they are bound to the cellular material is relatively unknown. It is believed that they may be tightly bound to cell wall material. A series of studies were conducted to determine how procyanidins interact with apple cell wall material (11, 26-27). The researchers found that isolated procyanidins bound readily to cell wall carbohydrates, particularly pectin, and binding increased with increasing DP of the procyanidin. Drying also increased the binding of procyanidins to the cell wall. Pinelo et al. (10) proposed that interactions between polyphenolics and cell wall material may be hydrophobic interactions between phenols and hydrophobic pockets or hydrogen bonding
between hydroxyl groups on phenolics and cell wall polysaccharides. It is also possible, though not proven, that procyanidins may be covalently linked to cell wall components similar to the way ferulic acid is linked to the cell wall of grains (18).

Hellström and Matilla (12) have developed a method to determine unextractable procyanidins in plant materials by acid-catalyzed depolymerization of the compounds into flavan-3-ols and benzylthioethers using thioacidolysis. They have used this method to determine the amount of unextractable procyanidins in several plant materials including cranberries (14). They found that a significant amount of procyanidins in many plant materials were “unextractable.” Other researchers have used butanol:HCl with heat to determine the amount of bound procyanidins (15, 28). This method is based on the principle that under heat and acid, procyanidins are converted to cyanidin which can be measured spectrophotometrically. Researchers found that apples, peaches, and nectarines contain higher levels of non-extractable procyanidins than extractable procyanidins (15).

These methods are effective in identifying the presence of bound procyanidins; however problems exist when using these methods for quantification because of the kinetics of the reactions. Thiolysis yields have been reported to be low (34 – 63%), and this may be due to impurities, thiolysis resistant bonds, or instability of reaction products (29). The butanol:HCl assay produces several side reactions which result in lower yields, and not all procyanidins react the same under the reaction conditions (30). Additionally, these methods do not preserve the integrity of the procyanidins; therefore, they are unrecoverable.
In contrast, treatment of the residue remaining after conventional extraction of phenolics from cranberry pomace resulted in release of procyanidins in the form of monomers, dimers, and other oligomers, which can be extracted and used in a variety of applications. Although the procyanidins were released as low molecular weight compounds, we do not believe that this indicates that it was the lower oligomers that were tightly bound and unextractable. Rather, given that researchers have shown that it is primarily polymeric procyanidins that bind strongly to cell wall material and therefore resist extraction (26), and the fact that procyanidins can be depolymerized under alkaline conditions, it is likely that treatment with NaOH resulted in release of the polymeric procyanidins in the form of lower oligomers by means of depolymerization. Since “free” procyanidins were removed from the residue before treatment with sodium hydroxide, by comparing the procyanidins released with those obtained by treatment of the whole pomace, we were able to differentiate between procyanidins that were truly bound to the cell matrix and those that were merely depolymerized from “free” polymeric procyanidins.

The mechanism by which sodium hydroxide enhances the extraction of procyanidins may also lie in its ability to solubilize cell wall material in the cranberry pomace. Dilute sodium hydroxide is commonly used to extract hemicellulose from cell wall material (31). It is possible that the solubilization of hemicellulose leads to the release of procyanidins that are entrapped or even esterified to the cell wall, but this needs to be confirmed in a follow-up study.

**Application of Alkaline Treatment of Cranberry Pomace.** Alkaline treatment of cranberry pomace provides a means of utilizing a waste material to produce valuable
procyanidins with potential health benefits that can be used for various nutraceutical purposes. We have demonstrated that sodium hydroxide releases bound procyanidins which can be subsequently recovered for a variety of applications. The most valuable application of this treatment would be to extract anthocyanins and other polyphenolics from the plant material first, and then treat the residue with sodium hydroxide to release the bound procyanidins in a usable form. Although in our experiment, treatment of the residue with NaOH resulted in lower yields than when the whole pomace was treated, a significant amount of procyanidins were further extracted from the residue. The lower yields were due to the fact that depolymerization of “free” polymeric procyanidins also occurred in the whole pomace, resulting in higher levels of monomers and dimers. The procyanidins extracted from the residue by NaOH treatment represents an estimate of the amount of procyanidins bound to the cell matrix.

Extraction with ethyl acetate provided a means of fractionation of low DP procyanidins from high DP procyanidins and other phenolic compounds. Although aqueous acetone is known to be the most effective solvent to extract procyanidins, the extracts require further purification steps to isolate procyanidins from other polyphenolics (e.g. anthocyanins, flavonols) prior to HPLC analysis. Ethyl acetate proved to be an effective extraction solvent for low DP procyanidins. This could be useful in an industrial application where it might be desired to separate procyanidins based on their molecular weight.

This application of this process is three-fold. It could be used as a means of estimating the amount of bound procyanidins in many plant materials, thus giving a better idea of the total procyanidins in the product since although they are unextractable, they
may still be biologically important. Secondly, treatment of procyanidin containing materials with sodium hydroxide could enhance the bioavailability of the compounds since DP1 and DP2 procyanidins were generated and released in the greatest quantities compared to higher oligomers. This is important because several researchers have noted that DP1, DP2, and to a lesser extent DP3 procyanidins are absorbed, whereas higher oligomers are not (8). The larger oligomers, however, may still confer health benefits due to their ability to be metabolized by colonic microflora, which in turn produce smaller molecules such as phenolic acids that may subsequently be absorbed (32). Additionally, free procyanidins may be more available for microbial metabolism than those bound within the cell wall. Lastly, treatment with NaOH could be used industrially as means of recovering procyanidins from plant material. Polyphenolics are often recovered from waste materials to be used in dietary supplements or fortification purposes. After anthocyanins, flavonols, and other phenolics have been extracted, the residue could be treated with NaOH, neutralized, and desalted. The released procyanidins could then be extracted for use in a variety of applications.

F. ACKNOWLEDGMENT

We thank Decas Cranberry Co., Inc., for providing the cranberry pomace used in this study.
G. LITERATURE CITED


H. FIGURE CAPTIONS

**Figure 1.** Changes in procyanidin monomer (DP1) composition of cranberry pomace treated with different concentrations of sodium hydroxide at different temperatures for varying amounts of time.

**Figure 2.** Changes in procyanidin dimer (DP2) composition of cranberry pomace treated with different concentrations of sodium hydroxide at different temperatures for varying amounts of time.

**Figure 3.** Changes in procyanidin trimer (DP3) composition of cranberry pomace treated with different concentrations of sodium hydroxide at different temperatures for varying amounts of time.

**Figure 4.** Procyanidin oligomer (DP1 – DP6) composition of cranberry pomace before and after treatment with sodium hydroxide. Treatment conditions were 2N NaOH at 60 °C for 15 m. Residue was collected following conventional extraction by homogenization with acetone/water/acetic acid (70:29.5:0.5). “DPn A” indicates a procyanidin containing at least one A-type linkage and “DPn B” indicates a procyanidin containing only B-type linkages.

**Figure 5.** HPLC chromatograms of procyanidins in cranberry pomace before (A) and after (B) treatment with sodium hydroxide. Treatment conditions were 2N NaOH at 60 °C for 15 m.

**Figure 6.** HPLC chromatograms of purified polymeric procyanidins from cranberry pomace before (dotted line) and after treatment with sodium hydroxide (solid line). Treatment conditions were 2N NaOH at 60 °C for 15 m.


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Table 2. Analysis of Variance for Procyanidin Extraction Using Sodium Hydroxide

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a Values represent means ± standard error
b Values within each column followed by the same letters are not significantly different (p>0.05)
Figure 1

Temperature

25 °C  40 °C  60 °C

Monomer Concentration (mg/100 g DW)
Figure 2
Figure 3
Figure 5
Figure 6
VII. RELEASE OF BOUND PROCYANIDINS FROM FRUIT BY-PRODUCTS BY ALKALINE HYDROLYSIS

A. ABSTRACT

Fruit by-products represent a potential source of bioactive compounds, including procyanidins. However, many of the procyanidins present in these by-products are ‘unextractable’ because they are tightly bound to other cellular components. Free and bound procyanidins were extracted from blueberry and grape pomace and seeds from three varieties of grapes. Free procyanidins were extracted by homogenization with acetone/water/acetic acid (70/29.5/0.5 v/v/v). Bound procyanidins were extracted by treating the resulting residue using alkaline hydrolysis and were released as monomers and dimers. Each of the by-products contained significant levels of polymeric procyanidins. Blueberry pomace contained low levels of oligomeric procyanidins, grape pomace contained high levels of monomeric catechin/epicatechin, and the grape seeds contained high levels of total free procyanidins. The by-products also contained significant levels of bound procyanidins. This research indicates that alkaline hydrolysis can be used to release bound procyanidins from fruit processing by-products as monomeric and dimeric procyanidins, which are known to be bioavailable.

**Keywords: Alkaline; blueberry; grape; hydrolysis; pomace; procyanidins; unextractable**
B. INTRODUCTION

The fruit processing industry produces a significant amount of by-products each year, primarily in the form of seeds and skins, which are left over from the pressing of fruit into juices. By-product utilization and disposal presents a major problem due to the cost of transportation and storage as well as the environmental impact of landfill disposal. Therefore, it is important that the fruit processing industries explore other potential uses for their by-products.

The seeds and skins of many fruits, including grapes and blueberries, are known to be rich sources of polyphenolic compounds, particularly procyanidins. Procyanidins are a class of oligomeric and polymeric compounds formed from condensation of flavan-3-ol monomeric units. The most common monomers are (+)-catechin and (-)-epicatechin, however (-)-gallocatechin and (-)-epigallocatechin are also found in some fruits such as grapes (1). Flavan-3-ols and procyanidins have been recognized for numerous health-benefiting properties, which include antioxidant, anticancer, anti-inflammatory, antiatherosclerotic, antimicrobial, and antiadhesive properties, among others (2, 3-6).

Procyanidins can vary in degree of polymerization (DP) from two (dimer) up to 200 monomeric units, and their biological properties and in vivo absorption, bioavailability, and metabolism are largely dependent upon the size of the molecule. Monomeric flavan-3-ols, dimers, and to a lesser extent trimers may be absorbed in the small intestine, whereas larger oligomers and polymeric compounds are not absorbed and proceed into the large intestine (7, 8). In the large intestine, procyanidins can be fermented by gut microflora to produce low molecular weight phenolic acids, which in
turn can be reabsorbed through the epithelial cells of the large intestine (9). Highly polymerized procyanidins, however, may be unavailable for gut fermentation because their ability to form complexes with proteins can inhibit extracellular microbial enzymes (10).

Most of the available qualitative and quantitative information about procyanidins in foods refers only to those that are readily extracted with organic solvents. A significant amount of procyanidins, however, may be unextractable because they have formed insoluble complexes with cell wall material through hydrophobic interactions, hydrogen bonding, or covalent linkages. Attempts to quantify unextractable procyanidins have focused primarily on their ability to be depolymerized in the presence of heat and acid. Butanol:HCl (Porter Method) has been used to convert unextractable procyanidins into cyanidin, which can be measured spectrophotometrically (11). Similarly, researchers have used thiolysis to depolymerize bound procyanidins and convert them into their corresponding thiol ethers which can be measured by HPLC (12). Absolute quantification of unextractable procyanidins using these methods is impossible due to low reaction yields and side reactions.

Recently, alkaline hydrolysis has been used to release bound procyanidins from cranberry pomace (13). It has been demonstrated that the interflavan bond is readily cleaved under alkaline conditions (14). Therefore, this process breaks polymeric procyanidins into low molecular weight monomers and oligomers and solubilizes cell wall material to provide release of bound procyanidins. When used on the residue remaining after solvent extraction of soluble procyanidins, alkaline hydrolysis can provide an estimation of the amount of nonextractable procyanidins in plant materials.
Additionally, the released procyanidins can be recovered and used for a variety of applications. This research uses alkaline hydrolysis to release bound procyanidins from various fruit by-products including grape seeds, grape pomace and blueberry pomace.

C. MATERIALS AND METHODS

Chemicals and standards. HPLC-grade acetone, methanol, acetonitrile, ethyl acetate, acetic acid, and formic acid were obtained from EMD Biosciences (Madison, WI). Sephadex LH-20 was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from Fisher Scientific (Pittsburg, PA). Procyanidin standards derived from cocoa (DP1-DP9) were obtained from Masterfoods Inc., (Hackettstown, NJ).

Samples. Sunbelt grape pomace contained skins, seeds, and stems and was obtained from the Enology program of the University of Arkansas (Fayetteville, AR). Blueberry pomace was obtained from Jasper Wyman & Son (Milbridge, MA). The pomaces were freeze dried using a Virtis Genesis freeze drier (Gardiner, NY) prior to grinding. Grape seeds of the varieties Merlot, Riesling, and Chardonnay were obtained from FruitSmart (Prosser, WA) and were ground using a coffee grinder.

Conventional Extraction of Procyanidins. Procyanidins were extracted by homogenizing 0.5 g of fruit byproduct with 20 mL acetone:water:acetic acid (70:29.5:0.5) using T18 Basic Ultra-Turrax® homogenizer (IKA WORKS, Wilmington, NC, USA). The extraction was repeated twice more, and the extracts were pooled. The residues resulting from extraction were collected by centrifugation for further analysis. Acetone was removed from 20 mL of the resulting extract using a SpeedVac®
concentrator and the remaining aqueous fraction was loaded onto a Sephadex LH-20 column for procyanidin isolation following the method described by Gu et al. (15).

**Alkaline Treatment of Fruit By-products.** Each of the fruit by-products, as well as their residues resulting from conventional extraction of polyphenolics, were subjected to alkaline hydrolysis as previously described with slight modifications (13). A schematic of the process is presented in Figure 1. Briefly, 0.5 g of fruit material was weighed into glass screw top tubes and 5 mL of 2N NaOH was added to the tubes. The tubes were then flushed with nitrogen, capped, vortexed, and placed into a 60 °C water bath with shaking (200 rpm) for 15 min. After the tubes were removed from the water bath, they were immediately placed into an ice bath to cool, and the pH of the mixture was adjusted to around 5 using 4M HCl. Procyanidins were then extracted by vortexing with hot water (60°C). The mixtures were centrifuged, the supernatant collected, and the residues rinsed with hot water two more times. The supernatants from each rinsing were pooled, subjected to LH-20 solid-phase extraction (SPE), and analyzed by HPLC.

**HPLC Analysis of Procyanidins.** Acetone and hot water extracts resulting from LH-20 isolation of procyanidins were evaporated to dryness using a SpeedVac® concentrator, resuspended in 2 mL of extraction solvent, and filtered through 0.45 μm filters for HPLC analysis. Procyanidins were separated on a 5-μm, 250- x 4.6-mm Luna Silica column (Phenomenex, Torrence, CA, USA) based on the method described by Hammerstone et al (16). Compounds were identified by fluorescence detection with an excitation of 276 nm and emission of 316 nm and quantified using a mixture of external procyanidin standards (DP1 – DP10) previously isolated from cocoa.
(17). To quantify polymeric procyanidins, a slope vs. degree of polymerization was
extrapolated to DP30 to approximate the slope of the polymer peak.

MALDI-TOF-MS Analysis of Procyanidins. Procyanidin extracts from
blueberry and grape by-products before and after alkaline hydrolysis were mixed in a 1:1
ratio with 1 M dihydroxybenzoic acid (DHB) in 90% methanol. The samples were then
spotted onto a ground stainless steel MALDI target using the dry droplet method.
Analysis was conducted using a Bruker Reflex III MALDI-TOF-MS, equipped with a
337 nm N₂ laser. Data were obtained in the positive ion reflectron mode.

D. RESULTS

Extraction of Free and Bound Procyanidins. Free and bound procyanidins
were extracted from fruit by-products including grape and blueberry pomace as well as
seeds from three varieties of grapes, and these results are presented in Table 1. Free
procyanidins are generally defined as those that can be extracted under normal extraction
conditions, which, in this case, was homogenization with aqueous acidified acetone. A
majority of the free procyanidins in each by-product were polymeric compounds (DP >
10). Consistent with previous analyses, blueberry pomace contained relatively low levels
of free procyanidin oligomers compared to the other by-products (18). Grape pomace
contained high levels of the monomers catechin and epicatechin compared to the other
oligomers, which is also consistent with other studies (19, 20). Of the grape seeds, those
from the Riesling variety of grapes contained the highest levels of both oligomeric and
polymeric procyanidins, followed by Chardonnay, whereas Merlot contained the lowest
levels. This is consistent with a previous study (21), but in contrast to another study that
found Riesling seeds to contain lower levels of procyanidins than Merlot and Chardonnay
(22). Other researchers, however, have found that Chardonnay seeds contain higher levels of total phenolics that Merlot seeds (23).

Total procyanidins were also extracted from the by-products using alkaline hydrolysis. This method depolymerized all of the procyanidins into monomers and dimers. Lower levels of total procyanidins were extracted by alkaline hydrolysis than by conventional extraction, indicating significant degradation of procyanidins under alkaline conditions. Bound procyanidins were also extracted from the fruit by-products by treating the residue remaining after conventional extraction using alkaline hydrolysis. Bound procyanidins were also released from the residue as monomers and dimers. Blueberry pomace contained higher levels of bound procyanidins than did the grape pomace. Of the grape seeds, Merlot contained the highest levels of bound procyanidins, followed by Chardonnay and Riesling, which is opposite of the results for free procyanidins.

**MALDI-TOF-MS analysis of procyanidins.** MALDI-TOF-MS was used to identify procyanidins in the fruit by-products, and this data is presented in Table 2. The DHB matrix masked the masses corresponding to monomeric catechin/epicatechin; however, procyanidins with DP ≥ 2 were observed by MALDI-TOF-MS in all of the by-products. The mass spectrum of the grape seed extracts indicated the presence of procyanidins ranging from DP2 to DP10 (Figure 2a). The observed masses are representative of sodium adducts [M + Na+] of each procyanidin oligomer. Several compounds were identified in the grape seeds with masses 152 amu greater than their corresponding procyanidin oligomer indicative of procyanidins esterified with gallic acid. The following equation has been developed to predict the masses of galloylated
procyanidins from grape seeds: 290 + 288c + 152g + 23, where 290 represents the mass of the terminal unit, c represents the number of extension units, g represents the number of galloyl esters, and 23 represents the molecular weight of sodium (24). Additionally, upon closer examination of the grape seed mass spectrums (Figure 3), compounds with mass 16 amu greater than the nearest procyanidin or procyanidin gallate were observed. The identity of these compounds is ambiguous. The masses could represent procyanidin di- and tri-gallates. For example, m/z 1482 could be a tetramer digallate and m/z 1634 could be a tetramer trigallate. Procyanidins with varying degrees of galloylation have been reported in grape seeds, so this is a likely explanation (25). A difference in 16 amu, however, could also represent replacement of hydrogen with a hydroxyl group and could correspond to oligomers containing one epigallocatechin unit in place of an epicatechin (26). If this were the case, m/z 1482 would represent a pentamer containing one epigallocatechin unit, and m/z 1634 would be a pentamer gallate containing one epigallocatechin unit. A third explanation could be that potassium [M + K+] adducts were formed alongside sodium adducts because the molecular weight difference in potassium and sodium is 16 amu. Additionally, B-type procyanidins ranging from DP2 to DP9 were identified in the blueberry pomace. The mass spectral profile of procyanidins from Sunbelt grape pomace was similar to the grape seeds with B-type procyanidins of DP2 – DP12 and galloyl esters of procyanidins identified.

Procyanidins resulting from alkaline hydrolysis of the by-products were also identified by MALDI-TOF-MS. The blueberry extract contained primarily dimeric procyanidins (m/z 601) after alkaline hydrolysis. In each of the grape seed extracts after alkaline hydrolysis, m/z’s of 601 and 617 were observed (Figure 2b). An m/z of 601 is
characteristic of a sodiated dimer; however, $m/z$ of 617 is again slightly ambiguous. It could correspond to a dimer containing one epigallocatechin unit in place of an epicatechin or a potassium adduct of a dimer. Presumably, it is also possible that $m/z$ 617 represents a monomeric catechin or epicatechin molecule that is esterified with two gallic acid moieties.

**E. DISCUSSION**

Recent research has demonstrated that a significant amount of procyanidins from plant foods are bound to other cellular components and are unextractable under normal conditions (11, 27). The means by which the procyanidins are bound, however, is not clearly understood. Procyanidins have a natural affinity for carbohydrates and proteins and can bind to them tightly through hydrogen bonding and hydrophobic interactions (28). These interactions, however, are largely reversible and cannot in entirety explain the high levels of unextractable procyanidins observed in plant foods. There has been additional evidence suggesting that procyanidins may be bound covalently to the cell wall (29). These covalent linkages could occur during biosynthesis of procyanidins in which an intermediate carbocation may be captured by nucleophilic groups on a protein or carbohydrate. Additional covalent linkages could be formed under oxidative conditions which might cause the procyanidin to be transformed into a quinone. This suggests that procyanidins can behave similarly to lignin within the cell wall matrix and be bound covalently to the cell wall (29). Due to an increase in the number of binding sites, polymeric procyanidins are more likely to be bound than low molecular weight monomers and oligomers.
Recently, researchers have investigated ways to quantify the amount of unextractable procyanidins in plant materials. These techniques have focused on acid catalyzed depolymerization of procyanidins from residues remaining after extraction with organic solvent. Hellström and Matilla (12) have used the principles of thiolysis to depolymerize procyanidins into benzylthioethers, which can be quantified by HPLC. Other researchers have used what is known as the Porter method, which depolymerizes procyanidins and converts the monomeric units to cyanidin which is measured spectrophotometrically (11, 30). These methods likely do not give absolute quantifications because reaction yields are low due to the instability of reaction products and side reactions (31, 32).

In the present study, alkaline hydrolysis was used to release bound procyanidins from by-products of the grape and blueberry processing industries. Some of the bound procyanidins in these products were likely inherently bound during biosynthesis; however, covalent binding might have also been perpetuated by oxidative conditions during the pressing of the fruit for juice. The procyanidins were likely released through a depolymerization mechanism. It has been demonstrated that the C-C interflavan bond which connects two monomeric units is labile under alkaline conditions (14). Bound procyanidins from cranberry pomace were recently extracted using alkaline hydrolysis and were released as a mixture of oligomeric (DP1 – DP6) procyanidins (13). However, in the present study, bound procyanidins were released only as monomers and dimers. This is likely because the fruit by-products used in this study contained procyanidins with only B-type linkages in comparison to cranberry pomace which contains several procyanidins with A-type linkages. B-type interflavan linkages are much more labile
under harsh conditions than A-type linkages (13, 15). This is likely the reason that we see depolymerization to a greater extent in grape and blueberry by-products when compared to the previous study on cranberry pomace. It has also been established that, in addition to depolymerization, procyanidin degradation through opening of the phenolic A-ring can occur under alkaline conditions in the presence of oxygen (14). In the present study, nitrogen flushing was used in attempt to minimize this degradation; however, a comparison of total procyanidins extracted conventionally and by alkaline hydrolysis clearly demonstrates that significant degradation occurs under our alkaline conditions. Given this, the levels of bound procyanidins reported likely only represents a fraction of the amount of procyanidins that are actually bound to the matrix.

This research should be of particular interest to industries exploring possible uses for their waste products. Fruit processing industries are aware of the presence of anthocyanins, flavonols, and procyanidins in their waste stream products, and many are exploring means to recover these health-benefiting phytochemicals. However, the material that results after other phytochemicals have been recovered may still be rich in procyanidins. Bound to the cellular matrix, these procyanidins would likely be facilitated through the gastrointestinal tract upon consumption and would not be absorbed or available for fermentation by gut microflora. Alkaline hydrolysis, however, may present an economically advantageous means of recovering the procyanidins from an otherwise useless waste material. The procyanidins would be in the form of monomers and dimers, which are known to be more bioavailable than their polymeric counterparts.


G. FIGURE CAPTIONS

**Figure 1.** Flow diagram of process of isolating extractable and non-extractable procyanidins from by-products of the fruit processing industry.

**Figure 2.** MALDI-TOF mass spectrum of a series of procyanidins and procyanidin gallates [M + Na\(^{+}\)] from the dimer (m/z 601) to the decamer gallate (m/z 3058) isolated from grape seeds.

**Figure 3.** Enlarged MALDI-TOF mass spectrum showing the pentamer (m/z 1466), hexamer (m/z 1754), pentamer gallate (m/z 1618), hexamer gallate (m/z 1906), tetramer digallate (m/z 1482), pentamer digallate (m/z 1770), tetramer trigallate (m/z 1634), and pentamer trigallate (m/z 1922).
Table 1. Concentrations (mg/100 g DW) of Free and Bound Procyanidins in Fruit By-Products Determined by HPLC Analysis

<table>
<thead>
<tr>
<th>By-Product</th>
<th></th>
<th>Free Procyanidins(^a)</th>
<th></th>
<th>Bound Procyanidins(^b)</th>
<th></th>
<th>Total Procyanidins(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DP1</td>
<td>DP2</td>
<td>DP3</td>
<td>DP4</td>
<td>DP5</td>
</tr>
<tr>
<td>Pomaces</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blueberry</td>
<td></td>
<td>11.4 ± 1.4(^d)</td>
<td>75.4 ± 4.5</td>
<td>23.3 ± 0.1</td>
<td>34.0 ± 1.1</td>
<td>39.8 ± 1.3</td>
</tr>
<tr>
<td>Grape</td>
<td></td>
<td>374.9 ± 12.8</td>
<td>120.4 ± 0.2</td>
<td>25.4 ± 1.2</td>
<td>18.7 ± 2.0</td>
<td>11.0 ± 0.5</td>
</tr>
<tr>
<td>Seeds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Merlot</td>
<td></td>
<td>163.6 ± 4.7</td>
<td>156.9 ± 0.9</td>
<td>96.2 ± 6.2</td>
<td>45.6 ± 1.5</td>
<td>26.4 ± 5.0</td>
</tr>
<tr>
<td>Chardonnay</td>
<td></td>
<td>428.5 ± 63.1</td>
<td>158.1 ± 19.4</td>
<td>161.2 ± 24.4</td>
<td>154.3 ± 22.9</td>
<td>118.3 ± 13.1</td>
</tr>
<tr>
<td>Riesling</td>
<td></td>
<td>577.4 ± 74.7</td>
<td>463.6 ± 29.3</td>
<td>344.7 ± 23.9</td>
<td>278.4 ± 15.6</td>
<td>185.8 ± 19.9</td>
</tr>
</tbody>
</table>

\(^a\) Extracted by homogenization with acetone/water/acetic acid (70/29.5/0.5 v/v/v)

\(^b\) Extracted by alkaline hydrolysis of the residue remaining after conventional extraction

\(^c\) Extracted by alkaline hydrolysis of the ground by-product

\(^d\) Values represent means ± standard error (n=3)
Table 2. Observed and Calculated Masses of Procyanidins from Fruit By-products by MALDI-TOF MS

<table>
<thead>
<tr>
<th>By-product</th>
<th>Procyanidin</th>
<th>Calculated [M + Na⁺]</th>
<th>Observed [M + Na⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grape Pomace</td>
<td>Dimer / Dimer gallate</td>
<td>601 / 753</td>
<td>601.1 / 753.4</td>
</tr>
<tr>
<td></td>
<td>Trimer / Trimer gallate</td>
<td>889 / 1041</td>
<td>889.4 / 1041.3</td>
</tr>
<tr>
<td></td>
<td>Tetramer / Tetramer gallate</td>
<td>1177 / 1329</td>
<td>1177.4 / 1329.4</td>
</tr>
<tr>
<td></td>
<td>Pentamer / Pentamer gallate</td>
<td>1465 / 1617</td>
<td>1465.4 / 1617.4</td>
</tr>
<tr>
<td></td>
<td>Hexamer / Hexamer gallate</td>
<td>1753 / 1905</td>
<td>1753.4 / 1905.4</td>
</tr>
<tr>
<td></td>
<td>Heptamer / Heptamer gallate</td>
<td>2041 / 2193</td>
<td>2041.5 / 2193.5</td>
</tr>
<tr>
<td></td>
<td>Octomer / Octomer gallate</td>
<td>2329 / 2481</td>
<td>2330.5 / 2482.5</td>
</tr>
<tr>
<td></td>
<td>Decamer / Decamer gallate</td>
<td>2905 / 3057</td>
<td>2906.5 / 3059.5</td>
</tr>
<tr>
<td></td>
<td>Undecamer / Undecamer gallate</td>
<td>3193 / 3345</td>
<td>3193.4 / 3346.3</td>
</tr>
<tr>
<td></td>
<td>Dodecamer</td>
<td>3481</td>
<td>3483.0</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
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<td>600.8</td>
</tr>
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<td></td>
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<td>889</td>
<td>889.2</td>
</tr>
<tr>
<td></td>
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</tr>
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</tr>
<tr>
<td></td>
<td>Heptamer</td>
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<tr>
<td></td>
<td>Octomer</td>
<td>2330</td>
<td>2329.4</td>
</tr>
<tr>
<td></td>
<td>Nonomer</td>
<td>2619</td>
<td>2619.6</td>
</tr>
<tr>
<td>Blueberry Pomace</td>
<td>Dimer / Dimer gallate</td>
<td>601 / 753</td>
<td>601.1 / 753.4</td>
</tr>
<tr>
<td></td>
<td>Trimer / Trimer gallate</td>
<td>889 / 1041</td>
<td>889.4 / 1041.6</td>
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<tr>
<td></td>
<td>Tetramer / Tetramer gallate</td>
<td>1177 / 1329</td>
<td>1177.6 / 1329.8</td>
</tr>
<tr>
<td></td>
<td>Pentamer / Pentamer gallate</td>
<td>1466 / 1618</td>
<td>1466.0 / 1618.2</td>
</tr>
<tr>
<td></td>
<td>Hexamer / Hexamer gallate</td>
<td>1754 / 1906</td>
<td>1754.2 / 1906.1</td>
</tr>
<tr>
<td></td>
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<td>2042 / 2194</td>
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<tr>
<td></td>
<td>Octomer / Octomer gallate</td>
<td>2330 / 2483</td>
<td>2329.4 / 2483.0</td>
</tr>
<tr>
<td></td>
<td>Nonomer / Nonomer gallate</td>
<td>2619 / 2771</td>
<td>2618.4 / 2771.2</td>
</tr>
<tr>
<td></td>
<td>Decamer / Decamer gallate</td>
<td>2907 / 3059</td>
<td>2907.4 / 3058.1</td>
</tr>
<tr>
<td>Grape Seeds</td>
<td>Dimer / Dimer gallate</td>
<td>601 / 753</td>
<td>601.1 / 753.4</td>
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<tr>
<td></td>
<td>Trimer / Trimer gallate</td>
<td>889 / 1041</td>
<td>889.4 / 1041.6</td>
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<td>Nonomer / Nonomer gallate</td>
<td>2619 / 2771</td>
<td>2618.4 / 2771.2</td>
</tr>
<tr>
<td></td>
<td>Decamer / Decamer gallate</td>
<td>2907 / 3059</td>
<td>2907.4 / 3058.1</td>
</tr>
</tbody>
</table>
Dried By-Product (0.5 g)

Homogenize with acetone/water/acetic acid (70/29.5/0.5 v/v/v)

Liquid Extract

Purification by Sephadex LH-20 SPE

NP-HPLC Analysis Extractable Procyanidins

Residue

Alkaline Hydrolysis 60°C, 15 min

Place in ice bath Neutralize with HCl

Centrifuge Rinse residue with hot water

Purification by Sephadex LH-20 SPE

NP-HPLC Analysis Non-extractable Procyanidins

Figure 1
Figure 2
VIII. VISUALIZATION OF PROCYANIDINS IN CRANBERRIES AND CRANBERRY POMACE BY LIGHT MICROSCOPY

A. ABSTRACT

Light microscopy was used to visualize flavan-3-ols and procyanidins present in fresh cranberries and cranberry pomace. The reagent p-dimethylaminocinnamaldehyde (DMAC) reacts specifically with flavonoids that contain a meta-substituted dihydroxybenzene ring, a single bond between C2 and C3, and lack a carbonyl at C4 to produce a blue color. Such requirements make the DMAC reagent highly specific for flavan-3-ols and procyanidins. The intense blue color of fresh cranberry skins when stained with DMAC indicated the presence of procyanidins throughout the skins. In the seeds, however, procyanidins appear to be located primarily in the seed coat. It was revealed by staining with DMAC that conventional solvent extraction by homogenization with acidified aqueous acetone does not completely extract all procyanidins from cranberry pomace. Treatment of the pomace by alkaline hydrolysis completely extracted all procyanidins, as indicated by the lack of blue color after staining with DMAC.

Keywords: Cranberry; dimethylaminocinnamaldehyde; localization; procyanidins; unextractable
B. INTRODUCTION

Cranberries (*Vaccinium macrocarpon*) are growing in popularity due to the increasing information regarding their health benefits. Cranberry juice has long been recognized for its ability to prevent urinary tract infections, however there are several other health benefits associated with cranberries which include antioxidant, anti-tumor, anti-ulcer, anti-inflammatory, and anti-atherosclerotic activities (1-4). Cranberry pomace is composed primarily of the seeds, skins, and stems which are leftover from the juicing and canning processes of the cranberry processing industry (5). The seeds and skins of berries are a rich source of polyphenolic compounds, which research has shown to be responsible for the numerous health benefits in which berries are associated.

Polyphenolics in plants vary in functionality according to their chemical structure. One of the most evident is the impartation of color to the plant, while others include protection from UV rays and parasites. Polyphenolics may also act in enzyme regulation and cell signaling. The astringency of many plant-derived foods is attributed to the ability of certain polyphenolics to precipitate proteins, which forms insoluble substances in the mouth (6). These compounds are primarily stored in vacuoles and released upon injury.

Procyanidins, specifically, are a class of polyphenolic compounds found in many plants. They provide protection to the plant from predators due to their astringent nature and may also play a part in many physiological and cell signaling processes (7). Procyanidins are oligomers and polymers of the flavan-3-ols, catechin or epicatechin (Figure 1). The catechin or epicatechin monomers may be linked together by one of two options. The most common linkages between the flavan rings are the $4\beta \rightarrow 8$ or $4\beta \rightarrow 6$
or B-type linkages. The A-type linkage is less common and consists of both $4\beta\rightarrow8$ and $2\beta\rightarrow\mathrm{O}\rightarrow7$ linkages. Recently, the ability of cranberries to prevent urinary tract infections has been attributed to the presence of procyanidins containing A-type linkages (8).

Very little histological work has been done to determine the localization of procyanidins in plant cells, especially cranberries. Flavan-3-ols and procyanidins may be visualized microscopically using $p$-dimethylaminocinnamaldehyde (DMAC), which reacts specifically with these compounds to impart a blue color (9). It is believed that DMAC reacts approximately 1:1 with a flavan-3-ol or procyanidin molecule. For the reaction to occur, the following structural features are required: (1) a meta-substituted dihydroxybenzene ring, in which the flavonoid A ring exhibits; (2) a single bond between C2 and C3, which eliminates reactions with anthocyanins; and (3) lack of a carbonyl at C4, which eliminates reactions with flavonols (10). Therefore, the DMAC reagent is very specific for flavan-3-ols and procyanidins compared to other polyphenolics.

It is widely recognized that procyanidins are best extracted from plant materials using aqueous acetone (11). However, a significant amount of procyanidins may be highly bound to other cellular components such as the cell wall carbohydrates or proteins and resist normal solvent extraction. Evidence of nonextractable procyanidins exists in that after normal solvent extraction, procyanidin degradation products are observed in the residue upon acid-catalyzed depolymerization by thiolysis (12) or treatment with butanol – HCl (13). Additionally, researchers have extensively studied the interactions between procyanidins and apple cell wall material. They found that procyanidins bind readily to the cell wall material through hydrogen bonding and hydrophobic interactions, and
binding affinity increases with increasing degree of polymerization (14-16). It is also possible that procyanidins may be covalently linked to cell wall components during flavonoid biosynthesis or under various oxidative conditions (17).

It has recently been demonstrated that alkaline hydrolysis can release bound procyanidins from cranberry pomace by depolymerization of polymeric procyanidins and solubilization of cell wall material (18). Following solvent extraction of procyanidins from cranberry pomace, the remaining residue was treated with sodium hydroxide, and this resulted in further release of procyanidins primarily as low molecular weight monomers and dimers. This is further evidence that many procyanidins, especially those with high DP, are bound to cell wall material and resist extraction.

A better understanding of localization of procyanidins within a cell and which components they are associated with could lead to the development of improved extraction methods. Therefore, this research aims to use light microscopy to evaluate the presence of procyanidins in fresh cranberries and cranberry pomace before and after solvent extraction and alkaline hydrolysis.

C. MATERIALS AND METHODS

**Plant material.** Dried cranberry pomace and fresh cranberries were provided by Decas Cranberry Company (Carver, MA). The pomace was a blend of several varieties, whereas the fresh cranberries were of the variety Early Black or Howe.

**Sample Preparation.** Whole pomace was stored at -20 °C until use. Ground pomace was prepared by passing through a 1000-µm sieve using an Udy Cyclone Sample Mill (Fort Collins, CO). Free hand sections of skins from fresh cranberries were prepared by manually peeling thin pieces from frozen berries using a razor blade. Pieces of skin from
cranberry pomace were selected and evaluated as is. Thin slices of seeds from fresh cranberries and cranberry pomace were cut with a razor blade for microscopic evaluation.

**Extraction of polyphenolics.** Polyphenolics from ground cranberry pomace were extracted three times by homogenization with acetone:water:acetic acid (70:29.5:0.5). The residue after extraction was collected for microscopic evaluation. Additionally, ground cranberry pomace was treated with sodium hydroxide to release bound procyanidins according to the method described by White et al. (18).

**Staining of samples.** Flavan-3-ols and procyanidins from fresh cranberry seeds and skins, pomace seeds and skins, and ground pomace were stained for 15 min with DMACA reagent (1 g DMACA dissolved in 100 mL 1.5N sulfuric acid) in micro-centrifuge tubes. Following staining, the samples were centrifuged, and the supernatant containing the stain was decanted. The residue was rinsed again with 1 mL of deionized (DI) water to removed excess stain, centrifuged, and the supernatant decanted. The DI water rinse was repeated once more.

**Light Microscopy.** Stained samples were mounted in either DI water or polyvinyl alcohol between a glass cover slip and slide and observed using a Zeiss Axioskop2 plus microscope (Peabody, MA). Digital images were taken with a JVC KY-F75U digital camera using Auto-Montage Pro v5.03 software. Samples were observed using either Bright Field or Differential Interference Contrast (DIC) microscopy at 10- or 20-times magnification.

**D. RESULTS AND DISCUSSION**

**Microscopic Evaluation of Fresh Cranberries.** Fresh cranberry skins were stained with DMAC to visualize procyanidins in cranberry skins. Procyanidins were
present in both unripe (Figure 2) and ripe (Figure 3) cranberry skins. The procyanidins did not appear to be localized in any particular part of the cell, but rather dispersed throughout the cell. However, this could be due to cellular disruption during sectioning of the skin pieces or procyanidin release during the staining procedure. Polyphenolic compounds are generally known to be present in the vacuoles of cells; however, upon damage to the cell, they may be released and dispersed throughout the cell. Additionally, the DMAC reagent may cause leaching of the procyanidins throughout the cell (9).

A cross-section of a cranberry seed was also stained with DMAC to visualize the location of procyanidins in the seed (Figure 4). It is evident from the image that procyanidins are only located in cells of the seed coat, as these are the only cells that were stained blue by DMAC. This is the first time that the localization of procyanidins is cranberry seeds has been reported; however, this finding is consistent with researchers who found that only the outer seed coat of Vitis vinifera L. cv Cabernet franc contained flavan-3-ols (19). Other researchers found that seeds coats of Pinot noir and Cabernet Sauvignon grapes contained a majority of both monomer and polymeric procyanidins; however, some were observed in the endosperm (20). The presence of procyanidins in the seed coat is indicative of their function as a protective barrier against potential predators (7).

**Microscopic Evaluation of Procyanidins in Cranberry Pomace.** Ground cranberry pomace (unstained) was observed under the light microscope (Figure 5). The cells appeared red, indicating the presence of anthocyanins remaining in the pomace. When the cranberry pomace was stained with DMAC for procyanidin visualization, all of the cells were stained blue, and no specific localization of procyanidins could be
determined (Figure 6). This is likely because the juicing process resulted in significant cellular disruption causing the procyanidins to disperse throughout the cell. Additionally, there appears to be a ring of blue surrounding each cell indicating the presence of procyanidins in the cell wall or middle lamella. The intense staining of procyanidins by DMAC in the cranberry pomace indicates that many of the procyanidins are not expressed in the juice, but instead remain in the skins and seeds. Polyphenolics from the ground pomace were then extracted by homogenization with acidified aqueous acetone, and the residue was observed microscopically (Figure 7). Most of the red pigment was removed from the cells, indicating efficient removal of anthocyanins during by polyphenolic extraction. When this residue was stained with DMAC, some cells were not stained, indicating that procyanidins had been effectively extracted from these cells (Figure 8). However, some cells were stained dark blue indicating the presence of procyanidins that were not extracted by homogenization with organic solvent. It is unclear why some cells retained procyanidins after polyphenolic extraction while others did not, but this demonstrates that under normal extraction conditions, procyanidins are not completely extracted from cranberry pomace. The pomace was also treated with sodium hydroxide to release bound procyanidins, and the residue was subsequently stained with DMAC (Figure 9). When these samples were observed under the microscope, none of the cells were stained blue, indicating complete removal of procyanidins by treatment with sodium hydroxide. Additionally, many of the cells were no longer intact but rather appeared to be fragments of cell wall material. It has been speculated that alkaline hydrolysis releases bound procyanidins by solubilization of cell wall material as well as depolymerization of polymeric procyanidins that are highly
bound to the cell wall (18). Sodium hydroxide is commonly used to extract hemicellulose from cell wall material (21). It is likely that the solubilization of hemicellulose leads to the release of procyanidins that are entrapped or even esterified to the cell wall. Additionally, depolymerization of procyanidins results in their release as smaller molecular weight monomers, dimers, and lower oligomers (18).

The occurrence of procyanidins during ripening of grapes has been extensively studied, and procyanidin content appears to decrease during ripening (22). The loss in procyanidins during ripening may be explained by the fact that these procyanidins become tightly bound to cell wall material and are rendered unextractable. This hypothesis is supported by the light microscopy pictures presented here. Additionally, if this holds true to other plant products, it is possible that there is a vast underestimation of procyanidin content in plants due to insufficient extraction methods.

This is the first time that procyanidins in fresh cranberries and cranberry pomace have been evaluated microscopically. In the cranberry seed, procyanidins are located primarily in the seed coat and are not present in the endosperm. Normal extraction procedures do not provide complete extraction of procyanidins from cranberry pomace. However, alkaline hydrolysis provides complete release of procyanidins from cranberry pomace and has potential for extraction of procyanidins from cranberries and other procyanidin containing plant materials.
E. LITERATURE CITED


F. FIGURE CAPTIONS

Figure 1. Structures of common flavan-3-ols and procyanidin dimers found in cranberries.

Figure 2. Bright field microscopy image of a section of unripe cranberry skin stained with dimethylaminocinnamaldehyde (DMAC) at 20x magnification.

Figure 3. Bright field microscopy image of a section of ripe cranberry skin stained with dimethylaminocinnamaldehyde (DMAC) at 20x magnification.

Figure 4. Differential interference contrast (DIC) microscopy image of a cross section of a cranberry seed stained with dimethylaminocinnamaldehyde (DMAC) at 10x magnification.

Figure 5. Differential interference contrast (DIC) microscopy image of ground cranberry pomace at 20x magnification.

Figure 6. Differential interference contrast (DIC) microscopy image of ground cranberry pomace stained with dimethylaminocinnamaldehyde (DMAC) at 20x magnification.

Figure 7. Differential interference contrast (DIC) microscopy image of residue remaining after ground cranberry pomace was extracted with acetone:water:acetic acid (70:29.5:0.5) at 20x magnification.

Figure 8. Differential interference contrast (DIC) microscopy image of residue remaining after ground cranberry pomace was extracted with acetone:water:acetic acid (70:29.5:0.5) and stained with dimethylaminocinnamaldehyde (DMAC) at 20x magnification.

Figure 9. Differential interference contrast (DIC) microscopy image of residue remaining after ground cranberry pomace was treated with 2 N NaOH at 60 °C for 15 m and stained with dimethylaminocinnamaldehyde (DMAC) at 20x magnification.
Figure 1
Figure 2

Figure 3
Figure 4

Figure 5
IX. CONCLUSIONS

Cranberry polyphenolics are relatively stable during processing compared to other berries and flavonols and procyanidins are more stable than anthocyanins. Hydrolysis of flavonol glycosides due to heat treatment led to an increase in levels of the flavonol aglycones, quercetin and myricetin, which are not present in fresh cranberries. A significant amount of polyphenolics are lost during processing as a result of pressing due to removal of the seeds and skins in the pomace. Therefore, cranberry pomace is a rich source of polyphenolics and should be explored as a source of these health benefiting compounds. This presents a potential economic advantage to the cranberry processing industry if they are able to recover valuable components from their waste material.

The cranberry pomace, in particular, contains high levels of A-type procyanidins, which have been attributed with cranberries’ ability to prevent urinary tract infections. A majority of the A-type procyanidins in cranberry pomace, however, are large molecular weight polymeric compounds, which are not absorbed because of their size. Smaller molecular weight monomers, dimers, and trimers have similar health benefits and are better absorbed. Additionally, a significant portion of procyanidins in cranberry pomace are bound tightly to other cellular components through hydrophobic interactions, hydrogen bonds, or covalent linkages, which renders them unextractable under normal solvent extraction conditions.

Extrusion processing is a high temperature, short time process that is capable of altering the procyanidin composition of cranberry pomace. An increase in procyanidin monomers and dimers and a decrease in high molecular weight procyanidins (DP > 4) as observed following extrusion of cranberry pomace. This indicates that extrusion may
enhance the bioavailability of procyanidins from cranberry pomace by converting large molecular weight polymers to monomers and dimers, which are better absorbed. Alkaline hydrolysis, under specific conditions, is also capable of altering the procyanidin composition of cranberry pomace by increasing the amount of low molecular weight procyanidins (DP1 – DP6) and decreasing the amount of polymers (DP > 10). It does so by breaking the interflavan bond linking the monomeric units of the procyanidins, thus decreasing their DP. Additionally, through depolymerization, alkaline hydrolysis is capable of releasing procyanidins that are bound to other cellular components. The end result is a significant increase in free procyanidins that are bioavailable. This process is also capable of releasing bound procyanidins from a variety of fruit by-products.

Although it is unclear which active component actually reaches the urinary tract to prevent infection, presumably absorption of the active A-type procyanidin or one of its metabolites is a necessary first step. Therefore, extrusion and alkaline hydrolysis should be explored by the cranberry processing industry as technologies to enhance the bioavailability of procyanidins from cranberry pomace. The lower molecular weight compounds could either be absorbed in the small intestine or passed into the colon. If they do reach the colon, the lower molecular weight compounds have a better chance of being fermented by colonic microflora than polymeric procyanidins to produce metabolites that could then be absorbed. The resulting extracts from extrusion or alkaline hydrolysis could be added back to juices to provide additional health benefits or explored as dietary supplements.