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Changes in Monomeric and Polymeric Pigments during Chokeberry Juice Processing

Changes in Monomeric and Polymeric Pigments during Chokeberry Juice Processing

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

By

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University of Arkansas
Bachelor of Science in Food Science, 2012

December 2014
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This thesis is approved for recommendation to the Graduate Council

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ABSTRACT

Chokeberry consumption has been increasing due to research exposing great potential of health-promoting compounds. However, chokeberries are highly astringent and are typically consumed in processed forms in which product heating is applied. Processing chokeberries has been reported to degrade bioactive compounds, thus limiting the potential for consumers to obtain their health-promoting benefits. The purpose of this research was to examine the effects of chokeberry juice processing and storage on anthocyanin, flavonol, proanthocyanidin, hydroxycinamic acid, and polymeric pigment content, as well as percent polymeric color. In addition, new analytical methodologies were explored to better understand possible outcomes of polymeric pigments due to juice processing and storage.

Chokeberries were processed into nonclarified juice with sampling at each stage of processing. Levels of anthocyanins, flavonols, proanthocyanidins, hydroxycinamic acids, and percent polymeric color were also analyzed each month throughout a 6 month storage period at ambient temperature. It was determined that anthocyanins readily degrade during juice processing, especially with heat applications during blanching and pasteurization. Comparatively, other compounds, such as proanthocyanidins were better retained during processing than anthocyanins, with significant levels remaining in the presscake. After pasteurization, lower levels of anthocyanins (7%), flavonols (52%), proanthocyanidins (55%), and hydroxycinamic acids (63%) remained in the juice. Alternatively, polymeric color values increased to 29% throughout processing. During juice storage, polyphenolic levels continued to decrease over 6 months while percent polymeric color values increased further to 44.5%. Little change occurred in levels of total flavonols (447.8 to 406 mg/100g), proanthocyanidins (19.7 to 16.5 mg/100g), and hydroxycinamic acids (72.7 to 48.9 mg/100g) over 6 months of storage.

After observing a 55% reduction in anthocyanins due to blanching frozen chokeberries, standard juice processing was altered by removing the blanch step and its effect on polyphenolics was evaluated. The effect of two different juice processes (with and without blanching) on anthocyanin, proanthocyanidin, flavonol, and hydroxycinnamic acid contents as well as percent polymeric color was evaluated at each stage of processing. Juice pasteurization times and temperatures were also evaluated to develop a statistical model that would predict optimal anthocyanin retention. In comparison of the two processes, there were no significant differences in anthocyanin content after pasteurization; however, samples receiving no blanch had higher levels of anthocyanins after enzyme treatment. Pasteurized juice samples receiving a blanch treatment had 37% and 16% higher levels of total proanthocyanidins and flavonols, respectively than pasteurized juice receiving no blanch treatment. A response surface model was designed for the prediction of anthocyanin retention with optimum pasteurization conditions of 74°C for 2.02 minutes.

MALDI-TOF-MS was used to identify large molecular weight proanthocyanidins and polymeric pigments throughout each stage of processing and over six months of juice storage. Proanthocyanidins and polymeric pigments having up to a degree of polymerization (DP) of 16 and 14, respectively, were identified in frozen berries, samples obtained throughout juice processing, and stored juices. In attempt to separate polymeric pigments, both normal phase and reverse phase TLC plates with various solvent systems were evaluated. However, only monomeric and polymeric fractions could be separated on a single plate, rather than separating each polymeric pigment by degree of polymerization. Further research is needed in order to isolate and purify polymeric pigments so that quantification methods can be developed and help explain the fate of anthocyanins during juice processing and storage.

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Chapter 3.

Wilkes, Kail; Howard, Luke R.; Brownmiller, Cindi; Prior, Ronald L. Changes in chokeberry (*Aronia melanocarpa* L.) polyphenolics during juice processing and storage. *Journal of Agricultural and Food Chemistry*. **2014**. *62*, 4018-4025.

I. INTRODUCTION

Berry fruits have been part of the human diet for centuries. Reasons for consumption of berries expand throughout culinary versatility and enhancement of life quality. Berries are even being utilized in the world of pet care in which they serve as key ingredients in high quality pet food. With this growing popularity, the demand for increased berry production is higher than it has ever been. According to the National Agricultural Statistical Services (*1*), the production values of blackberries, blueberries, cranberries, raspberries, and strawberries have been on the rise for the past several years. Flavor, functionality, and color are the characteristics involved in persuading consumers to purchase berries; the most important of these is color.

Color of berries is a strong representation of quality value and nutritional functionality. In addition to caloric content, dietary fiber, vitamin C, and folic acid, berries contain flavonoids which are being examined for their protection against chronic diseases (*2*). Anthocyanins are the pigment molecules responsible for the attractive colors of berries, ranging from reds to dark purple and black. Anthocyanins, as well as other phenolic compounds, are more concentrated in the skins and seeds of the fruit rather than fleshy material (*3*). These pigment compounds and other polyphenolics are responsible for the numerous health benefits claims. Recent nutrition studies are finding that polyphenolics may protect against chronic diseases by anti-cancer, anti-inflammatory, anti-obesity, and anti-heart disease effects (*4, 5, 6, 7, 8*). However, studies have shown that polyphenolics, especially anthocyanins, decrease dramatically throughout berry processing and storage of processed product (*9*).

Anthocyanin pigments are relatively unstable as they are affected by pH, storage temperature, enzymatic activity, light, oxygen, molecular structure, concentration, and molecular

interaction with other compounds (*10, 11*). Berry processing practices consist of several of these factors that can alter pigment and phenolic compounds. Since berries have such a short growing season, only summer months, a large portion of what are grown is typically processed to form shelf-stable products that are available to consumers throughout the year. Jams, jellies, juices, wines, and canned berries are just a few examples of processed products. Due to their instability, pigments change in structure to form polymers with proanthocyanidins or are transformed to other phenolic compounds, which can adversely affect the quality and potential health benefits of processed berries (*9*). The polyphenolic content of berries typically decreases when processed as a result of thermal treatments, juice pressing (physical removal of polyphenolic-rich seeds and skins), and clarification of juices. Pigments are susceptible to enzymatic degradation from peroxidase and polyphenoloxidase which have been shown to convert other polyphenolics to quinones, which in turn cause rapid degradation of anthocyanins (*12, 13, 14*).

One berry that is gaining much attention due to its exceptional content of polyphenolics is the chokeberry (*Aronia melanocarpa*). Several studies have been conducted to observe effects of processing and storage on chokeberry pigments and quality. For the most part, chokeberries are impalatable due to their astringent nature as a result of their exceptional procyanidin content (*15*), hence they are almost always processed to other forms to improve flavor and increase consumption. However, little knowledge is known about chokeberries (*Aronia melanocarpa*) which are becoming quite popular in health benefits research and general public consumption. Although more popular in European countries, chokeberries are native to the United States, and commercial production of the berries and derived products are increasing (*16*). The chokeberry is very dark in color due to its high anthocyanin content which is believed to provide major health benefits after consumption (*15*). Chokeberries are also rich in proanthocyanidins (*15*),

and the combination of elevated levels of anthocyanins and proanthocyanidins make chokeberries susceptible to the formation of polymeric pigments during juice processing and storage. As mentioned before, little information is known about the effect of processing, specifically juice processing, on changes in chokeberry polyphenolics. There exists a need to determine how each stage of chokeberry juice processing, as well as juice storage, impact the polyphenolic composition of chokeberries in order to gain a greater understanding of the mechanisms responsible for anthocyanin losses and pigment polymerization.

Objective 1: To identify and quantify anthocyanins, flavonols, hydroxycinnamic acids, proanthocyanidins, protocatechuic acid, phloroglucinaldehyde, and monitor percent polymeric color at each stage of chokeberry juice processing and throughout 6 months of storage.

Objective 2: To conduct blanching and juice pasteurization studies to determine the role of thermal treatments on anthocyanin degradation and formation of polymeric pigments.

Objective 3: To identify polymeric pigments at each stage of chokeberry juice processing and throughout 6 months of storage utilizing MALDI-TOF-MS.

Objective 4: To separate polymeric pigments by degree of polymerization using TLC and develop a MALDI-TLC analytical method to identify bands and fractions separated by TLC.

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II. LITERATURE REVIEW

A. BERRY FRUIT

Production. In the past several years, berry fruit production has been on the rise. In the U.S., strawberries are leading with almost 3 billion pounds being produced in 2011 (*1*), with a value ~ 2.4 billion dollars (*2*). Following strawberries is the production of raspberries which is close to 2 billion pounds, with a value over a quarter of a billion dollars in the U.S. (*1, 2*). Other berries that are important in U.S. production include blueberries, blackberries, cranberries, and black raspberries. The two markets for berry production are the fresh and processed, in which the fresh market receives most of the berry production. Within the processed market, berries are typically frozen directly after harvesting or processed in various forms before being frozen so that products can be produced throughout the entire year rather than being limited to growing season (*3*). One berry in particular that is more so consumed in processed form is the chokeberry.

Chokeberries. Chokeberries (scientific name *Aronia melanocarpa*) , are typically black in color and grow in clusters throughout the shrub (*4*). Chokeberry shrubs will bear fruit in late August and early September (*5*). Fore-mountain areas with high humidity and uniform annual rainfall are the most favorable environments for growth. After planting, chokeberries do not develop until the second year, but they are not fully mature. The shrubs produce fully developed berries with high phenolic content after the fourth year. Naturally, chokeberries are relatively resistant to disease and insects, but they are susceptible to other animals such as deer and birds (*6*). Chokeberries are native to the United States (Great Lakes, Northeast U.S., and Appalachian Mountain regions), but they have become more popular in European countries where most of the

commercial production occurs. Large-scale commercial production first started in the Soviet Union in the 1940's. Currently, Poland is one of the largest producers of chokeberries (*7*). Due to high concentrations of polyphenolics, which contribute health-promoting benefits, chokeberry products are marketed primarily as nutraceuticals and dietary supplements. The phenolic compounds' (namely, polyphenolics) of chokeberries, in respect to content and composition, are dependent on a series of factors such as cultivar, fertilization, berry maturation, time of harvest, or habitat/location (*8*).

Chokeberries are processed to create a wide variety of food products such as jams, jellies, wines, juices, and snack foods. They are also used for color enhancement in which the chokeberry pigments are extracted and added to products such as juice blends; this practice also increases phenolic content of the finished product.

Polyphenolic Content. Berry fruits contain high concentrations of polyphenolics like flavonoids and tannins (proanthocyanidins and ellagitannins). Flavonoids are a subgroup of polyphenolic compounds that include anthocyanidins, flavonols and flavanols, flavan-3-ols (catechins), and flavan-3,4-diols (*9*).

Anthocyanins. The anthocyanin group comprises the compounds that impart the black, blue, and red colors in berries (*10*). These compounds differ in the number and position of methoxyl and hydroxyl groups particularly on the B-ring in the flavilium ion skeleton (*11*). The substitution pattern of the B-ring and pattern of glycosylation of the flavan structure determine the reflected color (*12*). Figure 1 is the anthocyanidin basic structure derived from the flavylium ion and a list of the various types depending on the location of the methoxyl and hydroxyl groups.

Sugar moieties can attach to the anthocyanidin structure which changes them to anthocyanins (*11*). Anthocyanin structure consists of the aglycone base on the flavylium nucleus, sugar moietie(s), and often acyl acid(s) (*3*). Chemically, they are "glycosylated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and contain two benzoyl rings (A and B) separated by a heterocyclic (C) ring" (*13*). At the C-3 position, single sugars attach - most commonly glucose, galactose, arabinose, xylose, and rhamnose (*11*). When glycosylated with one or more sugar moieties at the C-3 position on the flavilium ion, they are called anthocyanins (*11*). Sugars and diglycosides can attach at other carbon positions of the anthocyanin structure (*11*). In addition, sugar moieties of anthocyanins can be acylated with aliphatic acids such as acetic, malonic, succinic, and oxalic (*11*). Anthocyanins located in the vacuoles of the cells are water-soluble and are concentrated in the skins of berry fruit (*14*). Anthocyanin content and composition varies between berries with blueberries having the most diverse composition (*11*). Chokeberries contain up to 6 different glycosylated anthocyanins. Sugar moieties are attached at the C-3 position of the cyanidin and pelargonidin structures including galactose, glucose, xylose, and arabinose (*15*). However, two cyanidin derviatives, galactoside and arabinoside, account for >80% of the total anthocyanin content of chokeberries (*15*).

Flavanols. Condensed non-hydrolysable tannins are proanthocyanidins, which are another subgroup of flavonoids that form oligomeric and polymeric structures made up of flavan-3-ols linked by C4 to C8 as well as C4 to C6 positions (*11*). Procyanidins are a class of proanthocyanidins which are only comprised of epicatechin or catechin flavan-3-ol units (*11*). (+)Catechin and/or (-)epicatechin units can link together through C4→C8 and C4→C6 which is the B-type, while A-type is the linkage between C2→C7 (*16*). The molecular size of

proanthocyanidins is measured by degree of polymerization in which oligomers have DP 10 units or less while polymers have DP greater than 10 units (*17*). The proanthocyanidins in chokeberries have been well characterized, ranging from monomers to decamers, but they also contain exceptionally high levels of polymers (*15*).

B. BERRY HEALTH BENEFITS

In the past several decades, studies shown that multiple health benefits are correlated with both fresh berry and processed product consumption. Recent research has been exploring berry polyphenolics and their biological properties of promoting health benefits in the areas of heart health, cardiovascular disease, neurodegenerative diseases, aging diseases, obesity, human cancers, symptoms of metabolic syndrome, and the enhancement of human performance (*18, 19*). Epidemiological research suggests that berry consumption, along with vegetables, helps delay the ageing process and reduces risk of lifestyle diseases (*20, 21, 22, 23*). Berries, in general, contain high amounts of phenolic compounds with various bioactive properties to promote human health. Chokeberries are among the leaders in phenolic compound contribution with some of the highest concentrations ever recorded above all edible berries (*14*). The most commonly researched health benefits associated with berry phenolic compounds include antioxidant capacity, anti-cancer, and anti-inflammatory effects. Most importantly, however, bioavailability and metabolism of the polyphenolics are determining factors influencing of health benefits.

Antioxidant Effects. Tannins, flavonoids, and phenolic acids are the major contributors of antioxidant activity in which they help protect unsaturated fatty acids, carotenoids, vitamin C, and human low-density lipoproteins against oxidation (*14*). The phenolic molecular structure comprising of an aromatic ring with hydroxyl group is what gives polyphenolics their

antioxidant capabilities (*24*). Antioxidants are hydrogen atom donors which allow them to scavenge free radicals, quench singlet oxygen, and complex with pro-oxidant metals (*25*). Oxidation within the human body results from the formation of free radicals and other reactive oxygen species which generally damage DNA, proteins, and lipids (*26*). When more reactive oxygen species exist than antioxidant defenses, oxidative stress occurs which can cause cell damage and cell death. The damage caused by oxidative stress can lead to adverse health effects such as inflammation, cardiovascular disease, cancers, and several other degenerative diseases (*27*). Numerous studies have shown strong correlations between berry consumption and the reduction of the risk of these adverse health effects as well as the onset of these effects by examining the antioxidant activity contributed by berry polyphenolics.

Antioxidant capacity shares a linear relationship to the phenolic content in berry fruits (*11*). Numerous *in vitro* studies have been conducted to assess the antioxidant capacity of various compounds, foods, and dietary supplements (*28*). Seeram and Nair (*29*) studied antioxidant capacities of several catechins, anthocyanins, and anthocyanidins within a liposomal oxidation model and compared the performance to synthetic antioxidants. Lipid peroxidation was induced by iron (Fe-II) ions within the models. They found that hydroxyl groups on the 3', 4', and 5' positions on the B-ring increased the ability to inhibit lipid peroxidation. Delphinidin had the greatest antioxidant effect among the anthocyanins and anthocyanidins, and (-)-catechin inhibited lipid peroxidation best among all catechins tested. However, the artificial antioxidants (TBHQ, BHT, and BHA) were superior above all tested compounds in antioxidant capacity (*29*).

Limitations exist when evaluating antioxidant capacity due to the fact that most studies are *in vitro* and may not "reflect the cellular physiological conditions" nor "consider bioavailability, metabolism, and other issues." Human *in vivo* studies generally evaluate

antioxidant capacity of blood plasma which correlates to the state or intensity of oxidative stress (*24*). Mazza et al. (*30*) observed increases of plasma antioxidant capacity in humans after consumption of freeze-dried blueberry powder. The study concluded that foods that demonstrate a high *in vitro* antioxidant capacity can be associated with diet-induced antioxidant capacity increases within the body. Several other studies have shown similar results in which increasing the consumption of high antioxidant capacity foods can increase that of blood plasma, a biomarker of oxidative stress (*31, 32, 33, 34*).

Other Health Benefits. Epidemiological studies have observed an inverse relationship between consumption of polyphenolics from fruits and vegetables and oxidative stress mediated diseases (*35*). Besides antioxidant effects, correlations have been observed that would suggest polyphenolics can prevent adverse health effects by regulating enzymes associated with xenobiotic and carcinogen metabolism, regulating nuclear receptors in cells, stimulating the immune system, reducing proliferation, protecting DNA from damage, and posing antiviral effects (*36, 37*).

Seeram et al. (*38*) conducted an *in vitro* study with tumor cell lines and cranberry. From the study, the cranberry extract containing proanthocyanidins, anthocyanins, and other flavonoids ceased the cell growth of tumor cells associated with oral, colon, and prostate cancers. Among other anticancer studies, berry phenolics correlated with apoptosis effects of cancer cells with anthocyanins being the major contributor of apoptosis effects on cancer cells. In addition, berry polyphenolics have been shown to prevent new blood vessels (angiogenesis) from forming in cancer cells. The ‘anti-angiogenic’ effect decreases the amount of oxygen and other nutrients needed for cell growth, thereby promoting tumor cell death (*39*).

In an animal study, blueberry supplementation was factored in mice diets that were part of an Alzheimer's disease model with the idea that the phenolic compounds would improve enzymatic activities that are directly related to memory signaling. The study showed that neuronal signaling was enhanced and that there is the possibility of preventing behavioral deficits with blueberry supplementation (*40*).

Bioavailability and Absorption. Despite the numerous health benefits associated with berry polyphenolics, not enough information is known about the bioavailability, absorption, and metabolism of these bioactive compounds throughout the human body. Through the course of digestion and metabolism, polyphenolics are exposed to variations in pH and interact with other chemicals. Human studies can observe amounts of phenolic compounds ingested and excreted via urine and fecal matter. The only information considering human absorption and metabolism is what can be detected in the blood plasma after withdrawal. After oral consumption, anthocyanins are subjected to the highly acidic environment of the stomach, and it is possible that sugar moieties can detach by acid hydrolysis in which the flavylium cation remains predominate. A relatively similar acidic environment continues while in the small intestine. Once in the intravenous system, plasma and blood are at a more neutral pH which would most likely cause the flavylium cation to change into quinonoids and chalcones (*13*). Most human studies have shown that anthocyanins have a relatively low bioavailability in which $< 0.1\%$ of the ingested dose is recovered in the excreted urine. Cao and Prior (*41*) observed 100 μg/L in human plasma 30 minutes after oral consumption of about 1.5 g of anthocyanins within an elderberry extract. Other studies have observed similar rapid absorption times which would suggest that anthocyanins, as well as other flavonoids, are absorbed in the stomach (*13, 42*). Most of these studies administer an unusually high dosage of polyphenolics, typically in the form

of concentrated extracts or pills. There still is uncertainty about possible chemical transformations or other breakdown products that may occur in the body.

Hollman and Katan (*43*) suggested that microflora within the colon may have the ability to hydrolyze flavonoids into simpler phenolic compounds and phenolic acids, allowing for reabsorption into the body which would contribute to health benefits. This would help explain the fate of larger polymeric pigments that are too large to be absorbed in the small intestine, as well as the very small amounts being detected in the urine. Other types of biotransformation occur in various locations within the human body. During metabolism, flavonoids can undergo glucuronidation, methylation, and sulfation which makes them more water soluble and decreases antioxidant activity (*44*).

C. CHOKEBERRIES AND HEALTH

Antioxidant capacity is assessed by the oxygen radical absorbing capacity (ORAC) assay that measures the "antioxidant scavenging activity against peroxyl radical initiated by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH)" (*15*). Chokeberries contain abundant levels of phenolics (proanthocyanidins, anthocyanins, hydroxycinnamic acids, and phenolic acids) that contribute to high ORAC value and numerous human health benefits (*8*). In a study that compared the antioxidant activity of several berry fruits (blueberry, cranberry, lingonberry, black currant, red currant, gooseberry, and elderberry), chokeberries were reported to be highest (*15*). Extensive research involving both *in vitro* and *in vivo* studies has been performed over the past 15 years. Multiple areas of potential health benefits associated with chokeberries have been examined, and new information is still being discovered. Chokeberry antioxidant capacity is most commonly studied due to the numerous possible adverse health effects that result from

oxidation. Beyond antioxidant capacity, research is branching out into newer areas such as anticancer effects, cardiovascular health, anti-obesity effects, and neurological health (*45*).

Hepatoprotection is a relatively new researched health benefit in which tests evaluate the ability to prevent damage to the liver. Carbon tetrachloride is a highly toxic compound and is capable of causing lethal liver injuries. In a rat study, chokeberry juice was administered before and during carbon tetrachloride-induced hepatoxicity. Rats that consumed the chokeberry juice did not experience damage to their livers nor did they experience any internal lipid oxidation (*46*).

Valcheva-Kuzmanova and Belcheva (*45, 47*) reported that chokeberry juice possessed both antibacterial and antiviral activities. The juice was observed to be bacteriostatic against *Staphylococcus aureus* and *Escherichia coli* and antiviral against *Influenza virus* type A. Evidence suggested flavonoids react with the bacteria membranes that led to a decrease in bacterial activity. In addition, positively charged anthocyanins formed complexes with the virus that prevented absorption through the human cell membrane.

Several other pharmacological effects have been associated with chokeberry consumption in both human and animal studies (mainly in the form of dry powder extract and juice). These include lipid-lowering (*48*), cardio-protective (*49*), antihypertensive (*50*), gastroprotective (*51*), antidiabetic (*52*), and anti-inflammatory (*53*). Polyphenolics, namely flavonoids, within chokeberries appear to play an important role in health promotion. The major issues still being examined include berry processing practices and their significance on chemical interaction and degradation that lead to changes in polyphenolic composition, which can alter the magnitude of these health benefits.

D. BERRY JUICE PROCESSING

Due to abbreviated growing season and their perishable state, berries are often processed into products including jams, jellies, nectars, wines, purees, canned and frozen products (*54*). After harvesting, a portion of the berries are frozen and periodically processed over time (*55*). Juice processing requires multiple steps including heat application, skin disruption, separation of liquid and solid material, and pasteurization. Figure 2 outlines the general juice processing steps.

Berries are generally thawed and treated with pectinase enzymes in order to release pigments and juice from within the cells and cell walls (*56, 57, 58*). Enzyme treatment usually occurs after berries have been blanched to inactivate browning enzymes such as polyphenoloxidase and peroxidase. The pulp is then pressed to remove solid material from the juice (*58*). Juice is then pasteurized to destroy spoilage microorganisms and extend shelf life. Juices are often concentrated by evaporation which can improve stability and reduce transportation costs. Unfortunately, processing leads to major reductions of phenolic compounds.

Anthocyanins. Anthocyanins are relatively unstable compounds. Glycosylation of the anthocyanidin structure as well as acylation of the sugar residues can greatly improve stability (*9*). Sugar moieties attached at C3 and C5 and anthocyanin acylation help stabilize anthocyanins from effects of light and heat. Anthocyanins are also more stable in relation to high concentrations in berry products (*59*). Anthocyanins can also form complex intermolecular structures with flavonols, metal ions, and other anthocyanins in order to improve stability (*9*). Several studies show major losses of anthocyanins after berry juice processing when converting back to a frozen berry basis after anthocyanin analysis. Anthocyanin losses of 62%, 67%, and 65% were reported for blueberries (*60*), blackberries (*61*) and black raspberries (*62*) when frozen

berries were processed into juice. Consistent with studies on other berries, Borowska et al. (*63*) reported anthocyanin loses of up to 85% when chokeberries were processed into juice compared to the original fresh berry content. From these and other studies, it appears that heat application during pasteurization and physical removal of solid material during pressing are the major factors responsible for the dramatic anthocyanin loss.

Thermal Application. According to the model juice process in **Figure 2**, heat is applied during blanching, enzyme treatment, and pasteurization (extensive heat applied during blanching and pasteurization). Heat is mainly believed to accelerate degradation due to oxidation, rather than being a direct cause of anthocyanin degradation. Oxidation can cleave various covalent bonds and result in several different phenolic compounds depending on the intensity of thermal application (*64*). Palamidis and Markakis (*65*) observed anthocyanin degradation rate increases as temperature increases during processing and storage of grape products. Figure 3 demonstrates possible pathways of anthocyanins being degraded into simple phenolic compounds.

Little knowledge is known about the exact mechanism(s) responsible for anthocyanin degradation in the presence of heat. It has been proposed, over the years, that the pyrylium ring opens forming chalcones (*66*), hydrolysis of sugar moieties forming aglycones, or loss of the B-ring (*67*). Rate of anthocyanin degradation has been studied by several researchers. Segal and Negutz (*68*) reported a logarithmic relationship among temperature and anthocyanin reduction, while Adams (*69*) reported a logarithmic reduction with applied heat held at a constant temperature.

Enzymatic Effects. Phenolase, polyphenolase, and glycosidase enzymes have been shown to play a significant role in anthocyanin degradation (*70, 71*). Polyphenol oxidase has been

known to catalyze oxidation of chlorogenic acid into quinones. The quinones can undergo a condensation reaction with anthocyanins to form brown products (*72*). Kader et al. (*73*) studied enzymatic effects and found that no degradation occurred in peroxide-free solutions which indicated that peroxidase contributes to browning as well as polyphenol oxidase. Skrede et al. (*26*) studied the effects of blanching highbush blueberries on anthocyanin retention after pasteurization. The study showed better retention occurred in the juice from blanched blueberries than non-blanched berries. This evidence supports that blanching reduces anthocyanin degradation effects from enzymes.

Copigmentation. Copigmentation refers to the pigment stabilization and/or enhancement reflected by bathochromic shift (change of the maximum absorption wavelength) of an anthocyanin when linked to another compound. For example, cyanidin-3-glucoside has a maximum absorbance of about 512 nm. When linked with rosmarinic acid, the maximum absorbance shifts to about 527 nm. Condensation between tannins and anthocyanins is common in stabilization, as well as catechins, amino acids, polysaccharides, and metal ions (*74*).

Oxygen Effects. Oxygen is a major problem for anthocyanins as it can accelerate oxidation mechanisms directly and/or can assist degradation from active oxidizing enzymes. Indirectly, degradation can occur by oxidation of ascorbic acid to produce hydrogen peroxide which can also be accelerated by metal ions (*70, 75*). Ascorbic acid has the ability to condense with anthocyanins which will degrade color (*70*).

pH Effects. pH has a major influence on the color of anthocyanins with the compounds displaying an intense color in low pH environments. However, when pH is above 4.5, the anthocyanins begin to degrade and original color is altered (*59*). At a pH less than 2, the

flavylium ion structure is predominant which reflects a bright red color. As pH increases, the molecular structure changes to form colorless chalcones and quinoidal bases which tend to be blue (*3*). Cabrita et al. (*76*) conducted a stability study in which anthocyanin solutions were stored for 60 days at 10°C and 23°C in various buffer solutions of pH 1-12. The study showed that over 70% of initial anthocyanins in the acidic solution (pH 1-3) were retained over the 60 days at 10°C, while more than 90% of the anthocyanins in the solutions of pH 5-6 were degraded after 8 days. At 23°C, anthocyanins degraded at higher rates than samples stored at 10°C.

Proanthocyanidins. The flavan-3-ols, catechins and epicatechins, are capable of condensing into oligomeric and polymeric proanthocyanidins during juice processing and storage (*77, 78*). These compounds are mostly located in the seeds of berries, and the greatest loss generally occurs at the pressing stage of processing in which the seeds are removed from the juice. Proanthocyanidins can stabilize color by linking with anthocyanins at C4 position. During storage, more condensation reactions occur, forming polymeric pigments (*54*). The formation of polymeric pigments is not entirely understood as to what mechanisms promote or catalyze the condensation reactions. Research studies on wine show that the process is slow requiring a long time for pigments to polymerize, and that the rate of polymerization is directly related to temperature of the surrounding environment (*79*).

Polymeric Color. During berry processing and storage, monomeric anthocyanins bind with other compounds, such as proanthocyanidins or other flavanols, to form polymers which help stabilize color. The bond occurs at the C4 position of the anthocyanin. Percent polymeric color is a measurement based on the ratio of bleached solution to a non-bleached solution. The spectrophotometric assay is designed to demonstrate the bleaching capacity of potassium metabisulfite which will only make an anthocyanin colorless if it is able to bind to the C4

position of the anthocyanin. The pigments that are not bleached once the potassium metabisulfite has been added to a berry sample are believed to represent anthocyanins linked to proanthocyanidins (polymeric pigments) at C4 (*54*). Studies have shown that polymeric color increases throughout berry juice processing and storage. A. Hager et al. (*62*) reported an increase of polymeric color between fresh black raspberry to 6 month juice from 8.6% to 32%. In a similar study, T. Hager et al. (*61*) observed major increases of polymeric color in stored blackberry juice starting with 12% and ending with 38%, respectively after 6 months. This study also observed that during the juice process, the presscake contained the highest amounts of polymeric color, most likely due to polymeric anthocyanins remaining in the solid material after pressing.

It must be noted that the percent polymeric color assay is not a quantitative method for the actual amount of polymeric pigments present in a sample. It is simply a ratio of anthocyanins susceptible to bleaching to those resistant to bleaching after addition of potassium metabisulfite. There is the possibility that flavan-3-ols are linked to C8 position of an anthocyanin which would technically be a polymeric pigment but would be bleached by potassium metabisulfite since the C4 would be exposed. In this case the percent polymeric color value would be underestimated. In addition, polymeric pigment standards do not exist because they cannot be separated by HPLC, so true quantification of polymeric pigments is an objective that needs further research to obtain (*54*).

E. JUICE STORAGE

Anthocyanins. Following processing, anthocyanins readily decrease in concentration over time when stored at ambient temperature (*6, 54, 60, 61, 62, 80*). Trost et al. (*81*) reported a linear relationship in the degradation rate of cyanidin-3-glucoside within blueberry-aronia nectar,

and also concluded that nectar stored in glass containers retained more anthocyanins than nectar stored in carton containers as a result of glass having a greater barrier to oxygen. The study also demonstrated that different anthocyanins varied in stability with cyanidin the least stable followed by peonidin, petunidin, then malvidin and delphidin. In addition, it was concluded that anthocyanin stability was greater with those glycosylated with glucose followed by galactose and arabinose. After 207 days of storage at 30°C, anthocyanin concentration decreased from 480.3 mg/L to 39.9 mg/L in nectars stored in cartons (183 days) and 481.3 mg/L to 51.2 mg/L in nectars stored in glass containers. Nectar stored in cartons showed a 22% higher anthocyanin degradation rate than the nectar stored in glass containers.

Wang and Xu (*82*) studied anthocyanin degradation kinetics of blackberry juice during storage at temperatures of 5, 25, and 37°C. Anthocyanins degraded in a linear fashion at 5 and 25°C while exponential degradation was observed at storage temperature of 37°C. Trends of decreasing anthocyanin content with the inverse relationship of increase in polymeric color values have been observed in recent juice storage studies (*54, 60, 61, 62, 80*). Brownmiller et al. (*60*) observed a significant reduction in anthocyanins, 68 and 64% in nonclarified and clarified blueberry juice, respectively, over 6 months of storage. The study also observed a linear increase in percent polymeric color of 10 to 25% for nonclarified juice and 8 to 17% for clarified juice stored at ambient temperature. Similar results were observed in a study involving stored blackberry juice at 25°C (*61*), as well as a study involving black raspberry juice (*62*). For chokeberries, an accelerated storage study was conducted in which pasteurized juice was stored at 40°C for 6 weeks (*54*). The results were a dramatic loss of anthocyanins, an increase of percent polymeric color from 20.2 to 67.8%, and a major increase of polymeric pigments that were analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry

(MALDI-TOF-MS). Fresh (pasteurized) and aged juices were compared to observe the increase in polymeric pigments. The pigments identified comprised of an anthocyanin with one to ten flavan-3-ol units attached via B-type linkage with associations of sodium and potassium adducts. Pigment polymerization may explain the loss of monomeric anthocyanins, however, polymeric pigment quantification cannot yet be determined due to the inability to separate the compounds by HPLC and lack of standards necessary for quantification. In order to prevent the dramatic loss of monomeric anthocyanins, juice should be subjected to refrigerated or frozen storage. Ancos et al. (*83*) conducted a study to observe the effects of frozen storage and anthocyanin retention of various raspberry cultivars. The raspberries were analyzed in raw form and after 1 year of frozen storage at -20°C. Virtually no change in anthocyanin concentration was observed after one year of frozen storage when compared to the raw berry.

Several reactions can occur during juice storage that can lead to dramatic decreases in anthocyanin content, depending on the storage temperature. A common observation is the condensation reactions between anthocyanins and flavan-3-ols to form polymeric pigments (*84*). Oxidation of anthocyanins and other phenolic compounds can also occur during juice storage depending on level of headspace within the container the juice is being held (*64*). Other common factors that affect anthocyanin retention must not be ruled out which include sugars, ascorbic acid, pH, light, metal ions, and possible presence of residual enzymatic activity (*85*).

Proanthocyanidins. In the accelerated storage study of chokeberry juice by Howard et al. (*54*), a decrease of cyaniding 3-galactoside linked to one, two and three flavan-3-ol units was observed while an increase in larger degree of polymerization polymeric pigments occurred. Behaving similarly to anthocyanins, dramatic loss of proanthocyanidins is generally observed throughout juice storage at ambient temperatures. The reduction is also accompanied by an

increase in percent polymeric color which may be inferred as indication of the formation of polymeric pigments. The idea of polymerization of pigment molecules was first studied in wines because it was initially believed that the reactions take long periods of time to occur such as aged red wine, which change from bright to dark red or mauve color (*54*). Polymeric pigments can be formed by various pathways. In fermented products such as wine, acetaldehyde can bind with an anthocyanin for pigment stability, which then can allow for a catechin(s) or epicatechin(s) to link with the compound to form polymeric pigments (*86*). Anthocyanins can also bind with flavan-3-ols by direct condensation to form larger polymers (*87*). In addition, anthocyanins can interact with hydroxycinnamic acids, pyruvic acid, and/or acetaldehyde to form pyranoanthocyanins during storage (*88*).

Anthocyanin and Procyanidin Mitigation during Processing and Storage. Several treatments exist to prevent the degradation of anthocyanins, but these practices may not be applicable or cost efficient to real, large-scale processing. For instance, degradation effects due to oxygen and/or enzymes could be prevented if berries were processed under vacuum or oxygen excluded environment by use of nitrogen atmosphere. In addition, condensation between anthocyanins and flavonols prevent harmful effects from ascorbic acid (*75*). Howard et al. (*54*) proposed that the use of enzyme inhibitors could help prevent polyphenolic loss during processing and storage if all enzymes are not inactivated by blanching and pasteurization. During juice processing, the physical removal of seeds and skins during pressing dramatically decreases polyphenolic content. Hence, an opportunity exists to recover polyphenolics from the pomace and add them back to the juice (*89*).

In regards to temporary color retention, a study conducted by Wrolstad (*59*) showed that sulfur dioxide reacts with anthocyanins making them colorless. This reaction could only be

partially reversed by heat application in which only some of the sulfur dioxide would release from the anthocyanins and bring back some of the original color.

Most berry juice processing and storage research has been on common berries such as strawberries, raspberries, blueberries, blackberries, and cranberries. These studies generally report major losses of anthocyanins and proanthocyanidins, which are believed to be major contributing factors for purported health benefits associated with berry consumption. The losses would therefore have an "adverse effect on bioactive properties and health outcomes" (*54*). Chokeberries are being utilized in mostly processed products such as fruit juice blends for color enhancement as they represent "natural colorants." Little research has been conducted on chokeberries in respect to anthocyanin retention, polymeric pigment formation, and stability of phenolic compounds during juice processing and storage. At present, refrigeration is the most efficient treatment to stabilize polyphenolics during storage of berry products. One of the challenges of analyzing polymeric pigments is that they have such a high polarity that separation is virtually impossible by reverse or normal phase HPLC. Therefore, quantification of the pigments is currently not possible, and only mass spectrometry techniques such as MALDI-TOF are able to identify the high molecular weight polymers (*54*).

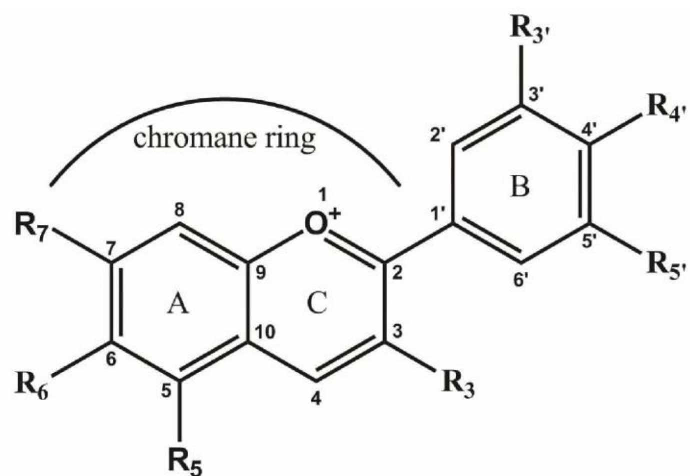
F. FIGURE CAPTIONS

Figure 1. Structure of common berry anthocyanidins (*90*).

Figure 2. Model juice process.

Figure 3. Thermal degradation mechanisms of two common anthocyanins (*64*).

Figure 1.



1 Pelargonidin (Pg) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=H; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=H

2 Cyanidin (Cy) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=OH; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=H

3 Delphinidin (Dp) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=OH; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=OH

4 Peonidin (Pn) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=OMe; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=H

5 Petunidin (Pt) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=OMe; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=OH

6 Malvidin (Mv) $\mathbf{R_3}$=OH; $\mathbf{R_5}$=OH; $\mathbf{R_6}$=H; $\mathbf{R_7}$=OH; $\mathbf{R_{3'}}$=OMe; $\mathbf{R_{4'}}$=OH; $\mathbf{R_{5'}}$=OMe

Figure 2.

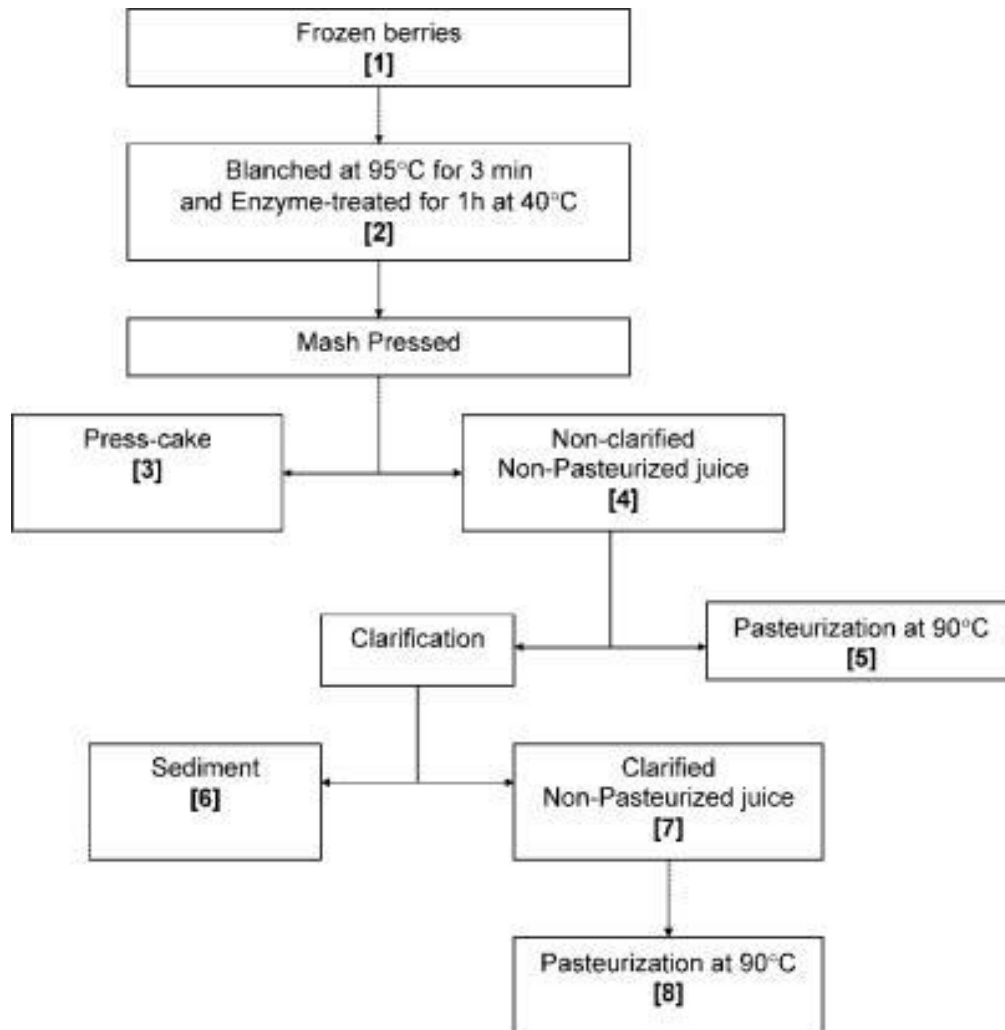
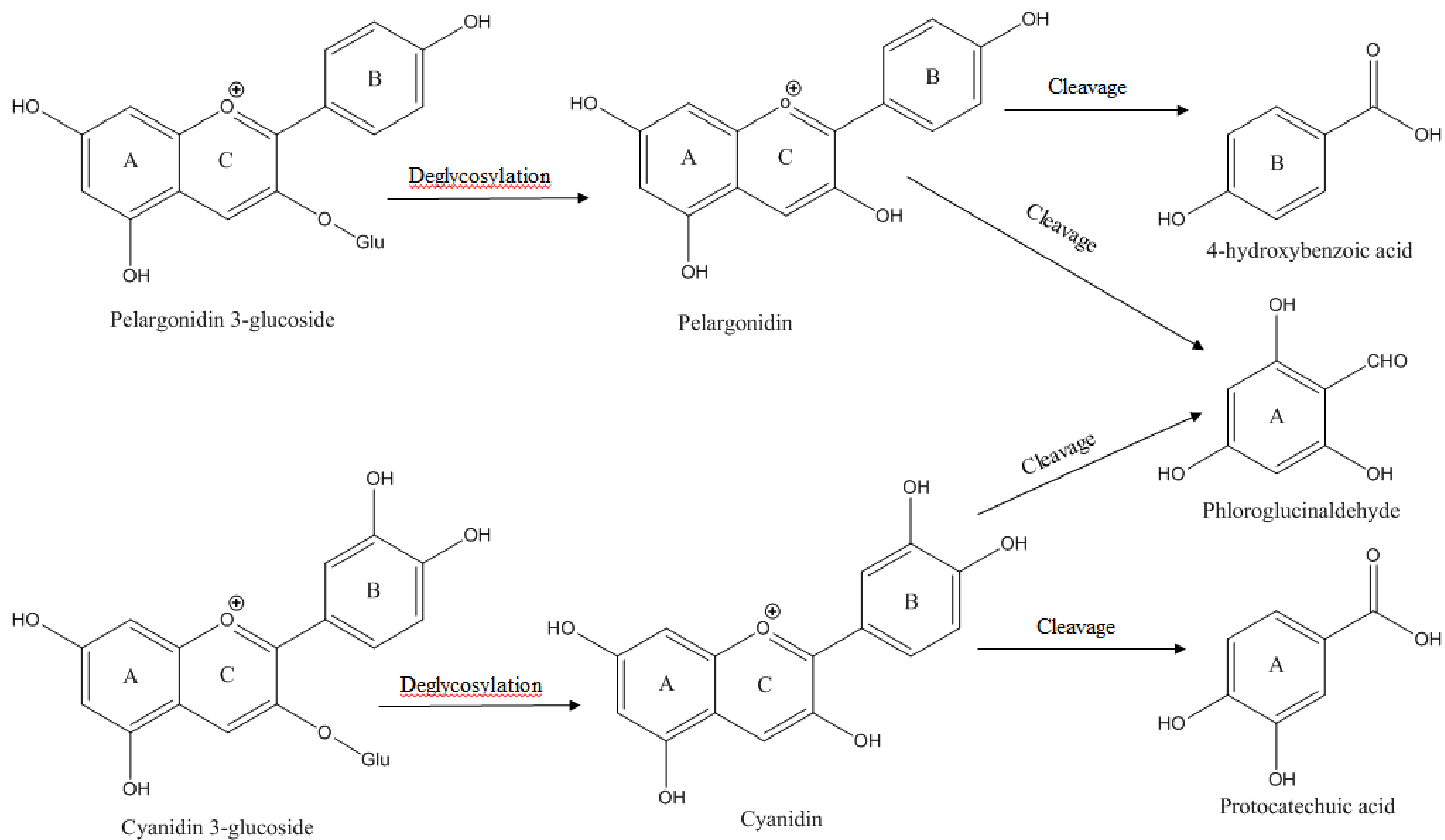


Figure 3.



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III. CHANGES IN CHOKEBERRY POLYPHENOLICS DURING JUICE PROCESSING AND STORAGE.

A. ABSTRACT

Chokeberries are an excellent source of polyphenolics, but their fate during juice processing and storage are unknown. The stability of anthocyanin's, total proanthocyanidins, hydroxycinnamic acids, and flavonols at various stages of juice processing and over six months of storage at 25 ^{o}C were determined. Flavonols, total proanthocyanidins and hydroxycinnamic acids were retained in the juice to a greater extent than anthocyanins, with losses mostly due to removal of seeds and skins following pressing. Anthocyanins were extensively degraded by thermal treatments during which time levels of protcatechuic acid and phloroglucinaldehyde increased, and additional losses occurred following pressing. Flavonols, total proanthocyanidins and hydroxycinnamic acids were well retained in juices stored for 6 months at 25 ^{o}C, while anthocyanins declined linearly. Anthocyanin losses during storage were paralleled by increased polymeric color values indicating that the small amounts of anthocyanins remaining were present in large part in polymeric forms.

B. INTRODUCTION

Chokeberries (*Aronia melanocarpa*) are gaining popularity as a result of their exceptional content of polyphenolics. The berries are exceptionally rich in anthocyanins (ACYs) that are responsible for the deep purple color. Chokeberry anthocyanins consist mainly of cyanidin glycosides (glucoside, xyloside, galactoside, and arabinoside), but the galactoside and arabinoside generally account for > 75% of total ACYs (*1*). Chokeberries are also rich in proanthocyanidins (PACs), which impart a very astringent taste. The proanthocyanidin profile of the berries is complex, ranging from monomers to decamers, to exceptionally high levels of

polymers (*1*). The berries also contain high levels of hydroxycinnamic acids (HCA) (chlorogenic acid and neochlorogenic acid), and considerable levels of flavonols, mostly quercetin derivatives (*2, 3, 4, 5*). As a result of their exceptional polyphenol composition, chokeberries are thought to play an important role in protection against many chronic diseases. There is accumulating evidence that chokeberries or polyphenolic-rich extracts from chokeberries possess cardio-protective, hepato-protective, anti-diabetic, and anticarcinogenic effects (*6, 7, 8, 9*).

Due to their astringent taste, chokeberries are commonly processed and consumed in various shelf-stable forms including juices, nectars, wines and liqueurs. Unfortunately, chokeberry processing has been shown to result in marked losses of ACYs (*10, 11*). Several studies have reported on the concentration of polyphenolics in chokeberries, pomace and juice, but results are expressed on a dry weight basis and do not provide any indications of actual concentrations in the three fractions as weights of the berries, juice and pomace were not measured or taken into account (*3, 12*).

In addition to anthocyanin losses during juice processing, further extensive losses of ACYs during storage have been reported in other anthocyanin-rich berries (*14, 15,* 16). Anthocyanin losses in these studies were paralleled by increased polymeric color values indicating anthocyanin-tannin polymers were formed during storage that are resistant to bleaching in the presence of potassium metabisulfite. Since chokeberries are particularly rich in polyphenolics, we suspect that similar losses of ACYs and possibly other polyphenolics occur during juice processing and storage. The objective of this study was to determine the fate of chokeberry ACYs, total PACs, HCA and flavonols during various stages of juice processing and throughout six months of storage at ambient temperature.

C. MATERIALS AND METHODS

Frozen Berries. Frozen chokeberries (*Aronia melanocarpa*, cv. Viking) were obtained from Maes' Health and Wellness (Omaha, NE). The berries grown outside of Davenport, NE were harvested at commercial ripeness and frozen within four hours after harvest. They were then transported overnight under refrigeration to the University of Arkansas, Food Science Department. Upon receipt, the berries were stored at -20 ^{o}C for less than two weeks prior to processing.

Chemicals and Reagents. A mixture of 3-O- β-glucoside standards of delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin was obtained from Polyphenolics Laboratories (Sandnes, Norway). HPLC grade methanol, acetone, formic acid, and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Chlorogenic acid, rutin, protocatechuic acid, phloroglucinaldehyde, (+) catechin, 4-dimethylaminocinnamaldehyde and quercetin were also obtained from Sigma-Aldrich (St. Louis, MO).

Juice Processing. Chokeberries were processed into non-clarified juice as previously described (*13*). The juice processing scheme with the five sampling points indicated is shown in Figure 1.1. Frozen berries were simultaneously heated and mixed with a Mixco Batch mixer (Avon, NY) in a large steam kettle until the berry mash reached a temperature of 95 ^{o}C. After 3 min incubation, the mash was allowed to cool to 40 ^{o}C prior to depectinization. Pectinex Smash XXL (Novozyme, Bagsvared, Denmark) was added at a concentration of 0.0827 mL/kg and the mash was incubated for 1 h at 40 ^{o}C. Complete depectinization was verified by the negative alcohol precipitation test where no flocculation of pectin was observed following the addition of two volumes of 96% ethanol (v/v) to one volume of juice. The mash was then pressed in a 25 L Enrossi bladder press (Enoagricol Rossi, s.r.l., Calzolaro, Italy) operated at 20 psi to separate the

juice from the presscake. The non-clarified juice was dispensed into 6 oz glass bottles and heated in a steam box (American Sterilizer Co., Erie, PA) until the juice temperature monitored using a thermocouple reached 90 °C. The bottle caps were tightened and the juices were allowed to cool overnight. Samples of each juice treatment were stored in the dark at 25 °C. Five samples were taken at each sampling point along the juicing process and five bottles of juice were sampled after 1, 2, 3, 4, 5, and 6 months of storage at 25 °C.

Extraction of Polyphenolics. For isolation of ACYs, HCA and flavonols, five samples each of frozen and blanched berries, enzyme-treated mash and presscake were homogenized with 20 mL of methanol/water/acetic acid (60:37:3, v/v/v) using a EuroTurrax T18 tissuemizer (Tekmar-Dohrman Corp., Mason, OH) and then filtered through MiraCloth (Calbiochem, La Jolla, CA). The residue was collected, the extraction repeated two more times and the volume of the extracts adjusted to 100 mL with extraction solvent. The same protocol was used to isolate PACs except acetone/water/acetic acid (70:29.5:0.5, v/v/v) was used as extraction solvent. Juice samples were analyzed directly and required no extraction.

HPLC Analysis of Anthocyanins and Hydroxcinnamic acids. Methanol/water/acetic acid **e**xtracts (8 mL) were dried using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY) and re-suspended in 1 mL of 3% formic acid in water. The reconstituted samples were passed through 0.45 µm PTFE syringe filters prior to HPLC analysis. Anthocyanin monoglycosides and HCA were separated by reverse phase HPLC on a Symmetry C_{18} column (Waters Corp., Milford, MA) according to the method described by Cho et al (*14*). Anthocyanins were monitored at 520 nm and HCA at 320 nm. Anthocyanin glycosides were quantified as cyanidin 3-glucoside equivalents and HCA as chlorogenic acid equivalents using external calibration

curves of authentic standards. Protocatechuic acid (PCA) and phloroglucinaldehyde (PGA) were monitored at 280 nm and quantified using external calibration curves of authentic standards.

HPLC and HPLC-MS Analysis of Flavonols. Methanol/water/acetic acid **e**xtracts extracts (8 mL) were dried using a SpeedVac® concentrator and re-suspended in 1 mL of 50% methanol in water. The reconstituted samples were passed through 0.45 μm PTFE syringe filters prior to HPLC analysis. Individual flavonols were separated by reverse phase HPLC on an Aqua C_{18} column (Phenomenex, Torrance, CA) according to the method described by Cho et al (*15*). Flavonol peaks were monitored at 360 nm and quantified as rutin equivalents using external calibration curves of an authentic standard. Quercetin was quantified using an external calibration curve of an authentic standard. The identification of flavonols was performed by HPLC-MS using the HPLC conditions described above, except the HPLC system was interfaced to a Bruker Esquire LC/MS ion trap mass spectrometer (Billerica, MA). Mass spectral analysis was conducted in negative ion electrospray mode using conditions previously described in Cho et al (*15*).

Percent Polymeric Color Analysis. Percent polymeric color of methanol/water/acetic acid **e**xtracts from samples taken throughout juice processing and juices was determined using the spectrophotometric assay of Giusti and Wrolstad (*16*). Absorbance values of two matching samples, one treated with water (control) and the other with potassium metabisulfite were recorded at 420, 520 and 700 nm. Potassium metabisulfite was used to bleach the monomeric anthocyanins present in the juices, while polymeric anthocyanins resistant to bleaching remain colored. The ratio of the absorbance value of the potassium metabisulfite bleached sample to the control sample (non-bleached) X 100 reflects percent polymeric color.

Total Proanthocyanidin Analysis. Total PACs were measured by the 4-dimethylaminocinnamaldehyde (DMAC) assay of Prior et al (*17*). The absorbance (640 nm) of samples in 96-well plates was measured every min for 30 min on a BioTek Synergy HT (Winooski, VT) plate reader. Catechin was used as standard with results expressed as mg of catechin equivalents per 100 g berry fresh weight.

Calculations. To account for dilution and concentration effects, anthocyanin, flavonol, proanthocyanidin, hydroxycinnamic acid and phenolic acid concentrations were converted to original berry weight to allow comparison of all samples on an equivalent basis. The following equation was used:

$$C_{berry} = C_{product} \times R$$

Where: C_{berry} = concentration on original berry weight basis, R = mass of the product divided by mass of original berry, and $C_{product}$ = concentration in the product.

Statistical Analysis. The effects of processing and storage on anthocyanin, hydroxycinnamic acid, flavonol, total proanthocyanidin, phenolic acid concentrations and percent polymeric color were determined by one way analysis of variance (ANOVA) using JMP Pro® 11 (Cary, NC). Differences between means (n=5) were determined by Student's *t*-test (α = 0.05).

D. RESULTS AND DISCUSSION

Polyphenol Composition of Chokeberries and Juice. Cyanidin 3-galactoside (cyd 3-gal), cyanidin 3-glucoside (cyd 3-glu), cyanidin 3-arabinoside (cyd 3-ara), and cyanidin 3-xyloside (cyd 3-xyl) were identified and quantified by HPLC (Figure 1.2) and the results are presented in Table 1.1. These ACYs were previously identified in chokeberry juice by HPLC-MS using identical HPLC conditions described above (*18*). Cyd 3-gal and cyd 3-ara were the

major ACYs in the chokeberry samples and juices confirming previous reports (*1, 2, 3, 4*). Two HCA, neochlorogenic acid (NCA) and chlorogenic acid (CA) were present in the berries and juices (Figure 1.3 and Table 1.1). The high concentrations of NCA and CA in chokeberry samples and juices confirm previous findings (*3, 4*). Eight flavonols were identified by HPLC and HPLC-MS (Figure 1.4) and included the following derivatives of quercetin: two dihexosides (*m/z* 625/301), vicianoside (arabinosyl-glucoside) (*m/z* 595/301), robinobioside (rhamnosyl-galactoside) (*m/z* 609/301), rutinoside (rhamnosyl-glucoside) (*m/z* 609/301), galactoside (*m/z* 463/301), glucoside (*m/z* 463/301) and aglycone (*m/z* 301). All of these compounds have previously been identified in chokeberries (*4, 5*). The quantities of individual flavonols in samples taken throughout juice processing are presented in Table 1.2. The relative abundance of quercetin derivatives in frozen berries was similar to findings of Mikulic-Petkovsek (*5*) who reported that galactoside was the predominant quercetin derivative followed by glucoside, rutinoside, vicianoside, robinobioside, and dihexosides. The amount of total flavonols in frozen chokeberries in our study 33.9 mg/100g FW falls within the range of values (26-71 mg/100g FW) previously reported for fresh chokeberries (*4, 5*).

Processing Changes in Chokeberry Polyphenolics and Percent Polymeric Color.

Anthocyanins. Anthocyanins were readily degraded during the two thermal treatments, blanching and pasteurization, as well as physical removal of the presscake (Table 1.1). Cyd pentosides (ara and xyl) were more susceptible to degradation during blanching (63% and 64% losses, respectively) than cyd hexosides (gal and glu, 52% and 45% losses, respectively). A similar trend was observed in response to pasteurization with cyd 3-ara and cyd 3-xyl each incurring 69% losses, while cyd 3-gal and cyd 3-glu showed 58% and 50% losses, respectively, compared with levels found in non-pasteurized juice. Our results are consistent with previous

studies reporting anthocyanin hexosides to be more stable than pentosides (*12, 20, 21*). Following pasteurization, the juices contained only 8%, 12%, 4%, 4% and 7% of the levels of cyd 3-gal, cyd 3-glu, cyd 3-ara and cyd 3-xyl and total ACYs found in frozen chokeberries. A major loss of ACYs also occurred during the pressing operation when presscake containing seeds and skins was removed from the mash. The presscake contained 52%, 51%, 54% and 54% of the levels of cyd 3-gal, cyd 3-glu, cyd 3ara and cyd 3-xyl found in the enzyme treated mash, respectively, with most of the balance transferred to the juice. These results indicate that chokeberry presscake is an excellent source of ACYs and may serve useful as a substrate for recovering natural pigments. The anthocyanin losses observed in response to thermal treatments were much higher than those previously reported for blueberry (*13*), blackberry (*19*), and black raspberry (*16*) juices processed under similar conditions. The extensive anthocyanin losses observed in this study could be due to several factors. It is clear that thermal treatments had a detrimental effect on ACYs. We observed significant increases in two HPLC peaks following blanching that were identified as protocatechuic acid (PCA) and phloroglucinaldehyde (PGA) (Figure 1.5), and the concentrations of these two compounds increased upon thermal treatments (Figure 1.6). Levels of PCA and PGA increased by 343% and 400% after blanching, 30% and 24% from blanched berries to enzyme treated mash and 29% and 7% from non-pasteurized to pasteurized juice. The formation of PCA and PGA in response to thermal treatments is consistent with the scheme of cyd 3-glycoside degradation proposed by Sadilova et al (*20*). According to Sadilova et al. (*20*), the first step of pH dependent thermal degradation of cyd at pH 3.5, which is similar to the pH of chokeberries used in this study, involves opening of the pyrilium ring and chalcone glycoside formation. The next step involves deglycosylation to yield chalcone, which degrades quickly in the presence of heat to protocatechuic acid, 4-

hydroxybenzoic acid and phloroglucinaldehyde. We did not detect 4-hydroxybenzoic acid in our chromatograms, which is consistent with results reported for heated elderberry extracts that are also rich in cyd glycosides (*20*). However, the increases in cyd degradation products PCA and PGA during processing only accounted for about 3% of the losses in total ACYs that occurred during processing. The presence of ascorbic acid and pro-oxidant metals in the chokeberries may have played a role in anthocyanin degradation (*21*). Enzymes such as polyphenol oxidase and peroxidase are also reported to cause degradation of ACYs (*22, 23, 24, 25, 26*), but the frozen chokeberries used in this study were immediately blanched to inactivate enzymes. However, we cannot eliminate the possibility that heat stable enzymes played a role in anthocyanin degradation prior to inactivation by the blanching treatment. The extensive loss of ACYs during blanching was most likely due to the long time it took the 23 kg batch of frozen chokeberries to heat to the blanching temperature of 95 ºC, and then cool down to 40 ºC. In an additional experiment involving a 5.4 kg batch of chokeberries, where it took 12 min for the frozen berries to heat up to 95 ºC , and 3 min to cool down to 40 ºC, we observed a 25% loss of ACYs. Since chokeberry ACYs appear to be very heat labile, use of a blanching treatment prior to depectinization may be counterproductive, especially if the berries contain low polyphenol oxidase activity.

Percent Polymeric Color. Percent polymeric color values decreased in response to blanching and enzyme treatments, which may indicate cleavage of some anthocyanin-tannin linkages (Table 1.1). The major change in polymeric color occurred when juice was pasteurized, 13.4% in non-pasteurized juice to 29.4% in pasteurized juice. According to Kunsagi-Mate et al. (*23*) the formation of anthocyanin-tannin polymers is slow at room temperature due to high activation energy of the reaction, but is accelerated at elevated temperatures. Hence, the

increased polymeric color values observed following pasteurization may reflect higher levels of anthocyanin-tannin polymers, which may account for some of the losses of monomeric anthocyanins.

Hydroxycinnamic acids. The level of NCA did not change in response to blanching while the level of CA acid increased by 14%, indicating CA was not completely extracted from frozen berries (Table 1.1). Significant losses of HCA occurred during the pressing operation. The presscake contained 33% and 37% of the levels of NCA and CA found in enzyme treated mash, respectively, while the NP juice contained 67% and 62% of NCA and CLA, respectively. Levels of NCA and CA were minimally impacted by pasteurization with CA and total HCA showing no losses and NCA showing a 7% loss compared with the value for NP juice. Following pasteurization, the juices contained 60%, 65%, and 63% of the levels of NCA, CA and total HCA found in frozen chokeberries, respectively.

Total proanthocyanidins. The levels of total PACs were stable upon blanching, but increased by 11% following the enzyme treatment (Table 1.1). Disruption of the cell wall polysaccharides presumably allowed for enhanced extraction of the PACs following enzyme treatment. The major loss of total PACs occurred during the pressing operation. The presscake contained 60% of the levels of total PACs found in enzyme treated mash, while the NP juice contained 46%. Surprisingly, PACs increased 17% in response to pasteurization. This may be due to disruption of cell wall polysaccharide-proanthocyanidin complexes, which allowed more PACs to react with the DMAC reagent. It is also possible that pasteurization resulted in depolymerization of large molecular weight PACs to monomers, which are reported to react more readily with the DMAC reagent than polymers (*17*).

Flavonols. Levels of quercetin dihexosides, vicianoside, robinobioside, and rutinoside showed modest increases (11%-20%) in response to either blanching or enzyme treatment, compared with values for frozen berries, while levels of the two major quercetin conjugates, galactoside and glucoside, and total flavonols were stable (Table 1.2). Significant losses of flavonol glycosides occurred during the pressing operation, with 39% to 49% of the compounds being retained in the presscake, while 54% to 64% were expressed into the juice. The less polar quercetin hexosides (galactoside and glucoside) were retained to a greater extent in the presscake (48% and 49%) than the more polar quercetin diglycosides (39% and 41%). Additionally, the majority of the non-polar quercetin aglycone was retained in the presscake, while only 27% was expressed into the juice. Pasteurization did not affect levels of the two quercetin hexosides, while other derivatives decreased by 16-21% in response to pasteurization. Although present at low level, the concentration of quercetin aglycone increased by 63% in response to pasteurization, indicating that some sugar conjugates were cleaved from the quercetin glycosides in response to thermal treatment. Overall, total flavonol levels decreased by 15% in response to pasteurization.

Storage Changes in Chokeberry Polyphenolics and Percent Polymeric Color.

Anthocyanins. Levels of cyd 3-gal, cyd 3-glu, cyd 3-ara, cyd 3-xyl and total ACYs in juices declined in a linear fashion over one to five months of storage at 25ºC, but levels appeared to stabilize from five to six months (Table 1.3). After six months of storage, the juices lost 75%, 75%, 76%, 64% and 75% of the levels of cyd 3-gal, cyd 3-glu, cyd 3-ara, cyd 3-xyl and total ACYs, respectively found in juices after one month of storage. These results are consistent with other studies reporting marked losses of ACYs during ambient temperature storage of chokeberry juice (*18, 24*), nectars and purees (*10*). The type of sugar attached had little effect on

anthocyanin stability during long-term storage, which contrasts with results observed in response to thermal treatments during processing, where hexosides showed greater retention than pentosides (Table 1.1). Consistent with our findings, Hellstrom et al. (*24*) reported no differences in stability of chokeberry anthocyanin glycosides stored at 4 ^{o}C, 9 ^{o}C, and 21 ^{o}C over 12 weeks of storage. The mechanism(s) responsible for anthocyanin degradation during storage have not been well elucidated, but may be associated with water nucleophilic attack at the 2-position of the anthocyanin nucleus resulting in decolorization (*25*), or the formation of anthocyanin-tannin polymers (*26*). Levels of PCA and PGA were stable up to three months of storage and then declined about 30% from 3 to 6 months of storage (data not shown) , indicating that ACYs were not degraded into phenolic acids during ambient temperature storage.

Percent Polymeric Color. Percent polymeric color values of the juices increased (34.8 to 44.5%) from one to six months of storage, and these values were inversely correlated with ACYs (r=-0.81) (Table 1.3). These changes indicate that some losses of ACYs throughout storage may be due to the formation of anthocyanin-flavan-3-ol polymers that are resistant to bleaching by potassium metabisulfite in the polymeric color assay. However, it is also possible that the anthocyanin-flavan-3-ol polymers formed in the juices during processing were more resistant to degradation during storage. The anthocyanin-flavan-3-ol polymers appeared to play an important role in color stability as the juices retained a dark purple color despite marked losses of monomeric ACYs.

Hydroxycinnamic acids. In contrast to anthocyanins, levels of NCL, CA and total HCA were stable from one to three months of storage (Table 1.3), but levels declined by about 30% from three to six months of storage. Levels of CA in blueberry and chokeberry juices were also found to be relatively stable over long term storage at ambient temperature (*28, 31*).

Total proanthocyanidins. Levels of total PACs were stable (> 90% retention) from one to six months of storage (Table 1.3). The stability of PAC's over storage contrasts with the marked decline in ACYs and increase in percent polymeric color values observed from one to six months of storage. These results indicate that PACs did not react with ACYs to any great extent to form polymeric pigments throughout storage. This finding supports the hypothesis that polymeric pigments formed during processing are more resistant to degradation than monomeric ACYs and play an important role in color stability.

Flavonols. Quercetin glycosides and aglycone were stable from one to four months of storage at 25 °C, but levels decreased by 17% to 29% from four to six months (Table 1.4). Total flavonol levels decreased by 21% from four to six months of storage at 25°C. Levels of quercetin followed the same trend as quercetin glycosides indicating that losses of glycosides from four to six months of storage were not due to cleavage of sugar moieties. The losses of flavonols late during storage may be due to physical binding to insoluble solids, resulting in precipitation, or oxidation to quinones.

In summary, juice processing had a much greater effect on polyphenolic losses in chokeberries than storage of juices at 25 °C. Anthocyanins were more susceptible to losses during processing than flavonols, total PACs, and HCA as a result of thermal degradation, evident by increased levels of PCA and PGA, and polymerization, evident by increased polymeric color values. The juice pressing step resulted in losses of all polyphenolics due to physical removal of skins, but ACYs and total PACs were retained in the presscake to a greater extent than HCA and flavonols. Flavonols, total PACs and HCA were well retained over six months of storage at 25 °C compared with ACYs, which degraded readily through the six months. Losses of ACYs during storage were accompanied by increased polymeric color values

indicating that the small amount of ACYs remaining after long-term storage were present largely in polymeric forms. The polymeric forms of ACYs appear to be more stable than monomeric ACYs and likely play an important role in color stability. Novel, cost-effective treatments are needed to stabilize ACYs during chokeberry juice processing and storage.

E. ACKNOWLEDGMENT

We thank Mae's Health and Wellness for providing the frozen chokeberries used in this study.

F. FIGURE CAPTIONS

Figure 1.1. Flowchart of chokeberry juice processing with sampling points indicated by asterisks.

Figure 1.2. Typical HPLC chromatogram (Abs 520 nm) of chokeberry anthocyanins. Peak 1 = cyanidin 3-galactoside, peak 2 = cyanidin 3-glucoside, peak three = cyanidin 3-arabinoside, and peak 4 = cyanidin 3-xyloside.

Figure 1.3. Typical HPLC chromatogram (Abs 320 nm) of chokeberry hydroxycinnamic acids. Peak 1 = neochlorogenic acid and peak 2 = chlorogenic acid.

Figure 1.4. Typical HPLC chromatogram (Abs 360 nm) of chokeberry flavonols. Peaks 1 and 2 = quercetin 3-dihexoside (*m/z* 625/463/301), peak 3 = quercetin 3-vicianoside (*m/z* 595/463/301), peak 4 = quercetin 3-robinobioside (*m/z* 609/463/301), peak 5 = quercetin 3-rutinoside (*m/z* 609/463/301), peak 6 = quercetin 3-galactoside (*m/z* 463/301), peak 7 = quercetin 3-glucoside (*m/z* 463/301), and peak 8 = quercetin (*m/z* 301).

Figure 1.5. HPLC chromatogram (260 nm) of frozen (solid line) and blanched (dashed line) chokeberry extracts. Peak 1 = protocatechuic acid and peak 2 = phloroglucinaldehyde.

Figure 1.6. Concentrations of protocatechuic acid and phloroglucinaldehyde throughout chokeberry juice processing. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 1.1

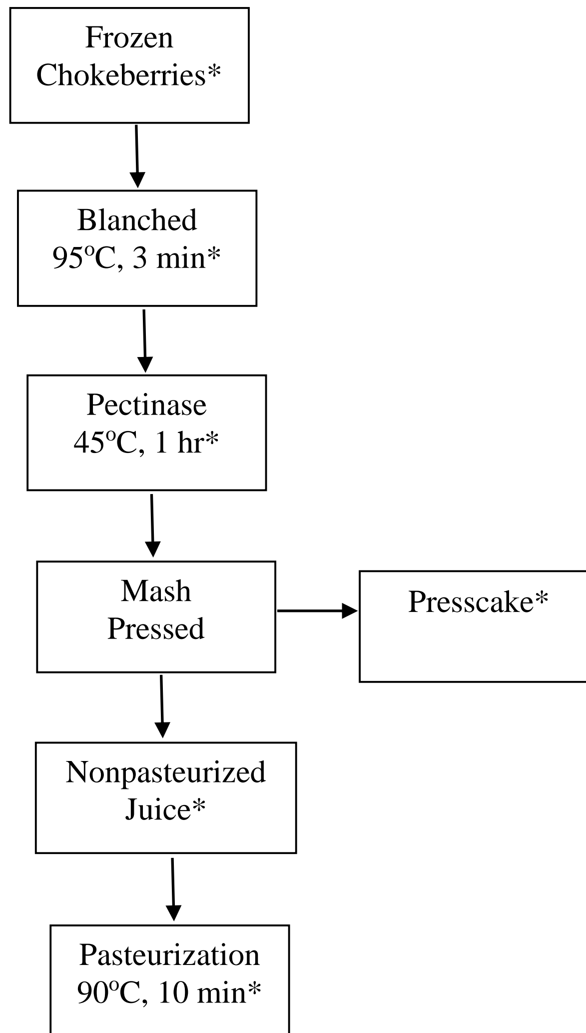

Figure 1.2

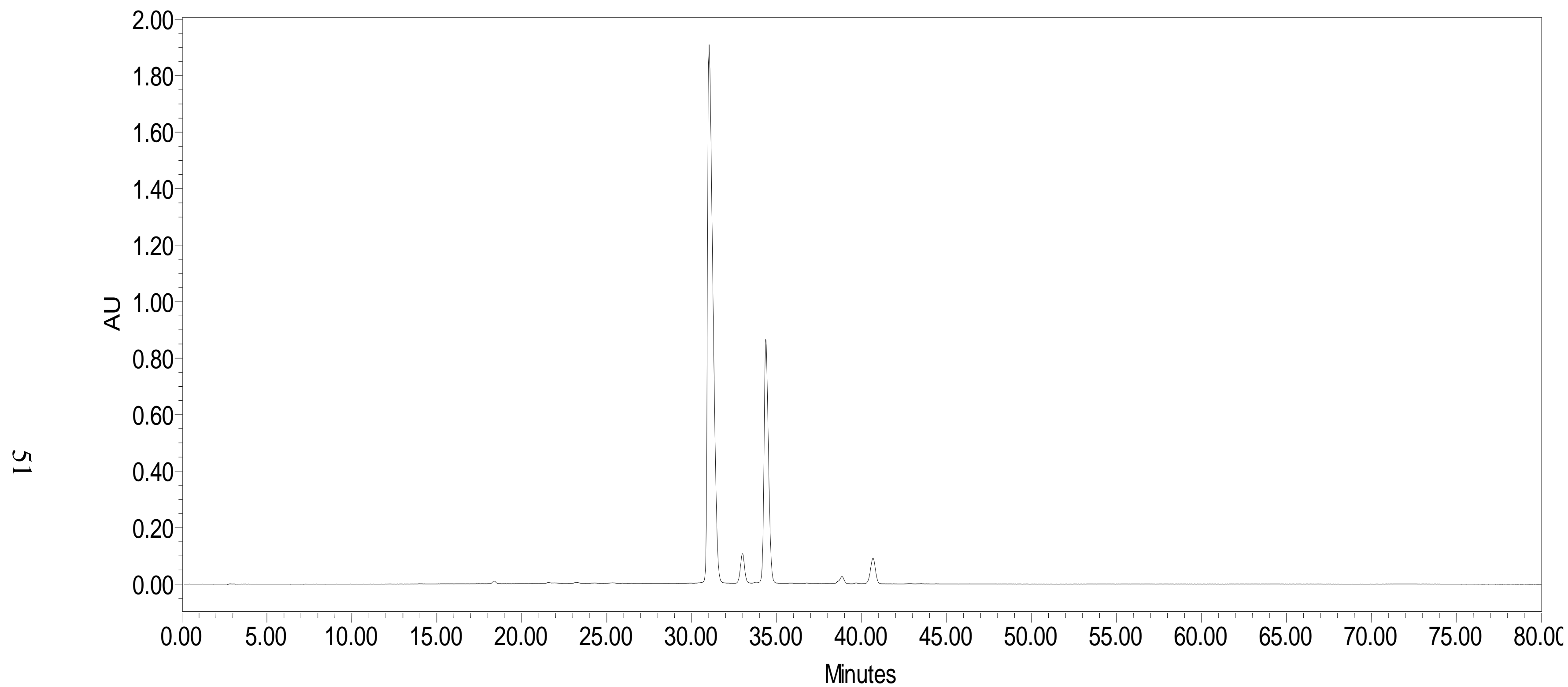

Figure 1.3

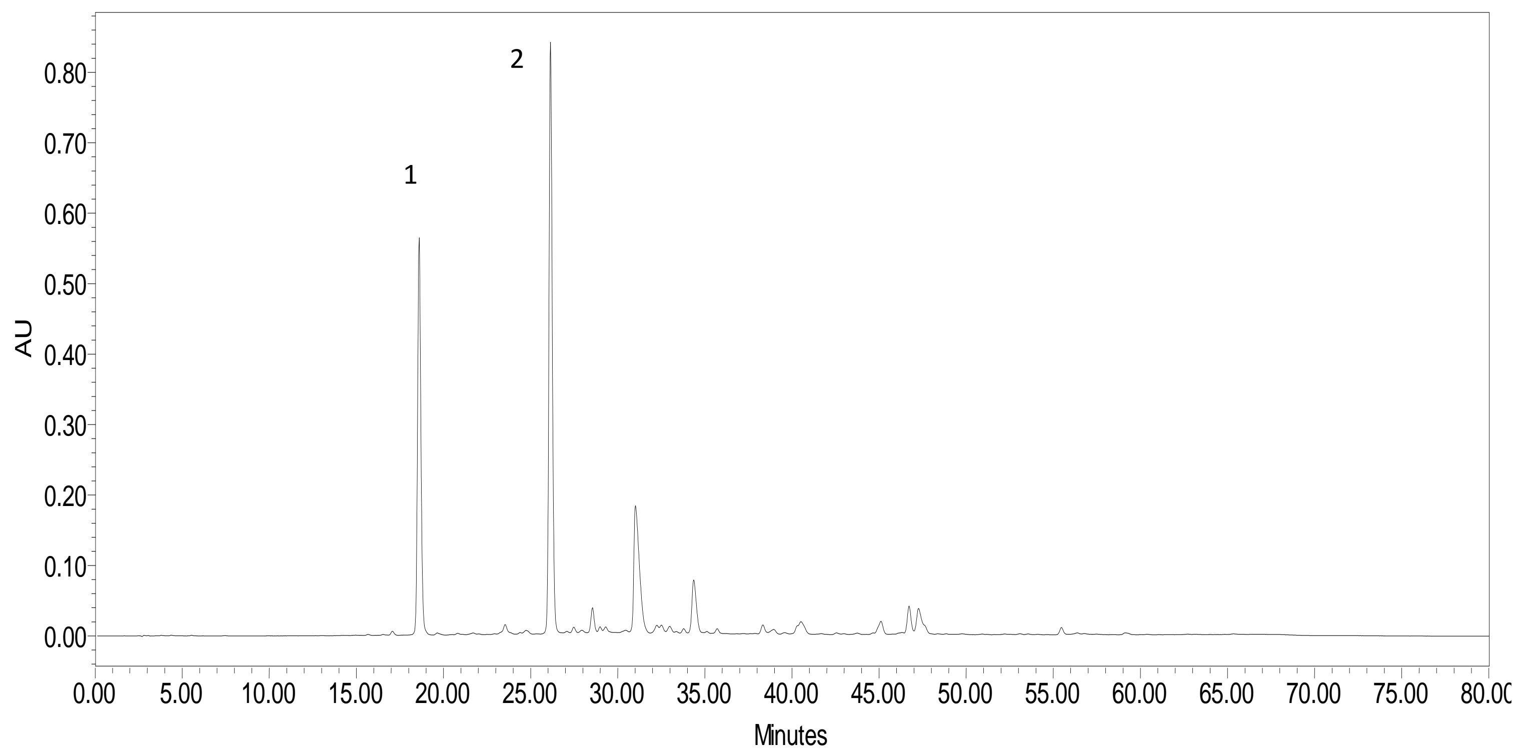

Figure 1.4

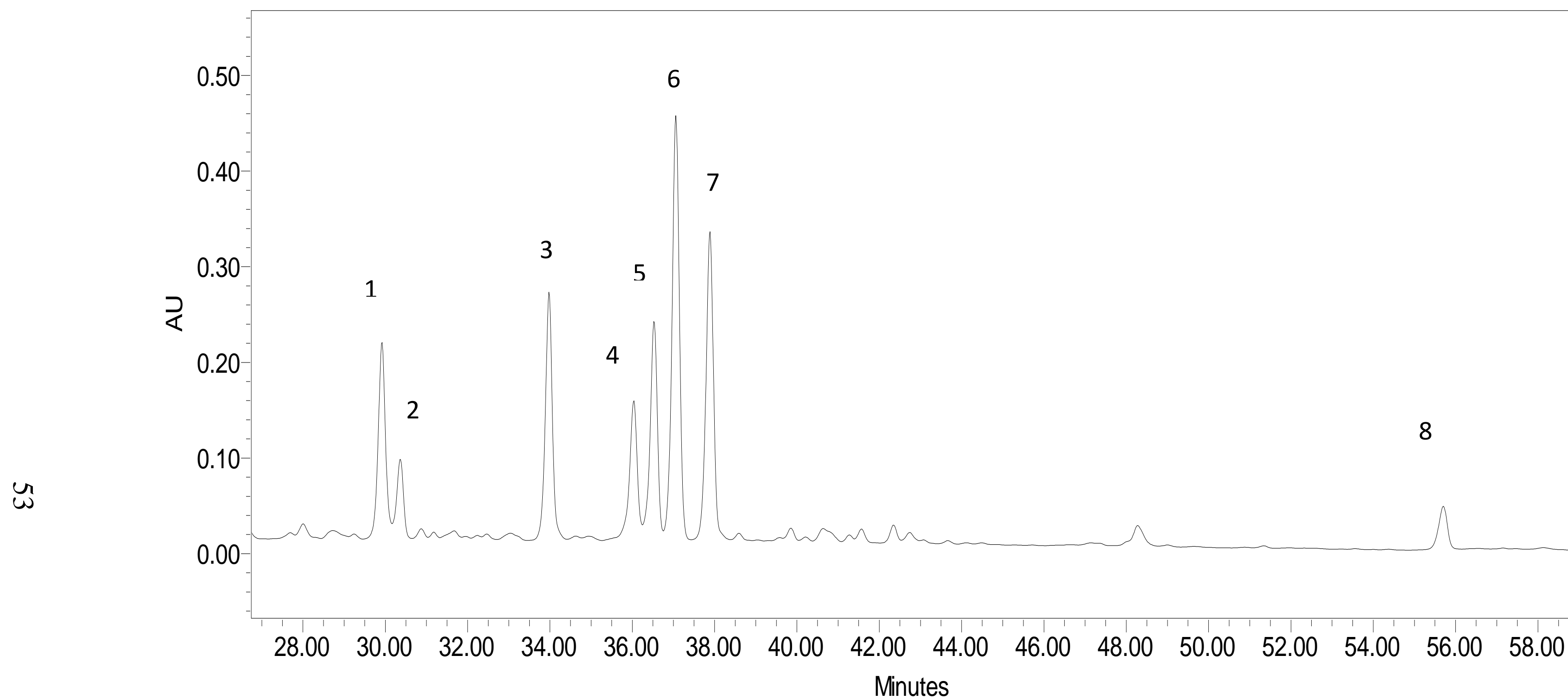

Figure 1.5

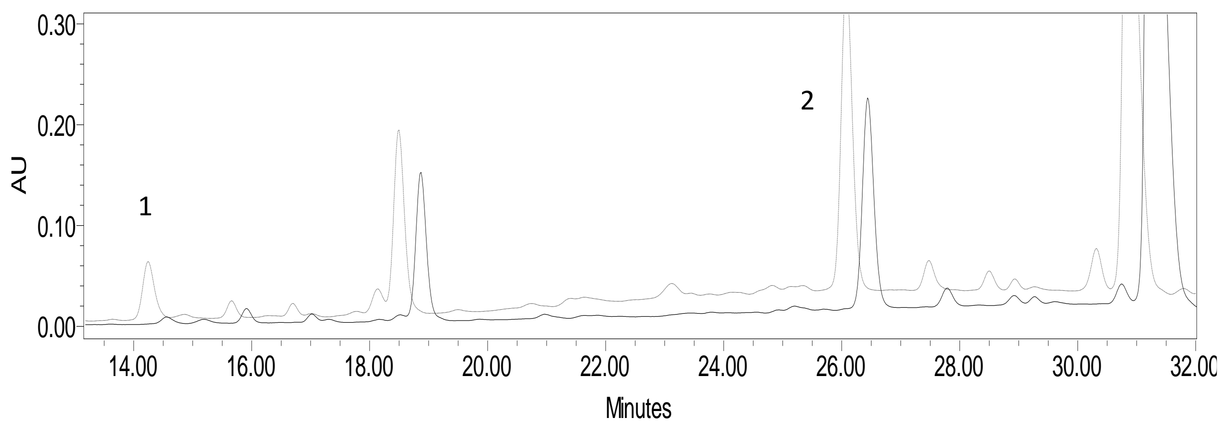

Figure 1.6

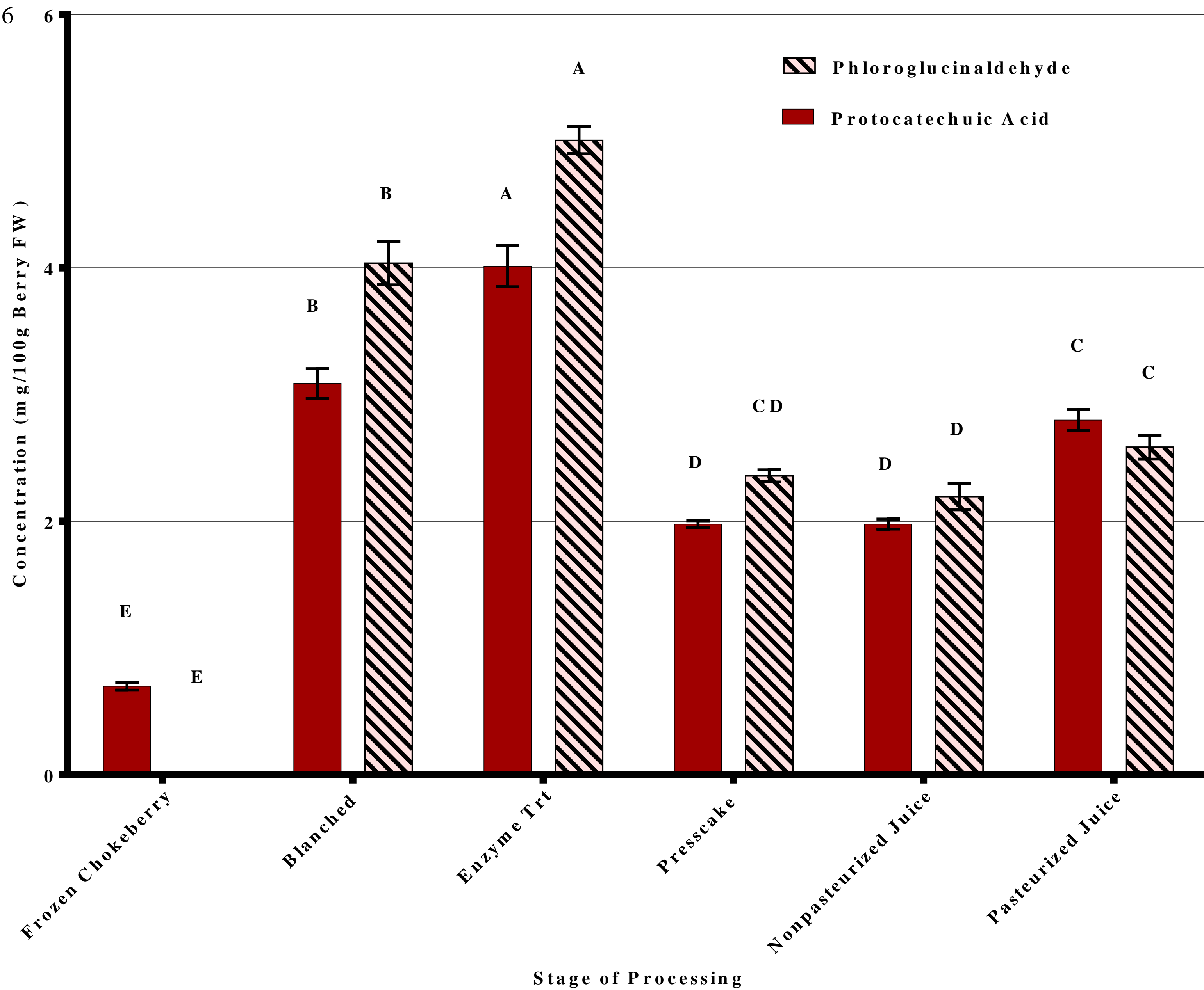

Table 1.1 Concentrations of Anthocyanins (mg/100 g berry FW) throughout Chokeberry Juice Processing.

	anthocyanins				
processing step	cyanidin 3-galactoside	cyanidin 3-glucoside	cyanidin 3-arabinoside	cyanidin 3-xyloside	total
frozen	424.7±9.6 a[c]	19.8 a	154.7 a	20.1±0.9 a	619.2±16.5 a
blanched	205.5±5.5 b	10.9±0.3 b	57.5±1.2 b	7.3±0.2 b	281.2±7.2 b
	(48%)	(55%)	(37%)	(36%)	(45%)
enzyme	190.7±0.8 c	10.5±0.1 b	54.2±0.5 b	6.9±0.0 b	262.4±1.4 b
	(45%)	(53%)	(35%)	(35%)	(42%)
juice - NP[b]	77.1±0.7 e	4.6±0.0 d	20.4±0.2 d	2.7±0.0 c	104.8±1.0 d
	(18%)	(23%)	(13%)	(13%)	(17%)
presscake	99.8±1.8 d	5.4±0.1 c	29.3±0.6 c	3.7±0.1 c	138.3±2.6 c
	(23%)	(27%)	(19%)	(18%)	(22%)
juice - P[b]	32.4±0.6 f	2.3±0.0 e	6.3±0.1 e	0.9±0.0 d	42.0±0.8 e
	(8%)	(12%)	(4%)	(4%)	(7%)

[b] NP = nonpasteurized, P = pasteurized

Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with frozen berries.

Table 1.2 Concentrations of Hydroxycinnamic acids and Total Proanthocyanidins (mg/100 g berry FW) and Percent Polymeric Color throughout Chokeberry Juice Processing.

	hydroxycinnamic acids				
processing step	neochlorogenic acid	chlorogenic acid	total	% polymeric color	total PACs[a]
frozen	46.2±1.0 a	70.2±1.9 b	116.4±2.7 b	16.2± b	845.2±25.6 b
blanched	46.8±1.1 a	80.2±1.6 a	127.0±2.6 a	7.4 e	868.6±7.4 b
	(101%)	(114%)	(109%)		(103%)
enzyme	45.1±0.2 a	77.2±0.4 a	122.2±0.5 a	10.1 de	940.7±29.1 a
	(98%)	(110%)	(105%)		(111%)
juice - NP[b]	30.1±0.0 b	47.7±0.1 c	77.9±0.2 c	13.4 bc	392.6±8.6 e
	(65%)	(68%)	(67%)		(62%)
presscake	14.9±0.1 d	28.8±0.3 d	43.7±0.3 d	12.2 cd	524.2±13.4 c
	(32%)	(41%)	(38%)		(46%)
juice - P[b]	27.9±0.7 c	45.9±1.2 c	73.9±1.9 c	29.4 a	464.8±20.8 d
	(61%)	(65%)	(63%)		(55%)

[a] PACs = proanthocyanidins.
[b] NP = nonpasteurized, P = pasteurized
[c] Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with frozen berries.

Table 1.3 Concentrations of Flavonols (mg/100 g berry FW) Throughout Chokeberry Juice Processing

	flavonols								
processing step	quercetin 3-dihexoside	quercetin 3-dihexoside	quercetin 3-vicianoside	quercetin 3-robinobioside	quercetin 3-rutinoside	quercetin 3-galactoside	quercetin 3-glucoside	quercetin	total
frozen	3.0±0.2 b[b]	1.4±0.1 b	4.0±0.2 b	3.5±0.2 a	3.9±0.1 b	10.6±0.5 a	7.6±0.3 a	0.74±0.0 a	34.7±1.4 a
blanched	3.6±0.1 a (120%)	1.6±0.0 a (114%)	4.5±0.1 a (115%)	3.1±0.1 b (89%)	4.1±0.1 ab (105%)	10.2±0.2 a (96%)	7.2±0.1 a (95%)	0.66±0.0 b (89%)	35.0±0.6 a (101%)
enzyme	4.1±0.2 a (137%)	1.7±0.0 a (121%)	4.80±0.0 a (123%)	3.3±0.0 ab (94%)	4.3±0.0 a (110%)	10.6±0.1 a (100%)	7.5±0.1 a (99%)	0.71±0.0 a (96%)	36.9±0.2 a (106%)
juice - NP[a]	2.2±0.0 c (73%)	1.0±0.0 c (71%)	3.1±0.0 c (79%)	2.1±0.0 c (60%)	2.8±0.0 c (72%)	5.7±0.0 b (54%)	4.2±0.0 b (55%)	0.19±0.0 d (26%)	21.3±0.0 b (61%)
presscake	1.6±0.0 d (53%)	0.7±0.0 d (50%)	1.9±0.0 e (49%)	1.4±0.0 e (40%)	1.7±0.0 e (44%)	5.2±0.1 bc (49%)	3.6±0.0 c (47%)	0.66±0.0 b (89%)	16.7±0.2 c (48%)
juice - P[a]	2.3±0.3 c (77%)	0.9±0.1 cd (64%)	2.6±0.2 d (67%)	1.7±0.2 d (49%)	2.3±0.2 d (59%)	4.5±0.4 c (42%)	3.5±0.3 c (46%)	0.31±0.0 c (42%)	18.2±1.8 c (52%)

[a] NP= nonpasteurized, P = pasteurized

[b] Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with frozen berries.

Table 1.4 Concentrations of Anthocyanins (mg/100 g berry FW) throughout Chokeberry Juice Storage at 25°C.

storage time months	anthocyanins				
	cyanidin 3-galactoside	cyanidin 3-glucoside	cyanidin 3-arabinoside	cyanidin 3-xyloside	total
1	22.2±0.8 a[b] (69%)	1.6±0.1 a (70%)	4.1±0.21 a (65%)	0.58±0.0 a (64%)	28.6±1.1 a (68%)
2	18.1±0.3 b (56%)	1.3±0.0 b (57%)	3.3±0.1 b (52%)	0.51±0.0 b (57%)	23.3±0.4 b (55%)
3	15.1±0.1 c (47%)	1.1±0.0 c (48%)	2.7±0.0 c (43%)	0.43±0.0 c (48%)	19.3±0.2 c (46%)
4	10.0±0.6 d (31%)	0.7±0.0 d (30%)	1.8±0.1 d (29%)	0.30±0.0 d (33%)	12.8±0.8 d (30%)
5	5.5±1.2 e (17%)	0.5±0.1 e (22%)	1.0±0.2 e (16%)	0.22±0.0 e (24%)	7.3±0.4 e (17%)
6	5.6±0.3 e (17%)	0.4±0.0 e (17%)	1.0±0.0 e (16%)	0.21±0.0 e (23%)	7.2±1.5 e (17%)

Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with pasteurized juice.

Table 1.5 Concentrations of Hydroxycinnamic acids and Total Proanthocyanidins (mg/100 g berry FW) and Percent Polymeric Color throughout Chokeberry Juice Storage at 25°C.

	hydroxycinnamic acids				
storage time months	neochlorogenic acid	chlorogenic acid	total	% polymeric color	total PACs[a]
1	27.5±1.1 a (99%)	45.2±1.7 a (98%)	72.7±2.8 a (98%)	34.8 d	447.8±8.7 ab (97%)
2	27.9±0.6 a (100%)	46.3±1.2 a (101%)	74.3±1.8 a (101%)	34.4 d	450.8±10.1 a (96%)
3	28.3±0.2 a (101%)	47.0±0.5 a (102%)	75.3±0.7 a (102%)	37.5 c	425.7±14.8 abc (92%)
4	22.9±1.2 b (82%)	37.7±2.0 b (82%)	60.6±3.2 b (82%)	40.4 b	413.2±13.8 bc (89%)
5	18.9±2.6 c (68%)	31.2±4.4 bc (68%)	50.1±7.0 bc (68%)	39.9 b	409.9±12.8 c (88%)
6	18.4±1.1 c (66%)	30.4±1.8 c (66%)	48.9±2.9c (66%)	44.5 a	406.0±9.2 c (87%)

[a] PACs = proanthocyanidins.
[b] Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with pasteurized juice

Table 1.6. Concentrations of Flavonols (mg/100 g berry FW) Throughout Chokeberry Juice Storage at 25°C

storage time months	flavonols (mg/100g berry FW)								
	quercetin 3-dihexoside	quercetin 3-dihexoside	quercetin 3-vicianoside	quercetin 3-robinobioside	quercetin 3-rutinoside	quercetin 3-galactoside	quercetin 3-glucoside	quercetin	total
1	2.3±0.2 a[a] (100%)	0.9±0.0 a (100%)	2.9±0.1 a (112%)	2.0±0.1 a (118%)	2.7±0.1 a (117%)	5.0±0.2 a (111%)	3.9±0.2 a (111%)	0.35±0.0 b (113%)	19.7±0.9a b (108%)
2	2.4±0.1 a (104%)	0.9±0.0 a (100%)	3.1±0.0 a (119%)	2.0±0.0 a (118%)	2.8±0.0 a (122%)	5.2±0.0 a (116%)	4.0±0.0 a (114%)	0.39±0.0 ab (126%)	20.9±0.2 a (115%)
3	2.3±0.1 a (100%)	1.0±0.0 a (111%)	3.2±0.0 a (123%)	2.1±0.0 a (124%)	2.9±0.0 a (126%)	5.3±0.0 a (118%)	4.1±0.0 a (117%)	0.40±0.0 a (129%)	21.3±0.2 a (117%)
4	2.1±0.1 a (91%)	1.0±0.0 a (111%)	3.1±0.0 a (119%)	2.1±0.0 a (124%)	2.9±0.0 a (126%)	5.2±0.0 a (116%)	4.1±0.0 a (117%)	0.40±0.0 a (129%)	20.9±0.1 a (115%)
5	1.6±0.2 b (70%)	0.8±0.1 b (89%)	2.5±0.3 b (96%)	1.7±0.2 b (100%)	2.4±0.3 b (104%)	4.2±0.5 b (93%)	3.3±0.3 b (94%)	0.35±0.0 b (113%)	16.7±1.8 b (92%)
6	1.5±0.2 b (65%)	0.8±0.1 b (89%)	2.5±0.3 b (96%)	1.6±0.2 c (94%)	2.4±0.3 b (104%)	4.1±0.4 b (91%)	3.2±0.3 b (91%)	0.35±0.0 b (113%)	16.5±1.8 b (91%)

[a] Mean values (n = 5) within a column with different letters are significantly different ($p \leq 0.05$). Values in parentheses represent percent retention compared with pasteurized juice.

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H. APPENDIX

Figure 1-A. Concentrations of total anthocyanins and percent polymeric color throughout chokeberry juice processing. Bars represent standard errors of the mean ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 1-B. Concentrations of each anthocyanin throughout juice processing.

Figure 1-C. Concentrations of hydroxycinnamic acids throughout juice processing. Bars represent standard errors of the mean ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 1-D. Concentrations of total procyanidins and flavonols throughout juice processing. Bars represent standard errors of the mean ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 1-E. Concentrations of total anthocyanins and percent polymeric color during juice storage.

Figure 1-F. Concentrations of each anthocyanin during juice storage.

Figure 1-G. Concentrations of protocatechuic acid and phloroglucinaldehyde during juice storage.

Figure 1-H. Concentrations of hydroxycinnamic acids during juice storage.

Figure 1-I. Concentrations of total procyanidins and flavonols during juice storage.

Figure 1-A

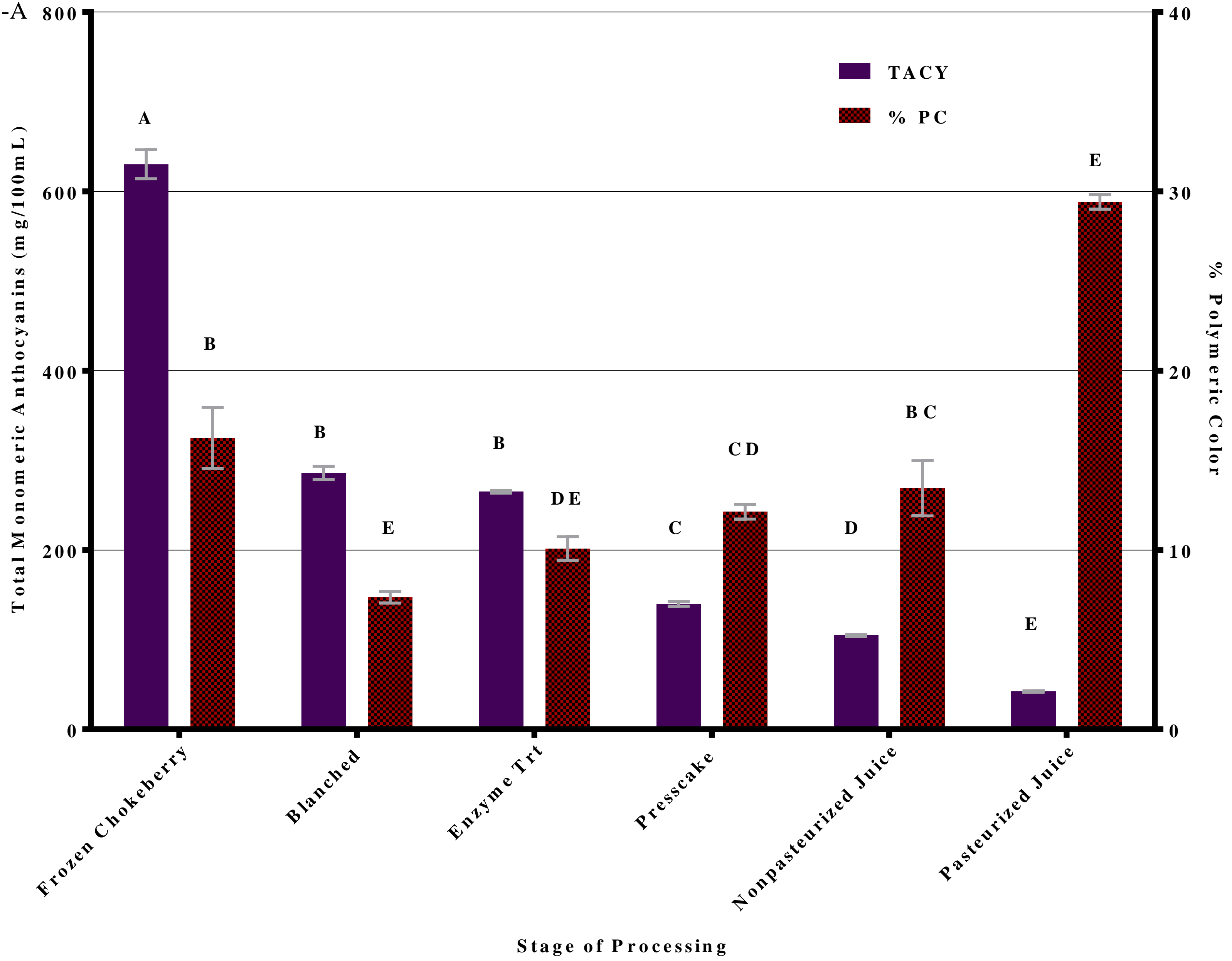


Figure 1-B

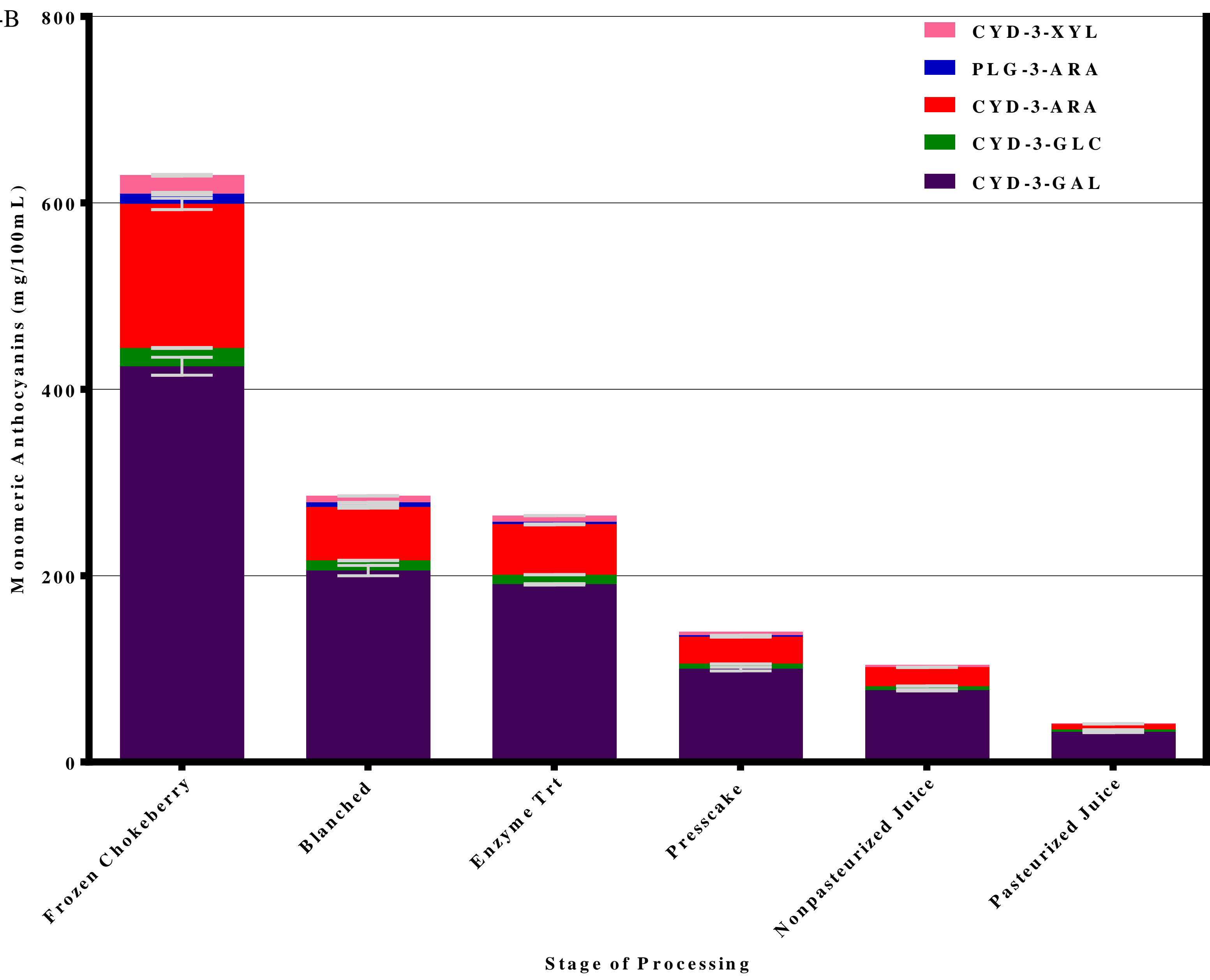
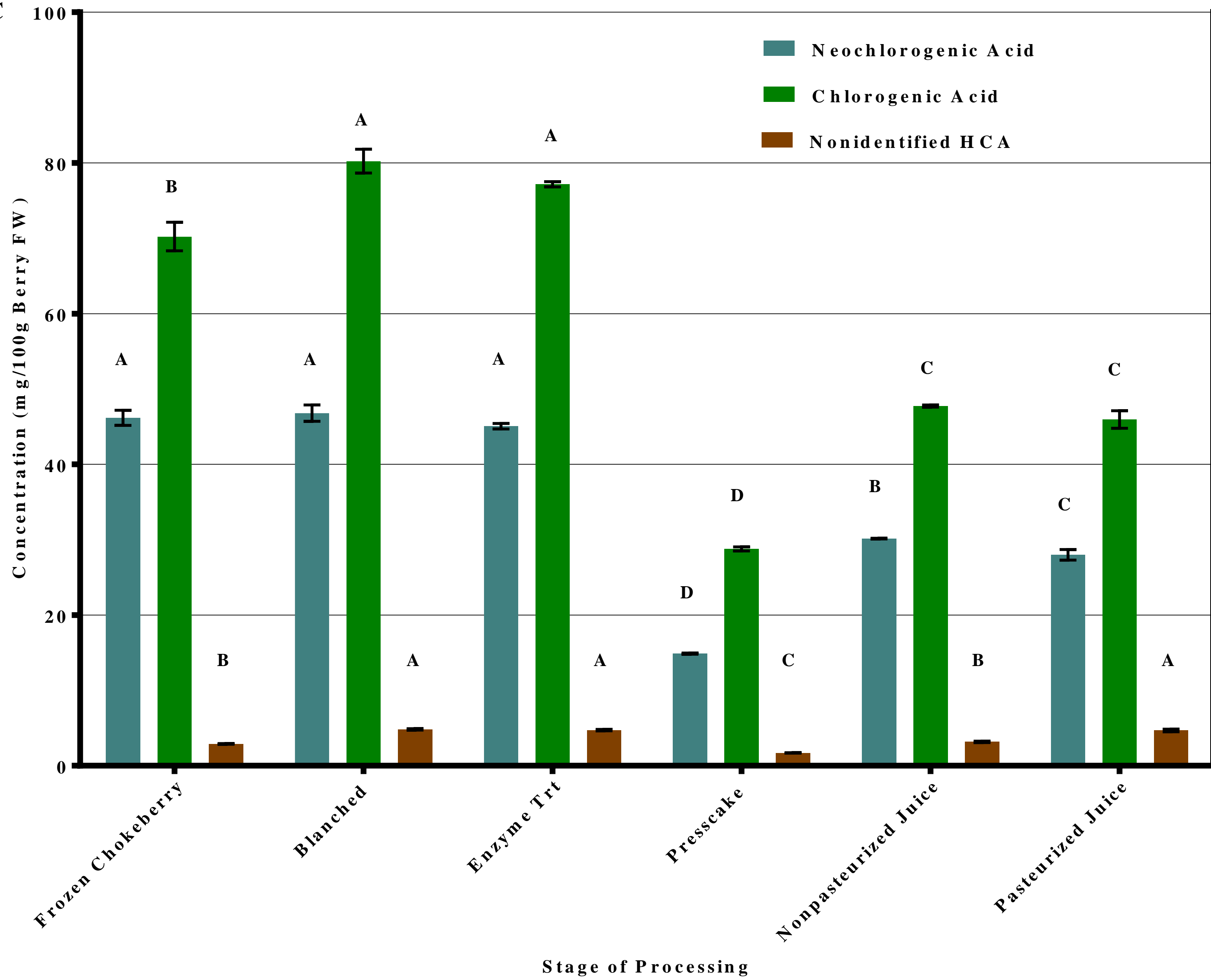


Figure 1-C



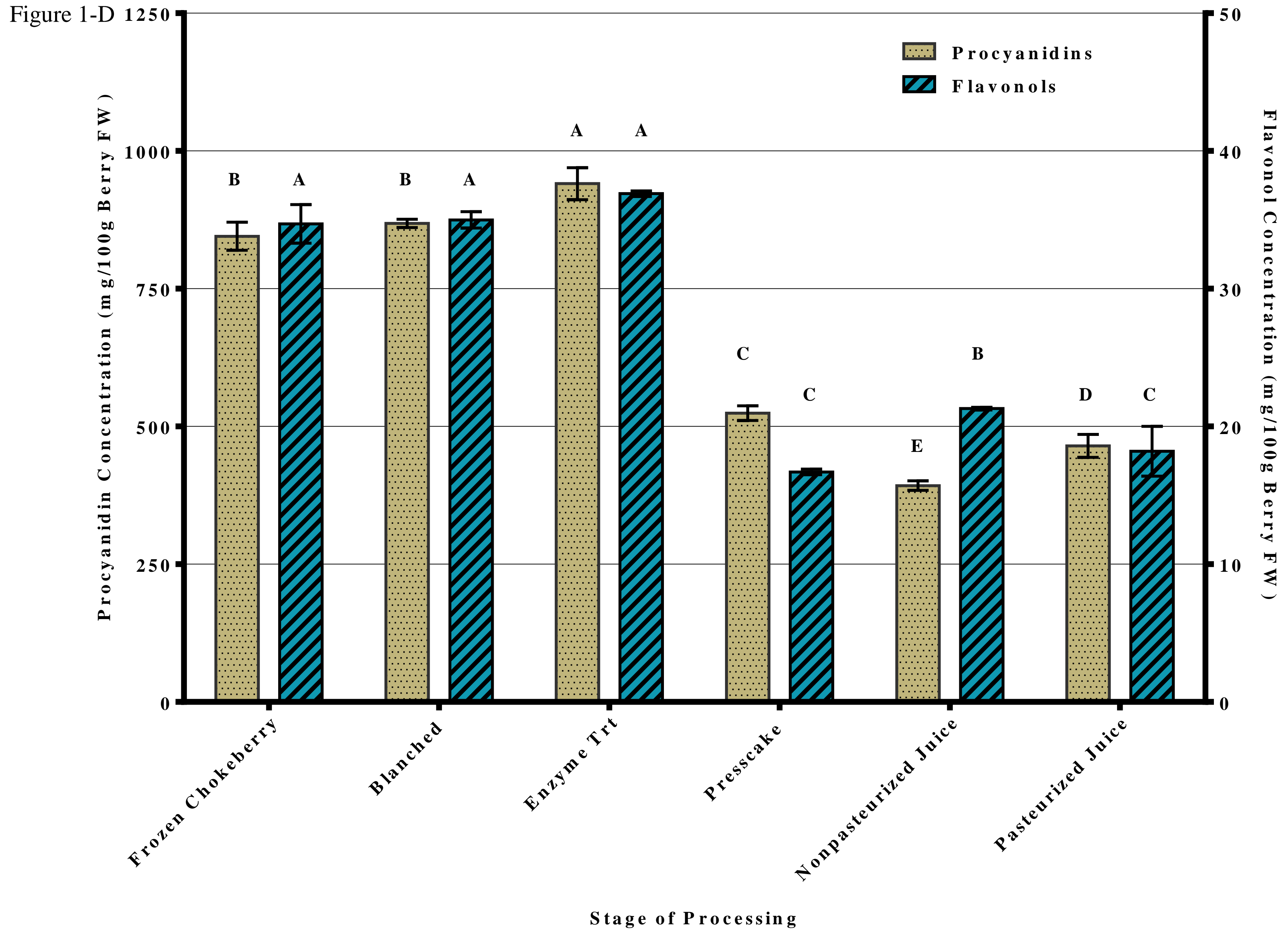


Figure 1-D

Figure 1-E

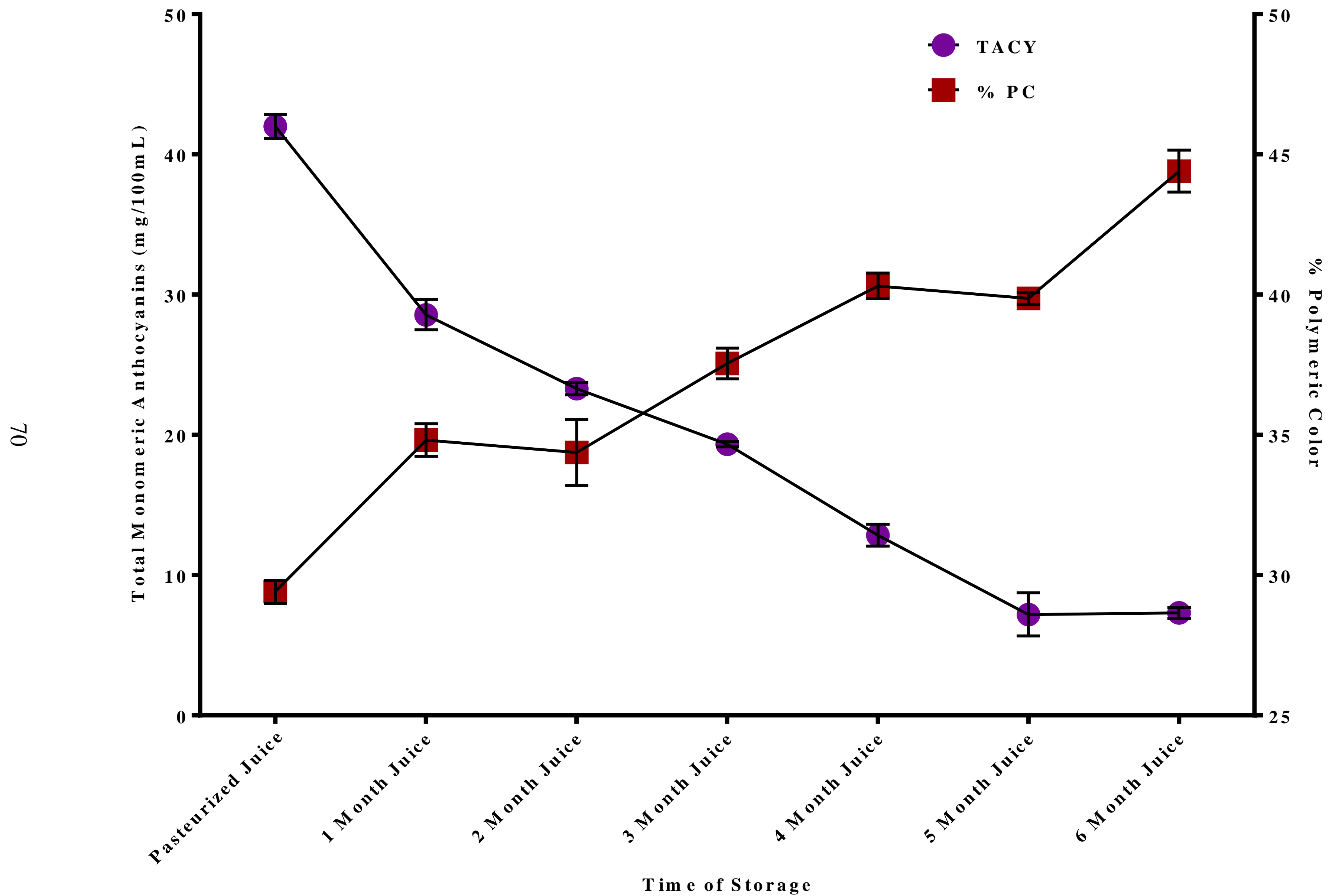


Figure 1-F

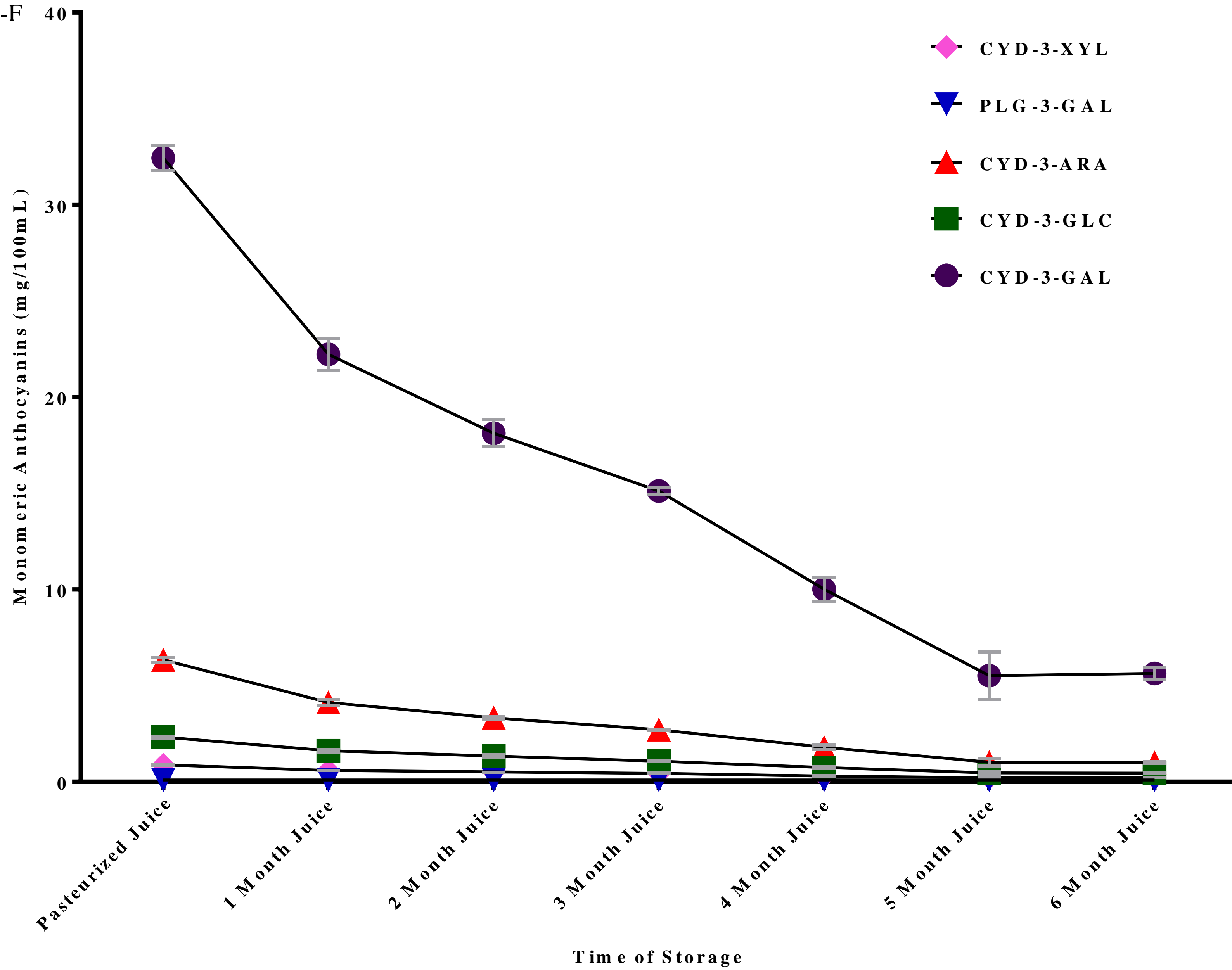


Figure 1-G

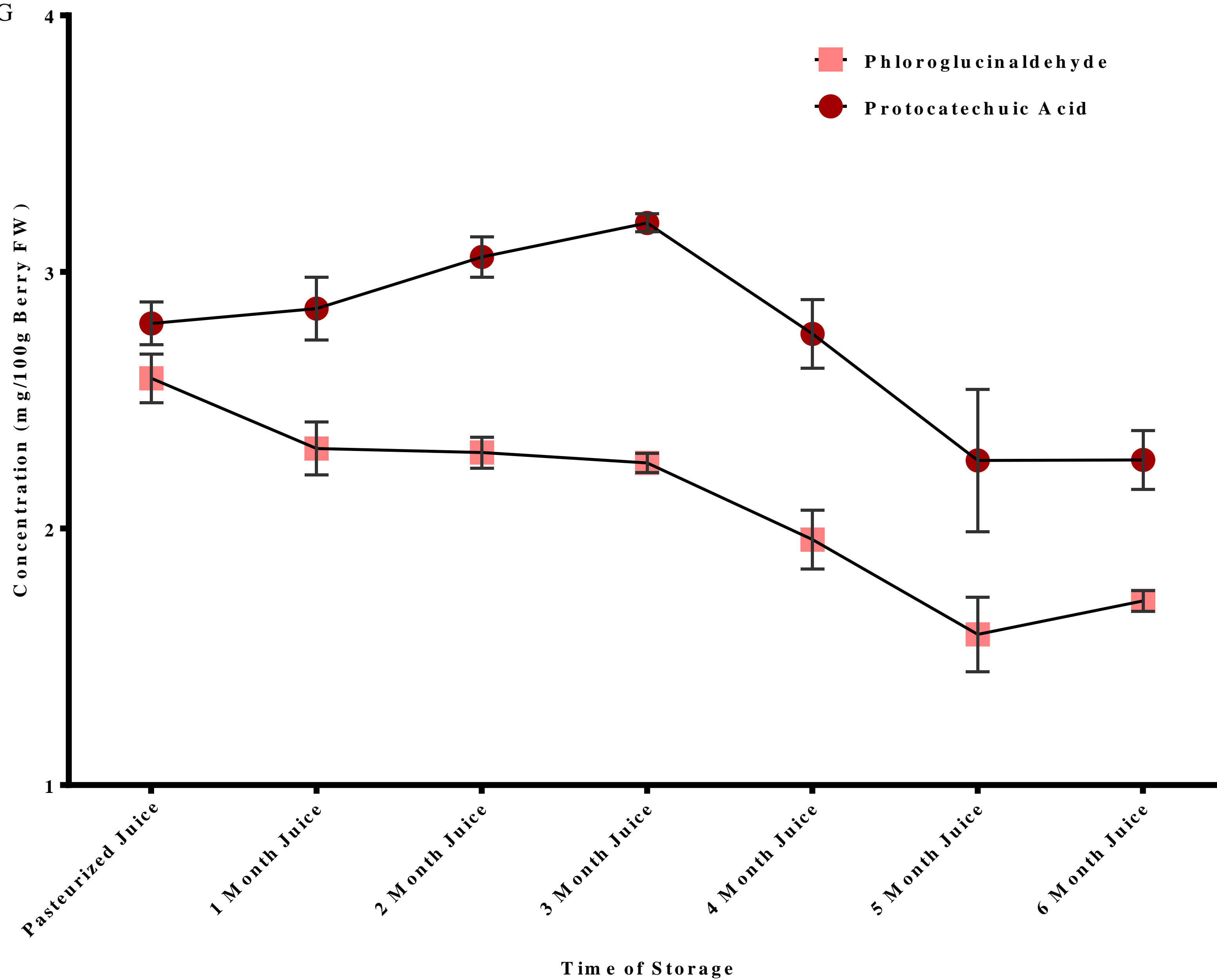


Figure 1-H

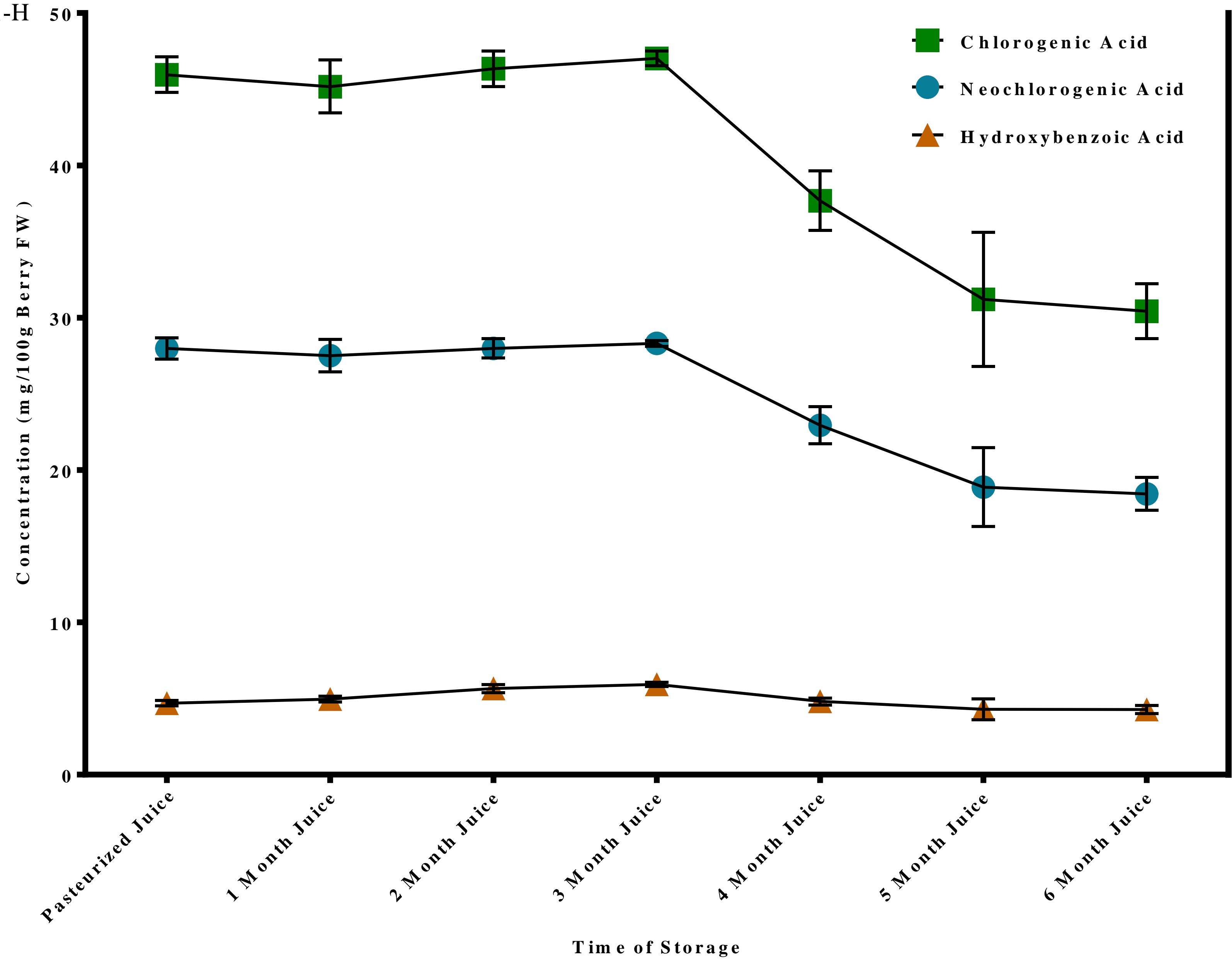
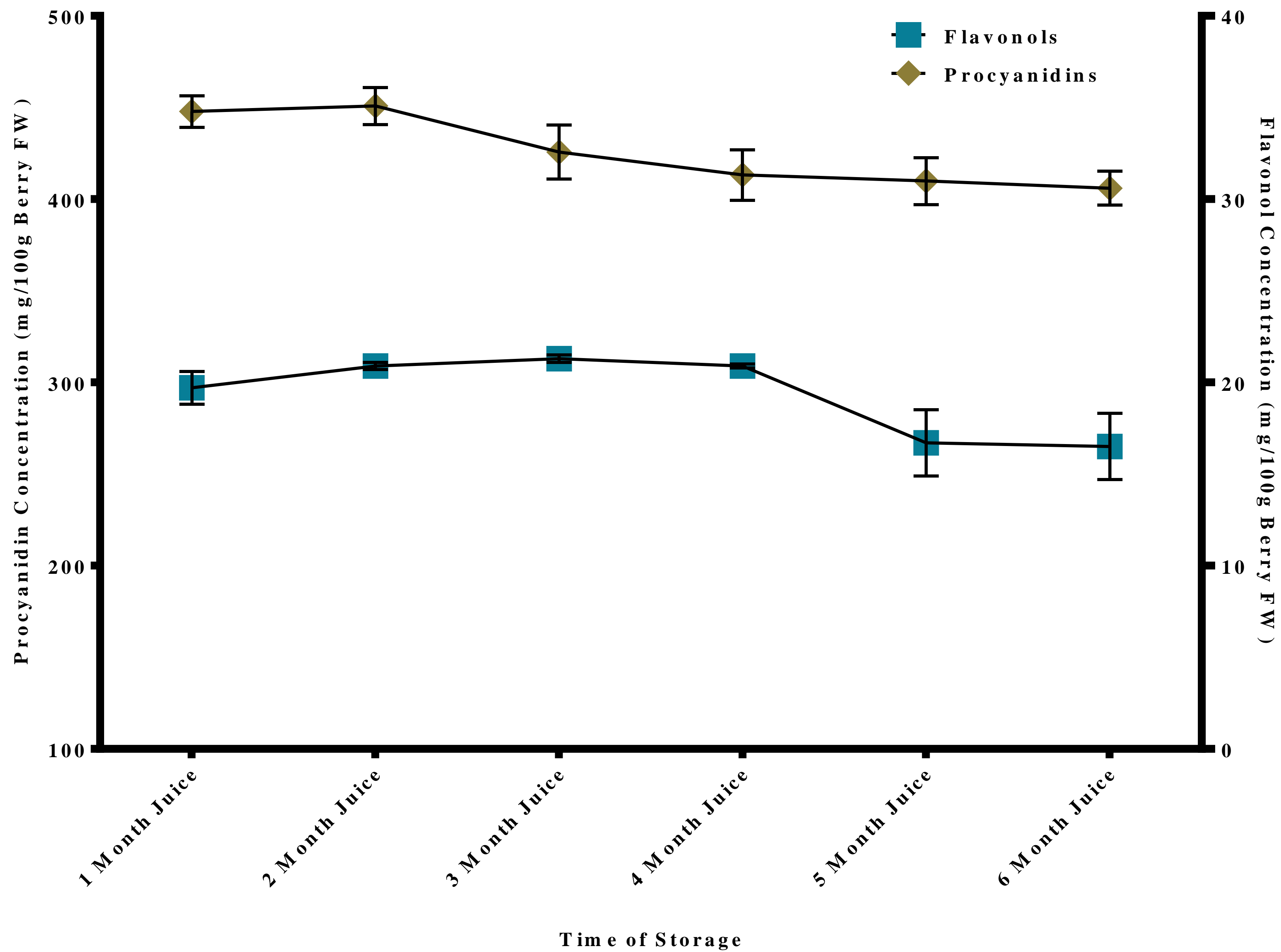


Figure 1-I



IV. COMPARISON OF TWO JUICE PROCESSES ON CHOKEBERRY POLYPHENOLICS AND OPTIMIZATION OF ANTHOCYANIN RETENTION DURING PASTEURIZATION.

A. ABSTRACT

Various forms of processed chokeberry products such as jams, extracts, wine, and juice are consumed more commonly than raw berry form due to their high astringency; however, little information is known as to how phenolic content can change in response to different processing procedures. In this study, the effect of two different processing methods (blanch vs no blanch) on changes in chokeberry anthocyanins, percent polymeric color, hydroxycinnamic acids, flavonols, and total proanthocyanidins at each stage of processing was determined. In addition, various pasteurization times and temperatures were evaluated using a response surface design to predict maximum anthocyanin retention. Anthocyanins experienced extensive losses, mostly due to physical removal of skins and seeds during pressing. Thermal applications resulted in further anthocyanin loss and were accompanied by increased levels of protocatechuic acid and phloroglucinaldehyde. Decreases in anthocyanin levels due to processing were opposite to increasing percent polymeric color values. Total proanthocyanidins, hydroxycinamic acids, and flavonols were better retained in pasteurized chokeberry juice than anthocyanins. Lower temperatures and shorter times (74°C for 2 minutes) resulted in the greatest anthocyanin retention during chokeberry juice pasteurization. Results from this study showed major differences in polyphenolic content during processing, in comparison of the two different methods. However, after the final stage of pasteurization, levels of each component analyzed were not as different as expected. Further research is needed for testing short time, high temperature pasteurization conditions to optimize anthocyanin retentions.

B. INTRODUCTION

During berry fruit juice processing, blanching is the first thermal application that can be used to deactivate browning enzymes, in particular peroxidase (POD) and polyphenol oxidase (PPO). Browning enzymes are believed to have a dramatic effect on the degradation of anthocyanins (ACYs) (*1*). Kader et al. (2002) (*2*) reported that within a peroxide-free solution, no anthocyanin degradation occurs, suggesting that POD plays an important role in anthocyanin degradation. Enzymatic degradation reactions of ACYs can occur directly with ACYs or include multiple steps involving other phenolics. Phenolase enzymes have been shown to oxidize pyrocatechol to ortho-benzoquinone which then oxidizes ACYs to colorless products (*3*). In addition, anthocyanin degradation is believed to be accelerated in the presence of chlorogenic acid and catechin. In association with ascorbic acid however, the oxidized orthoquinone is more prone to react with ascorbic acid rather than ACYs (*4*). Polyphenolase activity is optimal around a pH of 6.0 which is much higher than the chokeberry pH of 3.6. Cash et al. (1976) (*5*) reported that in Concord grape juice (pH of 3.4) phenolase activity was still near 50% of activity at the optimum pH. Several studies have shown that blanching berries prior to pressing results in greater anthocyanin retention after pasteurization compared to berries receiving no blanch treatment (*6, 7, 8*).

In the first study, 55% of total ACYs were lost after blanching chokeberries for 3 min/95°C.The marked loss of total ACYs in this study raised a question about the importance of blanching chokeberries, and the possibility of removing the step completely from juice processing. A marked loss of total ACYs was also observed during pasteurization at 90°C for 10 minutes (Chapter one). Results from Chapter one demonstrated that both thermal treatments, blanching and pasteurization were detrimental to retention of ACYs in the final juice product.

The objectives of this study were to validate the necessity of blanching based on analyzing POD and PPO activities of frozen berries used for processing, to compare polyphenolic content of juices processed with and without a blanch treatment, and to determine optimal pasteurization time and temperature to achieve the greatest anthocyanin retention. The anthocyanin retention optimization study was designed using various time and temperature combinations that surpassed FDA minimal pasteurization guidelines designed to achieve a five log reduction of *E. coli*.

C. MATERIALS AND METHODS

Frozen chokeberries, chemicals, sample preparations, and analytical methods described in Chapter 1 were used in this study. Two different juice processes were conducted, starting with 8.9 kg of frozen chokeberries each time. The first process was exactly the same as the one described in Chapter 1, which included a blanch step, while the second juice process differed only in elimination of the blanch step. Chokeberry material was weighed at each stage to account for moisture loss throughout the juice process. Determination of removing the blanch step was made after performing POD and PPO assays and not detecting any enzyme activity in frozen/thawed berries used for processing.

Phenolase and Peroxidase assay. Frozen chokeberries were allowed to thaw overnight under refrigeration. Triplicate samples of 5 grams were homogenized in 20 mL of phosphate buffer at a pH of 6.5 in an ice water bath. Samples were filtered through Miracloth (CalBiochem, La Jolla, CA, USA) and the liquid fraction was collected for enzyme assays.

Phenolase assay. A catechol substrate was made by dissolving 0.33 g of catechol in 50 mL of phosphate buffer at a pH of 6.5. This solution was allowed to stand 2 hours at ambient

temperature before use. 0.1 mL of enzyme extract was added to 2.9 mL of catechol substrate and mixed rapidly. The solution was transferred to a cuvette and quickly inserted in to a HP 5482A diode array spectrophotometer (Hewlett Packard, Palo, Alto, CA). Absorbance was measured at 420 nm for 2 minutes at 15 second intervals. Enzyme activity was expressed as change in absorbance per minute per gram fresh weight (gfw).

Peroxidase assay. A stock solution was prepared by pipetting 1 mL of 30% hydrogen peroxide in 100 mL of deionized water. 1 mL of the stock solution was diluted with 100 mL 0.05 M phosphate buffer. A 1% dianisidine in methanol solution was prepared, and 0.05 mL was added to 6 mL of the hydrogen peroxide substrate. 2.9 mL of the freshly prepared dianisidine solution was mixed with 0.1 mL of the enzyme extract in a cuvette and quickly inserted in to a HP 5482A diode array spectrophotometer (Hewlett Packard, Palo, Alto, CA). Absorbance was measured at 460 nm for 2 minutes at 15 second intervals. Enzyme activity was expressed as change in absorbance per minute per gram fresh weight (gfw).

After determining no enzymatic activity in thawed berries, berry conditions were altered to determine if heating might release enzymes possibly complexed with PACs. Frozen chokeberries were thawed to room temperature prior to blending in phosphate buffer. After filtering through Miracloth (CalBiochem, La Jolla, CA), the liquid fraction was heated to 40°C in a water bath and held for 30 minutes. Spectrophotometric analyses of the heated enzyme extract for PPO and POD activities as described above confirmed no enzymatic activity. These results indicated that either the enzymes were not released from the PAC complex upon heating, or chokeberries contain non-detectible POD and PPO activities.

Anthocyanin Optimization. Using the Design of Experiments function within JMP Pro 11, a response surface central composite design model was made with various time and temperatures to analyze their effects on anthocyanin retention during juice pasteurization. Limits of time were 0.5 – 5.0 minutes with temperatures ranging from 75.0 – 90.0°C. All twelve data points that were randomly selected are shown in Table 2.1. Statistical significance was tested with an alpha level of 0.05. Using a Precision Water Bath, glass test tubes containing 25 mL of nonpasteurized chokeberry juice of ambient temperature were heated and held at the temperatures and durations listed in the table. Temperatures were monitored using Enviro-Safe® thermometers (VWR) by placing one directly in the water bath and the other inside the control test tube representing each time and temperature combination. The water bath was set 5°C higher than each targeted temperature to reduce the juice come up time to temperature. Average time to raise the temperature of juice to desired value was 6.30 minutes. Samples were immediately placed in an ice bath to prevent further anthocyanin loss during cooling. Total ACYs were quantified by HPLC methods described in the previous chapter (*9*).

Statistical Analysis. Effects of processing on anthocyanin, hydroxycinnamic acid, flavonol, total proanthocyanidin, phenolic acid concentrations, and percent polymeric color were determined by one-way analysis of variance (ANOVA) using JMP Pro 11 (Cary, NC, USA). Differences between means (n=5) were determined by Tukey-Kramer HSD test (α=0.05). Linear and nonlinear regression analysis was also conducted to examine the effects of processing. Optimization of anthocyanin yield was analyzed by a full response surface model, examining factors of time and temperature under first order kinetics and possible higher order effects.

D. RESULTS AND DISCUSSION

Effects of Processing on Polyphenolics and Percent Polymeric Color. Levels of ACYs, HCA, and flavonols previously identified in Chapter one were quantified by HPLC. In addition to compounds identified in Chapter one, pelargonidin 3-arabinoside and a non-identified hydroxycinnamic acid (NI-HCA) were also identified and quantified. The polyphenolic composition of chokeberries was consistent with previous reports (*10, 11, 12, 13,* 14) and the results presented in Chapter one.

Changes in Chokeberry Polyphenolics and Percent Polymeric Color in Juice Process with Blanching.

Anthocyanins. Levels of total ACYs were significantly reduced after blanching and physical removal of skins and seeds with only 21% remaining in chokeberry juice following pasteurization (Table 2.2). Lee et al. (2002) (*7*) reported a similar result in which 23% of original ACYs remained following processing of blueberries into juice. Other studies reported higher anthocyanin retentions of pasteurized juice for black raspberry (31%) (*15*), blackberry (34%) (*16*), and blueberry (72%) (*17*). A 34% reduction in total ACYs occurred after blanching of frozen chokeberries, 62% were lost from physical removal of skins and seeds, and 18% were lost due to pasteurization. The presscake retained 38% of the original concentration of total ACYs in the frozen chokeberries, while most of the balance was transferred to nonpasteurized juice. Levels of cyd 3-glu and cyd 3-xyl were also significantly reduced at each stage of processing except following pasteurization. Plg 3-ara levels were significantly reduced only after blanching and pressing. Cyd pentosides (ara and xyl) were more susceptible to degradation following blanching (38 and 48% losses, respectively) than cyd hexosides (gal and glu, 32 and 39% losses, respectively). The same outcome occurred after pasteurization with cyd 3-ara and

cyd 3-xyl suffering losses of 22 and 20%, whereas cyd 3-gal and cyd 3-glc had losses of 17 and 14%. Plg 3-ara incurred a loss of only 21% after blanching and 16% after pasteurization. Previous studies show similar trends in which hexosides tend to be more thermally stable than pentosides (*18, 19, 6*). Following pasteurization, the juices contained only 22, 21, 18, 32, 16, and 21% of the levels of the cyd 3-gal, cyd 3-glu, cyd 3-ara, plg 3-ara, cyd 3-xyl, and total ACYs found in frozen chokeberries. Removal of the solid material (seeds and skins) during pressing significantly reduced the anthocyanin content in the final juice due to physical removal of the anthocyanin-rich berry skins. The presscake contained 38, 31, 37, 42, 29, and 37% of cyd 3-gal, cyd 3-glu, cyd 3-ara, plg 3-ara, cyd 3-xyl, and total anthocyanin levels found in frozen chokeberries. Compared to the study in the previous chapter, anthocyanin degradation was not as drastic, but once again, thermal application contributed significantly to anthocyanin degradation. As a result, significant increases in phloroglucinaldehyde (PGA) and protocatechuic acid (PCA) were evident from HPLC chromatograms. Levels of PCA and PGA increased 324 and 148% after blanching and 168 and 131% after pasteurization. Sadilova et al. (2007) (*19*) explained that thermal application of cyd glycosides at pH 3.5 causes the pyrilium ring to open, forming a chalcone glycoside. Chokeberries are almost entirely comprised of cyd glycosides and have a pH of 3.6. Next, deglycosylation occurs to yield a chalcone, which readily degrades to PCA and PGA in the presence of heat (*19*). However, the increasing levels of PCA and PGA only accounted for less than 5% of total anthocyanin losses during processing. The degradation of ACYs was probably related to the time required to heat 8.92 kg of frozen chokeberries to 95°C, and then cool the blanched berries to 40°C. The study in the previous chapter used 23 kg of frozen chokeberries to prepare juice which took longer to heat to 95°C and cool to 40°C and resulted in anthocyanin loss of nearly 55%. In a follow-up study, 5.4 kg of

frozen chokeberries blanched for 3 minutes at 95ºC, only incurred a 25% loss of ACYs. In this study the come-up time to blanch temperature of 95ºC was 12 minutes.

Percent Polymeric Color. Percent polymeric color values decreased from frozen berry state (8%) to blanching and enzyme treatment (6.6%) (Table 2.2). This might indicate that initial thermal application led to cleavage of some anthocyanin-tannin linkages that are already present in the frozen chokeberry. Previous research on blueberry (*17*) and blackberry (*16*) juice processing reported an increase of percent polymeric color due to blanching (0.6-8.6% for blueberry and 10.5-13.2% for blackberry). The presscake had a significantly higher percent polymeric color value (8.3%) than enzyme treated mash (6.6%), indicating polymers were possibly formed during the depectinization step at 40°C/1 hour and that they have a higher affinity to bind to cell wall polysaccharides/proteins (*17, 20*). The largest increase occurred in response to pasteurization, changing from 7.9% in nonpasteurized juice to 10.1% in pasteurized juice (Table 2.1). Lee et al. (2002) (*6*) also reported an increase in percent polymeric color due to blueberry juice pasteurization (37.6 to 50.4%). Previous studies indicate that anthocyanin-tannin polymers mainly form in wine during storage, which is slow and gradual at ambient temperature due to the reaction's high activation energy (*21*). The authors also reported that elevated temperatures accelerate the formation of anthocyanin-tannin linkages. This phenomenon was observed after pasteurization of chokeberry juice and may account for some of loss of ACYs.

Hydroxycinnamic Acids. Levels of neochlorogenic acid (NCA) and chlorogenic acid (CA) decreased following blanching, 15 and 14%, respectively (Table 2.3). The level of NI-HCA increased slightly (7%) following blanching and enzyme treatment. Total HCA decreased 13% after enzyme treatment when compared to frozen berries. Significant loss of all HCA's

occurred after pressing the chokeberry mash where 43-49% remained in the presscake, and 51-57% was transferred to nonpasteurized juice. Levels of NCA and CA were stable in response to pasteurization, however, the level of NI-HCA increased by 24%.

Total Proanthocyanidins. Levels of total PACs were unaffected by blanching and enzyme treatment (Table 2.3). Physical removal of the skins and seeds following enzyme treatment caused the greatest loss of PACs, where 57% remained in the presscake and 32% was transferred to nonpasteurized juice. An apparent 22% increase was observed following pasteurization, but was not statistically significant. Pasteurized juice retained 39% of the original total PACs found in frozen chokeberry, which was similar to 43% retention of PACs in pasteurized blueberry juice (*22*). However, these levels were much greater than the 19% retention of PACs in blueberry juice following pasteurization reported by Brownmiller et al. (2009) (*23*).

Flavonols. Total flavonol content of frozen chokeberries calculated as rutin equivalents was 38.6 mg/100 g. Eight different flavonols were identified by LC/MS (Chapter one) as quercetin derivatives and quantified by HPLC. Levels of quercetin dihexosides, vicianoside, galactoside, and glucoside increased following blanching and enzyme treatment (11-33%) when compared to frozen berries (Table 2.4). Levels of robinobioside decreased 7-8% following blanching compared to frozen berries, whereas rutinoside remained stable. Quercetin levels increased 86% after blanching, but decreased 15% after enzyme treatment. Total flavonol levels increased 14% after blanching and enzyme treatment. Significant flavonol losses occurred during the pressing stage with 32 to 50% of dihexoside-1, dihexoside-2, vicianoside, robinoside, rutinoside, galactoside, and glucoside retained in the presscake, whereas 52 to 62% was transferred to nonpasteurized juice. Quercetin showed 45% retention in the presscake while 18%

was transferred to nonpasteurized juice. Of the total flavonols, 41% was retained in the presscake while 53% was transferred to the nonpasteurized juice. After pasteurization, all flavonols except quercetin showed no significant change. Although present at low concentrations, quercetin content doubled from 0.2 to 0.5 mg/100g in response to pasteurization. The increased level of quercetin following pasteurization was most likely due to cleavage of glycosides in response to thermal treatment.

Changes in Chokeberry Polyphenolics and Percent Polymeric Color in Juice Process without Blanching.

Anthocyanins. Total anthocyanin content decreased significantly at each stage of processing, with 26% loss following enzyme treatment, and a further 46% loss following pressing (Table 2.5). The pasteurization step decreased total anthocyanin levels by 28%. Comparing the two different juice processes, with blanching, total ACYs declined 40% after enzyme treatment whereas without blanching, total ACYs declined only 27% after enzyme treatment. After separating solid material from nonpasteurized juice, presscake with and without blanch retained 53 and 62%, respectively of total ACYs. Pasteurized juice with a blanch step showed an anthocyanin loss of 18% whereas pasteurized juice without a blanch step experienced an anthocyanin loss of 28%. Surprisingly, pasteurized juice with blanching contained 204 mg/100 g total ACYs, whereas pasteurized juice without blanching contained 185 mg/100 g total ACYs. Although this difference was statistically insignificant, the result was unexpected. It was believed that removing the thermal blanching step would improve total anthocyanin retention in pasteurized juice. Removal of the blanch step resulted in greater retention of total ACYs after enzyme treatment, but this difference was negated in part due to greater loss of total ACYs during pasteurization of berries receiving no blanch.

Following enzyme treatment, levels of cyd 3-gal, cyd 3-glu, cyd 3-ara, plg 3-ara, and cyd 3-xyl decreased 25, 30, 28, 12, and 35% respectively. Levels of cyd 3-gal, cyd 3-glu, cyd 3-ara, plg 3-ara, and cyd 3-xyl were retained more in the presscake (61, 56, 65, 60, and 65%, respectively) rather than nonpasteurized juice (42, 45, 41, 54, and 46%, respectively). Cyd pentosides (ara and xyl) were more susceptible to degradation due to pasteurization (31 and 30%) than cyd hexosides (gal and glu) at 27 and 19%. PGA and PCA levels increased with thermal treatments in line with anthocyanin degradation. Initially not present in frozen berries, PCA increased 4-fold following enzyme treatment and increased 43% following pasteurization. PGA increased 21% from frozen berry to enzyme treated mash and increased 28% following pasteurization. Similar to the results in Chapter 1 and process with blanching, the increases in PGA and PCA only accounted for less than 5% of the total anthocyanin losses.

Percent Polymeric Color. No change in percent polymeric color occurred between frozen berry and enzyme treatment (Table 2.5). It is possible that the 40°C temperature used for enzyme treatment may not be high enough to break down anthocyanin-tannin polymers that are present in the frozen berries as did the blanch step from the previous process. Percent polymeric color values increased in presscake, nonpasteurized and pasteurized juice with significant increases between enzyme treatment and presscake and from nonpasteurized to pasteurized juice. Within the process having a blanch step, percent polymeric color decreased due to blanching, possibly due to high thermal application disrupting polymeric pigments already present in the frozen chokeberries. However, a larger increase in percent polymeric color occurred due to juice pasteurization. Between the two processes, presscake that was initially blanched contained a higher percent polymeric color value than the nonpasteurized juice. Without blanching, the presscake contained a lower value than the nonpasteurized juice. It appears ACYs are more

prone to polymerization in a process containing an initial blanch step, which is consistent with the reported high activation energy of the polymerization reaction (*21*).

Hydroxycinnamic Acids. Levels of total HCA were unaffected by enzyme treatment (Table 2.6). Only 33% of total HCA were retained in the presscake following pressing, with most of the balance transferred to nonpasteurized juice. In blueberries HCA are located in the pulp and are more easily released into the juice, compared to ACYs, which are located in the berry skin (*22*). It is possible that HCA in chokeberries are also present in the berry pulp, and may be released into the juice more readily than polyphenolics located in the skin and seeds. Levels of total HCA were unaffected by pasteurization. In comparison of the two processes, there was no difference in total HCA content in pasteurized juices. Levels of NCA and CA followed a similar pattern as total HCA, but the level of NI-HCA increased 9% following enzyme treatment. The presscake retained 34% of the NI-HCA, with the balance (64%) transferred to the nonpasteurized juice. The level of NI-HCA increased 16% following pasteurization.

Total Proanthocyanidins. From frozen chokeberry to enzyme treated mash, the level of total PACs increased 20%, presumably due to softening of tissues from heat application that allowed for better extraction (Table 2.6). Following pressing, 74% of the total PACs remained in the presscake, while 26% was transferred to the nonpasteurized juice. Levels of total PACs were unaffected by pasteurization. From the process with blanching, there were no significant differences between frozen berry, blanched, and enzyme treated mash. More PACs were retained in the presscake without blanching, resulting in 28% retention in pasteurized juice compared to 39% retention in pasteurized juice with blanch step.

Flavonols. No change occurred in the levels of total flavonols between frozen berry and enzyme treatment (Table 2.7). After pressing, 40% of total flavonols were retained in the presscake and 60% was transferred to nonpasteurized juice. Levels of total flavonols remained unchanged in response to pasteurization. Both dihexosides, vivianoside, and quercetin levels significantly increased (15, 29, 9, and 88% respectively) after enzyme treatment, whereas robinobioside decreased by 8%. Levels of rutinoside, galactoside, and glucoside remained unchanged after enzyme treatment. After pressing, levels of dihexosides, vicianosides, robinobiosides, and rutinosides remaining in the presscake ranged from 35-37%, while 41 and 43% of galactosides and glucosides, and 51% of quercetin remained in the presscake. Most of the balance of flavonols was transferred to nonpasteurized juice. Quercetin was the only flavonol that was affected by pasteurization, with the level increased by 59%. The process with blanching showed a significant increase in total flavonols between frozen berry and enzyme treated mash with 18 mg/100 g berry FW (47%) retained in the presscake. The process without blanching did not experience a significant increase after enzyme treatment with only 15.6 mg/100 g berry FW (40%) retained in the presscake. More flavonols were present in the nonpasteurized juice that received blanching during processing and therefore more flavonols were retained in pasteurized juice compared to the process without blanching.

Anthocyanin Retention Optimization. From the central composite response surface study, the quadratic factors of Time*Temperature and Temperature*Temperature were removed from the analysis because they did not show statistical significance to the overall model. The simplified model was statistically significant with a P-value of 0.0208 and explained 68.6% of the variability contributing to the overall model. Analysis showed significant linear effects of Time and Temperature parameters as well as a quadratic effect from Time*Time parameter (P-

values of 0.0416 for Time, 0.0453 for Temperature, and 0.0307 for Time*Time). Using the prediction profiler in JMP, the optimal anthocyanin retention level of 273.7 mg/100 g was predicted in which the pasteurization parameters were 74°C for 2.02 minutes. The prediction formula derived from the model was:

$$\text{Total ACY} = 265.97 + \left(-6.51 * \frac{(Time - 2.75)}{2.25}\right) + \left(-6.64 * \frac{(Temp. - 82.5)}{7.5}\right)$$

$$+ \left[\frac{\frac{(Time - 2.75)}{2.25} * (Time - 2.75)}{2.25}\right] * (-10.02)$$

Higher pasteurization temperatures (>80°C) were detrimental to anthocyanin retention. It was evident that holding chokeberry juice at 90°C for 10 minutes, as conducted in the previous processing studies, was too extreme for retaining ACYs. Factor constraints were included in the experimental design, however, data points of time and temperature that were randomly selected did not appear to contain any limitations. To improve this model, time and temperature ranges should be expanded (0.5-10 minutes for time and 70-100°C for temperature). Factor constraints can then be added to focus on short-time/high-temperature pasteurization as well as low-temperature/longer-time pasteurization.

E. CONCLUSION

Chokeberry juice processing dramatically diminishes the potential for consumers to obtain a significant level of health-promoting compounds. Major loss in ACYs due to processing is very problematic since chokeberries are readily consumed in various processed forms. At the end of juice processing, only 21 and 19% of the original, frozen chokeberry ACYs remained in pasteurized juice from processes with and without blanching. Flavonols, HCA, and

total proanthocyanidin retention levels were 59, 55, and 39%, respectively, after juice pasteurization with blanching, whereas 50, 57, and 28% were retained without blanching. Across all evaluated components, the majority of losses occurred during pressing due to physical removal of the polyphenolic-rich skins and seeds. Utilization of antioxidant-rich, presscake by-product opens possibilities for the development of nutraceutical ingredients and dietary supplements. Increases in PCA, PGA, and percent polymeric color only explain a fraction of total anthocyanin losses. More research is needed to identify other possible pathways of anthocyanin degradation or polymerization.

Table 2.1 Time and Temperature Variables used to Pasteurize Chokeberry Juice to Predict Optimal Anthocyanin Retention.

Time (min.)	**Temperature (Celsius)**
2.75	81.8
5	88
2.75	75
0.5	75
5	75
5	81.5
0.5	82.5
3	90
0.5	90
2.46	82.5
2.75	82.5
2.75	82.3

Table 2.2 Concentration of Anthocyanins and %Polymeric Color throughout Chokeberry Juice Processing with Blanch Step

	Anthocyanins (mg/100 g berry FW)						
Processing Step	Cyanidin 3-galactoside	Cyanidin 3-glucoside	Cyanidin 3-arabinoside	Pelargonidin 3-arabinoside	Cyanidin 3-xyloside	Total	% Polymeric Color
Frozen	644.6 ± 9.3 a	34.3 ± 0.9 a	253.8 ± 1.9 a	5.79 ± 0.18 a	35.3 ± 0.9 a	973.7 ± 12.8 a	8.0 ± 0.14 b
Blanch	436.8 ± 14.3 b	20.8 ± 0.6 b	158.0 ± 6.2 b	4.56 ± 0.40 b	18.3 ± 0.7 b	638.4 ± 21.6 b	6.3 ± 0.23 c
Enzyme	404.5 ± 9.5 b	18.6 ± 0.6 b	144.9 ± 3.1 b	4.08 ± 0.23 b	15.6 ± 0.6 c	587.8 ± 13.7 b	6.6 ± 0.56 c
Juice, NP	171.6 ± 2.3 d	8.4 ± 0.06 cd	60.1 ± 0.8 d	2.19 ± 0.09 c	7.1 ± 0.09 e	249.4 ± 3.3 d	7.9 ± 0.43 b
Presscake	247.0 ± 4.3 c	10.5 ± 0.5 c	94.1 ± 1.9 c	2.45 ± 0.12 c	10.2 ± 0.3 d	364.3 ± 6.8 c	8.3 ± 0.50 b
Juice, P	142.3 ± 0.8 d	7.3± 0.2 d	46.9 ± 0.6 d	1.85 ± 0.09 c	5.7 ± 0.07 e	203.9 ± 1.7 d	10.1 ± 0.05 a

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$). NP, nonpasteurized; P, pasteurized.

Table 2.3 Concentration of Hydroxycinnamic Acids and Total Proanthocyanidins (PACs) throughout Chokeberry Juice Processing with Blanch Step

	Hydroxycinnamic Acids (mg/100 g berry FW)				
Processing Step	Neochlorogenic acid	Chlorogenic acid	Nonidentified HCA	Total	Total PACs (mg/100 g berry FW)
Frozen	52.7 ± 0.75 a	66.2 ± 0.23 a	2.4 ± 0.08 a	121.3 ± 0.7 a	583.4 ± 35.4 a
Blanch	44.9 ± 0.47 b	57.0 ± 0.56 b	2.6 ± 0.07 a	104.5 ± 0.9 b	584.8 ± 26.7 a
Enzyme	45.9 ± 0.76 b	57.4 ± 1.40 b	2.6 ± 0.08 a	105.9 ± 2.1 b	582.4 ± 17.8 a
Juice, NP	29.9 ± 0.33 c	34.4 ± 0.30 c	1.6 ± 0.04 c	65.9 ± 0.6 c	186.2 ± 4.7 c
Presscake	22.8 ± 0.34 d	33.3 ± 0.32 c	1.4 ± 0.05 c	57.5 ± 0.7 d	332.3 ± 12.6 b
Juice, P	30.2 ± 0.02 c	35.0 ± 0.10 c	2.0 ± 0.08 b	67.2 ± 0.2 c	226.7 ± 5.1 c

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$). NP, nonpasteurized; P, pasteurized.

Table 2.4 Concentration of Flavonols throughout Chokeberry Juice Processing with Blanch Step

	Flavonols (mg/100 g berry FW)			
Processing Step	Quercetin 3-dihexoside	Quercetin 3-dihexoside	Quercetin 3-vicianoside	Quercetin 3-robinobioside
Frozen	3.0 ± 0.01 b	1.7 ± 0.01 b	3.7 ± 0.01 b	5.1 ± 0.04 a
Blanch	3.6 ± 0.1 a	2.1 ± 0.04 a	4.4 ± 0.2 a	4.6 ± 0.06 b
Enzyme	3.7 ± 0.3 a	2.2 ± 0.12 a	4.7 ± 0.3 a	4.7 ± 0.2 ab
Juice, NP	2.1 ± 0.07 c	1.1 ± 0.01 c	2.5 ± 0.04 c	2.7 ± 0.03 c
Presscake	1.4 ± 0.04 d	0.7 ± 0.02 d	1.7 ± 0.05 d	1.7 ± 0.02 d
Juice, P	2.0 ± 0.01 c	1.0 ± 0.004 c	2.4 ± 0.01 c	2.6 ± 0.01 c

	Flavonols (mg/100 g berry FW)				
Processing Step	Quercetin 3-rutinoside	Quercetin 3-galactoside	Quercetin 3-glucoside	Quercetin	Total
Frozen	5.2 ± 0.03 a	11.0 ± 0.1 b	8.3 ± 0.05 b	0.7 ± 0.01 c	38.6 ± 0.2 b
Blanch	5.1 ± 0.1 a	12.7 ± 0.4 a	8.7 ± 0.2 ab	1.3 ± 0.04 a	42.5 ± 0.9 a
Enzyme	5.1 ± 0.2 a	13.4 ± 0.8 a	9.1 ± 0.5 a	1.1 ± 0.08 b	44.1 ± 1.9 a
Juice, NP	3.1 ± 0.02 b	6.8 ± 0.1 c	4.8 ± 0.05 c	0.2 ± 0.001 e	23.2 ± 0.2 c
Presscake	1.9 ± 0.04 c	6.1 ± 0.1 c	3.9 ± 0.04 d	0.5 ± 0.02 cd	18.1 ± 0.2 d
Juice, P	3.1 ± 0.02 b	6.5 ± 0.04 c	4.6 ± 0.02 cd	0.5 ± 0.002 d	22.6 ± 0.1 c

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$). NP, nonpasteurized; P, pasteurized.

Table 2.5 Concentration of Anthocyanins and % Polymeric Color throughout Chokeberry Juice Processing without Blanch Step

	Anthocyanins (mg/100 g berry FW)						
Processing Step	Cyanidin 3-galactoside	Cyanidin 3-glucoside	Cyanidin 3-arabinoside	Pelargonidin 3-arabinoside	Cyanidin 3-xyloside	Total	% Polymeric Color
Frozen	644.6 ± 9.3 a	34.3 ± 0.9 a	253.8 ± 1.9 a	5.8 ± 0.2 a	35.3 ± 0.9 a	973.7 ± 12.8 a	8.0 ± 0.1 c
Enzyme	481.8 ± 13.5 b	23.9 ± 1.1 b	182.3 ± 4.7 b	5.1 ± 0.2 b	23.0 ± 0.7 b	716.1 ± 20.1 b	8.0 ± 0.1 c
Juice, NP	175.8 ± 4.9 d	8.3 ± 0.2 d	62.4 ± 1.8 d	2.5 ± 0.1 d	7.7 ± 0.2 d	256.7 ± 6.9 d	11.0 ± 0.1 ab
Presscake	259.1 ± 3.7 c	12.0 ± 0.2 c	102.8 ± 1.9 c	3.1 ± 0.1 c	12.4 ± 0.3 c	389.5 ± 6.2 c	10.1 ± 0.9 b
Juice, P	128.3 ± 8.3 e	6.8 ± 0.4 d	42.8 ± 2.8 e	1.9 ± 0.1 e	5.4 ± 0.3 e	185.0 ± 11.8 e	12.9 ± 1.1 a

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$). NP, nonpasteurized; P, pasteurized.

Table 2.6 Concentration of Hydroxycinnamic Acids and Total Proanthocyanidins (PACs) throughout Chokeberry Juice Processing without Blanch Step

	Hydroxycinnamic Acids (mg/100 g berry FW)				
Processing Step	Neochlorogenic acid	Chlorogenic acid	Nonidentified HCA	Total	Total PACs (mg/100 g berry FW)
Frozen	52.7 ± 0.7 a	66.2 ± 0.2 a	2.4 ± 0.1 a	121.3 ± 0.7 a	583.4 ± 35.4 b
Enzyme	52.3 ± 1.1 a	63.4 ± 1.3 a	2.6 ± 0.1 a	118.3 ± 2.4 a	702.8 ± 17.0 a
Juice, NP	33.1 ± 0.6 b	37.3 ± 0.7 b	1.8 ± 0.1 b	72.1 ± 1.4 b	152.0 ± 1.5 d
Presscake	14.6 ± 0.3 c	20.4 ± 0.1 c	0.9 ± 0.02 c	35.9 ± 0.3 c	438.9 ± 10.7 c
Juice, P	31.6 ± 1.4 b	35.0 ± 1.5 b	2.0 ± 0.1 b	68.6 ± 3.0 b	165.7 ± 10.3 d

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$). NP, nonpasteurized; P, pasteurized.

Table 2.7 Concentration of Flavonols throughout Chokeberry Juice Processing without Blanch Step

	Flavonols (mg/100 g berry FW)			
Processing Step	Quercetin 3-dihexoside	Quercetin 3-dihexoside	Quercetin 3-vicianoside	Quercetin 3-robinobioside
Frozen	3.0 ± 0.01 a	1.7 ± 0.01 b	3.7 ± 0.01 b	5.1 ± 0.04 a
Enzyme	3.4 ± 0.1 b	2.1 ± 0.05 a	4.1 ± 0.21 a	4.7 ± 0.08 b
Juice, NP	1.8 ± 0.003 c	1.0 ± 0.003 c	2.1 ± 0.002 c	2.5 ± 0.01 c
Presscake	1.2 ± 0.02 d	0.7 ± 0.01 d	1.4 ± 0.02 d	1.7 ± 0.01 d
Juice, P	1.7 ± 0.01 c	1.0 ± 0.01 c	2.1 ± 0.02 c	2.5 ± 0.01 c

	Flavonols (mg/100 g berry FW)				
Processing Step	Quercetin 3-rutinoside	Quercetin 3-galactoside	Quercetin 3-glucoside	Quercetin	Total
Frozen	5.2 ± 0.03 a	11.0 ± 0.1 a	8.3 ± 0.05 a	0.7 ± 0.01 b	38.6 ± 0.2 a
Enzyme	5.1 ± 0.1 a	10.9 ± 0.4 a	8.1 ± 0.2 a	1.2 ± 0.02 a	39.7 ± 1.1 a
Juice, NP	2.9 ± 0.01 b	5.3 ± 0.02 b	4.0 ± 0.01 b	0.3 ± 0.003 d	20.0 ± 0.1 b
Presscake	1.9 ± 0.01 c	4.7 ± 0.09 a	3.4 ± 0.04 c	0.6 ± 0.01 b	15.6 ± 0.2 c
Juice, P	2.9 ± 0.01 b	5.0 ± 0.09 bc	3.9 ± 0.07 b	0.5 ± 0.01 c	19.5 ± 0.2 b

Mean values ± std. error (n=5) within a column with different letters are significantly different ($p \leq 0.05$).
NP, nonpasteurized; P, pasteurized.

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G. FIGURE CAPTIONS

Figure 2-A. Concentrations of total anthocyanins and percent polymeric color throughout chokeberry juice processing with blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-B. Concentrations of each anthocyanin throughout juice processing with blanch step.

Figure 2-C. Concentrations of hydroxycinnamic acids throughout juice processing with blanch step. Bars represent standard errors of the means (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-D. Concentrations of total procyanidins and flavonols throughout juice processing with blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-E. Concentrations of protocatechuic acid and phloroglucinaldehyde during juice processing with blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-F. Concentrations of total anthocyanins and percent polymeric color throughout chokeberry juice processing without blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-G. Concentrations of each anthocyanin throughout juice processing without blanch step.

Figure 2-H. Concentrations of hydroxycinnamic acids throughout juice processing without blanch step. Bars represent standard errors of the means (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-I. Concentrations of total procyanidins and flavonols throughout juice processing without blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-J. Concentrations of protocatechuic acid and phloroglucinaldehyde during juice processing without blanch step. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-K. Comparing concentrations of total anthocyanin concentrations by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the mean (n = 5). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-L. Comparing percent polymeric color by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the mean ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-M. Comparing concentrations of total hydroxycinnamic acids by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the means ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

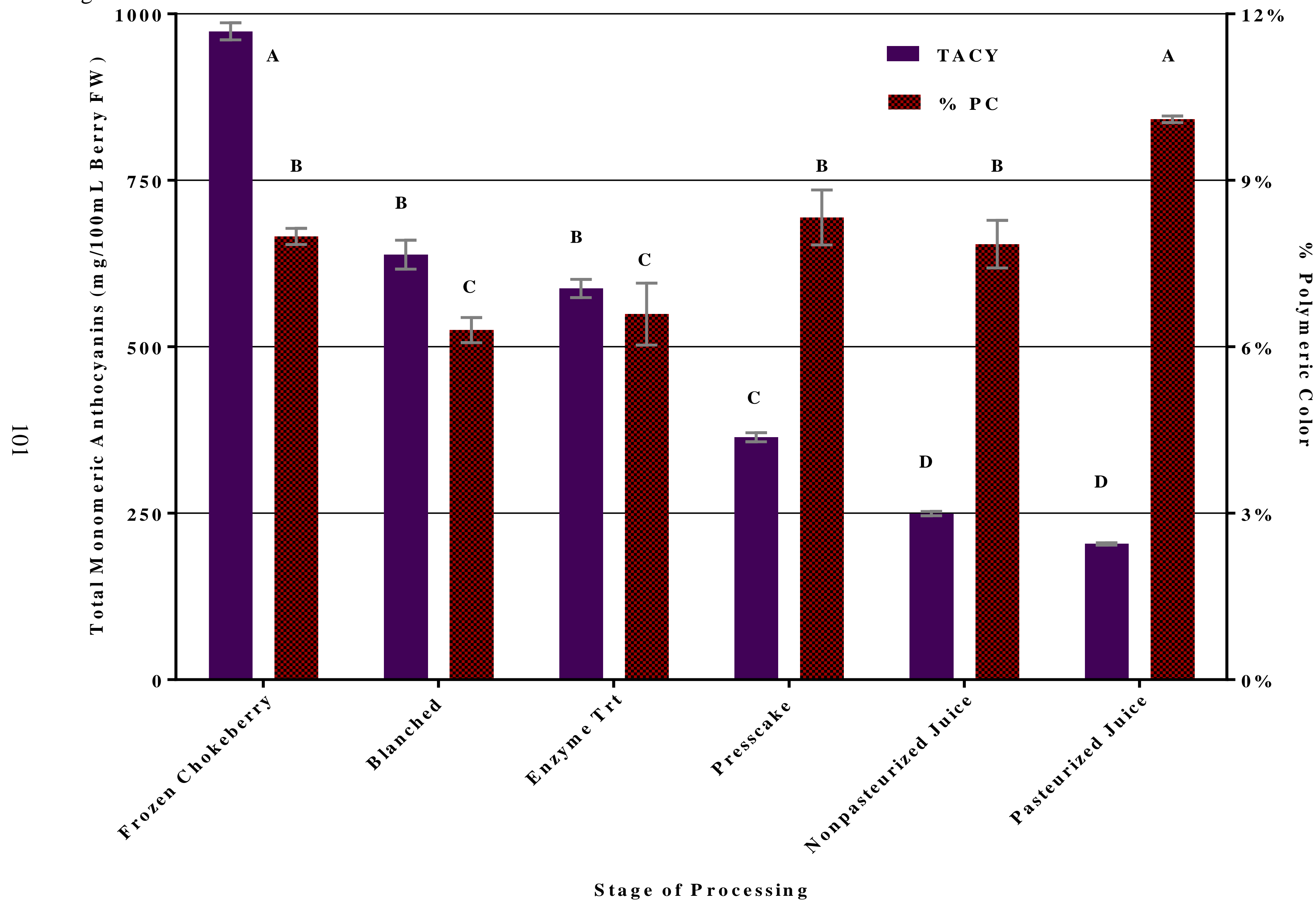
Figure 2-N. Comparing concentrations of total procyanidins by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the means ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-O. Comparing concentrations of total flavonols by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the means ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-P. Comparing concentrations of protocatechuic acid by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the means ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-Q. Comparing concentrations of phloroglucinaldehyde by each stage of both juice processes from enzyme treatment to pasteurized juice. Bars represent standard errors of the means ($n = 5$). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2-A



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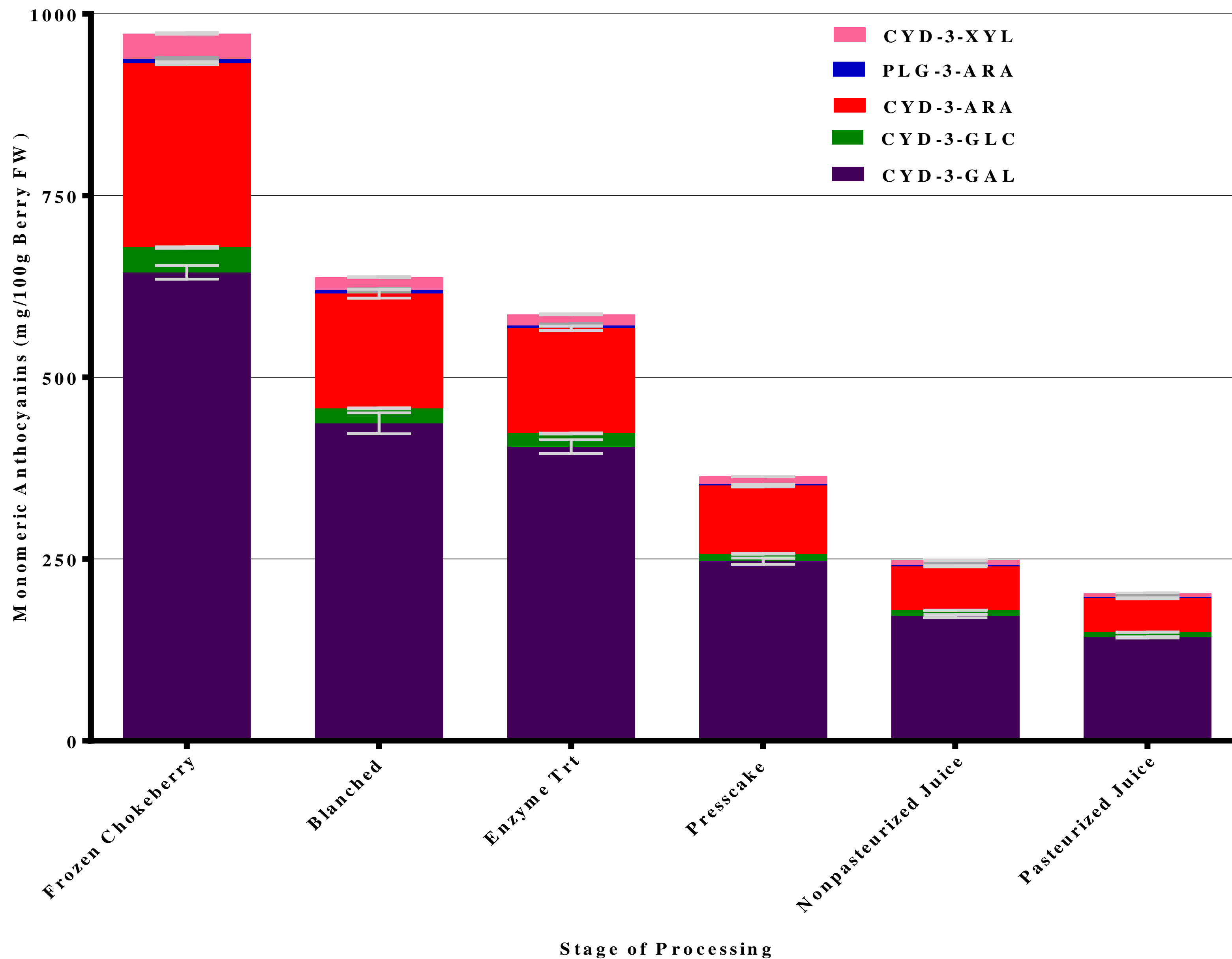

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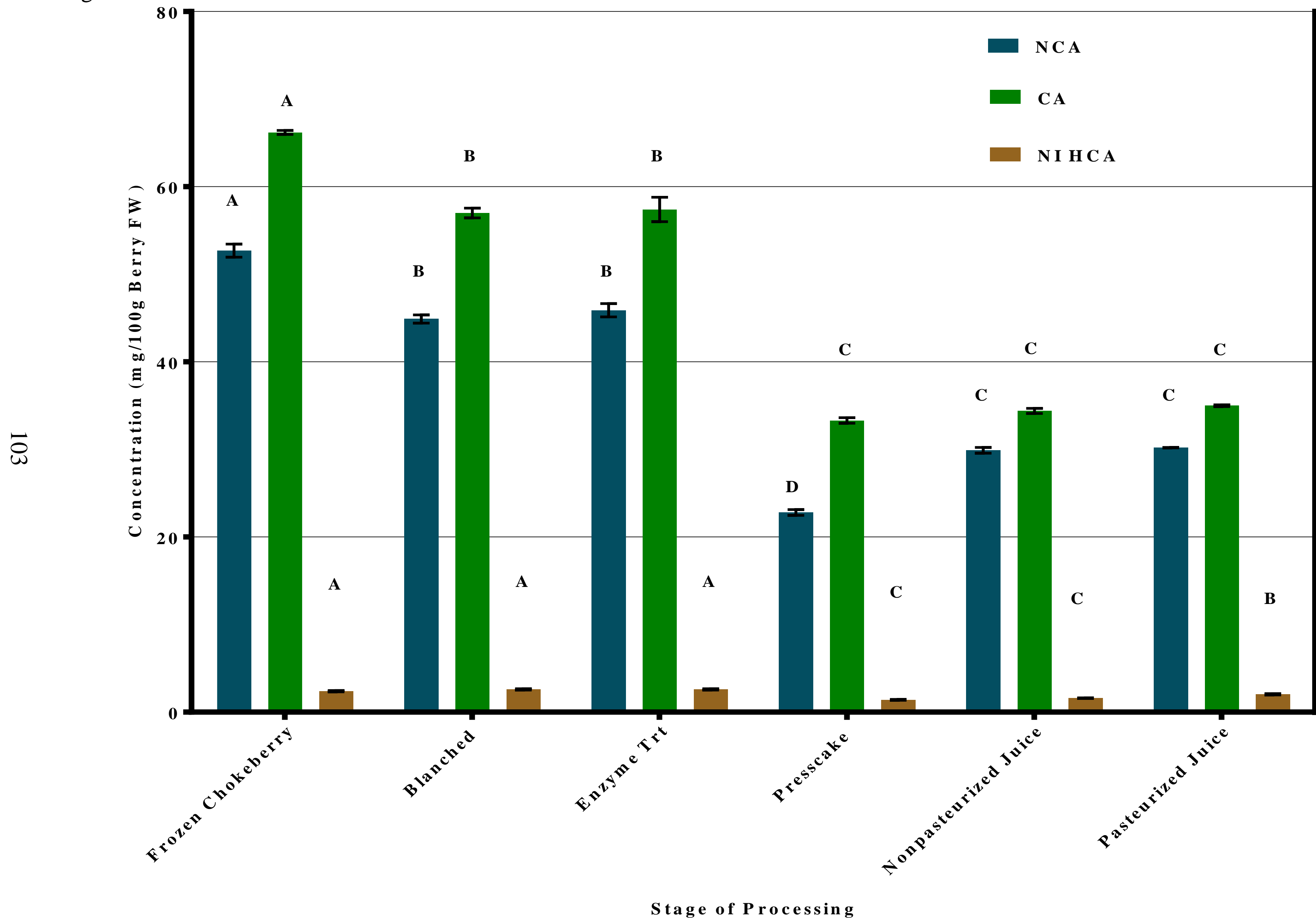

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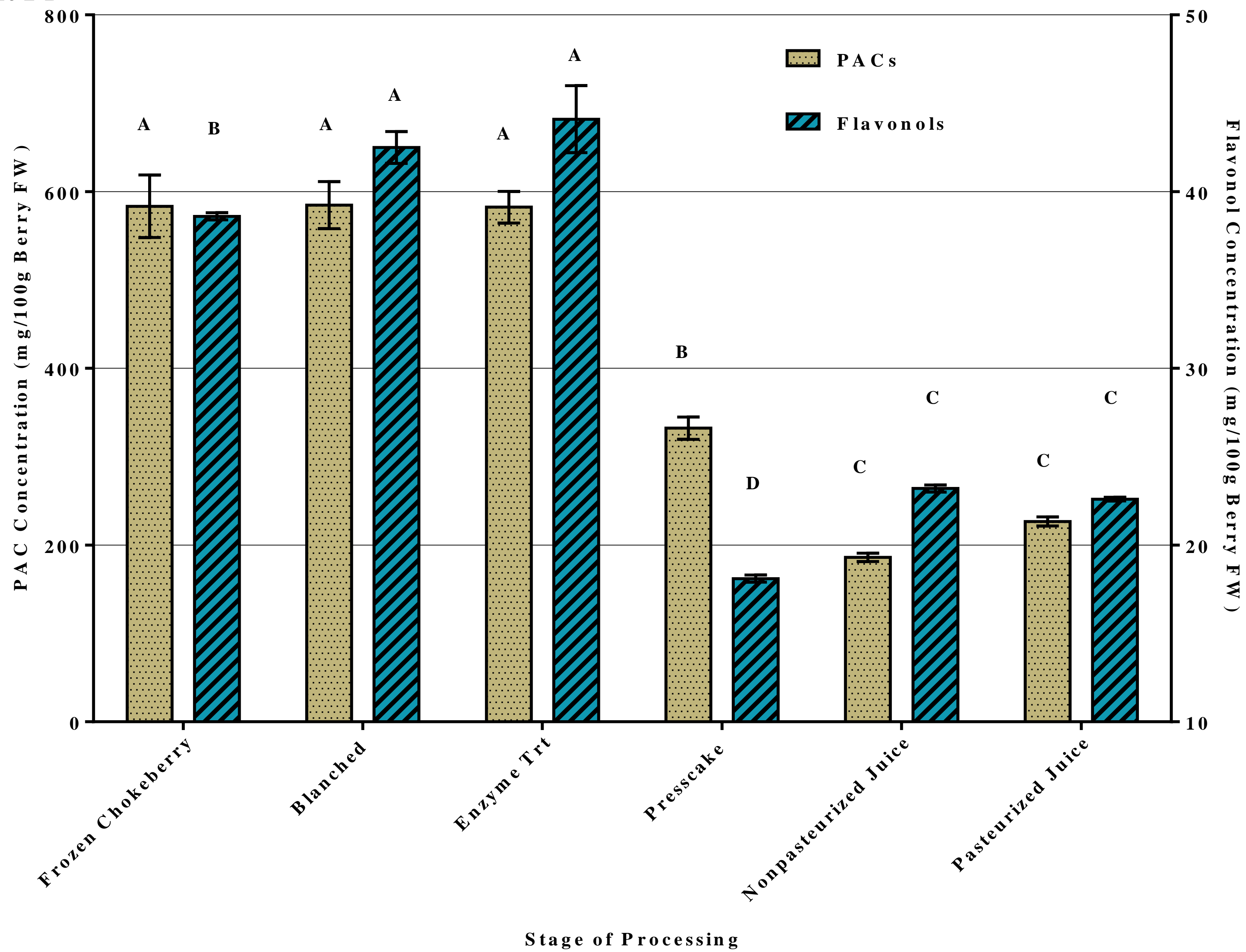

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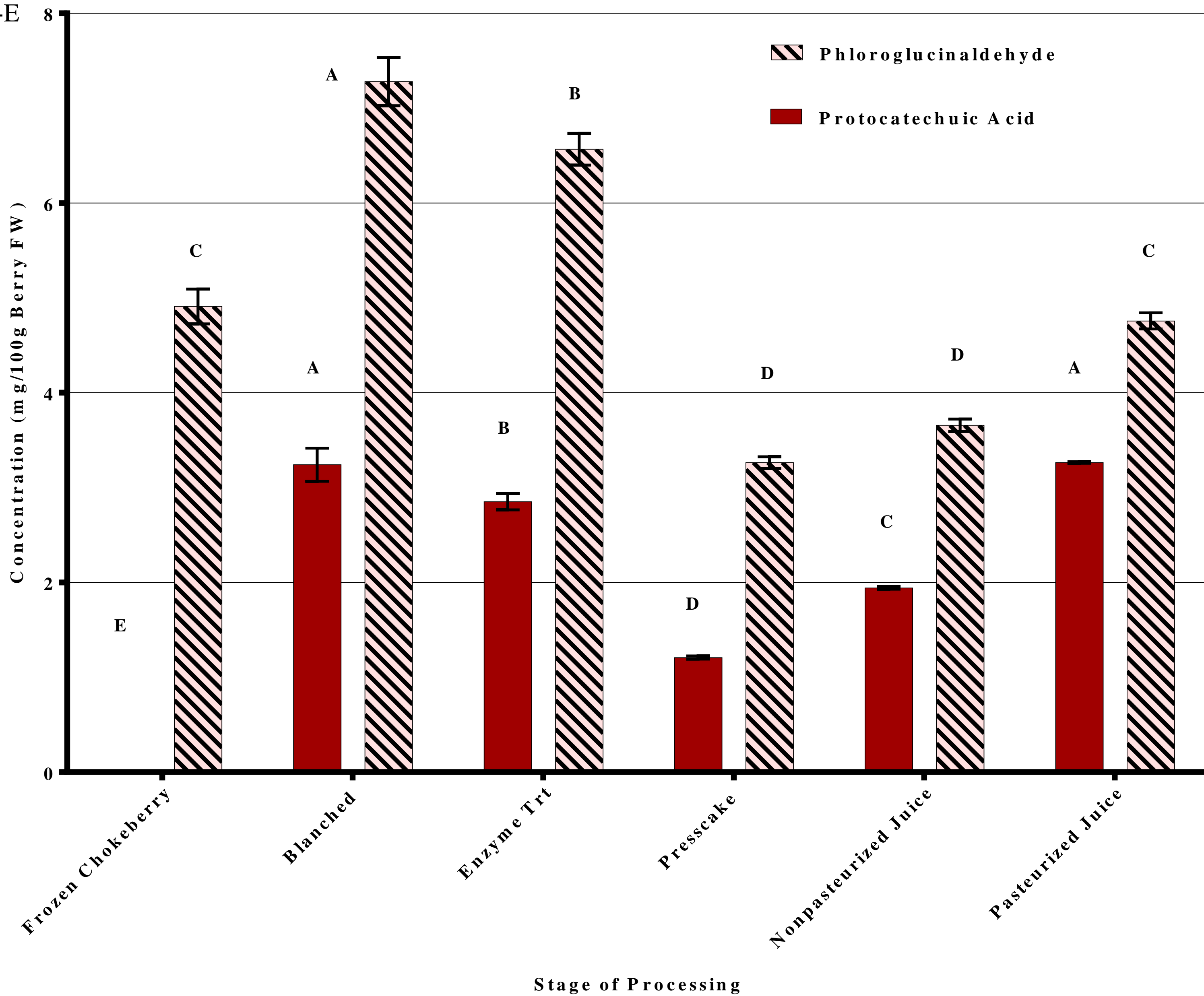

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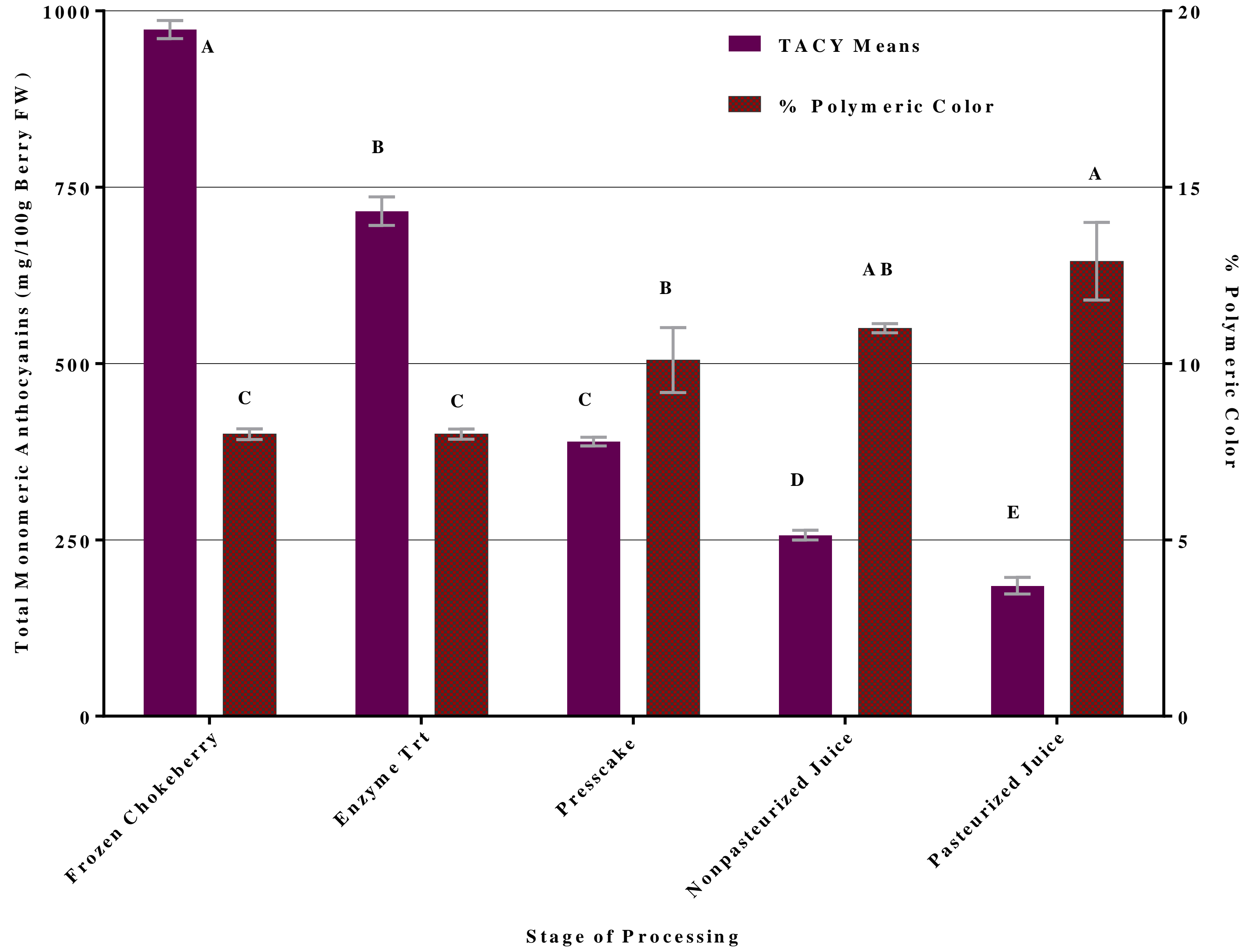

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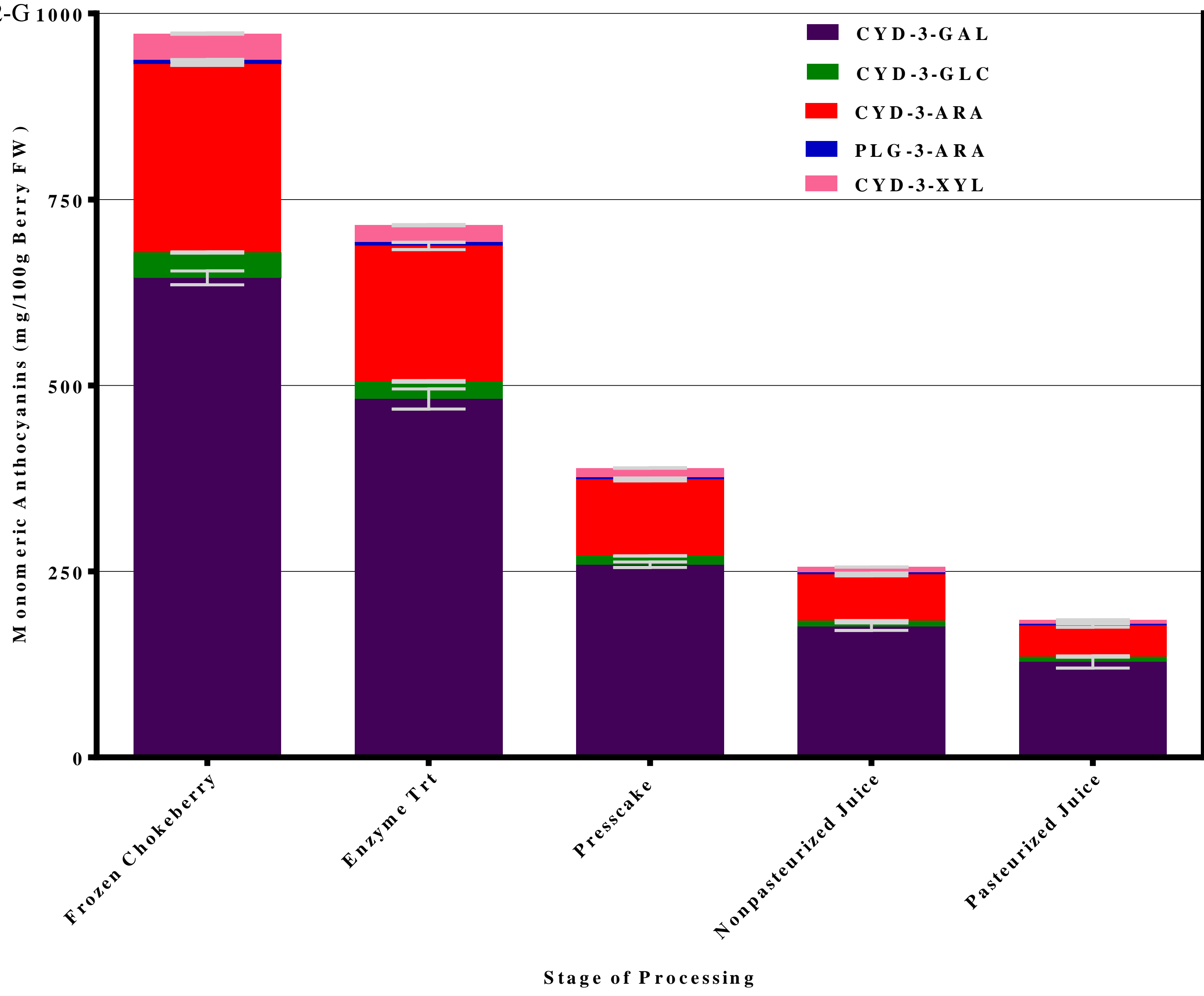

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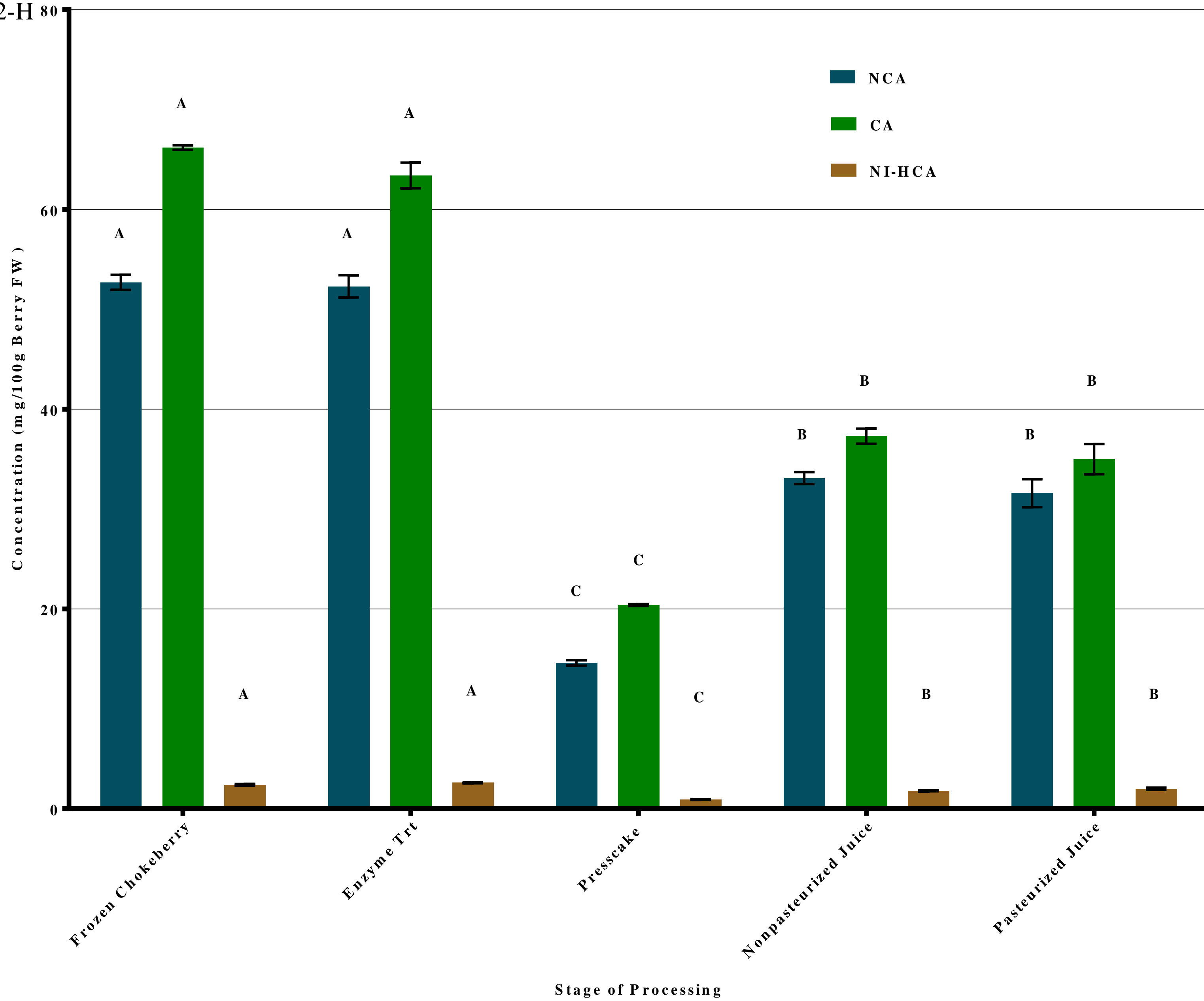

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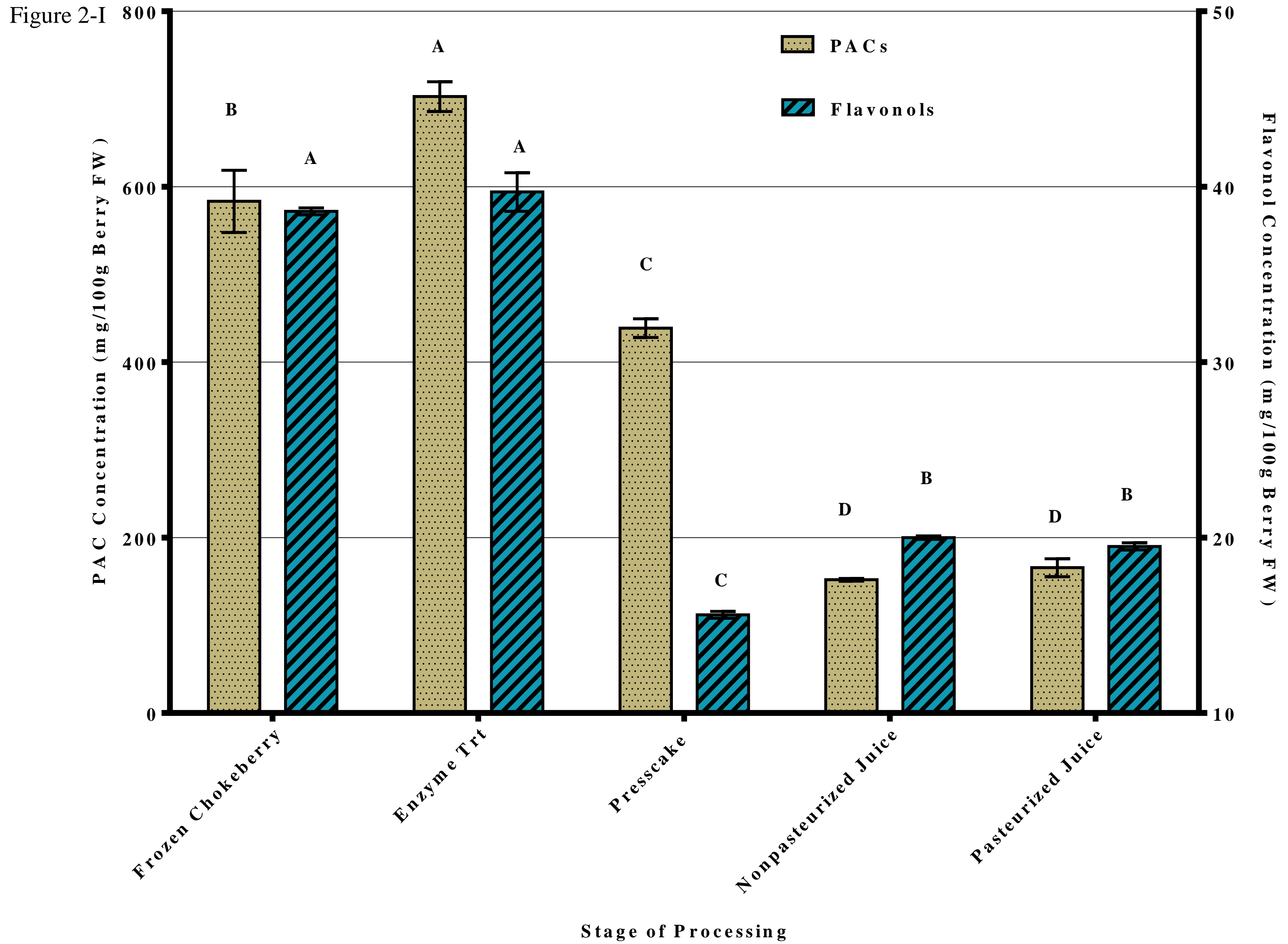

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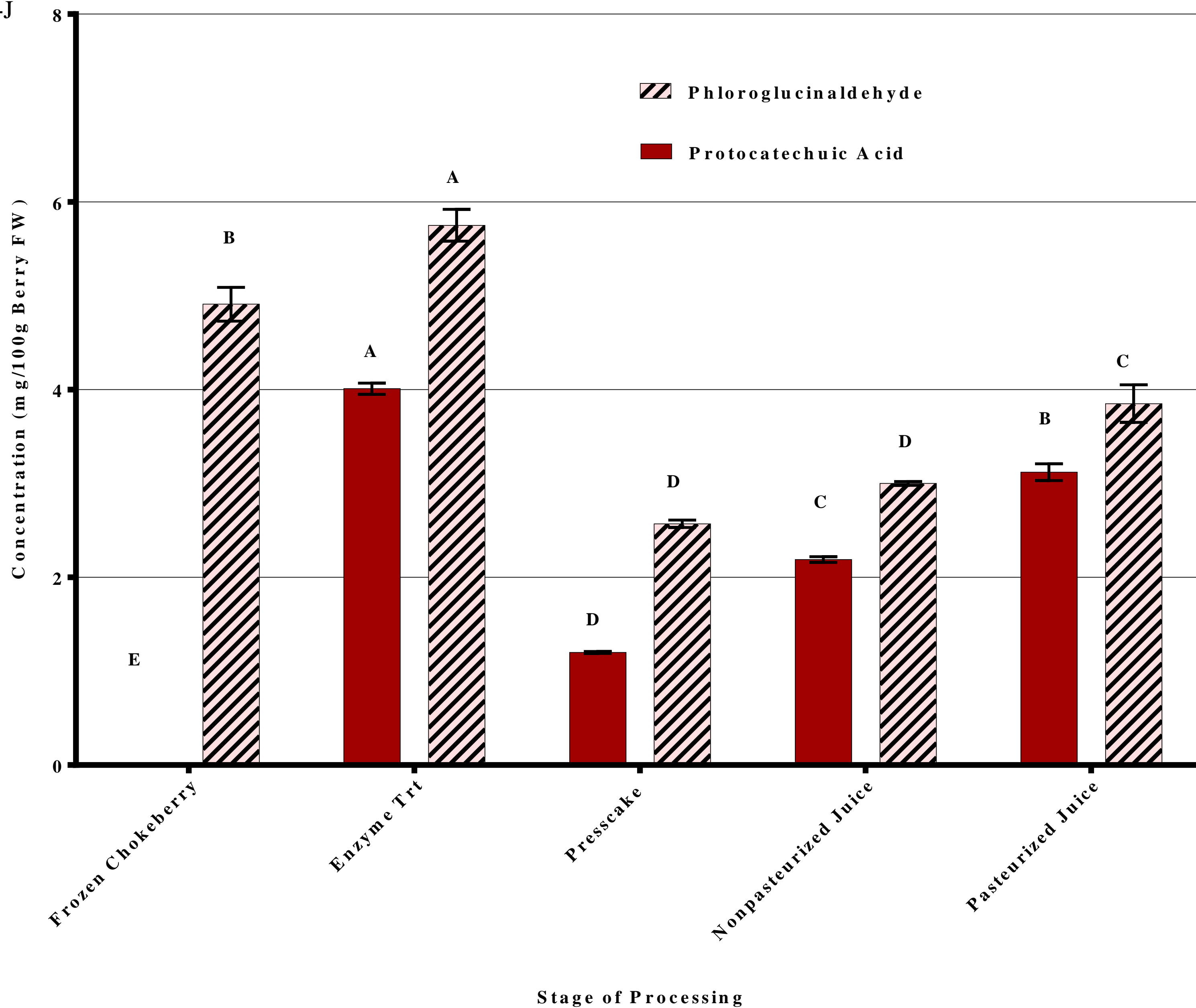

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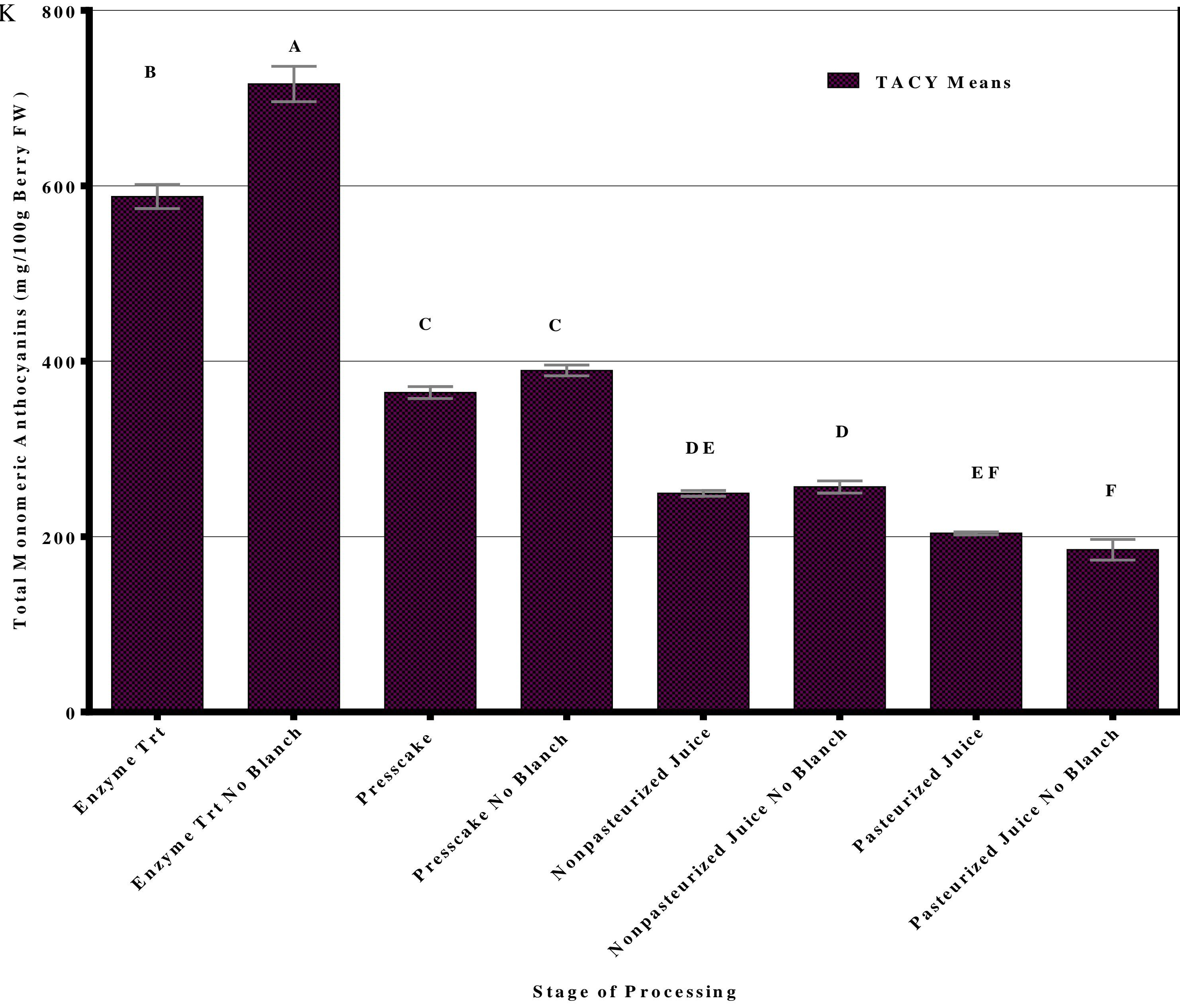

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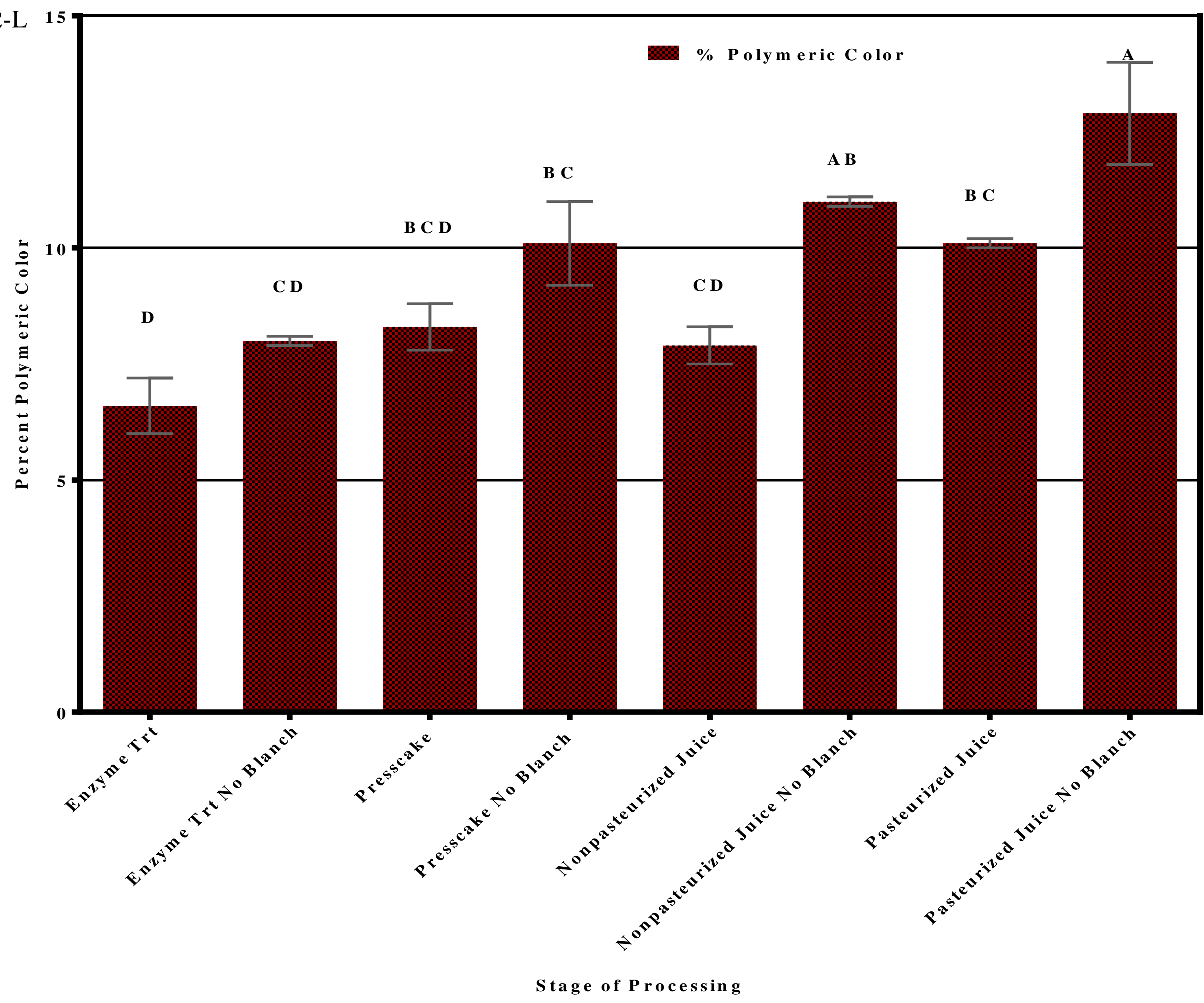

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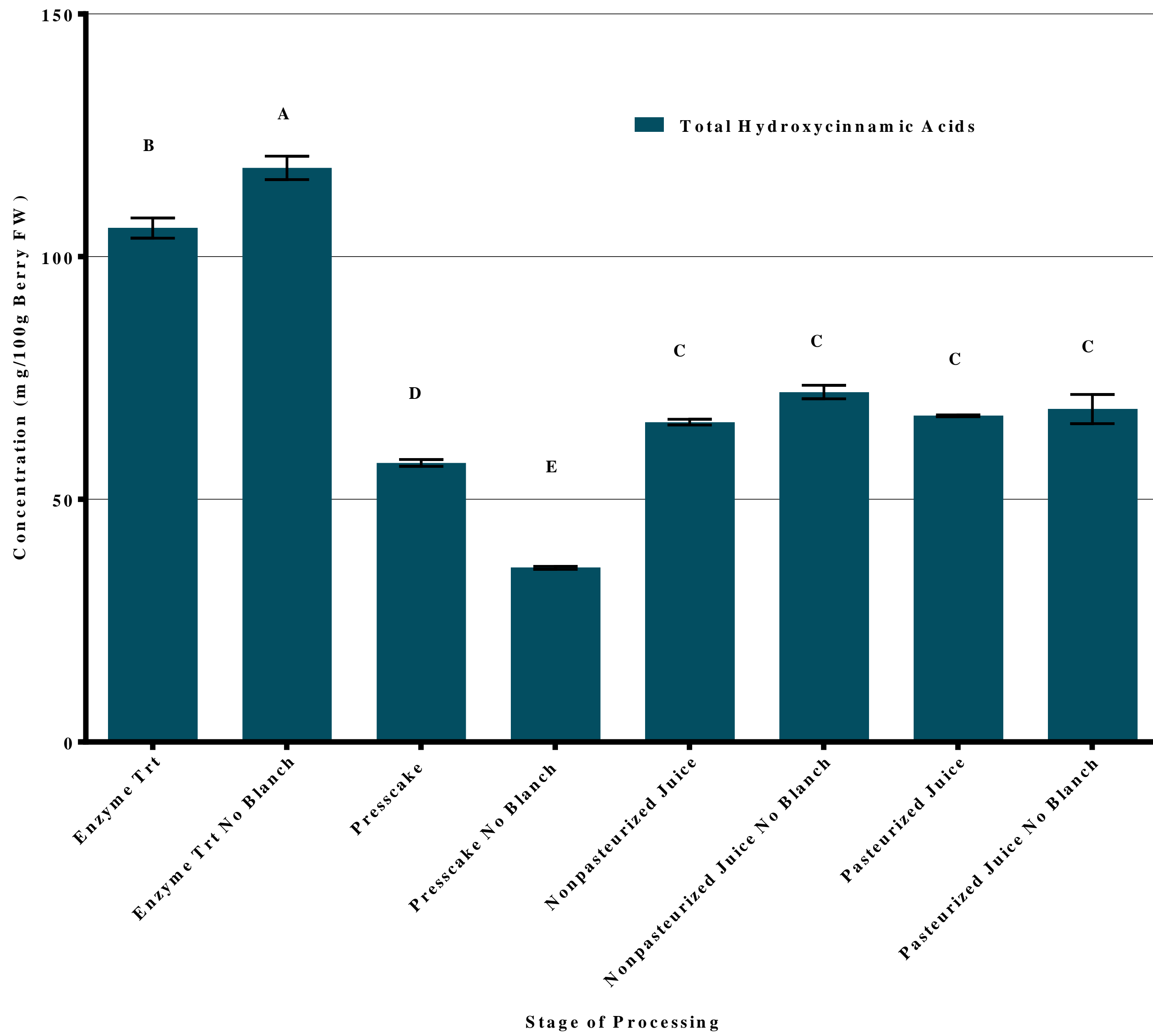

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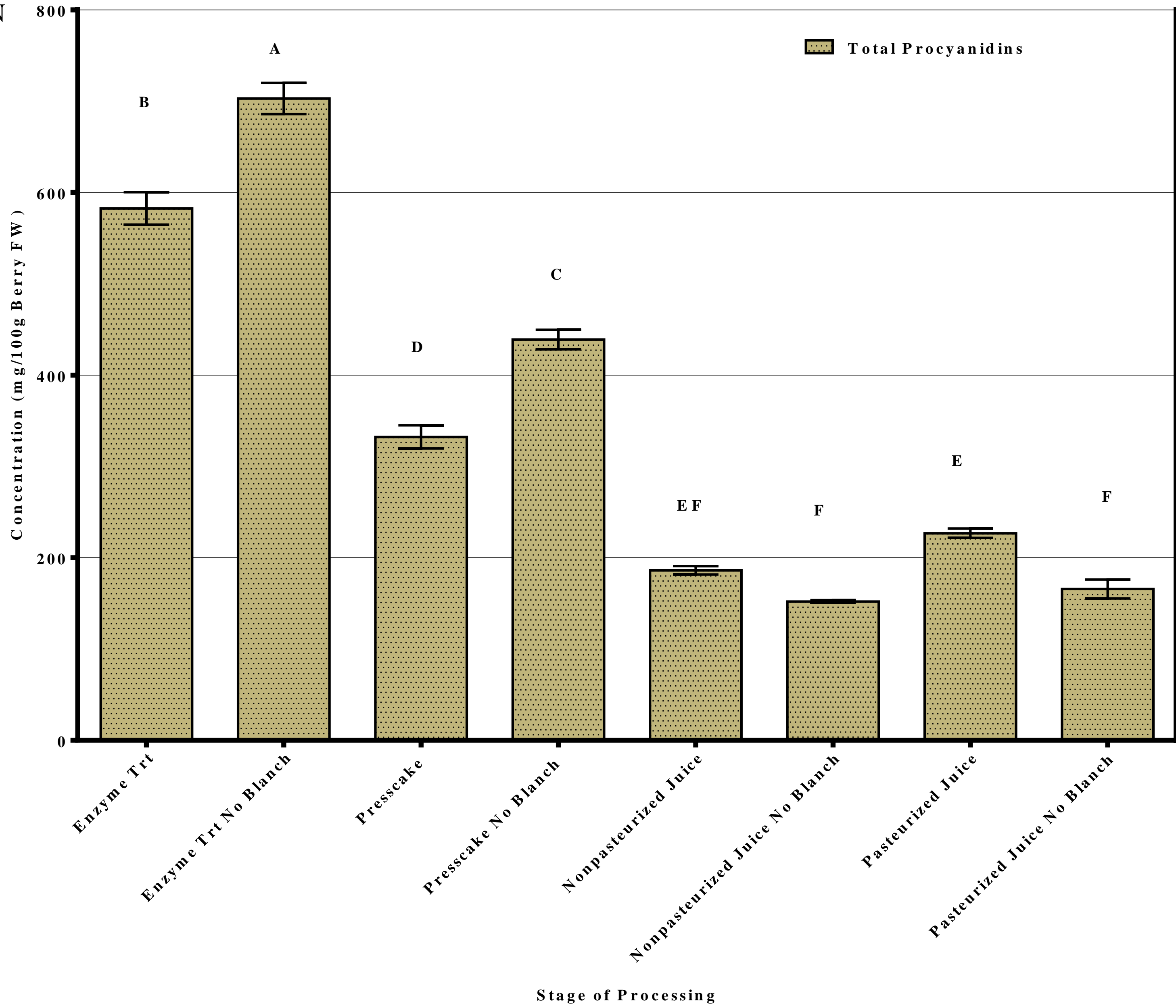

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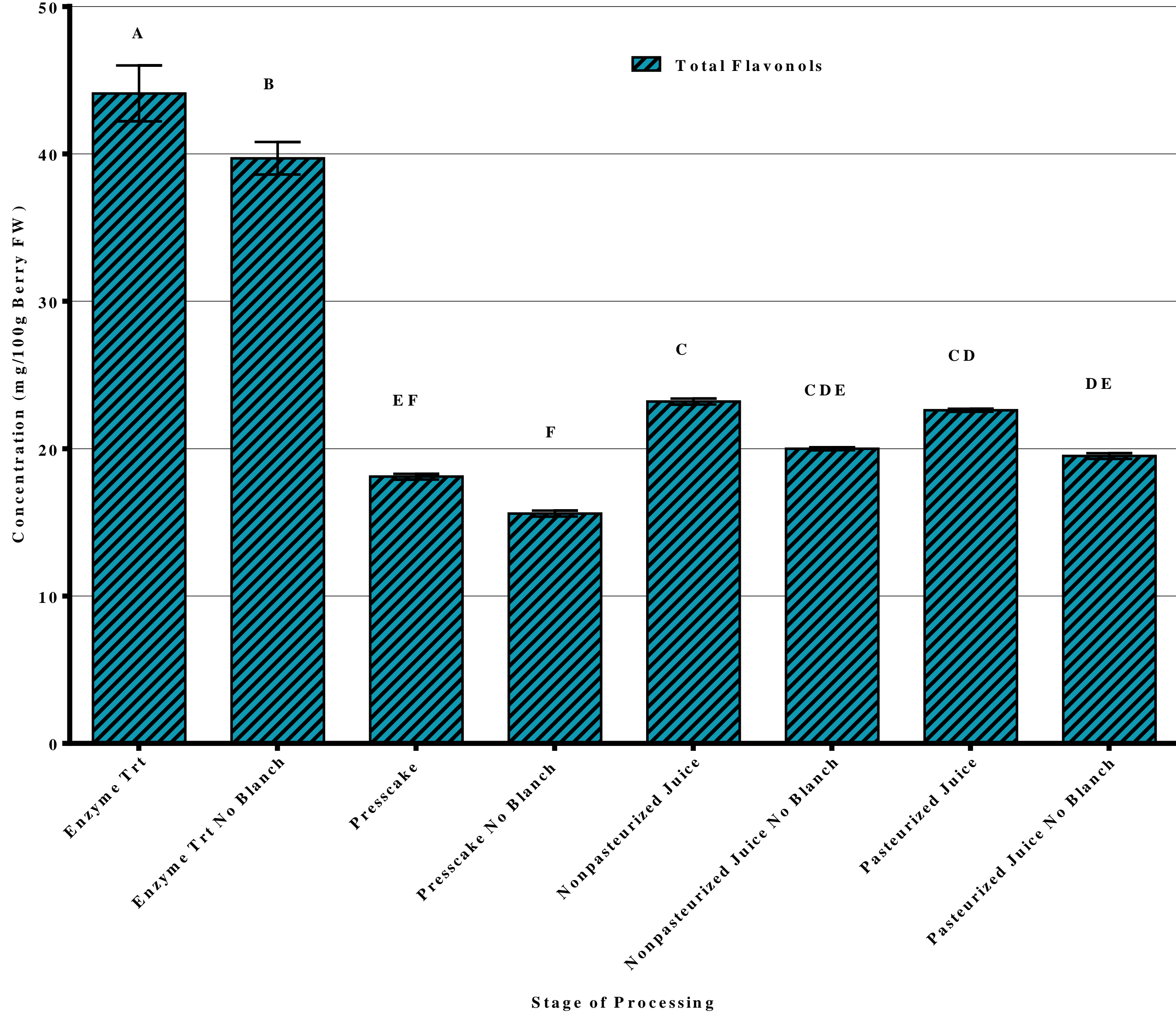

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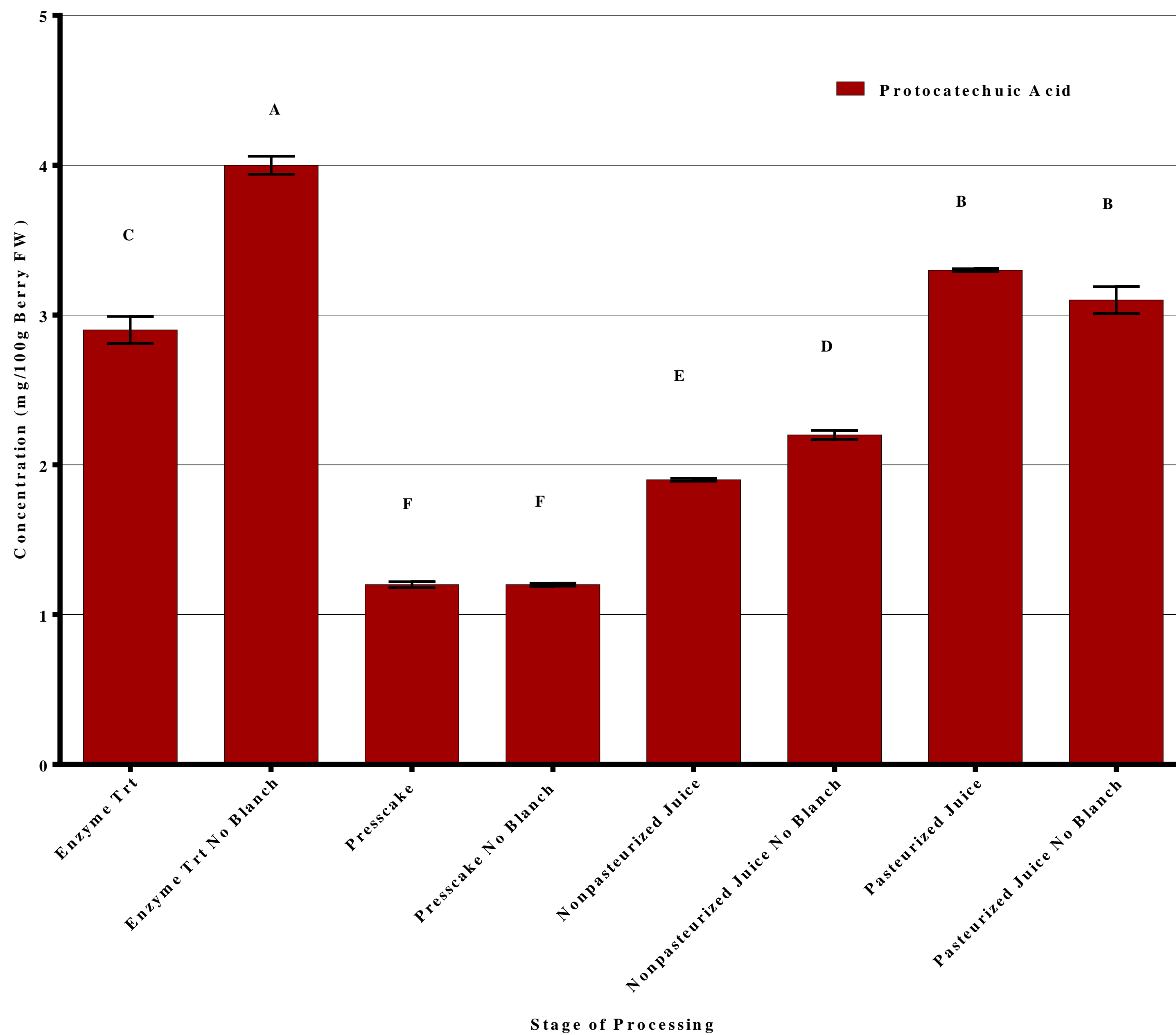

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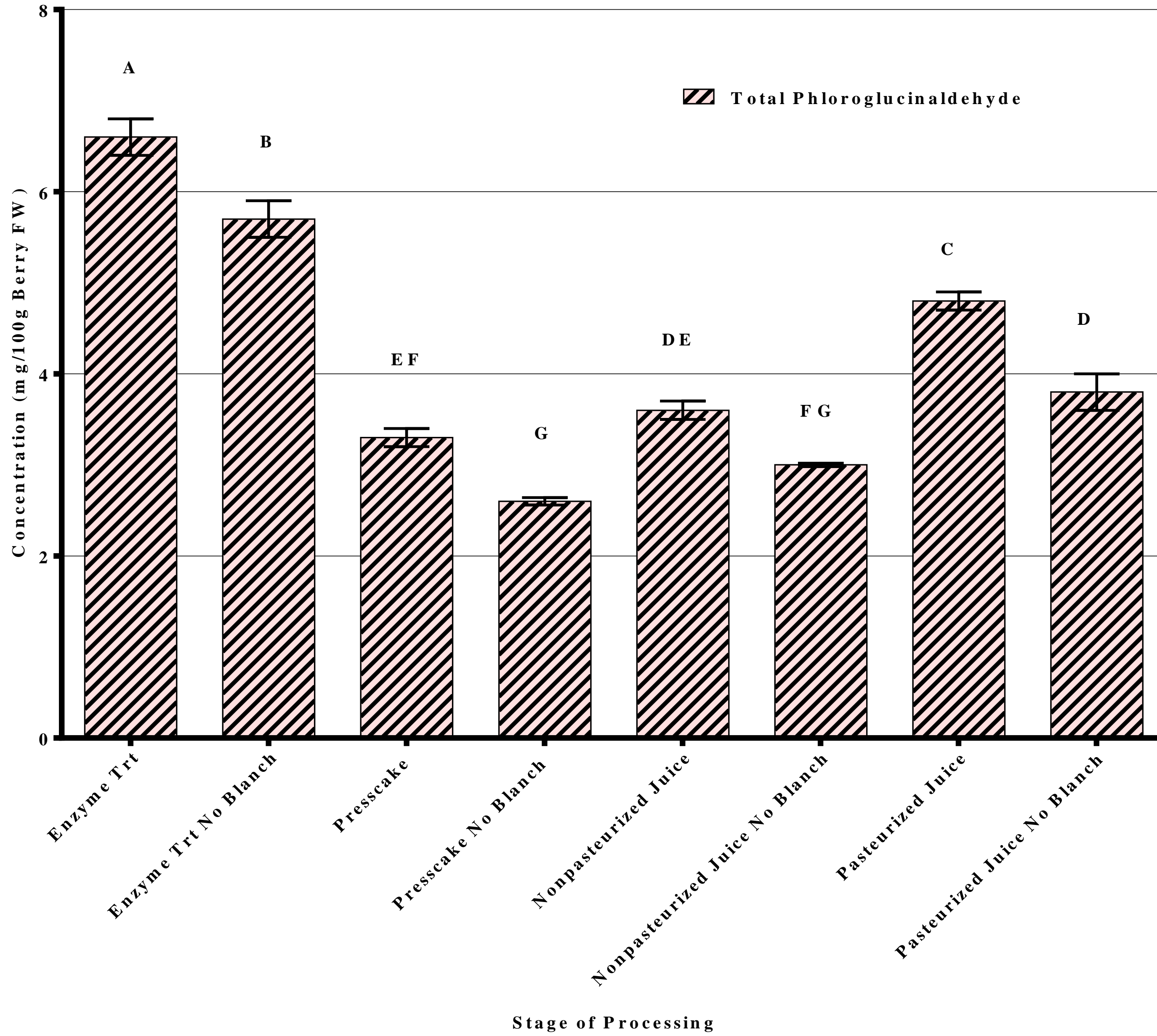

V. IDENTIFICATION AND SEPARATION OF POLYMERIC PIGMENTS AND PROANTHOCYANIDINS USING MATRIX ASSISTED LASER DESORPTION IONIZATION TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF-MS), THIN-LAYER CHROMATOGRAPHY (TLC), AND TLC-MALDI.

A. ABSTRACT

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) was used to identify proanthocyanidins (PACs) and polymeric pigments (PPs) throughout each stage of chokeberry juice processing and over six months of storage at 25°C. Extracts and juice samples were first purified by Sephadex LH-20 SPE followed by concentrating the recovered acetone fraction. Polymeric pigments comprised of cyanidin-3-galactoside + one flavan-3-ol unit up to 14 flavan-3-ols units attached (DP1-DP12) and cyanidin 3-arabinoside + one flavan-3-ol unit up to nine flavan-3-ols units attached (DP1-DP9) were identified by MALDI-TOF-MS. Additionally, PACs ranging from DP3 to DP15 were identified. Polymeric pigments were present in frozen berries used for processing, in samples obtained from each stage of processing (blanched berries, enzyme treated mash, presscake, non-pasteurized juice and pasteurized juice) and in all juices samples evaluated over six months of storage. A major shift in polymeric pigment composition occurred in response to juice pasteurization, with peak areas of cyd 3-galactoside linked to four, five and six flavan-3-ol units increasing, and peak areas of cyd 3-glactoside linked to one, two and three flavan-3-ol units decreasing. In an attempt to separate PPs by degree of polymerization, TLC plates (normal-phase and reverse-phase) with various mobile phase solvent systems were tested. The plates were then subjected to direct MALDI for mass spectral analysis to verify compound separations. Monomeric and polymeric fractions were easily separated using TLC plates, but separation of PPs by degree of polymerization remained difficult. The TLC plates showed fading in color within monomeric

fractions (from bright red to faint pink) throughout juice processing and storage, while polymeric fractions intensified in color (brick red). RP-C_{18} with methanol-water-trifluroacetic acid (TFA) (55/45/1/ v/v/v) as the mobile phase, produced the clearest separation of the two fractions as well as the clearest spectra obtained by TLC-MALDI and Brukerflex Imaging software.

B. INTRODUCTION

Winemaking operations contribute to anthocyanin degradation in grapes, starting with maceration. Once fermented, levels of ACYs decrease further due to chemical reactions that occur within the wine matrix. Generally, ACYs readily degrade by oxidation, bleaching with sulfites, polymerization with flavan-3-ols, complexing with metals, and co-pigmentation. Throughout the aging process, red wine's color changes from bright red to reddish-brown due to the formation of more stable PPs (*1*). This reaction can occur as a result of direct condensation between ACYs and flavanols, interaction with acetaldehyde, as well as co-pigmentation.

During chokeberry juice processing, ACYs degrade when heat is applied and further losses occur when juice is stored at ambient temperature. Anthocyanin losses are typically accompanied by increased polymeric color values, indicating a greater ratio of the ACYs remaining are in polymeric form (*2*). Even though monomeric ACYs dramatically degrade during juice processing and storage, the dark color still remains in the finished product. Anthocyanins become more stable when bonded with flavan-3-ols at carbon 4, forming PPs that can range from DP-2 to DP-30+ (*3*). Polymeric pigments and PACs larger than a degree of polymerization (DP) of 10 cannot currently be separated by degree of polymerization using standard HPLC methodology. This makes identifying and quantifying the compounds analysis challenging. Several studies have shown that PACs can be separated by normal phase and HILIC HPLC methods from DP1 up to DP10, but a single polymer peak (DP > 10) elutes at the

end of the chromatogram (*4*). Other studies using reverse phase HPLC methods have reported chromatograms that contain a hump underneath the anthocyanin peaks (indicative of PPs interacting strongly with the silica C_{18} stationary phase), which can alter peak integration and quantification (*4*). One direct mass spectrometry method involving soft ionization that can rapidly profile compound composition is MALDI-TOF-MS. This mass spectral analysis has the ability to identify larger molecular weight compounds (DP > 10) by single charge ionization and evaporation without any degradation of polymers. Data is gathered almost instantaneously with instrument usage which contributes to time-efficient analysis. MALDI-TOF-MS has been used to identify PPs and PACs in cranberry juice (*1, 3*).

Column liquid chromatography is the most widely used method to separate compounds in analytical chemistry (*5*). Consistent use is due to reproducible separation, chromatographic resolution, and versatility. However, thin layer chromatography (TLC) is still widely used in compound separation because it is convenient, simple to conduct, minimizes solvent usage, and multiple samples can be analyzed using one plate (*6*). The benefit of using berry extract and juice samples with TLC analysis is that separation of monomeric and polymeric pigment fractions is visible to the human eye. TLC separation techniques can be combined with MALDI-TOF mass spectrometry to clearly identify separated compounds and fractions. In order for this process to work, a solution containing a matrix (in this case 2,5-dihydroxybenzoic acid (DHB) dissolved in methanol) must be dispersed directly onto the TLC plate in order for the analyte of interest to be plumed and analyzed by the mass detector.

C. MATERIALS AND METHODS

Extraction of Polymeric Pigments and Proanthocyanidins. Samples collected from the first study (Chapter 1) stored at -70°C were used for this study. Five sample replications were

taken from each stage of chokeberry juice processing at each month of juice storage (12 sample sets total). Polymeric pigments and PACs were extracted from frozen berries, blanched berries, enzyme treated mash, and presscake using Acetone-Water-Acetic acid (AWA) solvent (70:29.5:0.5 v/v/v). About 15 g of frozen berries, blanched berries, and enzyme treated mash samples (5 g of presscake sample was used due to higher concentration of PPs and PACs) were homogenized with 20 mL of AWA solvent using a Euro Turrax T18 tissuemizer (Tekmar-Dohrman Corp., Mason, OH, USA) and then centrifuged to separate solid residue and liquid pigment fraction. The liquid fraction containing PPs and PACs was filtered through MiraCloth (Calbiochem, La Jolla, CA, USA) and the filtrate collected in a 250 ml volumetric flask. The residue was collected, and the extraction was repeated two more times, followed by adjusting all sample volumes to 250 mL with AWA solvent.

Sephadex LH-20 Solid Phase Extraction. Methods were adapted from Brownmiller et al. (*7*). Triplicate samples of each stage of processing and each month of stored juice were loaded on three separate columns containing 3 g of Sephadex LH-20 that was hydrated overnight with DI water. AWA sample extracts (23 mL) were dried down using a SpeedVac concentrator (ThermoSavant, Holbrook, NY, USA) and then re-suspended in 2 mL of AWA solvent prior to being applied to the LH-20 column. Juice samples (2 mL) were added directly to the columns without first being concentrated (determined based on extract dilution factor and mass balance calculations). Hydrated LH-20 was poured into the columns, and the excess water was allowed to drain out, but stopped when the meniscus barely touched the top of the packing material (this step is crucial in allowing the sample to be completely dispersed and bound throughout the lipophilic Sephadex LH20). Extracts and juices were pipetted into the columns and the excess water was removed under vacuum. Using 150 mL glass beakers to collect waste and eluted

pigments, columns were first washed with 40 mL of 30% methanol to remove residual sugars, phenolic acids, and other unnecessary compounds (no pigment was eluted). Next, columns were washed with 70 mL of 70% acetone, which eluted PPs and PACs. This yielded roughly 210 mL of acetone fraction (70 mL from 3 columns), which was completely dried using a SpeedVac concentrator and re-suspended in 2 mL of AWA. Samples were stored in a -70°C freezer prior to analysis.

Sample Preparation and MALDI spotting method for direct MALDI-TOF-MS studies. Methods were adapted from Hager et al. (*8*), White et al. (*9*), and Howard et al. (*2*). 1M DHB in methanol was used as the MALDI matrix solution in the direct MALDI studies. 1M DHB solution was prepared by dissolving 150 mg of 2,5-dihydroxybenzoic acid (DHB) in 1 mL of methanol. Concentrated extracts of frozen chokeberry, samples obtained throughout processing and stored juice samples, were diluted with the DHB matrix at a ratio of 1:10 (1 part sample and 10 parts matrix). The DHB matrix surrounds the PPs and PACs which improves the ability for a sample to be plumed, protonated and volatilized, once the nitrogen laser strikes a spotted sample. Molecular mass was analyzed based on mass to charge ratio (m/z) where the charge is M^{+1} most frequently. All the ions generated in the MALDI source have 25 kV potential, which eventually is translated or converted completely into kinetic energy in the MALDI-TOF instrument. Since all the ions travel the same distance to the MALDI-TOF detector, the time of flight will be different depending on the m/z ratio. In other words, masses were determined by the duration of time it took for the compound to reach the mass analyzer. The DHB matrix does not interfere with the mass detection due to its low molecular weight. Each sample/matrix solution (2 µL) was pipetted onto the MALDI target and allowed to evaporate. Sample volume of 0.5 µL at a time was pipetted on the ground stainless steel plate. A gentle stream of nitrogen gas was used to

facilitate rapid sample evaporation and prevent smearing on the target. Five replicates of each sample were spotted on the target (60 spots total) as well as 8 spots of a peptide standard (1:10 sample/matrix) for instrument calibration. Analysis was conducted using a Bruker Reflex III MALDI-TOF-MS (Billerica, MA) equipped with a 337 nm N_2 laser. BrukerDaltonics peptide standard consisting of angiotensin II, angiotensin I, substance P, bombesin, ACTH clip, and somatostatin was used for time of flight calibration. Spectra displayed polymeric pigment and proanthocyanidin masses up to 3500 daltons and their signal intensities were monitored using BrukerDaltonicsflexAnalysis software. Data was obtained in positive ion reflectron mode with an accelerating voltage of 25 kV and a reflectron voltage of 28 kV. The PPs and PACs were identified as the molecular ion and potassium and sodium adducts ($[M^+]$, $[M\text{-}H\text{+}K]^+$ and $[M\text{-}H\text{+}Na]^+$, respectively). Peaks were statistically evaluated using BrukerClinProTools software. Exact masses of PPs and PACs were obtained by drawing compound chemical structures using ChemDraw software. Peaks observed from MALDI-TOF-MS analysis were processed using BrukerflexAnalysis software and observed masses were compared to predicted masses. Whenever observed masses did not match up exactly to predicted masses, the difference in flavan-3-ol units (288 M^+) among each polymer series was calculated to verify identification. Statistical analysis was conducted using ClinProTools software. Each sample was analyzed in 20 replicates producing a total of 5 MALDI-TOF mass spectra per replicated sample.

TLC-MALDI. Reverse-phase (RP-18 F254s) and normal-phase (Silica gel 60 F254), 5 x 7.5 cm TLC plates with aluminum backing (EMD Chemicals Inc., Gibbstown, NJ; #5560-4, #5549-4) were used in this study. Samples evaluated included; frozen chokeberry, processing extracts, juices, and stored juices that were purified by Sephadex LH-20 SPE. 4 samples (4 lanes) at 2 μL each were spotted on each plate, by pipetting 0.5 μL of sample at a time, 10 mm from the base of

the plate. Pipetting 0.5 µL of sample 4 times (2 µLtotal) prevented the sample from spreading too far on the stationary phase and kept the lane of the pigment as narrow as possible (no overlap with other samples). Monomeric pigments can be easily separated by Thin-Layer Chromatography (TLC) on either C_{18} or silica plates, but little research has been conducted to effectively separate PPs using TLC. Different TLC mobile phase solvent systems were tested in an attempt to separate PPs by degree of polymerization (DP). Solvent systems for both TLC plates were adapted from Lambri et al. (*1*) and Oberholster (*10*) are displayed in Table 3.1. Each solvent used was poured into the TLC chamber just enough to cover the bottom (15-20 mL), but below the spot positions where the plates were placed. TLC chamber was equilibrated for 30 min with the lid closed before each TLC run. TLC plates were carefully placed into the chamber containing solvent and allowed to sit until the mobile phase traveled to the very top of the plate. Each application took 20-25 minutes to complete. The plates were then removed, dried at ambient temperature, and then subjected to a water/methanol/DHB matrix spray prior to MALDI analysis. TLC-MALDI runs were performed using Bruker TLC MALDI software and BrukerflexImaging software.

MALDI Matrix Spray Application. The MALDI matrix was sprayed directly onto the TLC plate with an airbrush using high purity nitrogen instead of air as the nebulizing gas. DHB matrix solution consisted of 10 mL of methanol, 5 mL of DI water, and 1 g of DHB. This solution was vortexed inside the glass bottle attachment for dissolution. TLC plates were pinned upright on a cardboard poster under a vent hood, and DHB matrix was applied to the TLC plate using the airbrush. Holding the airbrush 1 foot away from the TLC plate for even distribution, the entire volume of matrix solution was applied to the stationary phase surface containing sample lanes over 5 to 10 min, ensuring no smearing of color was apparent. The purpose of

spraying the DHB matrix onto the TLC plate was to allow the matrix to solubilize the compounds on the TLC plate and transfer them to the surface of the plate so that the nitrogen or the YAG laser used for MALDI could desorb and volatilize those compounds at any given position on the plate allowing detection by the time of flight mass spectrometer. TLC plates were secured onto a TLC MALDI target holder and inserted into a BrukerUltraflex II MALDI-TOF-MS (Billerica, MA) equipped with a 355 nm Nd:YAG laser. Data were processed and analyzed using TLC-MALDI and flexImaging (BrukerDaltonics) software, with spectra from the data output displayed as *m/z* on the x-axis and a relative length of the TLC plate (unitless) on the y-axis. Multiple samples (lanes) can be observed in the same graph output.

D. RESULTS AND DISCUSSION

Direct MALDI-TOF-MS studies. From the LH-20 SPE purification step, both methanol and acetone fractions were collected and analyzed using direct MALDI-TOF-MS without any further chromatographic separation. The methanol fraction did not contain PACs or PPs, so it was discarded. Several ratios of sample to DHB ratios were tested (1:1, 1:5, 1:7, 1:10, and 1:12) to obtain the best analyte/matrix ratio. A sample to DHB ratio of 1:7 produced the best MALDI-TOF mass spectra. The 1:1 and 1:5 sample/matrix ratios did not allow for larger molecular weight polymers (DP>10) to be detected. Sample/matrix ratios of 1:10 and 1:12 produced noisy baselines making peak integration difficult and inaccurate.

Before discussing results, it should be stressed that MADLI-TOF-MS cannot be used for absolute quantitation even if oligomer standards are available (*11*). Numerous PPs and PACs were identified (Table 3.2). Cyd 3-gal/glu with 1 to 12 flavan-3-ol units attached were identified as molecular ion (M^+) and/or sodium ($M\text{-}H\text{+}Na^+$) and potassium ($M\text{-}H\text{+}K^+$) adducts (Figure 3-1). In addition, cyd 3-xyl/ara with one to nine flavan-3-ol units attached was identified.

Proanthocyanidins that were identified ranged in DP from three to 16 flavan-3-ol-units, also in the structure of molecular ion (M+) and/or sodium ($M\text{-}H\text{+}Na^{+}$) and potassium ($M\text{-}H\text{+}K^{+}$) adducts (Figure 3-1). Even though larger molecular weight compounds were identified, the spectra began to lose resolution with compounds having an *m/z* ratio greater than 2500 daltons. Using MALDI-TOF-MS analysis, White (*9*) reported PACs containing A-type linkages as sodium adducts ranging from DP4 to DP13 in cranberries. In chokeberries, PACs are composed of flavan-3-ols units connected by B-type linkages. Reed et al. (*3*) reported higher molecular weight PACs in cranberries up to DP23, using MALDI-TOF-MS analysis in linear mode. Most of the PACs and PPs identifications in this study were confirmed by exact mass measurement studies using MALDI Fourier Transform Mass Spectrometry (MALDI-FTMS) performed on a Bruker Apex Ultra 9.4 T instrument.

Processing and Storage Effects on Chokeberry Polymeric Pigments. Marked loss of ACYs during juice processing and throughout six months juice storage was observed in Chapter 1. Despite dramatic loss of monomeric ACYs after six months of juice storage (1.2% retention from frozen berry), chokeberry juice was still very dark in color. Increasing percent polymeric color values during processing and throughout storage suggested that ACYs were stabilized in polymeric form with PACs. Polymeric pigments have yet to be effectively separated by standard HPLC methods, which circumvents there identification and quantification. MALDI-TOF-MS appears to be the only method currently available to identify PPs, although the method cannot be used for quantification.

Five sets of MALDI-TOF mass spectra of each replicated sample (60 samples total) were compared after normalization using Bruker ClinPro Tools software. Average normalized peak intensities for cyd 3-gal/glu PPs are shown in Figure 3-2. From frozen berry to nonpasteurized

juice, signal intensities of PPs from each processing stage, gradually increased. This indicated that there was a greater abundance of cyd 3-gal/glu attached to 1, 2, 3, and 4 flavan-3-ol units. After pasteurization, signal intensities of DP1 to DP3 PPs decreased while signal intensities of DP4 PPs increased. However, the distribution of PPs appeared to remain stable over six months of juice storage. Rather than seeing an overall increase in signal intensity of PPs throughout processing and storage, a redistribution effect was observed. A similar result occurred for PACs (Figure 3-3). After pasteurization, signal intensities of DP5, DP6, DP7 and DP 8 PACs increased, while signal intensities of DP3 PACs decreased. A shift in distribution of PACs also occurred in response to blanching, with blanched samples having higher peak intensities of DP5, DP6, DP7 and DP8 PACs than frozen berries. The distribution of PACs appeared to change little during juice storage, with the exception of DP3 PACs, where peak intensity decreased from two to three months of juice storage. Overall, the two thermal treatments, blanching and pasteurization appeared to be responsible for the redistribution of PPs and PACs.

Howard et al. (*2*) previously reported that signal intensities of higher DP PPs in chokeberry juice increased after accelerated storage conditions (40°C for 6 weeks), at the expense of lower DP PPs. These results contrast with findings from this study where signal intensities of PPs remained stable throughout six months of juice storage. In the Howard et al. (*2*) study, juice was stored under accelerated conditions (6 weeks at 40°C), whereas juice from this study was stored at ambient temperature. This gives reason to believe that heat application is the main cause or catalyst for polymeric pigment formation.

Reverse-Phase TLC Plates. The first solvent system used on the RP-C_{18} plates was methanol-water-trifluoroacetic acid (TFA) (55/45/1 v/v/v) with all 12 samples spotted on 2 plates (6 samples per plate) starting with frozen chokeberry on the far left and six month juice on the

far right (Figure 3-4). Methanol and water have polarity indexes of 5.1 and 10.2, respectively, making this solvent mixture relatively polar. The polymeric fraction immediately separated from the monomeric ACYs and moved up the plate with the mobile phase. Lambri et al. (*1*) also reported clear separation and band formation of monomeric ACYs as well as distinct polymeric pigment band in a wine sample using the same plate and solvent. Frozen chokeberry sample contained more monomeric ACYs than the six month juice sample. Looking at the plates from left to right, 3 distinct anthocyanin bands were easily observed near the middle of the plate in the frozen chokeberry sample and diminished in color intensity in the other lanes moving to the right. Just above these bands, the color changes from bright red to a brick red streak indicating the polymeric pigment fraction. No distinct separation occurred in this fraction, but the pigments formed a fork-like shape at the very top of the plate. The stationary phase was more nonpolar combined with a polar mobile phase in which the monomeric ACYs being less polar than the polymeric pigments, stopped on the plate before the polymeric pigment fraction.

Other solvent system combinations of methanol-water-TFA were applied to the RP-C_{18} plates. With solvent ratio 60/40/1 v/v/v, some monomeric anthocyanin separation occurred, but was not as well defined as results obtained using solvent ratio 55/45/1. In addition, the polymeric fraction was pushed further up the plate, shortening the length of streaking. Solvent ratios of 65/35/1 v/v/v; 70/30/1 v/v/v; and 75/25/1 v/v/v all resulted in most of the color migrating to the top of the plate without any clear separation of monomeric ACYs. The solvent ratio 60/40/2 v/v/v resulted in monomeric anthocyanin bands towards the center of the plate. The separated polymeric pigment fraction migrated to the top of the plate creating an arrow-like streak instead of a fork-like streak. The small increase in acid is most likely responsible for the arrow-like streak, causing the PPs to have a higher affinity for the C_{18} stationary phase. The

60/40/3 v/v/v solvent mixture resulted in tighter monomeric anthocyanin bands, but were too close together to visually see distinct bands formed. The polymeric fraction did not exhibit either a fork-like or arrow-like shape, only a colored streak at the top of the plate. A different solvent system was used on the RP-C_{18} TLC plate which consisted of acetonitrile-water-TFA (55/45/1 v/v/v). This solvent mixture resulted in poor separation of both monomeric ACYs and PPs, with most of the pigments moving up the plate together. Acetonitrile has a polarity index of 5.8, which is slightly higher than methanol. This solvent may have been too polar which contributed to more pigment movement along the TLC plate and no separation.

Normal-Phase TLC Plates. A complete list of solvent systems used for normal-phase (silica gel) plates are presented in Table 3.1. Two different toluene-acetone-formic (30/30/6 and 30/30/10 v/v/v) solvent systems were used in an attempt to separate monomeric ACYs from the PPs and to separate PPs by DP within samples of frozen chokeberry, and juices stored for 1, 2, 3, and 4 months. Toluene and acetone have polarity indexes of 2.4 and 5.1, respectively, which is more non-polar compared to methanol and water (5.1 and 10.2). Using these solvent mixtures, pigments only moved halfway up the plate from the origin, creating a more solid streak of color with minor monomeric anthocyanin band formation. 1-propanol-acetic acid-water (6/2/1 v/v/v) solvent mixture resulted in some separation, but was not as well defined as the reverse-phase plates with methanol-water-TFA (55/45/1 v/v/v). 1-propanol has a polarity index of 3.9 and when combined with water, the solvent is more polar than toluene-acetone-formic. Two weak bands began to form near the middle of the silica plate while darker red color remained at the origin. Oberholster (10) reported up to 10 different bands of a wine sample using propanol-acetic acid-water (6/2/1 v/v/v) solvent system on reverse phase silica gel TLC plates. According to the study's figure, color streaking is evident without any clear band separation occurring.

Butanol-acetone-water (3/1/1 v/v/v) solvent system was used on a silica gel plate containing nonpasteurized juice, pasteurized juice, and juice samples stored 2, 4, and 6 months. The result from this mobile phase system was not promising as pigments smeared up the plate without any clear separation or band formation. Butanol, acetone, and water have polarity indexes of 4.0, 5.1, and 10.2, making this solvent relatively polar for use on a normal-phase TLC plate. Solvents used for the normal-phase plates took between 45 and 60 minutes to cover the entire plate. Oberholster (*10*) used butanol-acetic acid-water (6/1/3 v/v/v) for wine samples on a normal phase TLC plate but only eluted 3 anthocyanins while most of the polymeric color remained at the origin.

The toluene-acetic acid-water (6/1/2 v/v/v) solvent mixture applied to all 12 samples resulted in weak, but noticeable bands not far from the origin of the silica plate with darker pigment retained at the origin, indicating strong affinity of the PPs to the silica gel (Figure 3-5). Normal-phase TLC plates are more polar and by using a more nonpolar mobile phase, the PPs do not associate with the mobile phase and therefore remain at the origin. After noticing the weak bands and some color movement following application of toluene-acetic acid-water (6/1/2 v/v/v), the mobile phase was applied four times to the same plate to increase pigment movement, allowing the plate to dry between each application. Multiple mobile phase applications resulted in further pigment movement as well as the formation of two distinct bands more than halfway up the TLC plate, while the PPs remained at the origin (Figure 3-6).

The same mobile phase multiple application theory was applied to two different silica gel plates, both containing samples of frozen chokeberry, and juices stored 1, 2, 3, and 4 months. The first plate received four mobile phase applications of toluene-acetone-formic acid (30/30/10 v/v/v) solvent mixture while the second received eight applications. After four mobile phase

applications, the first plate had distinct monomeric anthocyanin bands in the middle of the plate with slight movement of the polymeric pigment fraction, but most remained at the origin. The second plate resulted in further movement of the monomeric anthocyanin bands nearly ¾ the distance of the plate. Some of the polymeric pigment fraction moved towards the middle of the plate below the monomeric anthocyanin bands. The two plates were scanned under Epi-white conditioning using a UV filter 590 nm, grayscale, with an emission of 10 seconds (Figures 3-7 and 3-8). Oberholster (*10*) tested different solvent systems of toluene-acetone-formic acid (30/60/10 and 30/30/10 v/v/v) but observed color streaking with relatively little color separation.

TLC-MALDI-TOF-MS. TLC-MALDI data obtained were analyzed using Bruker TLC-MALDI software and were graphically presented using a standard gel view heat map. In the data output displayed, the x-axis represents *m/z ratio*, and the y-axis represents distance or height of the TLC plate. Figure 3-9 illustrates the frozen chokeberry sample from the RP-C_{18} plate with methanol-water-TFA (45/55/1 v/v/v) mobile phase, displaying the signal intensity of pigments in relation to the location on the TLC plate, supplemented with mass spectra. Figure 3-10 also illustrates the frozen chokeberry sample compared with 1, 2, and 3 month stored juice highlighting the polymeric pigment (PP) regions. The graph color display can be altered by the right axis color scale accordingly. The color brightness of the blue bars indicated compound signal intensities in relation to the MALDI spectra, which can be pulled from the flexImaging software by clicking on one of the bars (Figure 3-11). The locations of the bars indicate how far the compound(s) moved up the plate (y-axis), as well as the *m/z* of the compound(s) (x-axis). From the frozen berry sample, it is expected to see lower molecular weight compounds with higher signal intensities. However, large molecular weight compounds such as PPs and PACs were also observed. Polymeric pigments were clearly observed in the frozen chokeberry as well

as the 1, 2, and 3 months stored juice, as indicated in Figure 3-10. Comparing Figures 3-10 and 3-4, the notated polymeric pigment regions in Figure 3-10 appear to be directly related to the distance traveled of two distinct light red and brick red color regions located at the top half of the TLC plate in Figure 3-4. Comparing frozen chokeberry sample to 3 month stored juice sample on the RP-C_{18} TLC plate (Figure 3-4), the red region just above the monomeric pigment band (about ¾ up the TLC plate) was more prominent in red color in the frozen chokeberry sample than the softly faded red region in the 3 month juice sample. The opposite occurred with the brick red region just above, occupying the top quarter of the TLC plate surface (Figure 3-4). The frozen chokeberry sample showed relatively weak brick red color intensity whereas the same region from the 3 month juice was more pronounced with brick red color (Figure 3-4). These changes in color intensity of both light red and brick red regions of frozen chokeberry and 3 month stored juice on the TLC plates (Figure 3-4) behaved very similarly to the changes of polymeric pigment regions in Figure 3-10. The PP region in the frozen chokeberry sample (Figure 3-10) appeared to diminish in signal intensity when compared to the 3 month juice sample PP region. The upper PP region of the 3 month juice sample appeared to increase in signal intensity when compared to frozen chokeberry. This observed relationship connecting the changes in color intensity on TLC plates to the changes in signal intensities from TLC-MALDI, may suggest further evidence that during chokeberry juice processing and throughout juice storage, an increase in PP may be less likely to occur, but rather a PP redistribution effect.

The normal-phase TLC plates from Figures 3-7 and 3-8 were sprayed with the DHB matrix prior to TLC-MALDI analysis. From the output, the spectra remained too noisy until the top half of the plates were hit with the nitrogen laser. This resulted in having data for only small molecular weight pigments (data not shown). The polymeric pigment fraction had such a strong

affinity for the silica gel that when the DHB matrix was applied to the TLC plate the compounds were most likely not solubilized sufficiently to bring them to the surface of the plate for subsequent MALDI ionization. It is very important for the material be soluble and co-crystalize with the matrix for the MALDI process to work. Therefore normal phase TLC might not be amenable for TLC-MALDI for compounds which are relatively ionic to begin with. In an attempt to analyze the samples by MALDI, lanes from each plate were cut and separated, as well as each band and fraction. Cut strips were placed in 1 mL of AWA solvent within 3 mL vials and vortexed to remove silica gel from the aluminum plate and to remove sample from the silica gel. Sample solutions were immediately subjected to direct MALDI to observe if more separation occurred. This extra process did not succeed due to noisy spectra produced, but we believe this process has a greater potential to succeed than TLC-MALDI and warrants further testing.

E. CONCLUSION

MALDI-TOF-MS analysis demonstrated cyd 3-galactoside PPs ranging from DP1to DP13 were present in frozen berries used for processing, in all samples obtained throughout processing, and in all juice samples stored over six months at 25° C. The presence of PPs in frozen berries was unexpected as many researchers have speculated PPs are formed in berry juices or wines in response to processing or most likely during long-term storage of the processed products at ambient temperature. MALDI-TOF-MS analysis also showed a redistribution of PPs occurred in response to the thermal pasteurization step, with signal intensities of cyd 3-galactoside PPs with DP-1-3 decreasing and those of DP4-6 increasing. The distribution and signal intensities of PPs did not change throughout juice storage, indicating heat application is the main cause of polymeric pigment formation. Thermal treatments during juice

processing also altered the distribution of PACs. Following pasteurization, signal intensities of DP5, DP6, DP7 and DP 8 PACs increased, while signal intensities of DP3 PACs decreased. A shift in distribution of PACs also occurred in response to blanching, with blanched samples having higher peak intensities of DP5, DP6, DP7 and DP8 PACs than frozen berries. The distribution of PACs appeared to be stable over six months of juice storage.

Similar to the previous direct MALDI studies, smaller PPs (determined by distance on TLC plate and polarity) appear to decrease (determined by weakening color intensity), while an apparent increase in larger PPs occurred based on an increase in color intensity of the top brick red region of the TLC plate which was the location of the most polar compounds. Attempting to separate polymeric pigments using TLC methods further proved to be difficult, however, the TLC-MALDI technique proved useful in identifying PPs in the polymeric fraction present on the RP-C_{18} plate. More detailed research utilizing additional mobile phase systems and stationary materials are needed to effectively separate PPs by degree of polymerization. TLC-MALDI appears to be a promising technique to quickly isolate and identify PPs without the excessive use of organic solvents.

Table 3.1. TLC Mobile Phase Solvent Systems Evaluated.

Reverse-Phase (C_{18}) TLC plates (v/v/v)	**Normal-Phase (Silica) TLC plates (v/v/v)**
MeOH-H_2O-TFA (55/45/1)	Toluene-Acetone-Formic acid (30/30/6)
MeOH-H_2O-TFA (60/40/1, 2, 3)	Toluene-Acetone-Formic acid (30/30/10)
MeOH-H_2O-TFA (65/35/1)	1-Propanol-H_2O-Acetic acid (6/2/1)
MeOH-H_2O-TFA (70/30/1)	Butanol-Acetone-H_2O (3/1/1)
MeOH-H_2O-TFA (75/25/1)	Toluene-Acetic acid-Water (6/1/2)
Acetonitrile-H_2O-TFA (55/45/1)	

Table 3.2. MALDI-TOF and MALDI-FTMS Peak Assignments.

Compound	Observed m/z TOF (±200 ppm)	Observed m/z FTMS (±200 ppm)	Calculated m/z
Cyd 3-xyl/ara + 1 flavan 3-ol (M+)	706.79	707.161	707.161
Cyd 3-xyl/ara + 1 flavan 3-ol (Na)	729.74	729.143	729.143
Cyd 3-xyl/ara + 1 flavan 3-ol (K)	744.83	745.251	745.251
Cyd 3-gal/glu + 1 flavan 3-ol (M+)	736.84	737.171	737.171
Cyd 3-gal/glu + 1 flavan 3-ol (Na)	759.22	759.153	759.153
Cyd 3-gal/glu + 1 flavan 3-ol (K)	774.86	775.261	775.261
Proanthocyanidin trimer (M+)	865.98	866.206	866.206
Proanthocyanidin trimer (Na)	888.99	889.196	889.196
Proanthocyanidin trimer (K)	905	905.304	905.304
Cyd 3-xyl/ara + 2 flavan 3-ols (M+)	995.11	995.224	995.224
Cyd 3-xyl/ara + 2 flavan 3-ols (Na)	1017.1	1017.206	1017.206
Cyd 3-xyl/ara + 2 flavan 3-ols (K)	1033.57	1033.314	1033.314
Cyd 3-gal/glu + 2 flavan 3-ols (M+)	1025.15	1025.235	1025.235
Cyd 3-gal/glu + 2 flavan 3-ols (Na)	1047.14	1047.217	1047.217
Cyd 3-gal/glu + 2 flavan 3-ols (K)	1063.15	1063.325	1063.325
Proanthocyanidin tetramer (M+)	1154.17	1154.269	1154.269
Proanthocyanidin tetramer (Na)	1177.24	1177.259	1177.259
Proanthocyanidin tetramer (K)	1193.25	1193.367	1193.367
Cyd 3-xyl/ara + 3 flavan 3-ols (M+)	1283.36	1283.287	1283.287
Cyd 3-xyl/ara + 3 flavan 3-ols (Na)	1305.37	1305.269	1305.269
Cyd 3-xyl/ara + 3 flavan 3-ols (K)	1321.36	1321.377	1321.377
Cyd 3-gal/glu + 3 flavan 3-ols (M+)	1313.39	1313.298	1313.298
Cyd 3-gal/glu + 3 flavan 3-ols (Na)	1335.37	1335.28	1335.28
Cyd 3-gal/glu + 3 flavan 3-ols (K)	1351.38	1351.388	1351.388
Proanthocyanidin pentamer (M+)	1442.46	1442.333	1442.333
Proanthocyanidin pentamer (Na)	1465.44	1465.323	1465.323
Proanthocyanidin pentamer (K)	1481.43	1481.431	1481.431
Cyd 3-xyl/ara + 4 flavan 3-ols (M+)	1571.51	1571.351	1571.351
Cyd 3-xyl/ara + 4 flavan 3-ols (Na)	1593.52	1593.333	1593.333
Cyd 3-xyl/ara + 4 flavan 3-ols (K)	1609.5	1609.441	1609.441
Cyd 3-gal/glu + 4 flavan 3-ols (M+)	1601.55	1601.361	1601.361
Cyd 3-gal/glu + 4 flavan 3-ols (Na)	1623.55	1623.343	1623.343
Cyd 3-gal/glu + 4 flavan 3-ols (K)	1639.54	1639.451	1639.451
Proanthocyanidin hexamer (M+)	1730.52	1730.396	1730.396

Proanthocyanidin hexamer (Na)	1753.55	1753.386	1753.386
Proanthocyanidin hexamer (K)	1769.56	1769.494	1769.494
Cyd 3-xyl/ara + 5 flavan 3-ols (M+)	1859.64	1859.414	1859.414
Cyd 3-xyl/ara + 5 flavan 3-ols (Na)	1881.65	1881.396	1881.396
Cyd 3-xyl/ara + 5 flavan 3-ols (K)	1897.6	1897.504	1897.504
Cyd 3-gal/glu + 5 flavan 3-ols (M+)	1889.7	1889.425	1889.425
Cyd 3-gal/glu + 5 flavan 3-ols (Na)	1911.7	1911.407	1911.407
Cyd 3-gal/glu + 5 flavan 3-ols (K)	1927.66	1927.515	1927.515
Proanthocyanidin heptamer (M+)	2018.59	2018.459	2018.459
Proanthocyanidin heptamer (Na)	2041.62	2041.449	2041.449
Proanthocyanidin heptamer (K)	2057.57	2057.557	2057.557
Cyd 3-xyl/ara + 6 flavan 3-ols (M+)	2147.62	2147.478	2147.478
Cyd 3-xyl/ara + 6 flavan 3-ols (Na)	2169.6	2169.46	2169.46
Cyd 3-xyl/ara + 6 flavan 3-ols (K)	2185.53	2185.568	2185.568
Cyd 3-gal/glu + 6 flavan 3-ols (M+)	2177.58	2177.488	2177.488
Cyd 3-gal/glu + 6 flavan 3-ols (Na)	2199.62	2199.47	2199.47
Cyd 3-gal/glu + 6 flavan 3-ols (K)	2215.65	2215.578	2215.578
Proanthocyanidin octamer (M+)	2306.6	2306.523	2306.523
Proanthocyanidin octamer (Na)	2329.74	2329.513	2329.513
Proanthocyanidin octamer (K)	2345.31	2345.621	2345.621
Cyd 3-xyl/ara + 7 flavan 3-ols (K)	2473.48	2473.632	2473.632
Cyd 3-gal/glu + 7 flavan 3-ols (M+)	2465.57	2465.551	2465.551
Cyd 3-gal/glu + 7 flavan 3-ols (Na)	2487.51	2487.533	2487.533
Cyd 3-gal/glu + 7 flavan 3-ols (K)	2503.56	2503.641	2503.641
Proanthocyanidin nonamer (Na)	2617.66	2617.576	2617.576
Proanthocyanidin nonamer (K)	2633.57	2633.684	2633.684
Cyd 3-gal/glu + 8 flavan 3-ols (M+)	2753.48	2753.614	2753.614
Cyd 3-gal/glu + 8 flavan 3-ols (Na)	2775.55	2775.596	2775.596
Cyd 3-gal/glu + 8 flavan 3-ols (K)	2791.47	2791.704	2791.704
Proanthocyanidin decamer (Na)	2905.44	2905.64	2905.64
Proanthocyanidin decamer (K)	2921.39	2921.748	2921.748
Cyd 3-xyl/ara + 9 flavan 3-ols (Na)	3033.45	3033.652	3033.652
Cyd 3-gal/glu + 9 flavan 3-ols (Na)	3064.3	3063.659	3063.659
Cyd 3-gal/glu + 9 flavan 3-ols (K)	3080.17	3079.767	3079.767
Proanthocyanidin 11-mer (Na)	3194.12	3193.71	3193.71
Proanthocyanidin 11-mer (K)	3209.38	3209.818	3209.818
Cyd 3-gal/glu + 10 flavan 3-ols (M+)	3330.87	3329.74	3329.74
Cyd 3-gal/glu + 10 flavan 3-ols (Na)	3353.28	3351.722	3351.722

Cyd 3-gal/glu + 10 flavan 3-ols (K)	3369.59	3367.83	3367.83
Proanthocyanidin 12-mer (Na)	3482.07	3481.774	3481.774
Proanthocyanidin 12-mer (K)	3496.59	3497.882	3497.882
Cyd 3-gal/glu + 11 flavan 3-ols (Na)	3641.04	3639.786	3639.786
Proanthocyanidin 13-mer (Na)	3769.06	3769.838	3769.838
Proanthocyanidin 13-mer (K)	*	3785.946	3785.946
Cyd 3-gal/glu + 12 flavan 3-ol (Na)	3929.75	3927.849	3927.849
Proanthocyanidin 14-mer (Na)	4057.62	4057.899	4057.899
Proanthocyanidin 14-mer (K)	*	4074.007	4074.007
Cyd 3-gal/glu + 13 flavan 3-ol (Na)	*	4215.912	4215.912
Proanthocyanidin 15-mer (Na)	4346.12	4345.964	4345.964
Cyd 3-gal/glu + 14 flavan 3-ol (Na)	*	4503.975	4503.975
Proanthocyanidin 16-mer (Na)	*	4634.028	4634.028

* compound not observed in analysis.

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G. FIGURE CAPTIONS

Figure 3-1. MALDI spectra from 3 month chokeberry juice, notating the molecular ion (M+), sodium ($M-H+Na^+$), and potassium adducts ($M-H+K^+$) of cyd 3-gal/glu + 4 flavan 3-ols (PP-DP5) and procyanidin pentamer (Pro-DP5).

Figure 3-2. Signal intensities of Cyd 3-gal/glu ($M-H+Na^+$) derived from ClinPro Tools analysis after direct MALDI-TOF-MS of samples obtained at each stage of processing and over six months of storage.

Figure 3-3. Signal intensities of proanthocyanidins ($M-H+Na^+$) derived from ClinPro Tools analysis after direct MALDI-TOF-MS of samples obtained at each stage of processing and over six months of storage.

Figure 3-4. Reverse-phase C_{18} TLC plate with methanol-water-TFA as mobile phase. Samples spotted were frozen chokeberry (left) to juice stored 6 months (right).

Figure 3-5. Normal-phase TLC plate with toluene-acetic acid-water (6/1/2 v/v/v) as the mobile phase and single mobile phase application. Samples spotted were frozen chokeberry (left) to juice stored 6 months (right).

Figure 3-6. Normal-phase TLC plate with toluene-acetic acid-water (6/1/2 v/v/v) as mobile phase with 4 mobile phase applications. Samples spotted were frozen chokeberry (left) to juice stored 6 months (right).

Figure 3-7. Normal-phase TLC plate with toluene-acetone-formic acid (30/30/10 v/v/v) as mobile phase with 4 mobile phase applications. Samples spotted were frozen chokeberry (left), to juice stored 4 months (right).

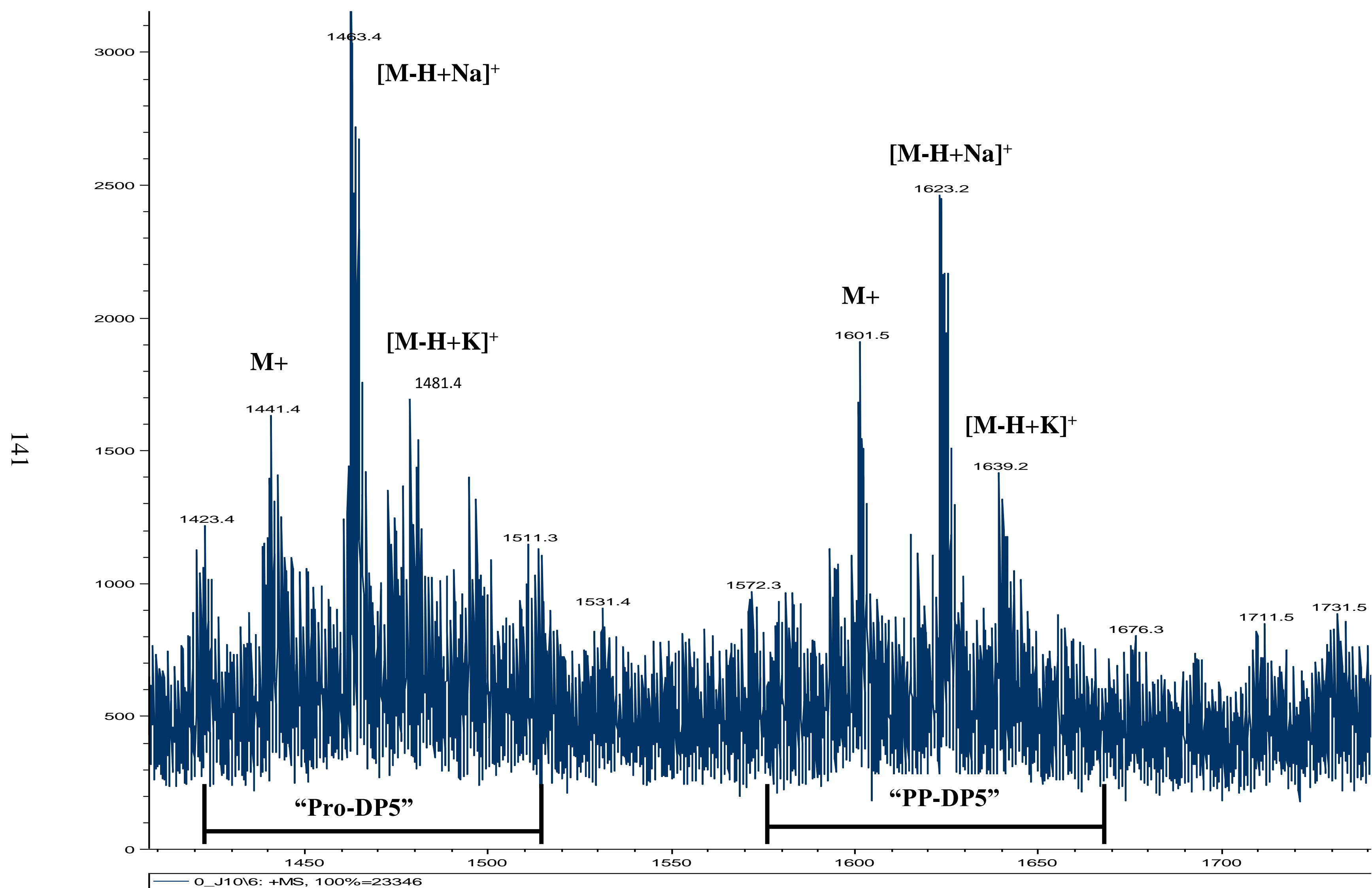
Figure 3-8. Normal-phase TLC plate with toluene-acetone-formic acid (30/30/10 v/v/v) with 8 mobile phase applications. Samples spotted were frozen chokeberry (left), to juice stored 4 months (right).

Figure 3-9. Data analysis output from TLC-MALDI for frozen chokeberry sample using a RP-C_{18} plate with methanol-water-TFA (55/45/1 v/v) mobile phase.

Figure 3-10. Data output of frozen chokeberry, 1, 2, and 3 month samples using RP-C_{18} plate with methanol-water-TFA (55/45/1 v/v) mobile phase.

Figure 3-11. TLC-MALDI spectra of 3 month stored juice on RP-C_{18} plate.

Figure 3-1.



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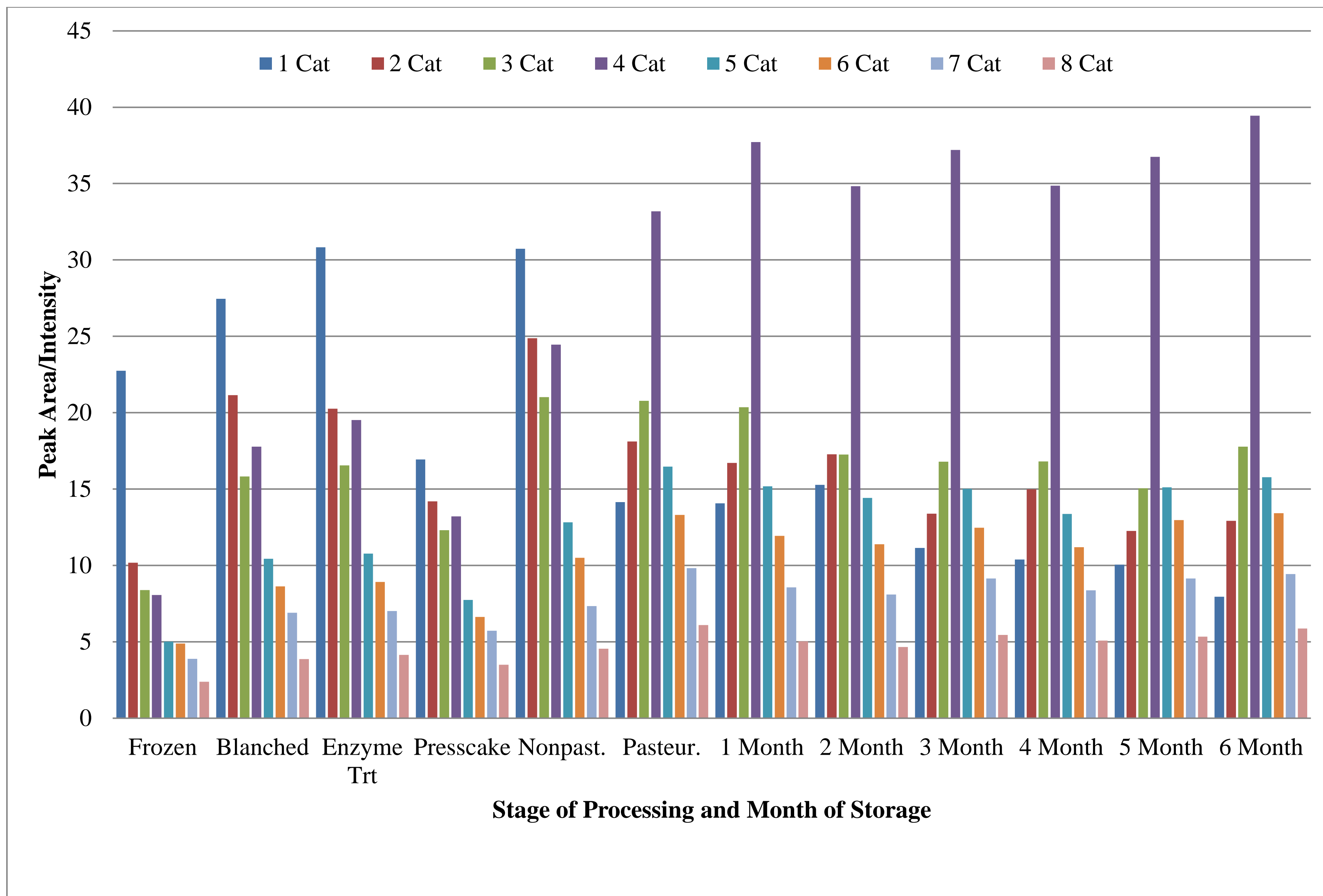

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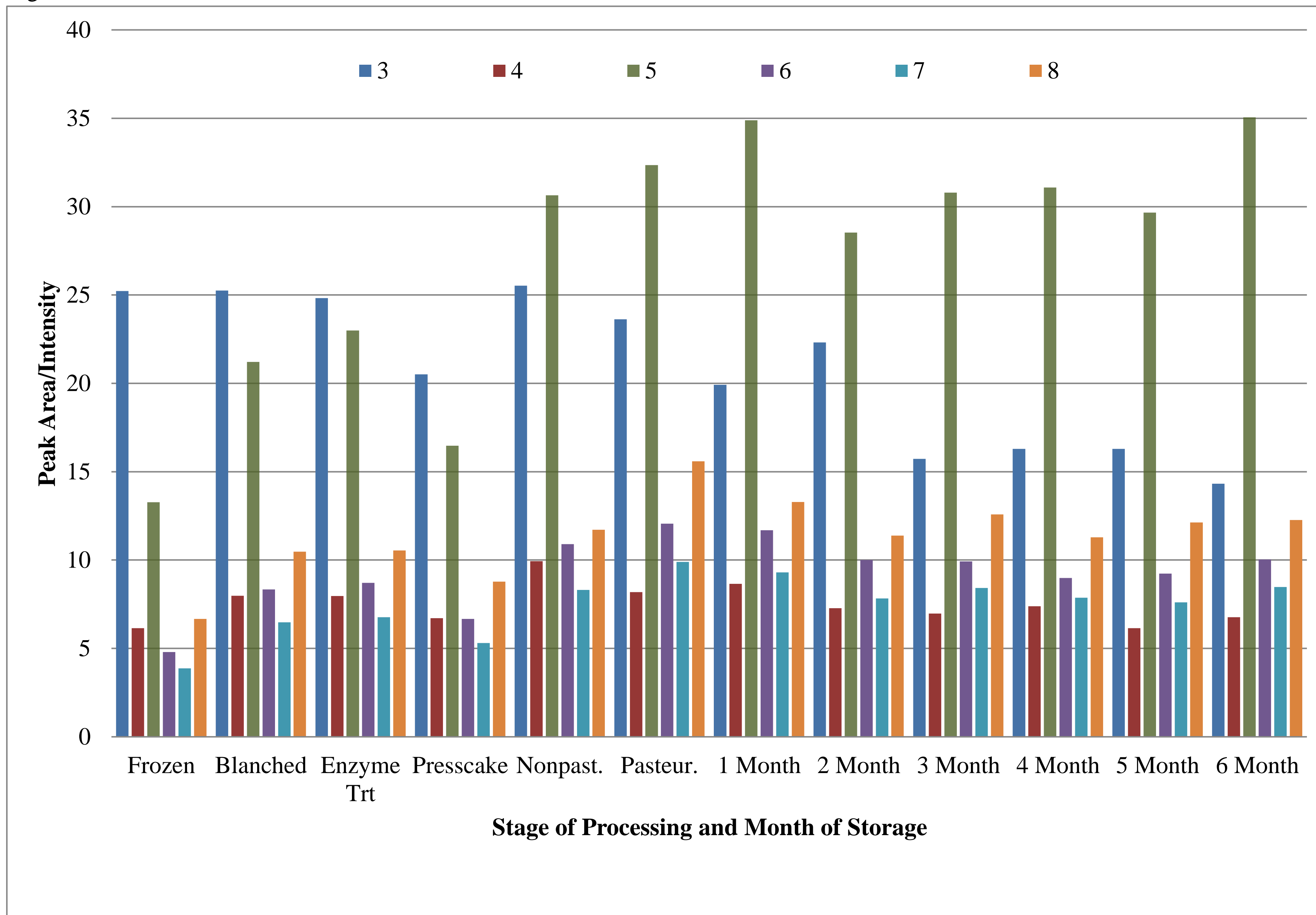

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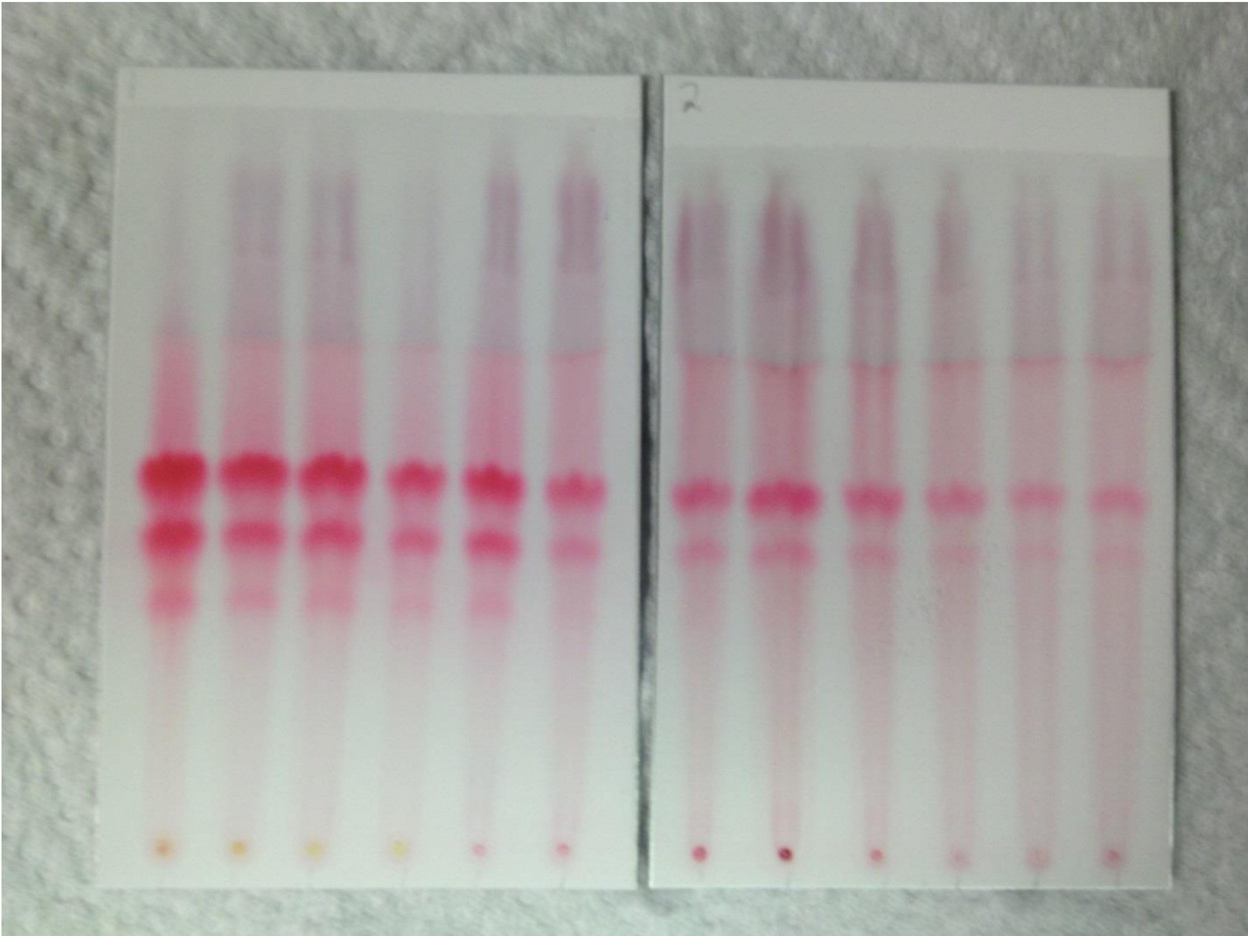

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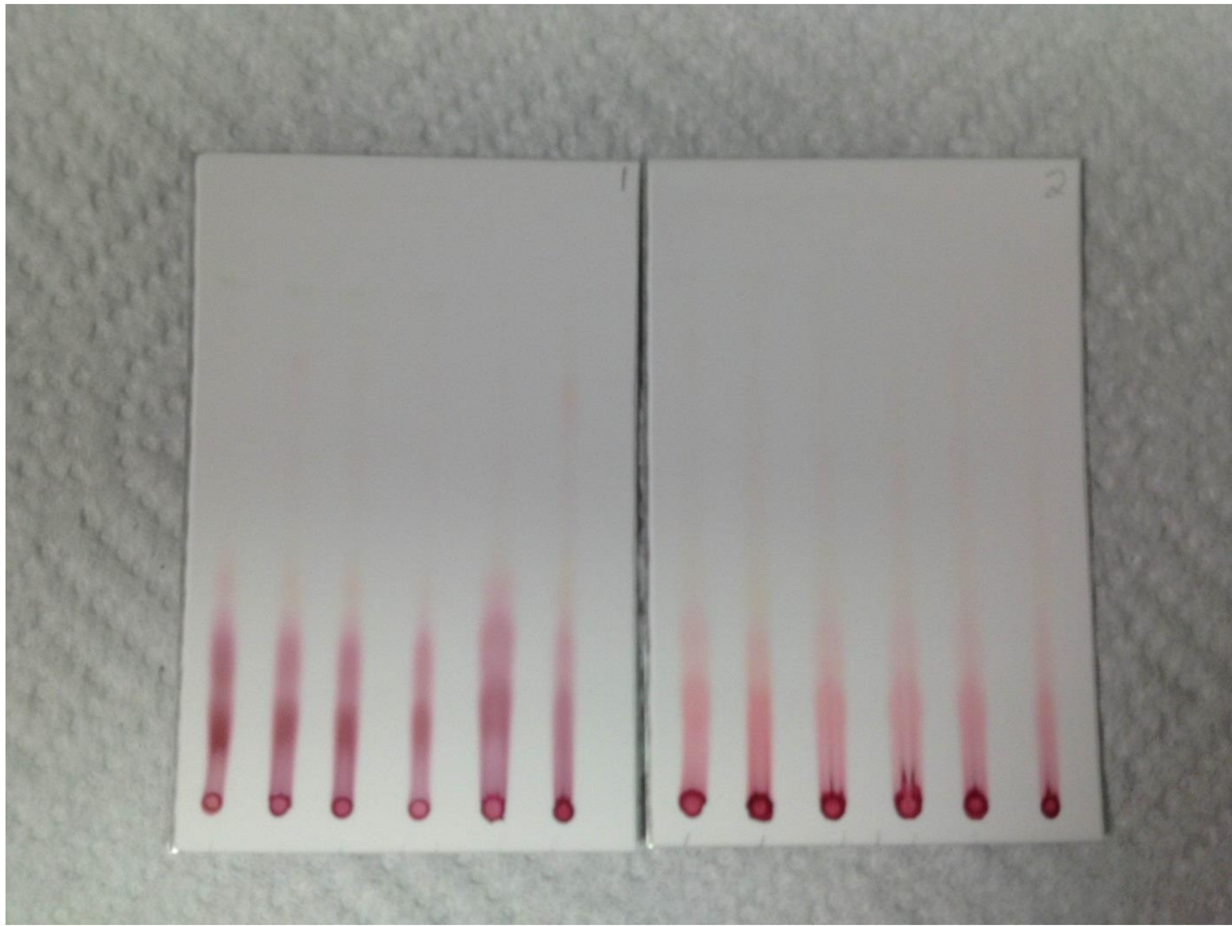

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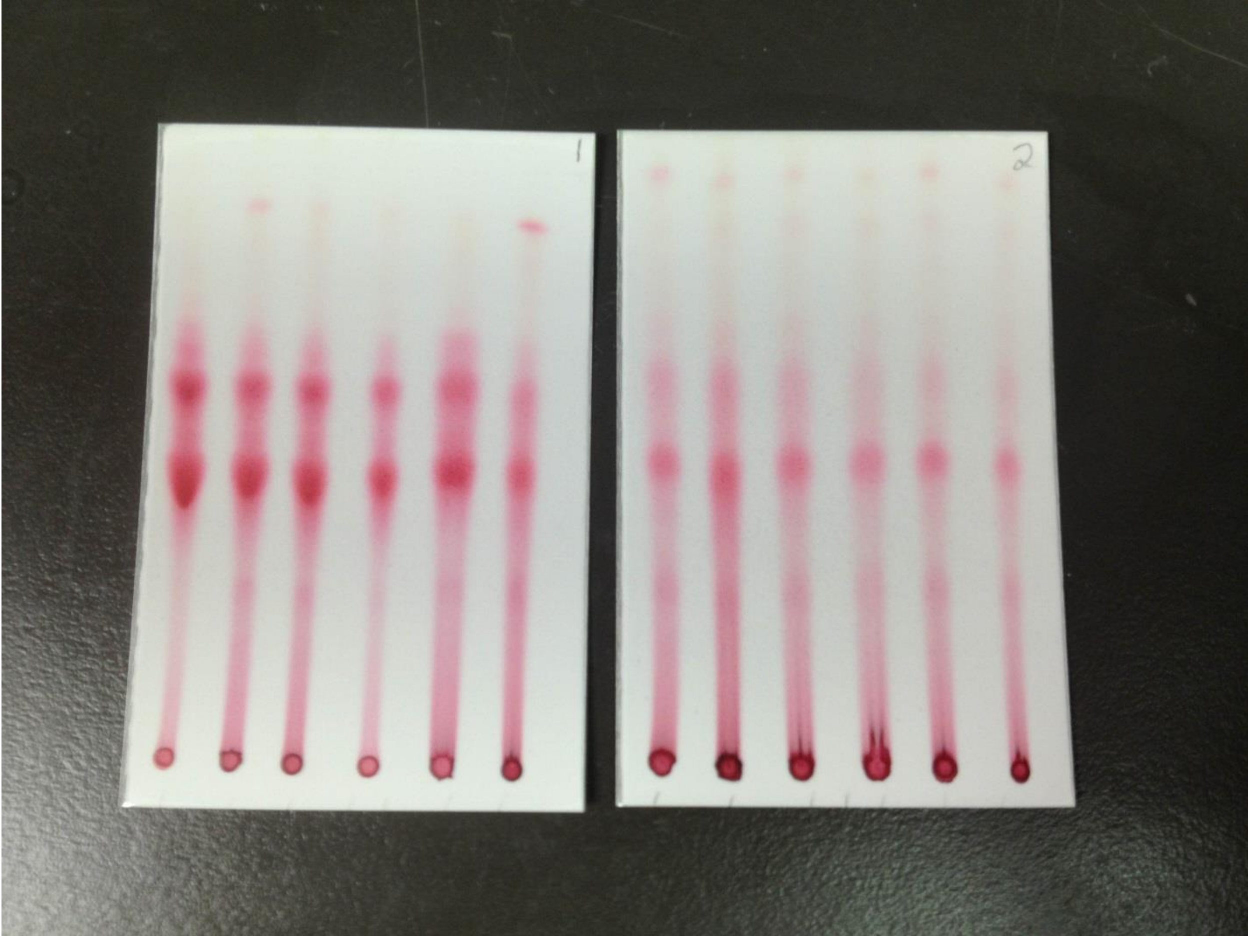

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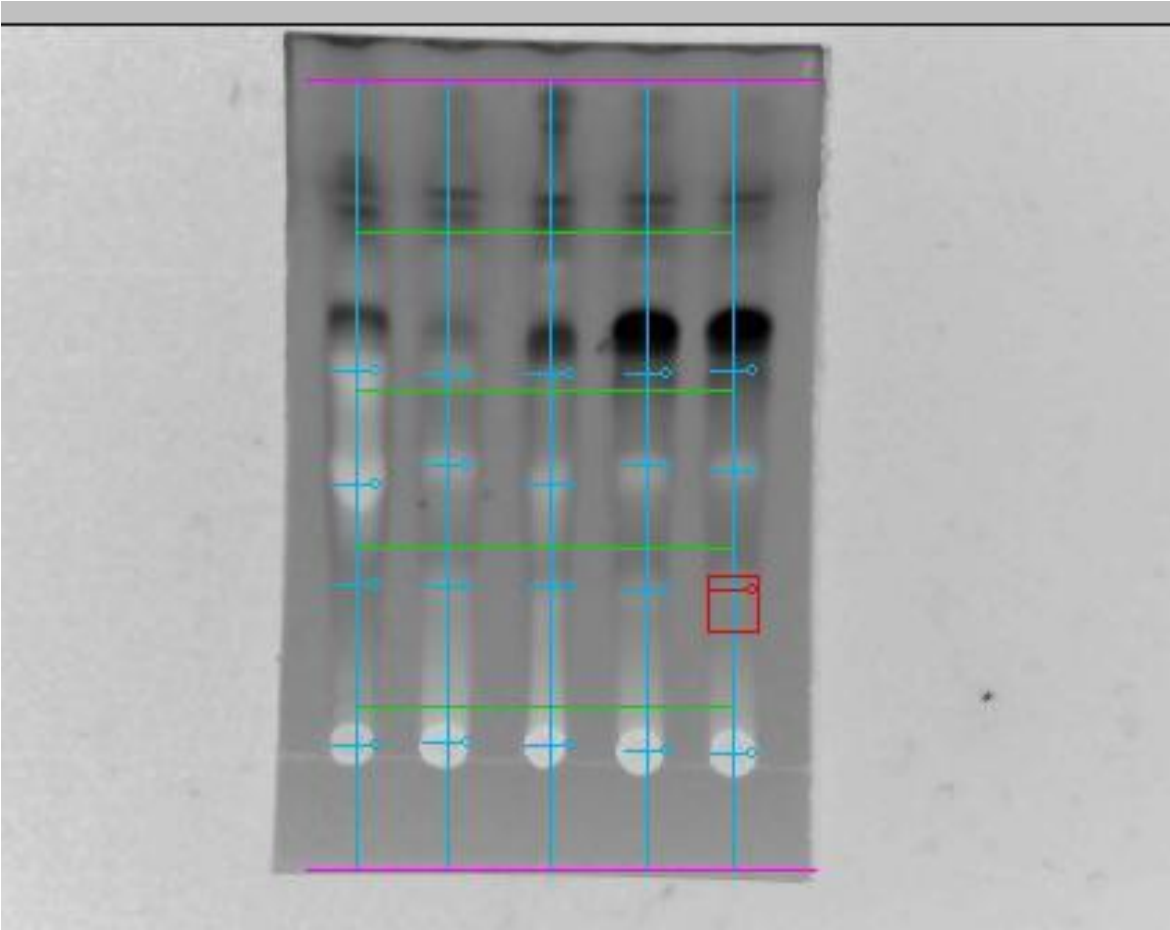

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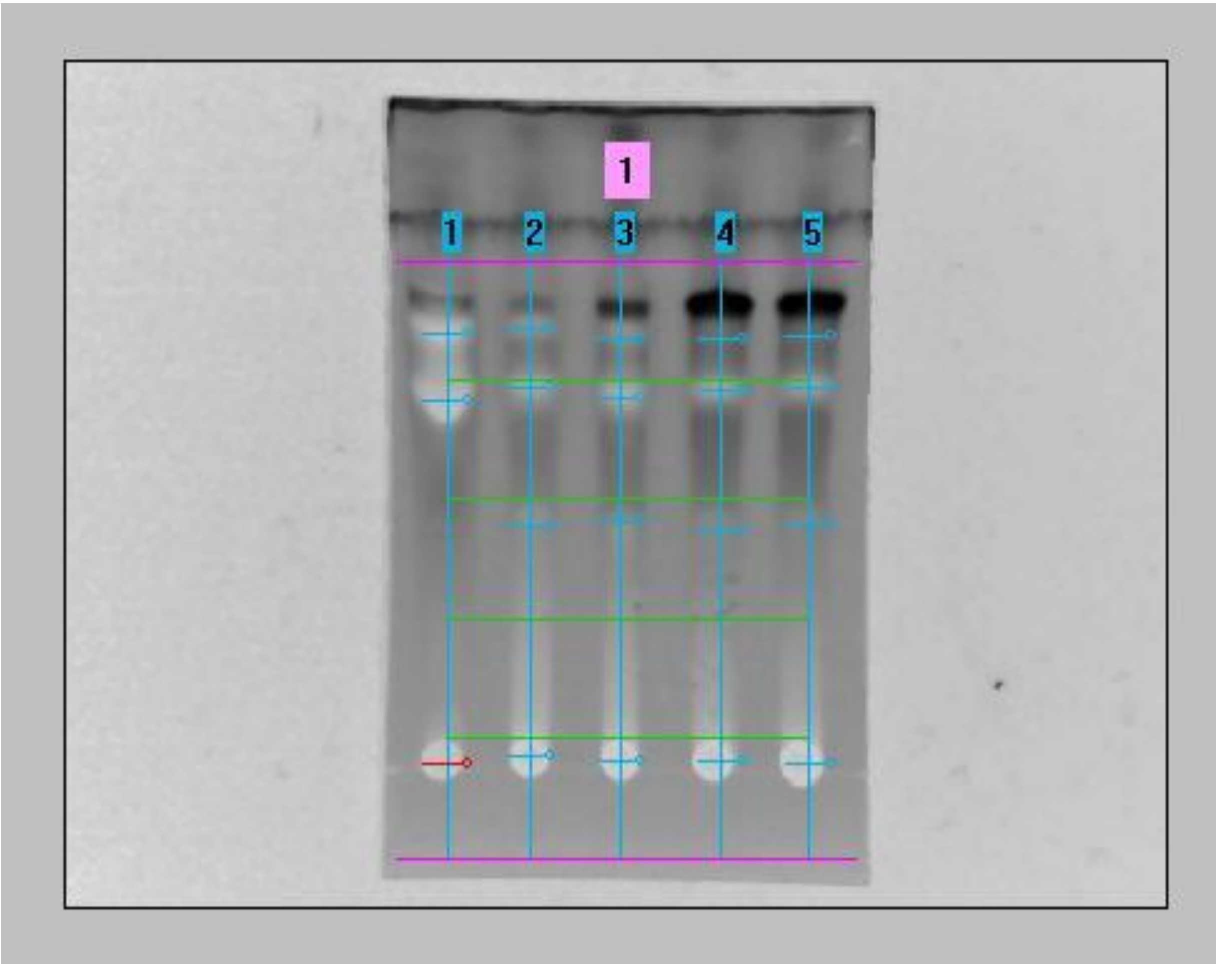

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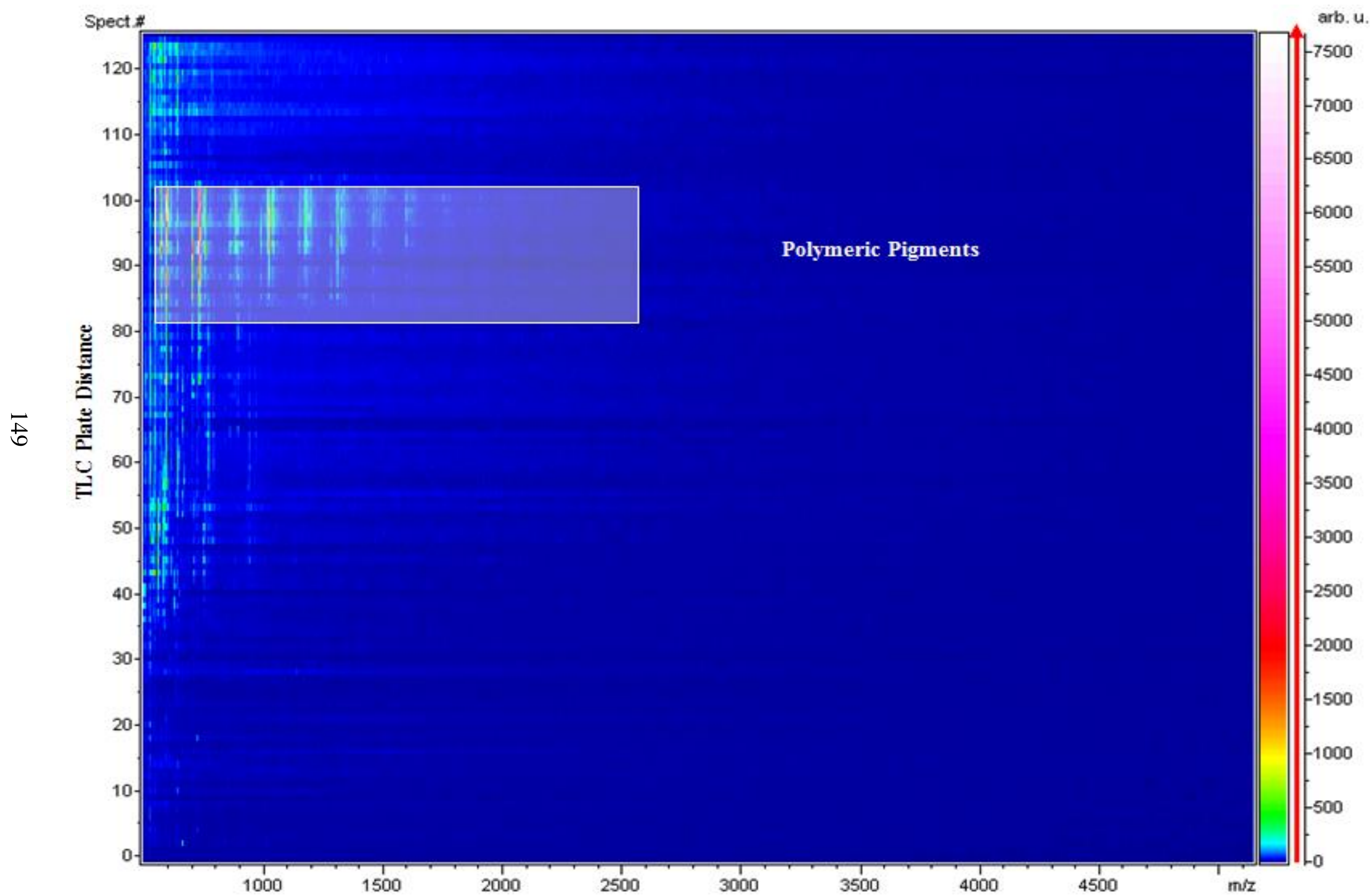


Figure 3-10.

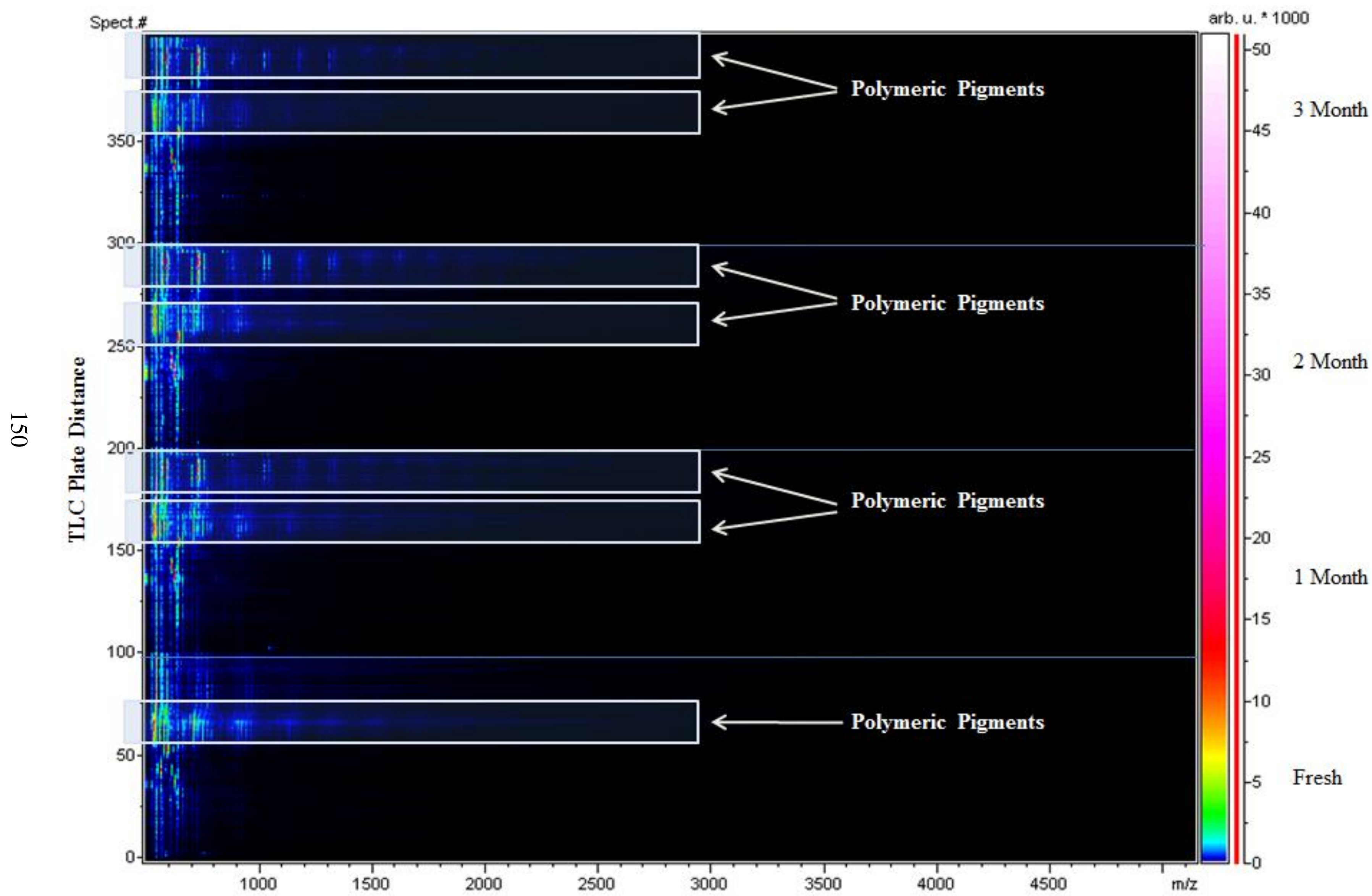
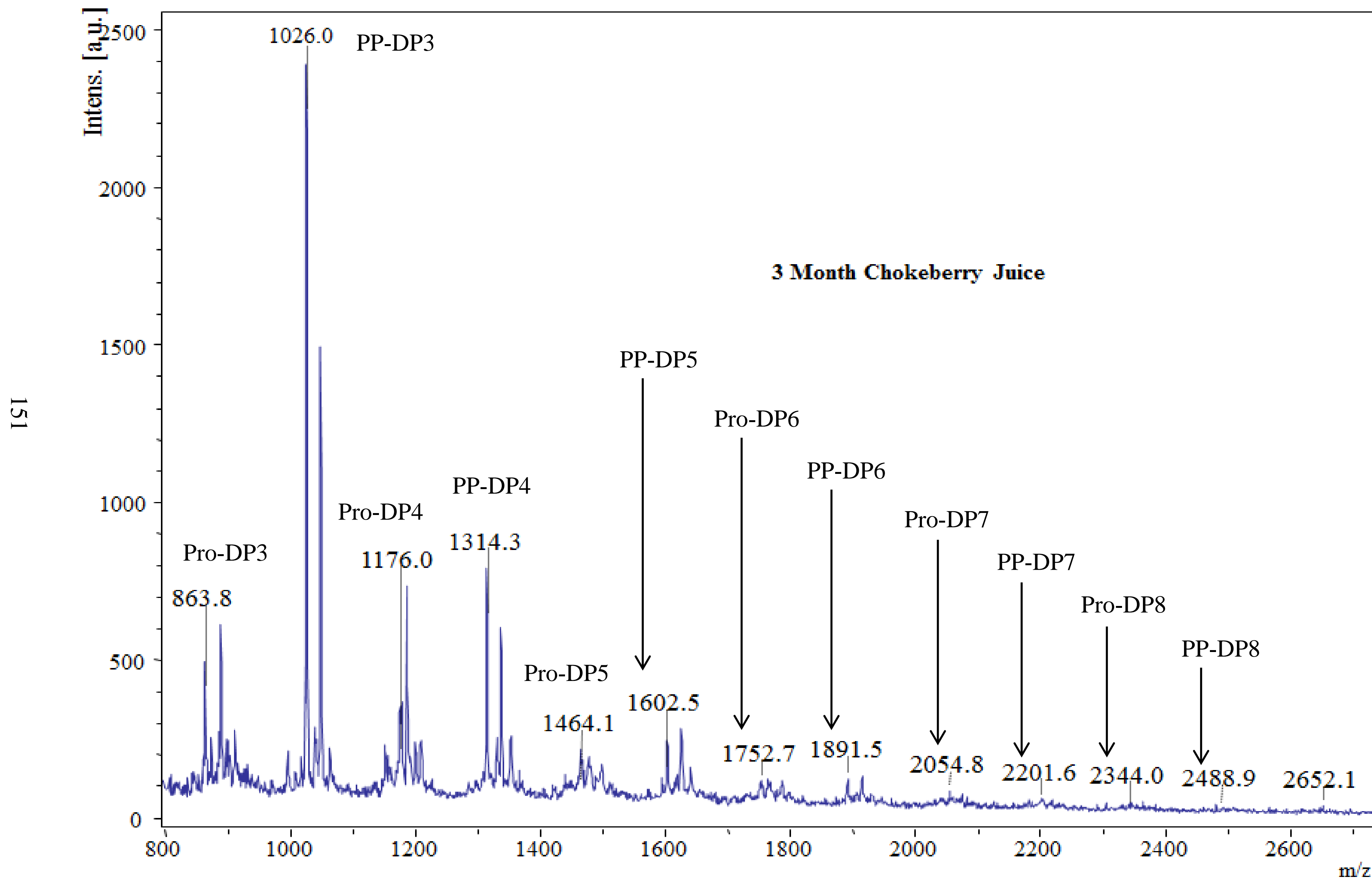


Figure 3-11.



VI. CONCLUSIONS

Thermal processing of chokeberries dramatically influenced total polyphenolic content as ACYs were most susceptible to degradation due to thermal application, whereas other polyphenolics such as flavonols, PACs, and HCA were more thermally stable, but experienced significant losses occurring during the pressing operation. Heat applications during blanching and pasteurization as well as physical removal of pressed pomace caused the most significant losses of ACYs during juice processing. By removing the blanch step from the process, polyphenolic levels were not as significantly different as predicted. Interestingly, anthocyanin pentosides were more prone to degradation than hexosides which suggests that molecular structural differences may play an important role in anthocyanin degradation. As a result of thermal degradation of ACYs, increasing levels of PCA and PGA occurred, but explained less than 5% of the overall anthocyanin degradation. Percent polymeric color values steadily increased due to processing which supports the idea of monomeric ACYs forming polymers with flavan-3-ols to stabilize color, even though almost all monomeric anthocyanin content was lost.

Long-term juice storage at ambient temperature also had detrimental effects on anthocyanin content, while less influential changes occurred in levels of other polyphenolics. The greatest losses of ACYs occurred after the first month of storage and continued to decrease until the sixth month where ACY's appeared to stabilize. Percent polymeric color values of the stored juice increased from one to six months which inversely correlated with anthocyanin degradation. Juice storage at ambient temperatures, however, did not have as strong of an effect on polyphenol losses as did juice processing.

Proanthocyanidin polymers and PPs were successfully identified and characterized by MALDI-TOF-MS. Surprisingly, PPs were present in frozen, whole chokeberries used for processing as well as all samples obtained throughout juice processing. A shift in polymeric pigment composition occurred in response to pasteurization, Pasteurized juice had lower levels of cyd 3-galactoside with one and two flavan-3-ols units attached, and higher levels of cyd 3-galactoside with four, five and six flavan-3-ols units attached than nonpasteurized juice, suggesting that heat applied during pasteurization resulted in formation of PPs with higher degree of polymerization. Separation of PPs by degree of polymerization using TLC plates was not successful, however, monomeric and polymeric pigment fractions were separated and identified by development of a TLC-MALDI-TOF analytical method. Further research is needed to examine more extensive methods for polymeric pigment isolation and purification. Additional TLC stationary phases and solvent systems should be tested that may require multiple procedures such as reapplication of solvents to a TLC plate, larger TLC plates allowing for longer pigment migration and separation, or multiple solvent systems applied to one plate.