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Blueberry Pectin-Anthocyanin Binding Kinetics and Stability Under In Vitro Digestion

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BLUEBERRY PECTIN-ANTHOCYANIN BINDING KINETICS AND STABILITY UNDER IN VITRO DIGESTION

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I would like to dedicate this dissertation to my family for their love, support, and encouragement.

A special gratitude to my parents and twin sister. Love you.

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Abstract

Blueberry pectins were sequentially extracted from highbush blueberry powder and precipitated in alcohol to obtain water soluble fraction (WSF) and chelator soluble fraction (CSF). WSF was high methoxyl pectin (degree of esterification: 53), rich in in neutral sugars (40 mol%), and had less negative charge (-21 mV) whereas CSF was low methoxyl pectin (degree of esterification: 21), rich in uronic acids (92 mol%), and had lower negative charge (-26 mV).

The stability of blueberry pectin-anthocyanin complex was investigated under simulated in vitro digestion. Binding of blueberry pectins with three anthocyanin standards (malvidin-3-glucoside; M3G, cyanidin-3-glucoside; C3G, and delphinidin-3-glucoside; D3G) and blueberry powder extract (BBE) were used. M3G was the most stable followed by C3G, whereas D3G completely disappeared after gastrointestinal digestion. CSF bound and prevented M3G and C3G degradation more than WSF. Increased stability of anthocyanins after simulated gastrointestinal digestion suggests that anthocyanins can be transported to colon where gut microbiota actively produce anthocyanin metabolites. The amount of bound anthocyanins that interacted with blueberry pectin increased as the number of hydroxyl groups increased on anthocyanins. This suggests that hydrogen bonding in addition to electrostatic interaction contribute to stability of pectin-anthocyanins complexes at pH 4.0.

A binding selectivity between blueberry pectin and anthocyanins was determined by Langmuir isotherm. CSF had four times higher ($R^2 > 0.94$) adsorption with anthocyanins whereas WSF had less and variable interaction with anthocyanins. Further binding study influenced by exogenous factors showed that blueberry pectin-anthocyanins adsorption was the highest at pH 3.0 and the least at pH 4.0 regardless of the ionic strength. Increasing ionic strength weakened the pectin-anthocyanin interaction between pH values 3.0 and 4.0, however, no

significant difference was observed at pH 2.6. As pH value changed from 4.0 to lower pH values, electrostatic interactions were less dominant while hydrophobic and hydrogen interactions contributed more to blueberry pectin and anthocyanins.

Chapter 1. Introduction and Literature Review

1.1. Introduction

Polysaccharide-polyphenol interactions receive much attention because of the functional and nutritional benefits. Upon examination of various polysaccharides (cellulose, starch, pectin, arabinoxylan, xyloglucan) and polyphenols (procyanidin, catechin, ferulic acid, anthocyanin), the greatest interaction is consistently reported for pectin, anthocyanin, or between pectin and (Le Bourvellec & Renard, 2005; Phan, Flanagan, D'Arcy, & Gidley, 2017). Pectin is a soluble polysaccharide mostly extracted from citrus peel, apple pomace, or sugar beet pulp as commercial sources (Chan, Choo, Young, & Loh, 2017). Anthocyanin is a phenolic compound which serves as a color pigment, and found in fruits and vegetables (Wrolstad, 2004). A majority of research shows the anthocyanin interaction after equilibration with citrus, apple, or sugar beet pectins (Buchweitz, Speth, Kammerer, & Carle, 2013a, 2013b; Fernandes, Brás, Mateus, & de Freitas, 2014). Recently, the binding of blueberry pectin to anthocyanin is reported, which contributes to understanding the beneficial health effects of blueberries (Z Lin, Fischer, & Wicker, 2016). The binding mechanism between blueberry pectin and anthocyanin is still lacking, so it is important to provide a better understanding of the interaction in order to manipulate a variety commercial areas and technologies to maximize the health benefit. In this literature review, pectin, anthocyanin, recent studies of blueberry pectins, polysaccharide-polyphenol interaction, and pectin-anthocyanin interaction are summarized.

The overall subjective of this study is to examine the effect of blueberry pectin structure on the anthocyanin stability under in vitro digestion, and to suggest the main contributor of blueberry pectin-anthocyanin interaction. In the second chapter, the sequential extraction and physico-chemical properties of blueberry pectins are described. The uronic acid content, degree

of esterification, protein content, ζ-potential, anthocyanin content, neutral sugars composition, molecular weight and viscosity are reported. In the third chapter, the stability of blueberry pectin-anthocyanin complexes under in vitro digestion is reported. Three anthocyanin standards (malvidin-3-glucoside, cyanidin-3-glucose, delphinidin-3-glucoside) and blueberry powder extract are used to form the blueberry pectin-anthocyanin complexes. In the fourth chapter, the effect of endogenous and exogenous factors on blueberry pectin-anthocyanin interaction are reported. The endogenous factors include the chemical composition and concentration of blueberry pectins; the exogenous factors include pH and ionic strength.

1.2. Pectin

Pectin is a soluble dietary fiber that exists in the middle lamella, primary and secondary plant cell walls (Voragen, Coenen, Verhoef, & Schols, 2009). It is mostly extracted from citrus peel, apple pomace, or sugar beet pulp as commercial sources (Chan et al., 2017). Pectin is an important ingredient used as a emulsifying, gelling, sugar replacing, and thickening agent in a variety of food products, jam, jelly, confectionery filling, beverage, and sauce (Thakur, Singh, Handa, & Rao, 1997). In addition, pectin is widely used a drug delivery vehicle in pharmaceutical, biodegradable film in textile, and a emulsion stabilizer in cosmetic industries (Thakur et al., 1997).

In the aspect of structure, pectin is composed of three main domains: homogalacturonan (HG, ~65%), rhamnogalacturonan I (RG-I, 20-35%), and substituted galacturonans such as rhamnogalacturonan II, xylogalacturonan, and apiogalacturonan (Figure 1.1) (Mohnen, 2008). HG is a linear structure composed of α-1,4-linked-D-galacturonic acid (GalA). The carboxyl groups of GalA can be methyl-esterified which determines the degree of esterification (DE) and further the functionality of pectin (Mohnen, 2008). Depending on the DE, pectins are grouped

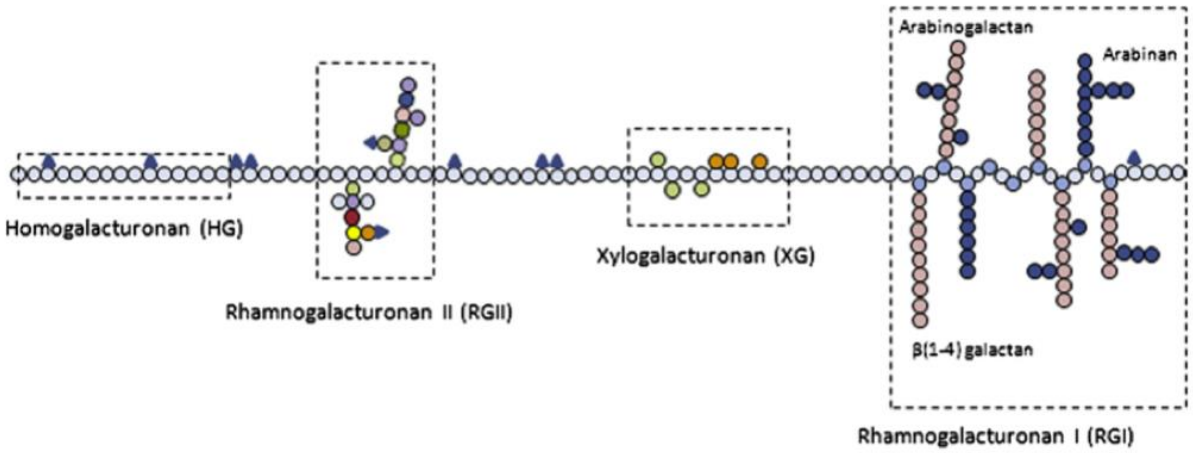

Figure 1.1. A general structure of pectin (Maxwell, Belshaw, Waldron, & Morris, 2012).

into high methoxyl (HM, DE above 50%) and low methoxyl (LM, DE below 50%) pectin (Willats, Knox, & Mikkelsen, 2006). Or the carboxyl groups of GalA can also be free carboxylated, thus renders the natural anionic charge of pectin in hydrate. RG-I has highly branched structures with a repeating backbone of GalA and rhamnose at which neutral sugar side chains attached such as arabinan, galactan, and arabiogalactan (Maxwell et al., 2012). The substituted galacturonans are complex branched structures same as RG-I, however, the backbone consist of HG and various rare neutral sugar side chains are attached to the GalA (Willats et al., 2006).

Many studies have showed various health benefits of consuming pectin; anti-obesity, anti-cancer, anti-diabetes, and positive modulator of the but microbiome (Islamova, Ogai, Abramenko, Lim, Abduazimov, Malikova, et al., 2017; Jenkins, Wolever, Jenkins, & Taylor, 1987; Wicker, Kim, Kim, Thirkield, Lin, & Jung, 2014). Pectin is typically resistant to gastrointestinal digestions, enters the colon and is fermented by gut microflora thus generating short chain fatty acids (acetate, butyrate, formate, lactate, and propionate) (Morrison & Preston, 2016). Interestingly, specific domains of pectin may have selected health benefits (Wicker et al., 2014). Pectin fragments rich in galactans bind galectin-3 protein, inhibit metastasis and

consequently give anti-cancer effect (Maxwell et al., 2012). Gut microbiota selectively ferments pectin fraction rich in arabinose and galactose and increased beneficial bacterial population, *Bifidobacterium* (Onumpai, Kolida, Bonnin, & Rastall, 2011). Highly branched pectins rich in arabinan side chains were rapidly fermented by human colonic bacteria and set a beneficial environment for fermentation by producing high concentrations of short chain fatty acids and lowering the pH (Gulfi, Arrigoni, & Amadò, 2007).

1.3. Blueberry Pectin

Compared to commercial pectins extracted from citrus peel and apple pomace since early 1900s, blueberry pectin has been studied somewhat recently. Proctor and Peng (1989) investigated the quantitative and qualitative changes of pectin in 'Bluetta' blueberries during fruit ripening. The total amount of pectin in 'Bluetta' blueberries was 15 mg pectin per g fruit on 30 days after full bloom and decreased to 7 mg pectin per g fruit when it was harvested. During fruit development, the amount of water- and chelator soluble pectins increased whereas that of dilute alkali soluble pectin decreased from 65 to 20% in alcohol insoluble solid (AIS). Since the type and amount of pectin notably determine the fruit texture, the softening can be predicted by the pectin transition. Pectin was extracted from cull lowbush blueberries using acidified ethanol with different concentrations of citric acid and stored for 9 months in frozen (H. C. Chen & Camire, 1997). The total amount of pectin during frozen storage ranged 2.7 to 4.1 mg pectin per g berries which was lower than that of Proctor et al. (1989). The highest pectin content was observed when it was extracted by 5% citric acid then stored for 7 months. The DE of blueberry pectins ranged from 66 to 77%. However, H. C. Chen et al. (1997) did not fractionate the pectin by solubility so that only reported the total pectin content in berries. AIS was prepared from blueberry fruit cv. Duke at five stages of ripeness and sequentially fractionated to water soluble

fraction (WSF), chelator soluble fraction (CSF), sodium carbonate soluble fraction (NSF), and two potassium hydroxide soluble fractions (4KSF and 24KSF) (Vicente, Ortugno, Rosli, Powell, Greve, & Labavitch, 2007). No changes in total pectin content was observed during blueberry ripening, ranged 311 to 344 μg GalA per mg AIS. Different changes of pectin content in each fractions were reported from green to 75% surface blue color: WSF (~60 μg GalA per mg AIS) and CSF (~30 μg GalA per mg AIS) increased whereas NSF (60-30 μg GalA per mg AIS) decreased. Arabinose was the major neutral sugar found in pectin fractions. The author concluded that softening in blueberry uncommonly proceeded without pectin depolymerization.

In recent years, AIS obtained from rabbiteye blueberries was sequentially fractionated to WSF, CSF, NSF, 4KSF, and 24KSF then fully characterized (Deng, Shi, Li, & Liu, 2012). The molecular weight of WSF, CSF, and NSF ranged from 33 to 122 kDa with high polydispersities from 1.86 to 2.66. Pectin fractions were expressed as molar ratio of GalA and neutral sugars (NS), thus the structural features were predicted. WSF was enriched in neutral sugars (GalA: 37% and NS: 63%) with a few of rhamnose whereas CSF was enriched in galacturonic acid (GalA: 64% and NS: 36%). The ratio of GalA and NS of NSF was 57% and 42%, respectively. Arabinose was the most abundant in WSF, CSF, and NSF. Six cultivars (Blueray, Duke, Northland, Patriot, Polaris, and Reka) of highbush blueberries were characterized physico-chemically, then two cultivars (Patriot and Polaris) were selected to study the effect of low temperature blanching on the blueberry pectin structure (Chevalier, Rioux, Angers, & Turgeon, 2017). Total pectin contents of six cultivars blueberries were estimated from 47 to 74 mg GalA per g AIS and the DE ranged 22 to 41%. Low temperature blanching (60 ºC/1 h) influenced the blueberry pectin properties; decreased the DE of pectin in AIS, changed the amount of pectin fractions (decreased WSF, increased CSF, and no change on NSF). However, the heat treatment

did not affect the monosaccharide composition of pectins. Therefore, it is concluded that low temperature blanching only decreased the DE of blueberry pectins without pectin depolymerization and degradation, consequently increased the functionality of pectin.

Contrary to other studies using fresh blueberry fruits, AIS was prepared from freeze-dried highbush blueberry powder, sequentially fractionated to WSF, CSF and NSF, and incubated with anthocyanins to investigate the pectin-anthocyanin interaction (Z Lin et al., 2016). The total amount of the pectin fractions ranged from 516 to 627 μg GalA per mg fraction (WSF, CSF, and NSF). However, these values were not comparable to those of other studies because they were on fraction weight basis, not AIS weight basis. The DE of pectin fractions ranged 26 to 36%, and molecular weight ranged from 169 to 602 kDa. From the same research group, the same freeze-dried highbush blueberry powder was fractionated to WSF, CSF, NSF, 4KSF, and 24KSF in order to characterize the blueberry pectins for better understanding of blueberry pectin-anthocyanin interaction (Zhuangsheng Lin, Pattathil, Hahn, & Wicker, 2019). The GalA content of WSF, CSF, NSF, 4KSF, and 24KSF was 25.0, 22.3, 27.6, 2, and 2 μg GalA per mg AIS, respectively, which indicated that WSF, CSF, and NSF were pectin-rich fractions whereas 4KSF and 24KSF were non-pectic fractions. The glycome profiling confirmed that the pectin-rich fractions (WSF, CSF, and NSF) had HG, arabinogalactan, and xyloglucan while 4KSF and 24KSF were hemicellulose-rich fractions with high amount of xylan. The increased flexibility of CSF and high amount of protein in NSF may facilitate the interaction with anthocyanins.

1.4. Anthocyanin

Anthocyanin is a water soluble color pigment appearing red, purple, and blue, and commonly found in fruits, vegetables, flowers, and cereal grains (Wrolstad, 2004). Anthocyanin is composed of anthocyanidin (aglycone) and sugar moiety. The structural feature of

anthocyanidin was an aromatic ring [A] connected to oxygen containing heterocyclic ring [C] which is bonded to another aromatic ring [B] (Figure 1.2) (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Jackman, Yada, Tung, & Speers, 1987). Depending on the number of hydroxyl and methoxyl groups attached to the [B]-

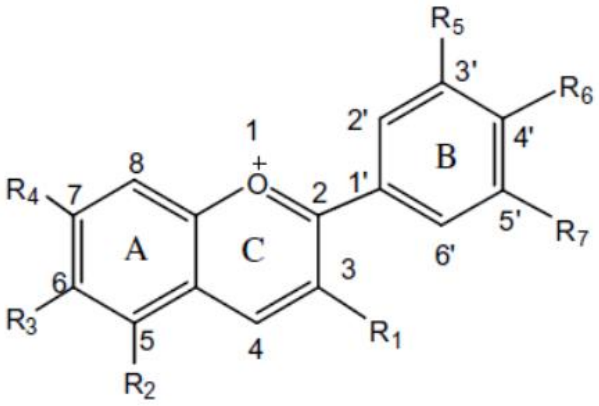

Figure 1.2. A general structure of anthocyanin (Castañeda-Ovando et al., 2009).

ring, six forms of anthocyanidin commonly exist; delphinidin, cyanidin, petunidin, malvidin, pelargonidin, and peonidin (Kong, Chia, Goh, Chia, & Brouillard, 2003). The structure and color of anthocyanin reversibly changes with pH values (Figure 1.3): flavylium cation (red) at pH 1-3,

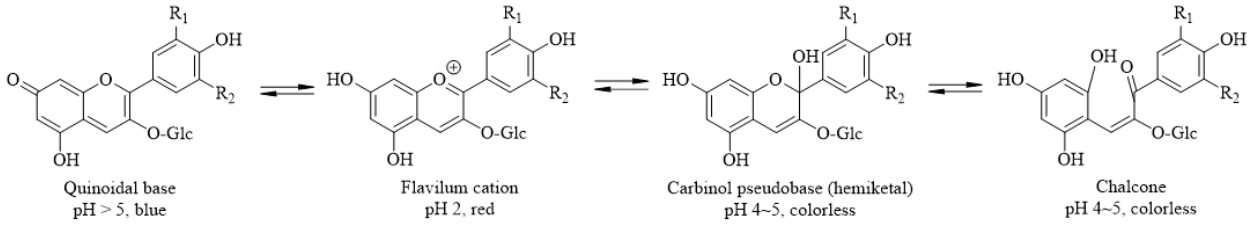

Figure 1.3. The structure and color of anthocyanin in different pH values.

hemiketal and chalcone (colorless) at pH 4-5, and quinoidal anhydrobase (blue) above pH 5 (Jackman et al., 1987; Wrolstad, 2004). In addition, the prevalent structures of the four anthocyanin in equilibrium condition varies by pH values (Figure 1.4): flavylium cation is dominant below pH 2.0 and decreases as pH increases whereas hemiketal is less in acidic pH values and increases as pH increases. The amount of chalcone and quinoidal anhydrobase increase as pH increases, however, the total amounts are much less than that of hemiketal form

(Jackman et al., 1987).

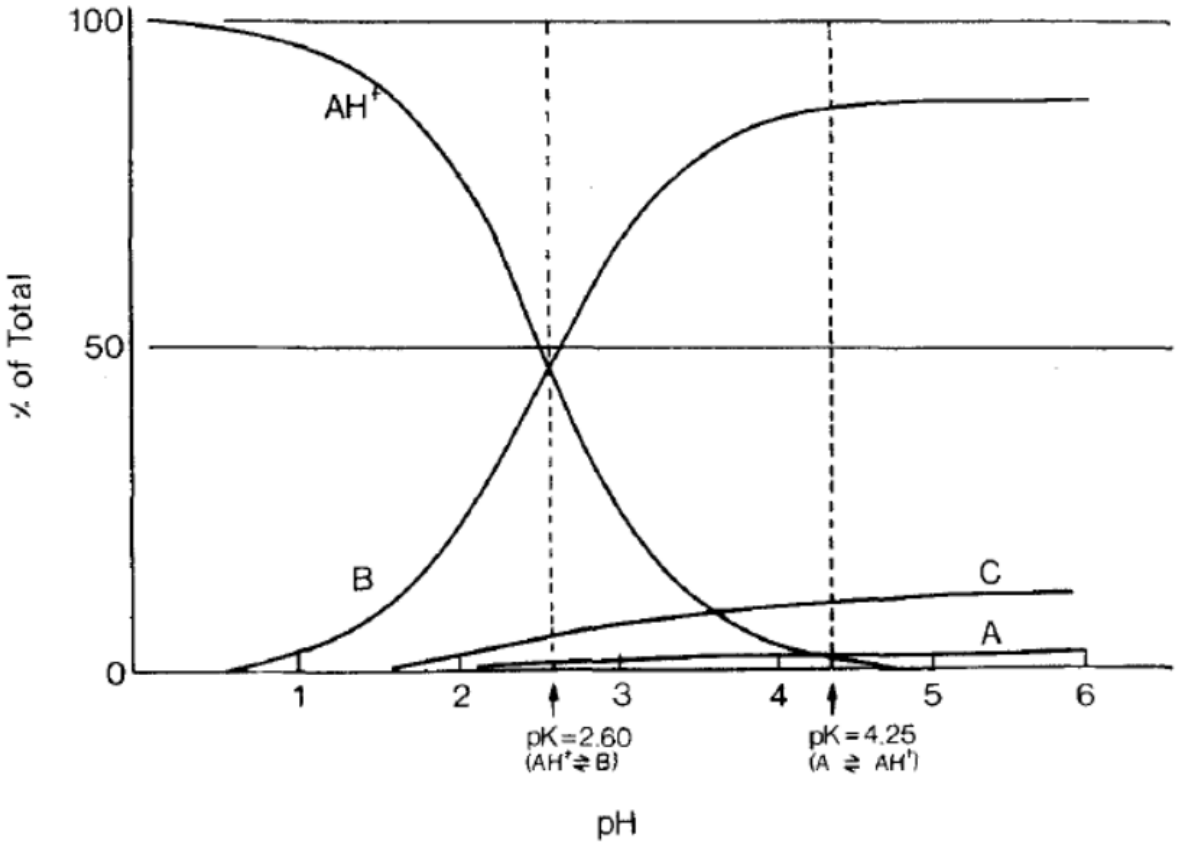

Figure 1.4. Distribution of the four different anthocyanin structures with pH under equilibrium conditions (Malvidin-3-glucoside: 25 ºC. AH+ = red flavylium cation, B = colorless carbinol pseudobase, C = colorless chalcone, A = blue quinoidal anhydrobase) (Jackman et al., 1987).

Anthocyanin is very susceptible to environmental factors such as pH, temperature, ascorbic acid, oxygen, enzymes, light and so on (Ongkowijoyo, Luna-Vital, & de Mejia, 2018). The heterocyclic ring containing oxygen [C] possibly have a partial positive charge and is easily attacked by nucleophilic water or sulfite ions, consequently leads to anthocyanin degradation (Jackman et al., 1987). The sugar moiety attached to the anthocyanidin ring prevents the degradation increasing stability. A glycosylation, attaching sugars mostly glucose to anthocyanidin, increases the stability of anthocyanin as well as increases the solubility (Jackman et al., 1987). Anthocyanin is also stabilized by metal ions or co-pigments. Co-pigmentation is a complex formation between color pigments and organic components which results in shift or

increase of pigment absorbance (Boulton, 2001).

Consuming anthocyanins revealed various health benefits, reducing oxidative stress, lowering the risk of cancer, diabetes, cardiovascular and neurological disorders (Castañeda-Ovando et al., 2009). In particular, intact anthocyanin and metabolites shows functionality as a pre-biotic and as a bioactive agent (Y. Chen, Li, Zhao, Zhang, Mao, Feng, et al., 2017; Faria, Fernandes, Norberto, Mateus, & Calhau, 2014; Lee, Keirsey, Kirkland, Grunewald, Fischer, & de La Serre, 2018). Deglycosylation of anthocyanin first occurred during microflora metabolism (Y. Chen et al., 2017). By UV-vis spectroscopy, [A,C] ring of anthocyanin shows an absorption peak at 520 nm whereas [B] ring has a peak at 260 nm (Jacob & Paliyath, 2008). After colonic fermentation, the absorption peak at 520 nm disappeared but that of 260 nm still remained, suggesting that the [A,C] ring of anthocyanin was cleaved and simple phenolic compounds were produced from the [B] ring as metabolites (Correa-Betanzo, Allen-Vercoe, McDonald, Schroeter, Corredig, & Paliyath, 2014). Anthocyanins and the metabolites modulated colonic bacteria populations enhancing the growth of beneficial Bifidobacterium spp. and Lactobacillus spp. (Hidalgo, Oruna-Concha, Kolida, Walton, Kallithraka, Spencer, et al., 2012)

1.5. Polysaccharide-Polyphenol Interaction

Due to the functional and nutritional benefits of plant-based foods, interactions between polysaccharide and polyphenol have received much attention and been investigated extensively. A number of research group used various polysaccharides and polyphenols with different sources, characteristics, and analysis methods; oat β-glucan and epigallocatechin-3-gallate (EGCG) (Gao, Liu, Peng, Wu, Wang, & Zhao, 2012), carrageenan and anthocyanin (Navikaite, Simanaviciute, Klimaviciute, Jakstas, & Ivanauskas, 2016), grape cell wall and proanthocyanidin (Ruiz-Garcia, Smith, & Bindon, 2014), gum arabic and anthocyanin (Chung, Rojanasasithara,

Mutilangi, & McClements, 2016) and so on.

Cell wall extracted from apple and six procyanidins extracted from apple, grape seed, and pear were used to study the binding effect of procyanidin structure and environment factors on the association (Le Bourvellec, Guyot, & Renard, 2004). High molecular size and degree of galloylation of procyanidin bound more with the apple cell wall. The interaction was not affected by pH, however, increased with increasing ionic strength and decreased by urea, dioxane, ethanol, and increasing temperature which suggested that non-covalent interactions, mostly hydrogen bond and hydrophobic force, were governed. The same six procyanidins were incubated with two apple cell walls which were obtained by mild and harsh drying, respectively, then the adsorption was quantified using Langmuir isotherms (Le Bourvellec & Renard, 2005). The harsh drying decreased the surface area of apple cell wall from 2.15 to 0.52 m^2/g so that decreased apparent affinity and increased apparent saturation levels were obtained when the values were calculated per cell wall weight. However, when the numbers were expressed per surface unit, both apparent affinity and saturation levels increased, indicating that the surface area measured in dry sample was not relevant for the adsorption analysis which was conducted in an aqueous media. The adsorptions between the six procyanidins mentioned above and four different polysaccharides (cross-linked pectin, cross-linked xyloglucan, cellulose and starch) were quantified using Langmuir isotherms (Le Bourvellec, Bouchet, & Renard, 2005). Higher affinity values were observed with pectin which possibly attributed to hydrophobic pockets encapsulating procyanidins whereas lower saturation levels were reported. While, cellulose and xyloglucan interacted procyanidins weakly, but, higher saturation levels were obtained resulting from procyanidin stacking which was facilitated by the structure of cellulose and xyloglucan. Because of the higher affinity between pectin and procyanidin, the pectin-procyanidin interaction

was further investigated. Isothermal titration calorimetry was used to determine the thermodynamic nature of the interaction and concluded that strong binding occurred between highly methylated pectin and long chain of procyanidin possibly due to hydrophobic interaction (Watrelot, Le Bourvellec, Imberty, & Renard, 2013). The following study reported that the highly branched pectin rich in neutral sugar side chains limited the pectin-procyanidin association which probably governed by hydrophobic interaction and hydrogen bond (Watrelot, Le Bourvellec, Imberty, & Renard, 2014).

Another research group comprehensively studied the binding between cellulose-based cell wall and polyphenols. Pure cellulose and two cellulose-pectin composites (LM and HM pectins) were incubated with anthocyanins and the rate of anthocyanin depletion was monitored over time (Padayachee, Netzel, Netzel, Day, Zabaras, Mikkelsen, et al., 2012). The interaction between cell walls and anthocyanins occurred in two phases, a rapid initial phase within the first thirty seconds and a slow additional phase from 2 to 14 days. The greatest anthocyanin binding was observed at the cell wall with the highest pectin content suggesting that ionic interactions involved. In another study from the same research group, five polyphenols (catechin, ferulic acid, chlorogenic acid, gallic acid, and cyanidin-3-glucoside) were incubated with cellulose and the adsorption was quantified using Langmuir isotherms (Phan, Netzel, Wang, Flanagan, D'Arcy, & Gidley, 2015). High molecular size of polyphenol (cyanidin-3-glucoside) had the highest binding and apparent affinity to cellulose, suggesting that molecular weight of polyphenol has a major impact on the interaction with native charge of polyphenols as a secondary factor. The cellulose-polyphenol interaction was further investigated under different environmental conditions (pH, temperature, and salt) (Phan, D'arcy, & Gidley, 2016). As in the previous study, the highest binding was observed in cyanidin-3-glucoside followed by catechin and ferulic acid.

The results revealed that pH was the most influential factor to the cellulose-polyphenol interaction followed by temperature; and salt had no effect. A binding selectivity was measured between cellulose-based composites (pure cellulose, cellulose-arabinoxylan, cellulose-xyloglucan, cellulose-pectin, and apple cell wall) and differently charged polyphenols (positively charged cyanidin-3-glucosise, negatively charged ferulic acid, and neutral catechin) using Langmuir isotherms (Phan et al., 2017). The greatest selectivity occurred between negatively charged pectin containing cell wall and positively charged cyanidin-3-glucoside whereas negatively charged ferulic acid bound cellulose-based composites the least probably due to charge repulsion.

1.6. Pectin-Anthocyanin Interaction

The interaction between pectin and anthocyanin has mostly focused on the anthocyanin stability in addition of commercially available pectin. Black currant anthocyanins were stabilized in addition to different pectins (low esterified amidated, low methoxyl, and high methoxyl citrus and apple pectins, and a sugar beet pectin) at pH 3.0 and 20 ºC (Buchweitz et al., 2013a). Regardless of the pectin source and type, pectin generally improved the stability of anthocyanins. Citrus pectins stabilized anthocyanins better than apple pectin if the degree of esterification and amidation were similar; low esterified amidated pectin was a better stabilizer than low and high methoxyl pectins. Different results were observed when strawberry anthocyanins were stabilized by the pectins (Buchweitz et al., 2013b). Enhanced anthocyanins stability was observed for apple and sugar beet pectins while citrus pectins had no effect. The discrepancy of this result to previously obtained results with black currant anthocyanins was attributed to anthocyanin composition. Pelargonidin-glycosides were the most abundant consisting strawberry anthocyanins and contained less amount of hydroxyl groups than cyanidin and delphinidin.

Therefore, hydrogen bond was suggested to be occurred between pectin and anthocyanin.

The similar suggestion of hydrogen bond between pectin and anthocyanin was reported between LM citrus pectin and anthocyanin (cyanidin-3-O-glucoside and delphinidin-3-O-glucoside) studied at pH 1.5 and 4.0 by saturation transfer difference (STD) NMR spectroscopy (Fernandes et al., 2014). The study showed that pectin had a stronger binding with delphinidin-3-O-glucoside than with cyanidin-3-O-glucoside at pH 4.0. Furthermore, interaction between pectin and flavylium cation at pH 1.5 was much stronger and faster than that of between pectin and pesudobase at pH 4.0. From the same research group, the effect of LM citrus pectin on the interaction between malvidin-3-O-glucoside and (+)-catechin was also evaluated at pH 3.5 (Fernandes, Brás, Oliveira, Mateus, & de Freitas, 2016). Interestingly, pectin limited the association between malvidin and catechin.

The binding between blueberry pectin and anthocyanin was evaluated at different pH from 2.0 to 4.5 (Z Lin et al., 2016). Anthocyanins bound to WSF the least at all pH values, to CSF the most at pH 2.0-3.6, and to NSF the most at pH 3.6-4.5. Ionic interaction along with anthocyanin stacking was suggested for the binding between blueberry pectin and anthocyanin.

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Chapter 2. Blueberry Pectin Extraction Methods Influence Physico-Chemical Properties

2.1. Introduction

Berries are a valuable source rich in natural antioxidants, mostly anthocyanin. Due to the nutritional and economic aspects, blueberries are widely consumed among berries and used in a variety of food products. Consumption of blueberry has health benefits, including reduced risk of cardiovascular diseases, improved eyesight and cognition, activity as anti-diabetic, anti-carcinogenic, and anti-inflammatory agents (Routray & Orsat, 2011; Wrolstad, 2004). Anthocyanin is a endogenous, water soluble color pigment found in fruits, vegetables, flowers, and cereal grains (Wrolstad, 2004). Even though the anthocyanin has distinctive functional and nutritional values in foods, anthocyanins are unstable and easily degraded by pH, heat, light, oxygen, temperature and other environmental factors (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009).

Pectin, a soluble dietary fiber, exists in plant cell wall and middle lamella of fruits and vegetables (Voragen, Coenen, Verhoef, & Schols, 2009). It consists of three main structural domains: homogalacturonan (HG, 60-65%), rhamnogalacturonan I (RG-I, 20-35%), and substituted galacturonans such as rhamnogalacturonan II, xylogalacturonan, and apiogalacturonan (Mohnen, 2008). HG is a linear structure mainly composed of α-1,4-linked-D-galacturonic acid (UA) which can be methyl-esterified and/or acetylated. RG-I has a branched structure consisting of a backbone repeating UA and rhamnose residues, at which side chains, arabinans, galactans, arabinogalactans and other sugars are attached (Maxwell, Belshaw, Waldron, & Morris, 2012). The structure of the substituted galacturonans is highly complex

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containing a backbone of HG with various rare neutral sugar side chains (Willats, Knox, & Mikkelsen, 2006).

Even though blueberries contain pectin, there is little information on the composition or structure. A decline in dilute alkali soluble pectin and an increase in water soluble pectin were observed in ripening Bluetta blueberries (Proctor & Peng, 1989). Lowbush blueberry pectin extracted by 5% citric acid-ethanol had highest yield at 7 months frozen storage (Chen & Camire, 1997). During ripening from green to 75% surface blue color, an increase of UA was observed in water and chelator soluble pectin fractions in Duke blueberry (Vicente, Ortugno, Rosli, Powell, Greve, & Labavitch, 2007). Pectin polysaccharides from Rabbiteye were characterized; arabinose was the predominant neutral sugar in pectin fractions and chelator soluble fraction had highest UA content compared to water and sodium carbonate soluble fractions (Deng, Shi, Li, & Liu, 2012). Three pectin-rich fractions from mixed cultivar Highbush blueberry powder were extracted and the interaction with anthocyanins was measured (Lin, Fischer, & Wicker, 2016). Low temperature blanching at 60 °C, a desirable temperature to activate pectinmethylesterase, decreased the degree of esterification of pectins from blueberry purees (Chevalier, Rioux, Angers, & Turgeon, 2017).

Interaction of phenolic compound and fiber may have beneficial effects in gut health and is typically ascribed to transport of bioactives to the colon and colonic fermentation (Quirós-Sauceda, Palafox-Carlos, Sáyago-Ayerdi, Ayala-Zavala, Bello-Perez, Alvarez-Parrilla, et al., 2014). Anthocyanin containing three hydroxyl groups and at acidic pH had stronger interaction with pectin than anthocyanin with two hydroxyl groups and less acidic condition (Fernandes, Brás, Mateus, & de Freitas, 2014). Chelator soluble pectin from blueberry bound more anthocyanin at pH 2.0-3.6, than higher pH of 3.6-4.5, whereas water soluble pectin had less

binding than chelator soluble pectin at any pH (Lin et al., 2016).

The previous work by Lin et al. (2016) observed non-extractable, bound anthocyanin in blueberry water soluble pectin. The presence of the bound anthocyanin may influence estimates of binding sites and binding kinetics. Therefore, it is necessary to know the physicochemical properties and functionality of blueberry pectins. The objective of this study was to evaluate extraction methods to remove endogenous anthocyanin and to characterize physico-chemical and functional properties of blueberry pectin fractions.

2.2. Materials and Methods

2.2.1. Material

Freeze-dried blueberry powder (Tifblue/Rubel 50/50 blend) was donated by the U.S. Highbush Blueberry Council (Folsom, Ca., U.S.A.); blueberry powder is not standardized but each lot is characterized. Chemicals were obtained as described: 190 proof ethyl alcohol (Pharmco-Aaper, Brookfield, Ct., U.S.A.), acetone (EMD Millipore Corp., Billerica, Ma., U.S.A.), sodium acetate (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.), ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), sodium carbonate (Sigma-Aldrich), sodium borohydride (Sigma-Aldrich), sodium azide (EMD), acetic acid (EMD), seven monosaccharides (arabinose, fucose, glucose, galactose, mannose, rhamnose, xylose from Sigma-Aldrich), and myo-inositol (Sigma-Aldrich).

2.2.2. Extraction of blueberry pectin fractions

To obtain alcohol insoluble solid (AIS), 150 g of freeze-dried blueberry powder was mixed in 10 volumes of boiling ethanol for 10 min with stirring. The mixture was cooled to room temperature, centrifuged (Sorvall® RC 6 Plus, Thermo Fisher Scientific, Waltham, Ma., U.S.A.) at 6000 × *g*, 4 °C for 10 min, and filtered through Miracloth (EMD) under vacuum to obtain the

pellet. The pellet was washed with four volumes of ethanol, centrifuged, and filtered four times, then washed with four volumes of acetone, centrifuged, and filtered as before. The pellet was dried under the fume hood overnight and denoted as AIS.

AIS fractionation (Figure 2.1) was followed based on the modified method of Lin et al. (2016) and Deng et al. (2012). An aliquot of 40 g of AIS was dispersed in 400 mL of 50 mM

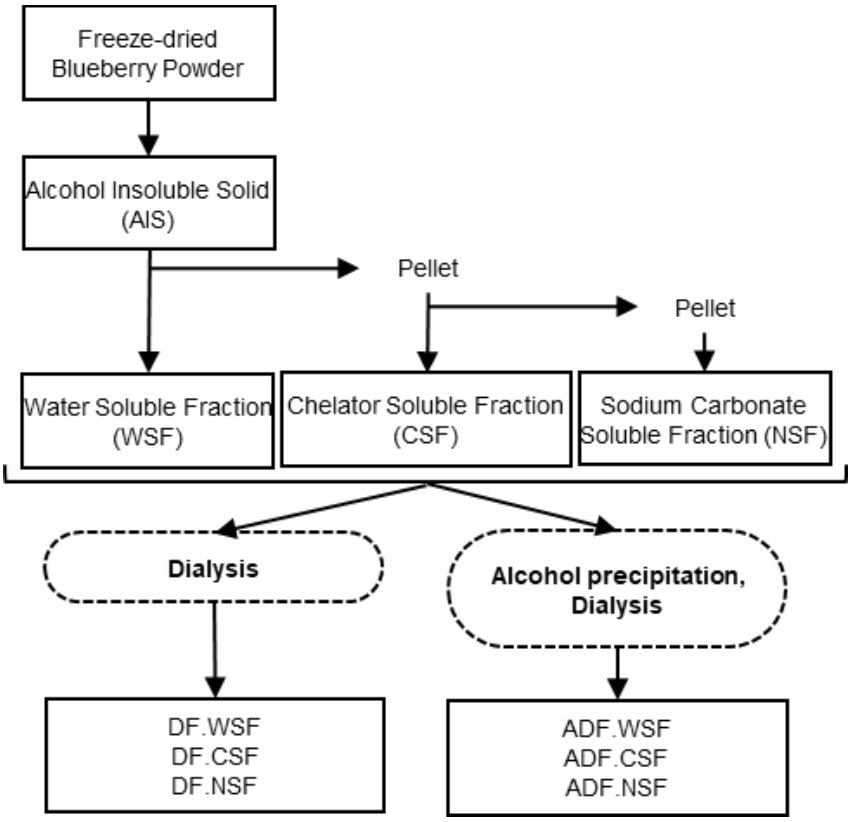

Figure 2.1. A flowchart of the blueberry pectins extraction.

sodium acetate buffer (pH 5.2) containing 0.02% sodium azide for 24 h at room temperature. The dispersion was centrifuged at 6000 × *g*, 4 °C for 20 min and filtered through Miracloth to separate supernatant and pellet. The supernatant was denoted as water soluble fraction (WSF) and stored at 4 °C. The pellet was then washed by deionized water once and dispersed in 400 mL of 50 mM sodium acetate buffer (pH 5.2) containing 50 mM EDTA and 0.02% sodium azide for 24 h at room temperature. The supernatant and pellet were separated as above. The supernatant

was denoted as chelator soluble fraction (CSF) and stored at 4 °C. The separated pellet was washed by deionized water and dispersed in 400 mL of 50 mM sodium carbonate containing 20 mM sodium borohydride and 0.02% sodium azide solution for 24 h at room temperature. After centrifugation and filtration, the supernatant was adjusted to pH 5.2 with acetic acid, denoted as sodium carbonate soluble fraction (NSF), and stored at 4 °C.

All fractions were freeze-dried for 24 h to decrease volume. The fractions were divided into two parts. One part was dialyzed (Spectra/Por®1 6-8 kD MWCO, Spectrum®, Rancho Dominguez, Ca., U.S.A.) against deionized water for 48 h and freeze-dried for 72 h. The factions were denoted as DF.WSF, DF.CSF, and DF.NSF. Another part was precipitated by three volumes of ethanol, equilibrated at room temperature for 4 h and centrifuged to obtain the pellet. The pellet was dissolved in deionized water, dialyzed against deionized water for 48 h, and freeze-dried for 72 h. The fractions were denoted as ADF.WSF, ADF.CSF, and ADF.NSF. All fractions were stored at 4 °C until analysis.

2.2.3. Degree of esterification

Degree of esterification (DE) was measured by Fourier Transform Infrared (FTIR) spectrometer (Bruker Alpha, Billerica, Ma., U.S.A.) equipped with an Alpha Pt-Diamond ATR cell. Powder was placed on the ATR and scanned from 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. A total of 64 scans were collected. After the baseline was corrected by OPUS software (Version 7.2, Bruker, Billerica, MA), peak heights at 1750 cm^{-1} and 1630 cm^{-1} were measured to calculate DE as described by Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis, and Polissiou (1998).

2.2.4. Uronic acid content

Uronic acid (UA) content was determined as described by Lin et al. (2016). Each fraction was dissolved in Type I water (ASTM D1193-91) and hydrolyzed in concentrated

sulfuric acid. An aliquot of each hydrolysate was mixed with 4 M sulfamic acid/potassium sulfamate (pH 1.6) and transferred slowly into pre-chilled sulfuric acid containing 75 mM sodium tetraborate. The mixture was incubated in the water bath at 100 °C for 20 min and cooled on ice for 10 min. After cooling, 0.15% (w/v) 3-hydroxybiphenyl in 0.5% (w/v) sodium hydroxide, was added into the mixture and equilibrated for three min for color development. Absorbance was measured by spectrophotometer (GENESYS^{TM} 20, Thermo Scientific) at 525 nm. D-(+)-Galacturonic acid monohydrate was used as the standard.

2.2.5. Protein content

Protein content was determined by Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). Each fraction was dissolved in Type I water, mixed with the Bradford reagent, and incubated at room temperature for 10 min. Absorbance was measured at 595 nm. Bovine serum albumin was used as the standard.

2.2.6. ζ–potential

ζ–potential was measured by Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK). To maintain consistent pH of sample without addition of acid or base, each fraction was dispersed in 10 mM sodium phosphate buffer, (pH 7.0), which was filtered through 0.2 μm (GH Polypro, Pall Laboratory, Mexico) (Kim & Wicker, 2009). An aliquot of each fraction was filtered through 5.0 μm Durapore® PVDF membrane filter (Millex®-SV, EMD) and directly injected into the disposable folded capillary cell (Malvern Instruments). Measurements were performed at 25 °C.

2.2.7. Anthocyanin content

Acid hydrolysis with heating was applied to release bound anthocyanin efficiently from the fiber matrix by the method of Bener, Shen, Apak, Finley, and Xu (2013). The freeze-dried

blueberry powder, AIS and fractions were dissolved in pH 1.0 deionized water, which was adjusted with hydrochloric acid. An aliquot of the hydrates was heated in a heating block at 100 °C for 15 min and filtered through 0.45 μm polyethersulfone (PES) syringe filter (Whatman, Maidstone, UK) to remove insoluble material. The high pressure liquid chromatography system consisted of Waters 2690 separation module, 996 photodiode array detector (Waters, Torrance, Ca., U.S.A.) and Luna C18 column (i.d. 250 × 4.60 mm, 5 μm, Phenomenex, Torrance, Ca., U.S.A.). Mobile phases were acetonitrile (A) and 10% acetic acid (B). The separation was performed by increasing A from 0 to 15% for 15 min, ramping A from 15 to 100% in 1 min, holding A 100% for 18 min, decreasing A from 100 to 0% in 1 min, and holding A for 5 min. The injection volume was 50 μL and the flow rate was set at 0.8 mL/min. The analysis was performed at room temperature and anthocyanin was monitored at 520 nm by Millenium32 chromatography manager (Waters). The concentration of individual anthocyanins was calculated using the calibration curve constructed from cyanidin chloride.

2.2.8. Neutral sugar composition

Each fraction was hydrolyzed in 2 N trifluoroacetic acid at 100 °C for 1 h and converted to alditol acetates by the modified method of Albersheim, Nevins, English, and Karr (1967) and Blakeney, Harris, Henry, and Stone (1983). An aliquot of 1 μL of the derivatized fraction was injected into gas chromatography-mass spectrometry (7890B-5977A MSD, Agilent Technologies, Santa Clara, Ca., U.S.A.) equipped with an Agilent J&W DB-5 column (30m × 0.15 mm, Agilent). The carrier gas was helium and the oven temperature gradually increased from 120 to 300 °C in 4 °C increment. Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc) were calculated relative to the internal standard, myo-inositol, using an individual monosaccharide standard curve. Total sugar content

was determined as the sum of the individual sugars on weight basis.

2.2.9. Molar mass (MM)

Molecular characteristics were carried out by high performance size exclusion chromatography (HPSEC) equipped with an inline degasser (Gastorr BG-34, JM Science Inc., Grand Island, Ny., U.S.A.) and 1100 HPLC system (1100 isocratic pump, 1100 autosampler, Agilent). The two detectors were coupled with the system: a Dawn Heleos multi-angle laser light scattering (LS) (Wyatt Technology Corporation, Santa Barbara, Ca., U.S.A.) and a Waters 410 differential refractometer (DRI) (Millipore, Bedford, Ma., U.S.A.). PL Aquagel-OH Guard (50 × 7.5mm, Agilent) and PL Aquagel-OH Mixed-H (300 × 7.5 mm, Agilent) columns were used, with a separation range of 6.0×10^3 to 1.0×10^7 g/mol. The mobile phase consisted of 100 mM sodium nitrate, 10 mM pH 7 sodium phosphate, and 0.02% sodium azide. The flow rate was set at 1.0 mL/min and the analysis was performed at room temperature. Each fraction (3.0 mg/mL) was dissolved in the mobile phase and filtered through 13 mm 0.45 μm polyethersulfone (PES) filter (Whatman) before injection. Peak selection was based on LS/RI signal and was held constant between samples and depicted by grey area on chromatograms. The chromatograms were analyzed by ASTRA software (Version 6.1., Wyatt) and a dn/dc value of 0.131 was used (Jung & Wicker, 2012).

2.2.10. Rheological measurement

Each fraction was dispersed at 1% (w/v), in Type I water at room temperature for 4 h by magnetic stirring and stored at 4 °C before measurement. The rheological measurement was determined using a strain-controlled rheometer (AR 2000, TA Instruments, New Castle, De., U.S.A.) equipped with a cone and plate geometry (2° cone angle, 40 mm diameter, 56 μm truncation) at 25 °C. For the viscosity, 0.6 mL of each solution was placed on the rheometer plate

and shear was increased from 0.01 to 100 s^{-1}. The power law model ($\tau = k\gamma^n$) was used for the data analysis and the flow behavior (n) were calculated using TA Data Analysis (TA instruments).

2.2.11. Statistical analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC., U.S.A.). Analysis of variance (ANOVA) was performed on the data and Duncan was used to compare the means at significant level of 0.05. The differences between the means were denoted as significant if $p \leq 0.05$.

2.3. Results and Discussion

The yield was determined gravimetrically. Based on dry weight, 31% of AIS was obtained from freeze-dried blueberry powder, which was greater than the dietary fiber content, 22%, provided by the U.S. Highbush Blueberry Council and those of AIS obtained from 6 blueberry cultivars (Chevalier et al., 2017). Mild laboratory extraction may have co-extracted pectin with different structures and other low molecular weight proteins or phenol; variance may also be attributed to different methods used. The color of the freeze-dried blueberry powder was intense purple, containing 22.6 μg/mg total anthocyanin content which was consistent with the value provided by the U.S. Highbush Blueberry Council, 21.3 μg/mg. After three-time washings, the AIS was less purple compared to the freeze-dried blueberry powder and still contained 2.5 μg/mg total anthocyanin content. Ethanol-acetone washing reduced anthocyanin content by about ten-fold but did not remove anthocyanins below the limit of detection.

The yield and physicochemical characteristics of blueberry pectin fractions after sequential fractionation by solubility and precipitation in ethanol are reported in Table 2.1. On AIS dry weight basis, DF.NSF and ADF.NSF had high yield, 5.9 and 6.9%, respectively. Less

than 4% yield of DF.WSF, ADF.WSF, DF.CSF, and ADF.CSF were obtained. At greater than 50% DE, DF.WSF and ADF.WSF are classified as high methoxyl (HM) pectins, whereas other fractions were low methoxyl (LM) pectins with DE less than 50%. The sequential buffer fractionation extracts weakly bound HM pectin by water, ionically bound LM pectin by chelators, and protopectin by weak acid or alkali solution (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011; Saulnier & Thibault, 1987). The DE of DF.WSF and ADF.WSF were 51% and 58%, respectively. Earlier, Lin et al. (2016) reported 36% DE in blueberry WSF, compared to 51 to 58% DE in this study. The low DE and solubility in buffer without chelators is unusual. The DE of DF.CSF and ADF.CSF was near 30% DE, similar to the value for CSF reported by Lin et al. (2016) and Chevalier et al. (2017). DF.NSF and ADF.NSF had the lowest DE value of 11%, reflecting the impact of alkaline sodium carbonate and saponification of methoxyl groups of pectins. The DE of water- and chelator soluble fractions, 51% and 28%, was slightly lower after dialysis, compared to DE of water- and chelator soluble fractions after alcohol precipitation and dialysis, 58% and 31%, respectively. Pectin exists in different populations and are polydisperse in molecular weight, degree of branching and total DE and distribution of charge (Willats et al., 2006). Pectin forms aggregates which are difficult to separate. In these samples ADF.WSF and ADF.CSF had higher molar mass and higher DE than DF.WSF and DF.CSF. It is possible pectin with higher molar mass and high DE formed aggregates that were not removed by dialysis. Hence, the population measured showed higher DE in ADF.WSF and ADF.CSF than DF.WSF and DF.CSF.

The uronic acid (UA) content of DF.WSF and ADF.WSF was 498 and 575 μg/mg fraction; DF.CSF and ADF.CSF were 553 and 658 μg/mg fraction; DF.NSF and ADF.NSF were 295 and 317 μg/mg fraction, respectively. Alcohol precipitation was more effective than dialysis

only, to remove non-UA. However, in each fractionation, the yield of ADF.WSF and ADF.CSF was lower than those of DF.WSF and DF.CSF by about 0.4-0.8% w/w. To estimate the UA content in AIS, the yield ratio of fraction was considered; UA content of DF.WSF and ADF.WSF was 19 and 17 μg/mg AIS. The content of UA in DF.CSF and ADF.CSF were both 18 μg/mg AIS; UA content in DF.NSF and ADF.NSF was 18 and 21 μg/mg AIS, respectively. The yield of UA depends on cultivar and ranged from 4.7-7.4 g/100 g dry weight in frozen blueberries (Chevalier et al., 2017). In frozen blueberries, the yield of UA in WSF and CSF was 70 and 30 μg/mg AIS (Vicente et al., 2007) and 85-123 and 42-73 μg/mg AIS (Chevalier et al., 2017), respectively. In this study, the yield of UA from freeze dried blueberry powder derived from mixed Tifblue/Rubel cultivar was 17-21 μg/mg AIS which was two to six times lower than previously reported by (Chevalier et al., 2017; Vicente et al., 2007).

Water- and chelator soluble fractions had a protein content around 40 μg/mg. Interestingly, sodium carbonate soluble fractions were richer in protein, 180 μg/mg. Also, DF.NSF and ADF.NSF had higher absorbance between 1570-1515 cm^{-1}, on FTIR spectra (data not shown). This region represents amide II from protein (Gannasin, Adzahan, Hamzah, Mustafa, & Muhammad, 2015). In contrast, no peaks were detected between 1570-1515 cm^{-1} in water or chelator soluble fractions, in confirmation of colorimetric protein assay.

All blueberry pectin fractions were negatively charged at pH 7.0. Water soluble fractions were least negatively charged at -18 and -19 mV; whereas chelator soluble fractions were more negatively charged than water soluble fractions at -25 and -26 mV, respectively. Significantly, ADF.NSF was the most negatively charged at pH 7.0 and ranged from -42 to -45 mV. This can be attributed to de-esterification of UA backbone by saponification in congruence with the low DE.

Alcohol precipitation is a technique to purify pectin on the industrial scale (Garna,

Mabon, Robert, Cornet, Nott, Legros, et al., 2007) and alcohol facilitated removal of anthocyanin from pectin in this study. Freeze-dried ADF.WSF was visibly lighter purple than DF.WSF; anthocyanin contents were 0.1 and 1.4 μg/mg, respectively. Freeze-dried DF.CSF and ADF.CSF had light pink color and were pale pink when dissolved in water. The anthocyanin contents of freeze-dried DF.CSF and ADF.CSF were 0.8 and 0.1 μg/mg, respectively. Thus, alcohol precipitation removed tightly bound anthocyanins from blueberry pectin fractions more effectively than dialysis alone. The anthocyanin content of WSF was reported to be 12.5 μg/mg (Lin et al., 2016). Both freeze-dried sodium carbonate soluble fractions were brown with no detectable anthocyanins (data not shown).

The total neutral sugar amounts of DF.WSF and ADF.WSF were 269 and 252 μg/mg fraction; those of DF.CSF, and ADF.CSF were 47 and 50 μg/mg fraction, and DF.NSF and ADF.NSF were 231 and 192 μg/mg fraction, respectively (Table 2.1). Neutral sugars in sodium carbonate soluble fractions were lower in alcohol precipitated fractions than dialyzed fractions ($p < 0.05$). Total neutral sugars content between DF.WSF and ADF.WSF had no difference; the same trend was observed in chelator soluble fractions (Table 2.1). The molar composition of UA and neutral sugars in blueberry pectin fractions is presented in Figure 2.2. Arabinose and galactose were the predominant neutral sugars in the fractions. Around 30% of arabinose and 7% of galactose were found in water soluble fractions with lower amount of glucose (1-2%), rhamnose (1%), xylose (1%) and mannose (< 1%). Chelator soluble fractions contained only neutral sugars of arabinose, galactose, and rhamnose. A rare monosaccharide, fucose, was found in small amount at 1%, in sodium carbonate soluble fractions. Moreover, the fractions, DF.NSF and ADF.NSF, were rich in neutral sugars, accounting for 48 and 42% of the total monosaccharides,

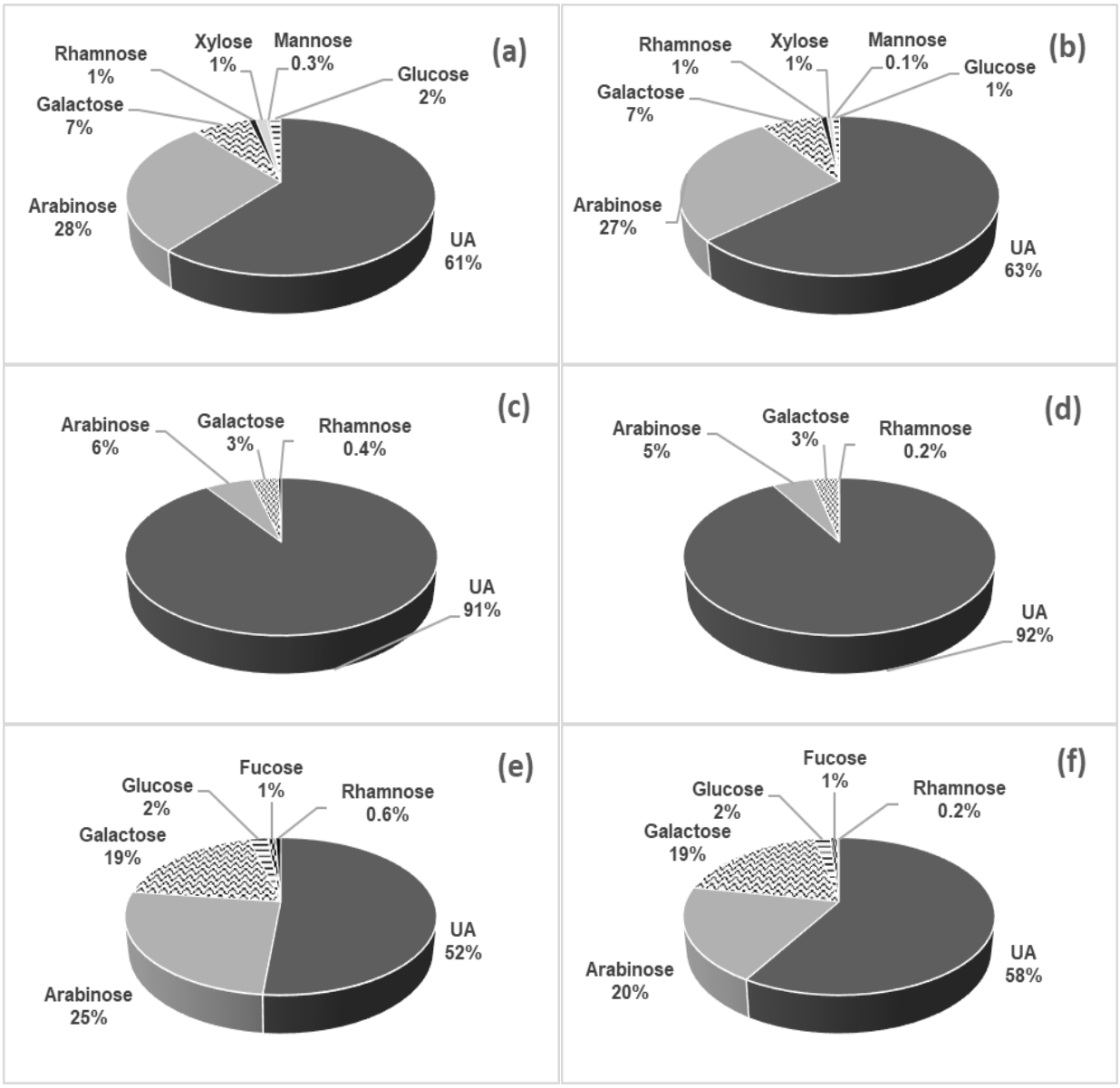

Figure 2.2. Molar ratio of uronic acid (UA) and neutral sugars of blueberry pectin fractions (a) dialyzed water soluble fraction (DF.WSF), (b) alcohol-precipitated and dialyzed water soluble fraction (ADF.WSF), (c) dialyzed chelator soluble fraction (DF.CSF), (d) alcohol-precipitated and dialyzed chelator soluble fraction (ADF.CSF), (e) dialyzed sodium carbonate soluble fraction (DF.NSF), and (f) alcohol-precipitated and dialyzed sodium carbonate soluble fraction (ADF.NSF).

and the most of them were arabinose (20-25%) and galactose (19%). Because GalA-Rha linkage is resistant to acid (Renard, Crépeau, & Thibault, 1995), rhamnose may be underestimated. In accordance with highbush blueberry in this study, arabinose was the most abundant monosaccharide in Rabbiteye blueberry (15-30%) and Duke blueberry (45-60%) (Deng et al.,

2012; Vicente et al., 2007).

The neutral sugar composition data can be further used for inference of pectin structure. 'The linearity of pectin' is suggested by the molar ratio of UA to neutral sugars; neutral sugars comprise the branched region of pectin including fucose, arabinose, rhamnose, galactose, and xylose (Houben et al., 2011). Also, the molar ratio of the sum of arabinose and galactose to UA can be used to predict the degree of neutral sugar branching. DF.WSF and ADF.WSF, had linearity values of 1.6 and 1.8, and branching of 0.58 and 0.55, respectively, which indicated that these fractions were rich in neutral sugars containing highly branched regions. Considering the high proportion of arabinose and galactose in water soluble fractions, these may be rhamnogalacturonan I (RG-I) regions which are associated with arabinan, galactan, or arabinogalactan. In contrast, chelator soluble fractions were more linear and less branched than water soluble fractions. The linearity and branching of DF.CSF was 9.6 and 0.1. Notably, ADF.CSF was the most linear with fewest neutral sugar side chains than any other fraction, presenting ratio 11.1 for linearity and 0.09 for branching. Earlier Houben et al. (2011) showed that CSF from tomato fruits had linearity of 12.8. It is likely that ADF.CSF is primarily homogalacturonan region. Whereas, sodium carbonate soluble fractions, DF.NSF and ADF.NSF, were rich in neutral sugars and had low linearity, 1.1 and 1.5, and high degree of branching, 0.87 and 0.66, respectively. The data suggests that short chains of UA backbone, with high extent of branching points, resulting from the β-eliminative depolymerization. High concentrations of arabinose and galactose in DF.NSF and ADF.NSF, in addition to the high protein content suggest the presence of arabinogalactan II, which is associated with arabinogalactan protein containing polysaccharides. The linearity of DF.WSF and ADF.WSF ranged between 1.6 and 1.8; linearity of DF.CSF and ADF.CSF was 9.6 and 11.1, respectively; the linearity of DF.NSF and ADF.NSF

was 1.1 and 1.5, respectively. UA/sugar ranged between 0.55-0.58, 0.09-0.10, and 0.66-0.87 for WSF, CSF and NSF fractions, respectively. Alcohol precipitation more effectively removed soluble sugars and other low molecular compounds and linearity tended to be higher. The linearity and the degree of branching of commercial citrus pectin were 6.6 and 0.14, while those of commercial apple pectin were 2.9 and 0.27 (Taboada, Fisher, Jara, Zúñiga, Gidekel, Cabrera, et al., 2010).

The elution profiles of blueberry pectin fractions obtained by multi-angle laser light scattering (LS) and differential refractometer (DRI) are shown in Figure 2.3. The area selected for analysis is depicted in the grey square was selected by the signal to noise of LS (ending point) and DRI (starting point) and used for the molar mass calculation. Both water- and chelator soluble fractions had high weight average molar mass with high polydispersity, as evidenced by the dissimilarities of the elution profiles between the LS and DRI detectors. The molar mass of DF.WSF and ADF.WSF was 7.01×10^5 and 2.71×10^6 g/mol, respectively; the molar mass of DF.WSF was similar to that of WSF, 6.02×10^5 g/mol, reported by Lin et al. (2016). The MM of ADF.WSF was higher than those of any other fractions, and it was atypically out of the molecular weight range for commercial pectins (8-800 kDa) (Corredig, Kerr, & Wicker, 2000). The MM of DF.CSF and ADF.CSF were 1.59×10^6 and 2.06×10^6 g/mol, respectively. Blueberry chelator soluble fraction had higher molar mass than water soluble fraction from tomato fruit (Houben et al., 2011) and blueberry (Deng et al., 2012), respectively. There was no effect of alcohol precipitation on MM of DF.CSF and ADF.CSF (Table 2.1). Sodium carbonate soluble fractions had significantly lower molar mass of 1.08×10^5 and 1.27×10^5 g/mol, respectively. Weak alkali condition likely degraded the pectin backbone and resulted in short chain of small pectin molecules. The polydispersity was above 1.7 for all pectin fractions (Table 2.1), indicating that

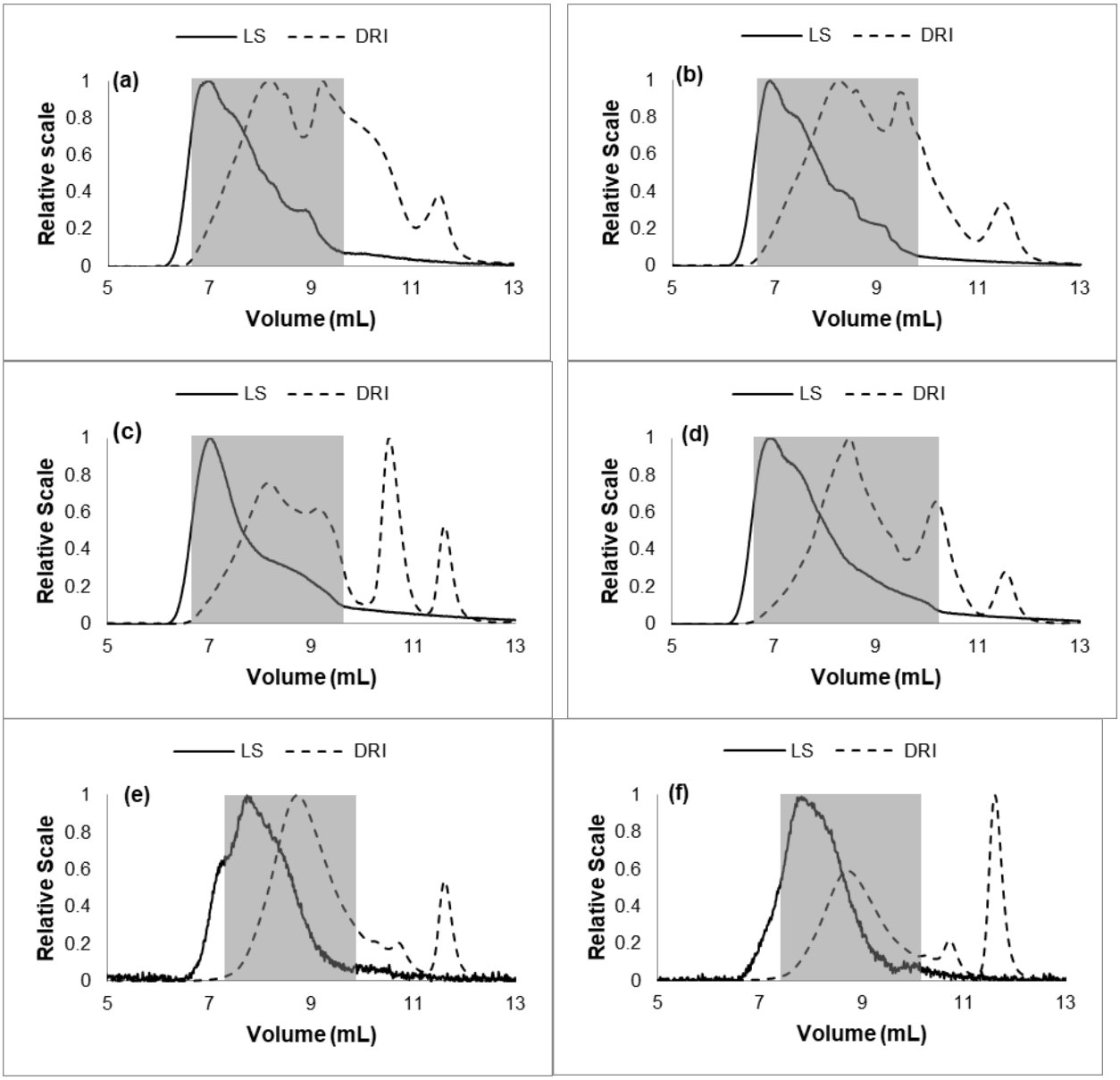

Figure 2.3. HPSEC-multi-angle laser light scattering (LS)-differential refractometer (DRI) elution profiles of blueberry pectin fractions (a) dialyzed water soluble fraction (DF.WSF), (b) alcohol-precipitated and dialyzed water soluble fraction (ADF.WSF), (c) dialyzed chelator soluble fraction (DF.CSF), (d) alcohol-precipitated and dialyzed chelator soluble fraction (ADF.CSF), (e) dialyzed sodium carbonate soluble fraction (DF.NSF), and (f) alcohol-precipitated and dialyzed sodium carbonate soluble fraction (ADF.NSF).

blueberry pectins are heterogenous. The profiles of the LS and DRI detectors indicate there is a small amount of a very large population of pectin and a larger amount of a smaller molar mass pectin (Figure 2.3).

Viscosity is an important functional property widely applied in the food industry. The

behavior of 1% blueberry pectin dispersions is depicted in Figure 2.4. The viscosities of water- and chelator soluble fractions decreased as shear rate increased between 0.1 and 100 sec^{-1}. This is a typical behavior of pseudoplastic fluid. The flow behavior (*n*) also confirms the type of viscous behavior. Newtonian fluid has the value of 1; pseudoplastic fluid ($n < 1$) and dilatant fluid ($n > 1$) (Bourne, 2002). The *n* values of DF.WSF, ADF.WSF, DF.CSF, and ADF.CSF were 0.55, 0.53, 0.80, and 0.82 which indicated that blueberry water- and chelator soluble fractions exhibited pseudoplastic behavior. Whereas, sodium carbonate soluble fractions were shear rate independent with the flow behavior of 0.98 which showed nearly Newtonian behavior.

The viscosity of DF.WSF ranged from 1683 to 154 mPa.s and that of ADF.WSF ranged 2423 to 233 mPa.s. over the shear rate of 0.1 to 100 sec^{-1}. ADF.WSF had higher viscosity compared to DF.WSF, which can be attributed to higher molar mass. Viscosity is the tendency to resist flow so that high molecular weight pectin tends to flow with more difficulty than small molecular weight pectin (Bourne, 2002). Moreover, the viscosities of blueberry water soluble fractions were much higher than those of apple and citrus pectins at the same concentration (1%, w/w) (Morales-Contreras, Contreras-Esquivel, Wicker, Ochoa-Martínez, & Morales-Castro, 2017). This suggests that blueberry water soluble fractions can be used as a thickener in high viscous foods or drinks.

The viscosity of DF.CSF and ADF.CSF ranged 167-70 and 157-79 mPa.s, respectively, in the shear rate which were lower than that of DF.WSF. This might be due to the branched structure of DF.WSF. Viscosity is also affected by the degree of branching (Yuliarti, Goh, Matia-Merino, Mawson, & Brennan, 2015). Sodium carbonate soluble fractions had the viscosities of 5 mPa.s which was close to that of water at 25 °C. The dispersions of water- and chelator soluble fractions were light blueish and turbid whereas those of sodium carbonate soluble fraction were

brown and clear.

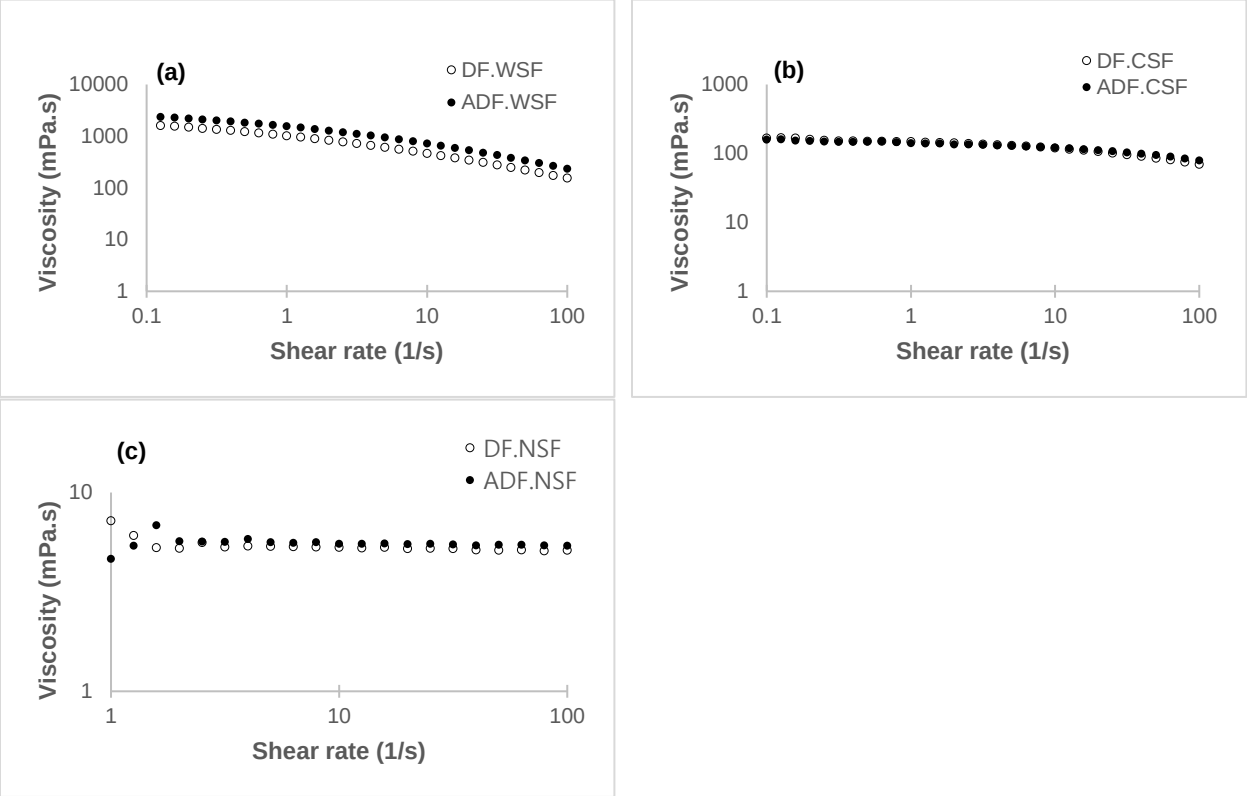

Figure 2.4. Viscosity behavior of 1% blueberry pectin dispersion as a function of shear rate (a) dialyzed water soluble fraction (DF.WSF), alcohol-precipitated and dialyzed water soluble fraction (ADF.WSF) (b) dialyzed chelator soluble fraction (DF.CSF), alcohol-precipitated and dialyzed chelator soluble fraction (ADF.CSF), and (c) dialyzed sodium carbonate soluble fraction (DF.NSF), alcohol-precipitated and dialyzed sodium carbonate soluble fraction (ADF.NSF).

2.4. Conclusion

In this study, blueberry pectins were extracted by sequential buffers and characterized. Blueberry water soluble fractions had high degree of branching, molar mass, and viscosity. Blueberry chelator soluble fractions were mainly composed of uronic acid with low neutral sugar side chains and rich in homogalacturonan region. Blueberry sodium carbonate soluble fractions were extensively branched structures rich in neutral sugars and protein, most likely arabinogalactans with arabinogalactan proteins. Alcohol precipitation was more effective than dialysis alone to remove small molecular weight compounds such as sugars, phenolic compounds. Compared to commercial citrus or apple pectin, ADF.WSF had higher viscosity and

ADF.CSF was more linear. Depending on the functionality and specific applications, blueberry pectins can be considered as potential industrial ingredients in various food products.

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Table 2.1. Yield and physicochemical characteristics of blueberry pectin fractions; dialyzed water soluble fraction (DF.WSF), alcohol-precipitated and dialyzed water soluble fraction (ADF.WSF), dialyzed chelator soluble fraction (DF.CSF), alcohol-precipitated and dialyzed chelator soluble fraction (ADF.CSF), dialyzed sodium carbonate soluble fraction (DF.NSF), and alcohol-precipitated and dialyzed sodium carbonate soluble fraction (ADF.NSF).

	DF.WSF	ADF.WSF	DF.CSF	ADF.CSF	DF.NSF	ADF.NSF
Yield (w/w, %)	3.8 ± 0.1	3.0 ± 0.1	3.2 ± 0.3	2.8 ± 0.3	5.9 ± 0.4	6.9 ± 1.3
DE (%)	51^{b}	58^{a}	27^{d}	31^{c}	11^{e}	11^{e}
UA (μg/mg)	498^{c} ± 19	575^{b} ± 26	553^{bc} ± 25	658^{a} ± 62	295^{d} ± 12	317^{d} ± 91
Protein (μg/mg)	39^{b} ± 1	39^{b} ± 2	41^{b} ± 0	39^{b} ± 2	178^{a} ± 4	178^{a} ± 3
ζ– potential(mV)	-18^{a} ± 1	-19^{a} ± 1	-25^{b} ± 1	-26^{b} ± 1	-42^{c} ± 1	-45^{d} ± 1
ACN (μg/mg)	1.4^{a} ± 0.5	0.1^{b} ± 0.0	0.8^{a} ± 0.1	0.1^{b} ± 0.0	–	–
Neutral sugars (μg/mg)						
Rhamnose	6 ± 2	6 ± 2	2 ± 0	1 ± 0	3 ± 1	1 ± 0
Fucose	–	–	–	–	5 ± 1	4 ± 1
Arabinose	177 ± 21	192 ± 10	27 ± 7	28 ± 3	113 ± 18	82 ± 4
Xylose	9 ± 1	4 ± 1	–	–	–	–
Mannose	2 ± 0	1 ± 0	–	–	–	–
Glucose	11 ± 2	7 ± 2	–	–	12 ± 0	10 ± 2
Galactose	54 ± 10	62 ± 7	19 ± 5	21 ± 4	101 ± 16	95 ± 8
Total sugars (μg/mg)	269^{a} ± 3	252^{a} ± 14	47^{d} ± 11	50^{d} ± 6	231^{b} ± 16	192^{c} ± 14
Linearity UA/(Rha+Fuc+ Ara+Xyl+Gal)	1.6	1.8	9.6	11.1	1.1	1.5
Degree of branching (Ara+Gal)/UA	0.58	0.55	0.1	0.09	0.87	0.66

(table cont'd)

	DF.WSF	ADF.WSF	DF.CSF	ADF.CSF	DF.NSF	ADF.NSF
MM (g/mol)	$7.01\times10^{5c} \pm 5.4\times10^{4}$	$2.71\times10^{6a} \pm 1.69\times10^{5}$	$1.59\times10^{6b} \pm 4.13\times10^{5}$	$2.06\times10^{6b} \pm 3.31\times10^{5}$	$1.08\times10^{5d} \pm 7.1\times10^{3}$	$1.27\times10^{5d} \pm 4.3\times10^{4}$
Polydispersity	2.1 ± 0.2	2.1 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	1.8 ± 0.2

Average ± standard deviation. Averages of yield, degree of esterification (DE), uronic acid (UA), protein, ζ-potential, anthocyanin (ACN), total sugar, and molar mass (MM) are significantly different ($p < 0.05$) if they have different superscript in the same row.

Chapter 3. Blueberry Pectin and Increased Anthocyanin Stability Under In Vitro Digestion

3.1. Introduction

Anthocyanin is a water soluble colorant naturally existing in fruits, flowers, and cereal grains, presenting orange, red, purple and blue colors (Giusti & Wrolstad, 2003). Anthocyanin reduces oxidative stress and may lower the risk of cancer, diabetes, cardiovascular and neurological disorders (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). While a potent anti-oxidant with health promoting properties, anthocyanin stability is influenced by pH, heat, light, oxygen, enzymes, and other compounds (Ongkowijoyo, Luna-Vital, & de Mejia, 2018).

Anthocyanin is composed of anthocyanidin (aglycone) and sugar moiety with structural features of an aromatic ring [A] connected to oxygen containing heterocyclic ring [C] which is bonded to another aromatic ring [B] (Castañeda-Ovando et al., 2009; Jackman, Yada, Tung, & Speers, 1987). Depending on the number of hydroxyl and methoxyl groups attached to the B-ring, anthocyanidin commonly exists in six forms; delphinidin, cyanidin, petunidin, malvidin, pelargonidin, and peonidin (Kong, Chia, Goh, Chia, & Brouillard, 2003). The structure and color of anthocyanin reversibly changes with pH change: flavylium cation (red) at pH 1-3, carbinol pesudobase and chalcone (colorless) at pH 4-5, and quinoidal anhydrobase (blue) above pH 5 (Jackman et al., 1987; Wrolstad, 2004).

Pectin, a value-added food ingredient mostly extracted commercially from citrus peel and apple pomace, is used as an emulsifying, gelling, stabilizing and thickening agents in versatile foods, beverages, confectionery/bakery products and sauces (Chan, Choo, Young, & Loh, 2017; Thakur, Singh, Handa, & Rao, 1997). Pectin is a complex polysaccharide composed of linear and branched regions (De Vries, Den Uijl, Voragen, Rombouts, & Pilnik, 1983). Homogalacturonan

(HG), a linear backbone, consists of α-1,4-linked-galacturonic acid (GalA) residues which can be methyl-esterified, carboxyl or acetylated determining the anionic nature of pectin (Voragen, Coenen, Verhoef, & Schols, 2009). Rhamnogalacturonan I and II (RGI and RGII) and xylogalacturonan (XG) are branched structures with various neutral sugar side chains and covalently connected to HG (Ellen G Maxwell, Nigel J Belshaw, Keith W Waldron, & Victor J Morris, 2012). As a soluble fiber with diverse structure, pectin exhibits health benefits including anti-carcinogenic, anti-hypertensive, positive modulator of the gut microbiome (Islamova, Ogai, Abramenko, Lim, Abduazimov, Malikova, et al., 2017; E. G. Maxwell, N. J. Belshaw, K. W. Waldron, & V. J. Morris, 2012; Wicker, Kim, Kim, Thirkield, Lin, & Jung, 2014). Moreover, pectin is stable under gastrointestinal (GI) conditions and used as a drug delivery vehicle in pharmaceutical area to carry bioactives to colon where gut microbiota readily ferment soluble dietary fibers (Jung, Arnold, & Wicker, 2013; Mueller, Jung, Winter, Rogoll, Melcher, Kulozik, et al., 2018; A Padayachee, Day, Howell, & Gidley, 2017).

Anthocyanin and pectin complexes offer several technological and health benefits. Plant cell walls containing pectin had the greatest anthocyanin binding than cellulose or cellulose-based other composites (Anneline Padayachee, Netzel, Netzel, Day, Zabaras, Mikkelsen, et al., 2012; Phan, Flanagan, D'Arcy, & Gidley, 2017). Pectin and isolated pectic fractions from sugar beet prevented the precipitation of anthocyanin-metal chelates, thus increased the anthocyanin color intensity and stability (Buchweitz, Carle, & Kammerer, 2012; Buchweitz, Nagel, Carle, & Kammerer, 2012). From the same research group, anthocyanins from black currant and strawberry were stabilized by adding different pectins from apple, citrus, and sugar beet (Buchweitz, Speth, Kammerer, & Carle, 2013a, 2013b). Binding constants between anthocyanin and pectin increase as the number of hydroxyl group on the anthocyanin B-ring increased (A.

Fernandes, Brás, Mateus, & de Freitas, 2014). The interaction between blueberry pectin anthocyanin showed that the chelator soluble fraction had strong anthocyanin binding at pH 2.0-3.6, possibly governed by electrostatic interaction and anthocyanin stacking (Z Lin, Fischer, & Wicker, 2016) and flexibility of the pectin chain (Zhuangsheng Lin, Pattathil, Hahn, & Wicker, 2019).

Pectin is fermented in the colon by gut microflora, especially, the branched regions of pectin rich in neutral sugars were selectively metabolized, produced short chain fatty acids and increased the beneficial *Bifidobacteria* population (Gulfi, Arrigoni, & Amadò, 2007; Onumpai, Kolida, Bonnin, & Rastall, 2011). Likewise anthocyanin shows functionality as a pre-biotic and as a bioactive agent with intact anthocyanin and metabolites showing activity (Chen, Li, Zhao, Zhang, Mao, Feng, et al., 2017; Faria, Fernandes, Norberto, Mateus, & Calhau, 2014; Lee, Keirsey, Kirkland, Grunewald, Fischer, & de La Serre, 2018). Anthocyanins and metabolites modulated colonic bacteria populations enhancing the growth of beneficial Bifidobacterium spp. and Lactobacillus spp. (Hidalgo, Oruna-Concha, Kolida, Walton, Kallithraka, Spencer, et al., 2012). Considering the systemic favorable effects of anthocyanin and pectin in the gastrointestinal system, the increased presence of anthocyanins stabilized by pectin and transport to the distal colon may have further synergistic effect on improving gut health.

Blueberry is a good source of anthocyanin and soluble fiber, like pectin; the interaction between blueberry anthocyanin and blueberry pectin is likely to affect anthocyanin stability and pectin functionality. The highly charged chelator soluble fraction of blueberry pectin bound anthocyanin more effectively than water soluble pectin and was attributed to electrostatic interaction between the anthocyanin flavylium and ionized carboxyl, followed by anthocyanin stacking (Z Lin et al., 2016). Yet, little is known about the anthocyanin-pectin stability under in

vitro digestion and the structural domains of pectin that facilitate anthocyanin binding. In this study, blueberry pectin has been extracted and characterized, equilibrated with anthocyanin standards and blueberry extract, and the stability has been estimated under in vitro digestion conditions. The objective of this study was to determine the effect of blueberry pectin structure on stability of three anthocyanin standards and a mixture of anthocyanins in a blueberry extract under in vitro digestion.

3.2. Material and Methods

3.2.1. Material

Freeze-dried blueberry powder (Tifblue/Rubel 50/50 blend, lot# 30711) was donated by the U.S. Highbush Blueberry Council (Folsom, CA, USA) in 2016. Pepsin from porcine gastric mucosa (Lot# SLBL1993V, ≥ 250 units/mg solid), pancreatin from porcine pancreas (Lot# SLBJ7293V, 8 × USP) and bile salts (Lot# MKBT5711V) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anthocyanins were obtained as described: Cyanidin-3-O-glucoside chloride from Chromadex (Lot# 00011606-516, Irvine, CA, USA), Malvidin-3-O-β-glucoside (Product# 1601-1) and Delphinidin-3-glucoside (Product# 1401-1) from Polyphenols (Sandnes, Norway). All other chemicals and solvents used in this study were analytical grade.

3.2.2. Blueberry pectin extraction

Blueberry pectins, water soluble fraction (WSF) and chelator soluble fraction (CSF), were obtained by previously described (Koh, Xu, & Wicker, 2018). Briefly, alcohol insoluble solid (AIS) was obtained by mixing freeze-dried blueberry powder in boiling ethanol for 10 min. The pellet was washed four times with ethanol and once with acetone to precipitate AIS. The AIS was sequentially fractionated with acetate buffer (50 mM, pH 5.2) to obtain WSF and acetate buffer (50 mM, pH 5.2) containing 50 mM ethylenediaminetetraacetic acid (EDTA) to obtain

CSF. Supernatants were collected, freeze-dried to decrease the volume, and precipitated by ethanol. The precipitated pellets were dialyzed, freeze-dried, and stored at -20 °C until analysis.

The physicochemical properties of blueberry pectins were characterized: uronic acid (UA) by colorimetric assay, degree of esterification (DE) by FTIR, ζ-potential by dynamic light scattering, molecular weight (MW) by size exclusion with light scattering and concentration detectors, bound anthocyanins content (ACN) by liquid chromatography with photodiode detector, individual and total sugar content (TS) by gas chromatography. Linearity [= UA / (rhamnose + fucose + arabinose + xylose + galactose)] and degree of branching [= (arabinose + galactose) / UA] were calculated as previously described by (Koh et al., 2018).

3.2.3. Blueberry extract

Blueberry extract (BBE) was extracted by modified method of Bener, Shen, Apak, Finley, and Xu (2013) to release bound anthocyanin efficiently from the fiber matrix. Freeze-dried blueberry powder (125 mg) was dissolved in 5 mL of acetate buffer (25 mM, pH 4.0) buffer and mixed for 10 min at room temperature. Then the mixture was heated in a water bath for 15 min at 100 °C, filtered through a 5.0 μm syringe filter (Millipore corporation, Billerica, MA, USA), and stored in a dark vial at 4 °C. BBE was prepared fresh the day before analysis.

3.2.4. Preparation of blueberry pectin and anthocyanin mixture

WSF and CSF were dissolved in acetate buffer (25 mM, pH 4.0) with stirring at room temperature to make 0.6 mg/mL. Anthocyanin standards, malvidin-3-glucoside (M3G), cyanidin-3-glucoside (C3G), and delphinidin-3-glucoside (D3G), were dissolved in Type I water (ASTM D1193-91) to make stock solutions. Each stock solution was diluted to 0.4 mg/mL with acetate buffer (25 mM, pH 4.0). Equal volumes (1000 μL) of blueberry pectin and anthocyanin for a total of 2000 μL, was mixed in a dark vial and incubated at 4 °C for 18 h. The final concentration

of blueberry pectin and anthocyanin was chosen to simulate the ratio of soluble fiber to anthocyanin provided by U.S. Highbush Blueberry Council. A control was mixed with equal volumes (1000 μL) of acetate buffer and anthocyanin and incubated as above.

3.2.5. In vitro digestion

After 18 h incubation, an aliquot (500 μL) of the blueberry pectin-anthocyanin mixture was transferred to a centrifugal filter (Amicon Ultra-0.5, NMWL: 30K, Millipore) and centrifuged at 14,000 x *g*, 4 °C for 20 min to separate free and bound anthocyanin (Z Lin et al., 2016). Incubation was timed so that an aliquot of the filtrate was injected into HPLC immediately after incubation as the initial sampling.

The simulated in vitro gastrointestinal digestion was carried out by a modified method of Anneline Padayachee, Netzel, Netzel, Day, Mikkelsen, and Gidley (2013). For the gastric digestion, an aliquot (600 μL) of the blueberry pectin-anthocyanin mixture was adjusted to pH 2.0 by adding 1 M HCl to simulate the gastric condition. Then, 30 μL pepsin solution (40 mg/mL pepsin dissolved in 0.1 M HCl) was added to the mixture and incubated in a shaking water bath (MAXQ 7000, Thermo Scientific, Marietta, OH, USA) at 100 rpm for 1 h at 37 °C. An aliquot (500 μL) of the mixture was centrifuged, filtered and injected into the HPLC as described above.

For the small intestinal digestion, a second aliquot of the equilibrated blueberry pectin-anthocyanin mixture (900 μL) underwent in vitro 1 h gastric digestion. The pH was increased 5.7 with 1 M $NaHCO_3$ and the mixture was incubated for 20 min at 37 °C. Then, 1 M NaOH was added to adjust the pH 7.0 prior to the addition of 163 μL pancreatin-bile salt solution (2 mg/mL pancreatin and 12 mg/mL bile salts dissolved in 0.1 M $NaHCO_3$). The mixture was further incubated for another 2 h at 37 °C, 6 M HCl was added to deactivate enzymes, and an aliquot (500 μL) was taken for centrifugation. The aliquot of 500 μL of the mixture was centrifuged and

the filtrate was analyzed by HPLC.

3.2.6. HPLC-PAD analysis

Anthocyanins were quantified using a high-performance liquid chromatograph (HPLC) consisting of 2690 separation module (Waters, Torrance, CA, USA) and photodiode array detector (PAD), and Luna C18 column (i.d. 250 × 4.60 mm, 5 μm, Phenomenex, Torrance, CA, USA). The injection volume was 50 μL and the flow rate was 0.8 mL/min. Mobile phases were acetonitrile (A) and 10% (v/v) acetic acid (B). The elution gradients were as described: 0-15min, 0-15% A; 15-16 min, 15-100% A; 16-34 min, 100-100% A; 34-35 min, 100-0% A; 35-40 min, 0-0% A. The analysis was performed at room temperature and anthocyanins were monitored at 520 nm by Millenium 32 chromatography manager (Waters). The concentration of anthocyanin was calculated using the calibration curve constructed from cyanidin chloride. The anthocyanin in the filtrate was denoted as free anthocyanin. Bound anthocyanin content was estimated by subtracting free anthocyanin content of treatment (with WSF or CSF) from free anthocyanin content of corresponding control.

3.2.7. Statistical analysis

Data were presented as mean ± standard deviation and in vitro simulation was conducted in triplicate. Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Two sample t-test was conducted for blueberry pectin characterization. One-way ANOVA was used to analyze the rest of anthocyanin content data with Duncan's multiple range test. Means were considered significantly different if $p < 0.05$.

3.3. Results and Discussion

3.3.1 Physico-chemical properties of blueberry pectins

The physicochemical properties of blueberry pectins indicate unique structural features

compared to commercially available citrus, apple or sugar beet pectin as well as unique properties compared to other blueberry pectins. The uronic acid (UA) content, degree of esterification (DE), ζ-potential, molecular weight (MW), bound anthocyanin (ACN) content, individual and total sugar (TS) content, linearity, and degree of branching of blueberry pectins, WSF and CSF, are summarized in Table 3.1 & 3.2. The uronic acid content for WSF and CSF was 552 and 567 μg/mg, ($p > 0.05$), respectively; values are comparable to those reported earlier (Koh et al., 2018; Zhuangsheng Lin et al., 2019). The degree of esterification for WSF and CSF was 53% and 21%, respectively, and the ζ-potential values were -21 and -26 mV, respectively. Notably, the total neutral sugar content was significantly higher ($p < 0.05$) in WSF at 313 μg/mg, than CSF at 41 μg/mg. The degree of branching as the ratio of the sum of Ara and Gal to UA (Taboada, Fisher, Jara, Zúñiga, Gidekel, Cabrera, et al., 2010), was lower in CSF than WSF (Table 3.1 & 3.2). The ratio of UA to the sum of rhamnose, fucose, arabinose, xylose, and galactose was used as an estimation of linearity (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011) and linearity was much higher in CSF than WSF (Table 3.1 & 3.2), reflecting the low neutral sugar content in CSF. The molecular weight of WSF and CSF was 2452 and 2538 kDa ($p > 0.05$), respectively, similar to that reported by Koh et al. (2018) and much higher than typical for citrus, apple or sugar beet pectins. WSF and CSF had less than 0.4 μg/mg of bound anthocyanin.

3.3.2. Pectin-anthocyanin binding

3.3.2.1. Bound anthocyanin after equilibration

The amount of bound anthocyanin for M3G, C3G, D3G or BBE after 18 h equilibration with WSF or CSF at pH 4.0 is presented in Figure 3.1. The amount bound for any of the anthocyanins was higher with CSF than WSF. M3G bound WSF and CSF at 9 and 43 μg/mL,

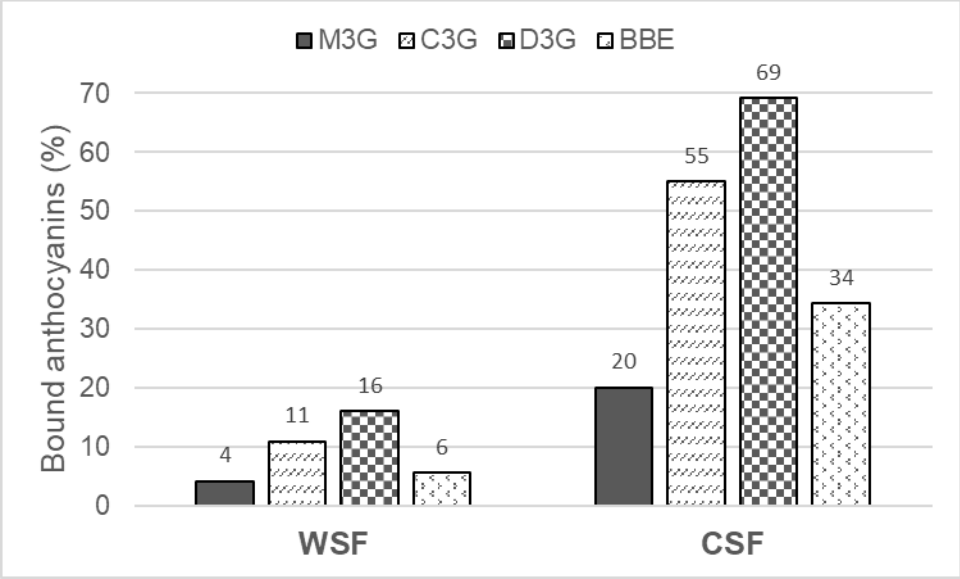

Figure 3.1. Bound anthocyanins percentage to blueberry pectin at pH 4.0. Water soluble fraction (WSF) and chelator soluble fraction (CSF). Malvidin-3-glucoside (M3G), cyanidin-3-glucoside (C3G), delphinidin-3-glucoside (D3G), and blueberry extract (BBE).

which were 4% and 20%, respectively (Figure 3.1). Approximately, five-fold more M3G bound to CSF compared to WSF during 18 h incubation at 4 °C, pH 4.0. The amount of bound C3G to WSF and CSF was 30 μg/mL (11%) and 148 μg/mL (55%), respectively; the amount bound by CSF was about five-fold higher than WSF. The amount of D3G in the no pectin control was 148 μg/mL. The amount of bound D3G to WSF and CSF was 124 μg/mL (16%) and 46 μg/mL (69%), respectively, about four times more with CSF than WSF. The amount of anthocyanins in BBE in the no pectin control was 163 μg/mL. The amount of bound anthocyanin was 9 μg/mL (6%) for WSF and 56 μg/mL (34%) for CSF. The amount of bound, mixed anthocyanins in BBE and CSF is intermediate between M3G and C3G or D3G.

The greater binding of anthocyanin by CSF than WSF was mainly attributed to the compositional difference of blueberry pectins. The linear, more negatively charged structure of CSF, compared to WSF, facilitated anthocyanin binding approximately by fivefold at pH 4.0. Pectin charge and extent of charge influence anthocyanin binding. A greater magnitude of

negative charge on pectin in plant cell walls had greater binding and less anthocyanin release under in vitro digestion, than pectin with less magnitude of negative charge (Anneline Padayachee et al., 2013; Anneline Padayachee et al., 2012). Even with pectins with a total degree of esterification less than 50%, the lower the degree of charge, the greater the propensity to bind anthocyanin (Z Lin et al., 2016). However, other factors are important, such as the total amount of galacturonic acid (Anneline Padayachee et al., 2013) and physico-chemical nature of pectin (Zhuangsheng Lin et al., 2019). CSF with high ratio of neutral sugar (Z Lin et al., 2016) or low ratio of neutral sugar (this study) showed higher binding compared to WSF.

3.3.2.2. M3G

The theoretical amount of pectin and anthocyanin in the equilibrated mixture before in vitro gastric conditions was 300 μg/mL and 200 μg/mL, respectively. The measured M3G content of the no pectin added control at 0 h, after 18 h incubation, before gastric conditions, was slightly higher at 216 μg/mL (Figure 3.2). When M3G was mixed with WSF or CSF, the measured M3G content was 207 μg/mL ($p > 0.05$) and 173 μg/mL, ($p < 0.05$), respectively, indicating that M3G was bound by WSF and CSF during 18 h equilibration.

After the simulated gastric digestion, free M3G content in no pectin added control decreased ($p < 0.05$) from 216 to 191 μg/mL. Free anthocyanin in M3G from the WSF+M3G complex decreased ($p < 0.05$) from 207 to 186 μg/mL. Free anthocyanin in CSF+M3G complex increased ($p < 0.05$) from 173 to 189 μg/mL after in vitro gastric digestion. There was no difference ($p > 0.05$) in free anthocyanin content in M3G between the no pectin control, WSF or CSF treatment groups after in vitro stomach digestion. Under gastric conditions, about 87% of the M3G remains free.

Under gastric conditions, pH 2.0, anthocyanin was transformed from chalcone/hemiketal

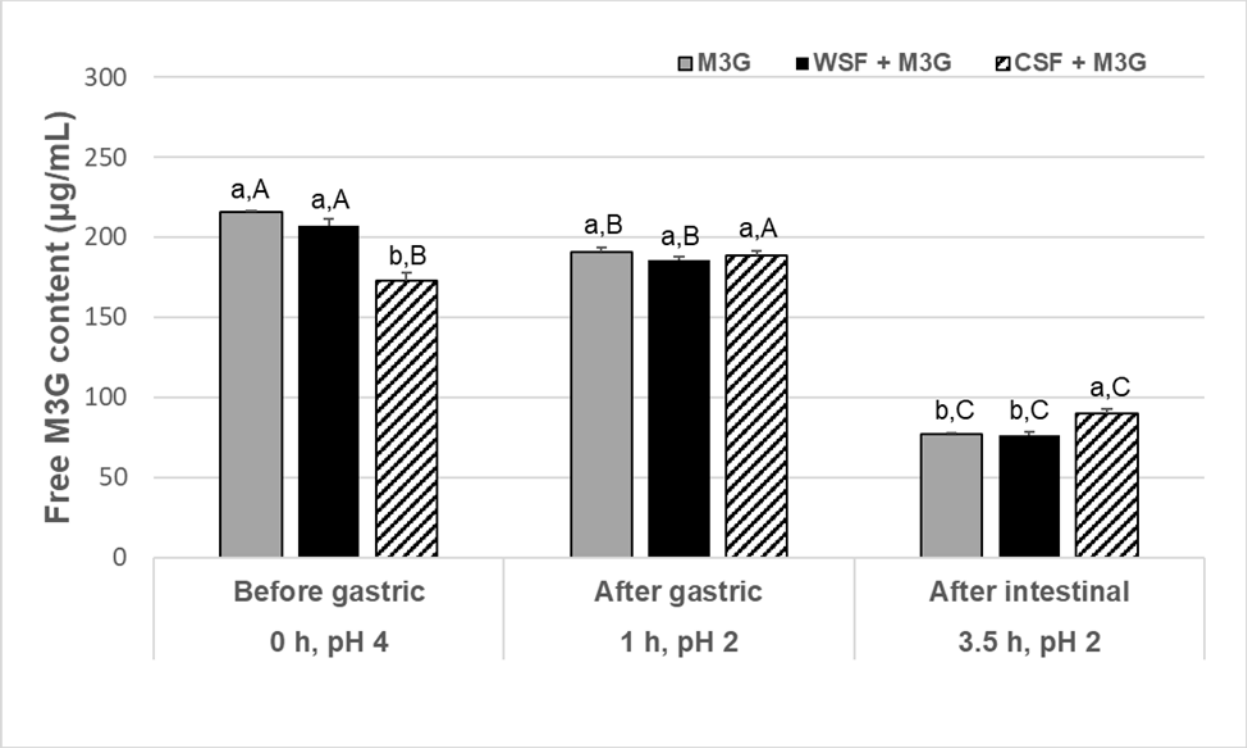

Figure 3.2. Free malvidin-3-glucoside (M3G) content measured in filtrate from blueberry pectin-M3G complex after 18 h incubation and before and after sequential in vitro digestion. Water soluble fraction (WSF) and chelator soluble fraction (CSF). Different uppercase letters denote significant differences across the in vitro digestions and different lowercase letters denote significant differences within the same digestion step. Letters denote significant difference if $p < 0.05$.

to flavylium cation form, which is relatively stable. The reduction of anthocyanin content under gastric conditions for M3G and WSF+M3G may be due to short exposure to some environmental stresses during experimental steps. The increase in CSF+M3G under gastric condition relative to the CSF+M3G before gastric condition suggests that CSF provided some protection to M3G even if the majority of M3G was not bound. The decline in free M3G in WSF+M3G indicates less protective capacity. The similar amount of free anthocyanin in M3G, WSF+M3G, and CSF+M3G under gastric conditions suggests electrostatic and non-electrostatic interactions are involved in binding. At pH 2.0, most M3G is in the flavylium form; pectin loses the net negative charge as pH decreases below the apparent pKa near 3.5 (Voragen et al., 2009). Reactive blocks of charge can be clustered on the homogalacturon region in pectin even with overall low total

charge (Kim & Wicker, 2009), which may initiate localized electrostatic interactions. Further, hydrogen bonding is possible between the pectin carboxyl group and anthocyanin hydroxyls. Anthocyanins interacted with proteins mainly through hydrogen bonds (Cahyana & Gordon, 2013; Chung, Rojanasasithara, Mutilangi, & McClements, 2016; Zhu, 2018).

After intestinal digestion, free M3G content in no pectin added control decreased from 191 to 77 μg/mL, which was a 60% reduction ($p < 0.05$). Likewise, free M3G content from WSF+M3G complex decreased from 186 to 76 μg/mL ($p < 0.05$) and that of CSF+M3G decreased from 189 to 90 μg/mL ($p < 0.05$), which were 59% and 52% decreases, respectively. No difference ($p > 0.05$) was observed on free M3G content between no pectin added control and WSF+M3G treatment group, demonstrating that WSF had no effect on preventing M3G degradation; anthocyanin content in CSF+M3G was higher ($p < 0.05$) than no pectin control or WSF+M3G. Notably, free M3G content from CSF+M3G complex after intestinal digestion was 90 μg/mL, which was significantly higher than those of M3G in control and from WSF+M3G complex, at 76-77 μg/mL ($p < 0.05$).

3.3.2.3. C3G

The impact of anthocyanin-pectin binding for C3G under in vitro gastrointestinal conditions is presented in Figure 3.3. Free C3G content in no pectin added control at 0 h before gastric digestion was 269 μg/mL. The C3G content in the filtrate from WSF+C3G and CSF+C3G complex at 0 h before gastric digestion was 239 and 121 μg/mL, respectively. During the 18 h equilibration with WSF or CSF, the free C3G decreased significantly ($p < 0.05$) with greater binding by CSF.

After the simulated gastric digestion, there was a slight decrease of free C3G content in no pectin added control from 269 to 251 μg/mL ($p < 0.05$) which was in accordance with

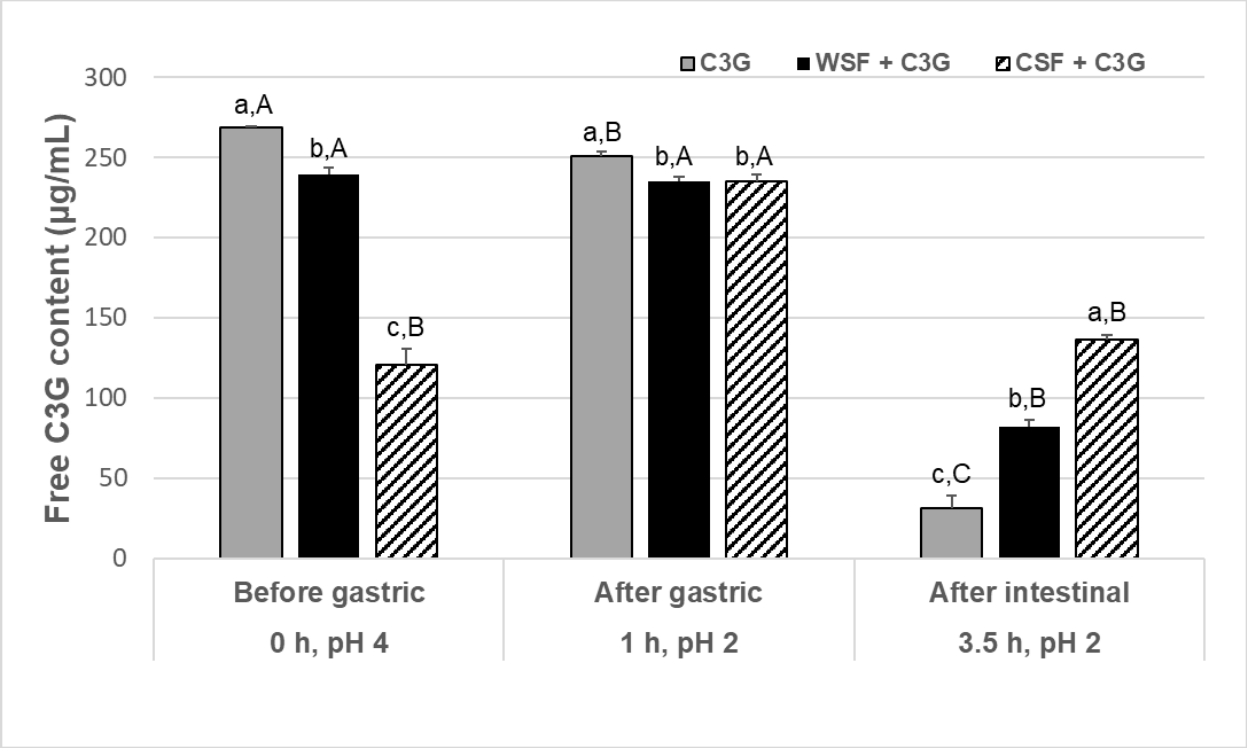

Figure 3.3. Free cyanidin-3-glucoside (C3G) content measured in filtrate from blueberry pectin-C3G complex after 18 h incubation and before and after sequential in vitro digestion. Water soluble fraction (WSF) and chelator soluble fraction (CSF). Different uppercase letters denote significant differences across the in vitro digestions and different lowercase letters denote significant differences within the same digestion step. Letters denote significant difference if $p < 0.05$.

observations of studies used C3G standard under in vitro condition (A. Fernandes, Rocha, Santos, Brás, Oliveira, Mateus, et al., 2018; Oliveira & Pintado, 2015). Free C3G content in WSF+C3G complex was similar ($p > 0.05$) between 239 and 235 µg/mL before and after in vitro gastric digestion. Free anthocyanin in CSF+C3G complex significantly increased from 121 to 235 µg/mL ($p < 0.05$). Under gastric conditions, the free C3G in no pectin control was significantly higher than free C3G in samples with WSF or CSF ($p < 0.05$) in contrast to results for M3G where no difference was observed in free M3G was observed under gastric condition.

After simulated small intestinal digestion, free C3G content in no pectin added control substantially decreased from 251 to 31 µg/mL, an 88% reduction ($p < 0.05$). A 90% decrease of C3G was previously reported earlier Oliveira et al. (2015). Free C3G content from WSF+C3G

complex decreased from 235 to 82 μg/mL and free C3G in CSF+C3G decreased from 235 to 135 μg/mL, ($p < 0.05$), which were 65% and 42% reduction, respectively. Remarkably, the free C3G content released from CSF+C3G complex after intestinal digestion was 135 μg/mL which was significantly higher than those of C3G from WSF+C3G complex and no pectin added control, 82 and 31 μg/mL, respectively ($p < 0.05$).

3.3.2.4. D3G

The result for D3G anthocyanin and pectin binding is presented in Figure 3.4. Free D3G

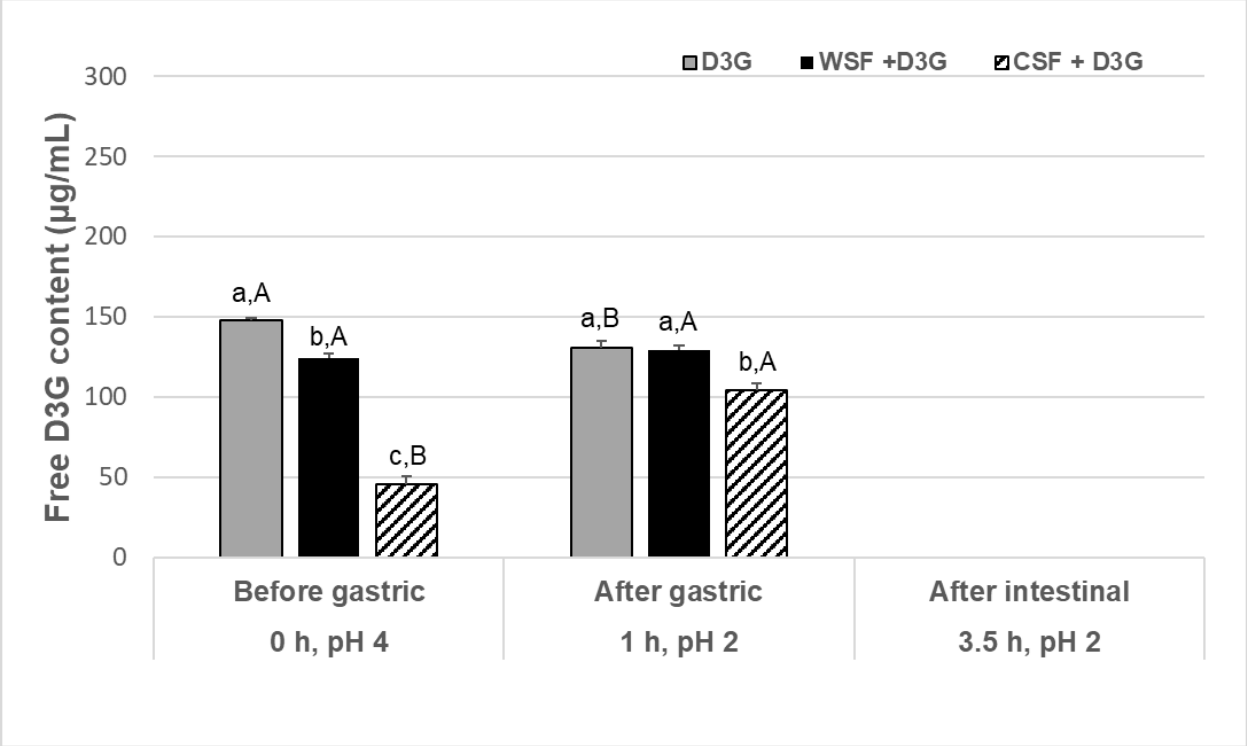

Figure 3.4. Free delphinidin-3-glucoside (D3G) content measured in filtrate from blueberry pectin-D3G complex after 18 h incubation and before and after sequential in vitro digestion. Water soluble fraction (WSF) and chelator soluble fraction (CSF). Different uppercase letters denote significant differences across the in vitro digestions and different lowercase letters denote significant differences within the same digestion step. Letters denote significant difference if $p < 0.05$.

content in no pectin added control at 0 h before gastric digestion was 148 μg/mL. The free D3G content in the filtrate from WSF+D3G and CSF+D3G complex at 0 h was 124 and 46 μg/mL, respectively. After the simulated gastric digestion, free D3G content in no pectin added control

decreased from 148 to 130 μg/mL ($p < 0.05$). Free D3G content in the WSF+D3G complex was about the same ($p > 0.05$) from 124 to 129 μg/mL before and after in vitro gastric digestion. Free D3G content in CSF+D3G complex increased from 46 to 104 μg/mL after in vitro gastric digestion ($p < 0.05$). Unlike to M3G and C3G, free D3G content in WSF+D3G after gastric condition, 129 μg/mL, was higher than that from CSF+D3G, 104 μg/mL. After simulated small intestinal digestion, no D3G was detected in the no pectin control, nor WSF or CSF treatment groups, indicating that D3G was degraded during small intestinal condition. Compared to M3G and C3G standard, D3G was less soluble and partially precipitated on the filter, which could underestimate the actual values.

3.3.2.5. Blueberry extract (BBE)

Anthocyanins from blueberry are typically composed of delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, and malvidin-3-arabinoside. Free anthocyanins in BBE content in no pectin control and WSF+BBE or CSF+BBE is shown in Figure 3.5. Free BBE content in no pectin added control at 0 h before gastric digestion was 163 μg/mL. The free BBE content in the filtrate from WSF+BBE and CSF+BBE complex after 18 h equilibration was 154 and 107 μg/mL, respectively. The results show that CSF binds anthocyanins in BBE more effectively than WSF.

After 18 h equilibration and simulated gastric digestion, the free BBE content in no pectin added control was similar ($p > 0.05$) at 163 to 172 μg/mL, respectively, indicating that gastric conditions had no effect on anthocyanins content in no pectin control. Free BBE content in WSF+BBE increased ($p < 0.05$) 154 to 172 μg/mL and free BBE in CSF+BBE increased ($p < 0.05$) from 107 to 150 μg/mL under gastric conditions. The increase in free anthocyanins

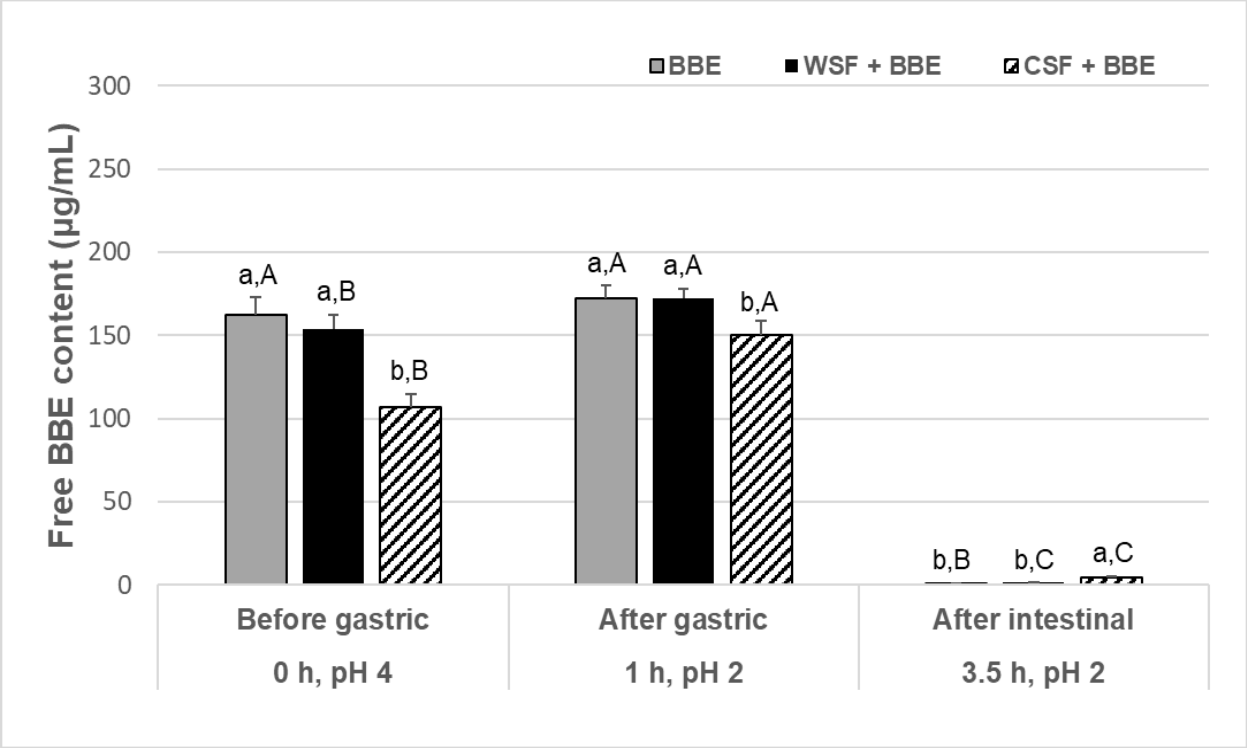

Figure 3.5. Free blueberry extract (BBE) content measured in filtrate from blueberry pectin-BBE complex after 18 h incubation and before and after sequential in vitro digestion. Water soluble fraction (WSF) and chelator soluble fraction (CSF). Different uppercase letters denote significant differences across the in vitro digestions and different lowercase letters denote significant differences within the same digestion step. Letters denote significant difference if $p < 0.05$.

suggests that the non-covalent forces involved in binding are not as strong under gastric conditions. The differences in expected mass balance are probably related to co-pigmentation.

After the simulated small intestinal digestion, free BBE content in no pectin added control decreased from 172 to 1.1 µg/mL which represented a 99% reduction ($p < 0.05$) under intestinal conditions. Likewise, free anthocyanin from BBE in the WSF+BBE complex decreased from 172 to 1.5 µg/mL ($p < 0.05$) under intestinal conditions. Free anthocyanin content in the CSF+BBE complex decreased from 150 to 4.6 µg/mL ($p < 0.05$) under intestinal conditions. The values represent 99% and 97% reduction, respectively.

The free BBE content in the CSF+BBE complex after intestinal digestion, 4.6 µg/mL, was significantly higher ($p < 0.05$) than those of BBE from WSF+BBE complex and no pectin

added control, 1.5 and 1.1 μg/mL, respectively. CSF more effectively prevented BBE degradation under gastrointestinal digestion. However, the stability of anthocyanin in BBE was lower than observed for M3G and C3G standards.

Contrary to the anthocyanin standards, BBE extracted from blueberry powder contains not only anthocyanins but also residual phenolic acids such as gallic acid, ellagic acid, protocatechuic acid, chlorogenic acid, caffeic acid, syringic acid, p-hydroxybenzoic acid, p-coumaric acid, ferulic acid, quercetin, and myricetin (Bener et al., 2013). Co-pigmentation is a complex formation between pigments and organic components which results in shift or increase absorbance (Boulton, 2001). Phenolic acids can be co-pigments that stabilize anthocyanin and increase color stability (Eiro & Heinonen, 2002). Therefore, the stability of anthocyanins in BBE content after gastric digestion was presumably due to co-pigmentation with phenolic acids. Anthocyanins content in BBE after in vitro gastric digestion is relatively stable. A 10% increase from pomegranate juice (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002), 1% decrease from bokbunja extract (Ryu & Koh, 2018), and 3% decrease from blueberry extract (Correa-Betanzo, Allen-Vercoe, McDonald, Schroeter, Corredig, & Paliyath, 2014) have been reported for anthocyanins after gastric conditions. If BBE contained phenolic compounds as well as anthocyanins, co-pigmentation might occur and prevent anthocyanins degradation under gastric condition. The amount of bound CSF after 18 h equilibration was 20%, 65%, 69% and 34% in M3G, C3G, D3G, and BBE, respectively. The amount of bound WSF after 18 h equilibration was 4%, 11%, 16% and 6% in M3G, C3G, D3G, and BBE, respectively. The binding by CSF likely conferred stability throughout the gastrointestinal simulation as evidenced by the higher anthocyanin in CSF containing extracts. Electrostatic interactions between anionic charged pectin and the flavylium cation form of anthocyanin certainly contribute to binding at some pH

values, but if electrostatic interaction is the major effect for the binding between blueberry pectin and anthocyanin, the extent of anthocyanins bound to blueberry pectin should have been constant as the number of hydroxyl group on B-ring of anthocyanin increase.

However, when additional hydroxyl group attached to the B-ring from M3G to D3G, the amount of bound anthocyanin increased. It was possible that hydrogen bond formed between hydroxyl groups of anthocyanin and carboxyl or hydroxyl groups of GalA backbone of blueberry pectin. At pH 4.0, most of anthocyanins exist in chalcone and hemiketal forms with less amount of flavylium and quinoidal (I. Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Jackman et al., 1987). Consequently, hydrogen bond might be dominant in the binding between blueberry pectin and anthocyanin along with electrostatic interaction at pH 4.0. While, ionic interaction would be dominant between blueberry pectin and anthocyanin at lower pH values since flavylium cation is prevalent at more acidic condition.

3.3.3. Color stability

Color can be used to qualify the type of anthocyanin and stability. The color of M3G, C3G, D3G and BBE in the filtrate before gastric digestion (pH 4.0) was pink and existed in hemiketal or chalcone forms primarily. After the gastric digestion at pH 2.0, the color changed to red, as the anthocyanins transformed to flavylium cation. After the small intestinal digestion at pH 7.0, M3G, C3G, D3G and BBE were dark blue and became red when HCl was added to pH 2.0. At pH 7.0, the quinoidal base form only is responsible for the blue color even though the hemiketal is dominant (Jackman et al., 1987). After small intestinal digestions, the dark blue color of M3G at 7.0 faded to light brown at room temperature slowly over time (Figure 3.6). Likewise, the dark blue of C3G faded slowly to light brown when stored at room temperature under light (Figure 3.7). The dark blue C3G after small intestinal digestion became red when

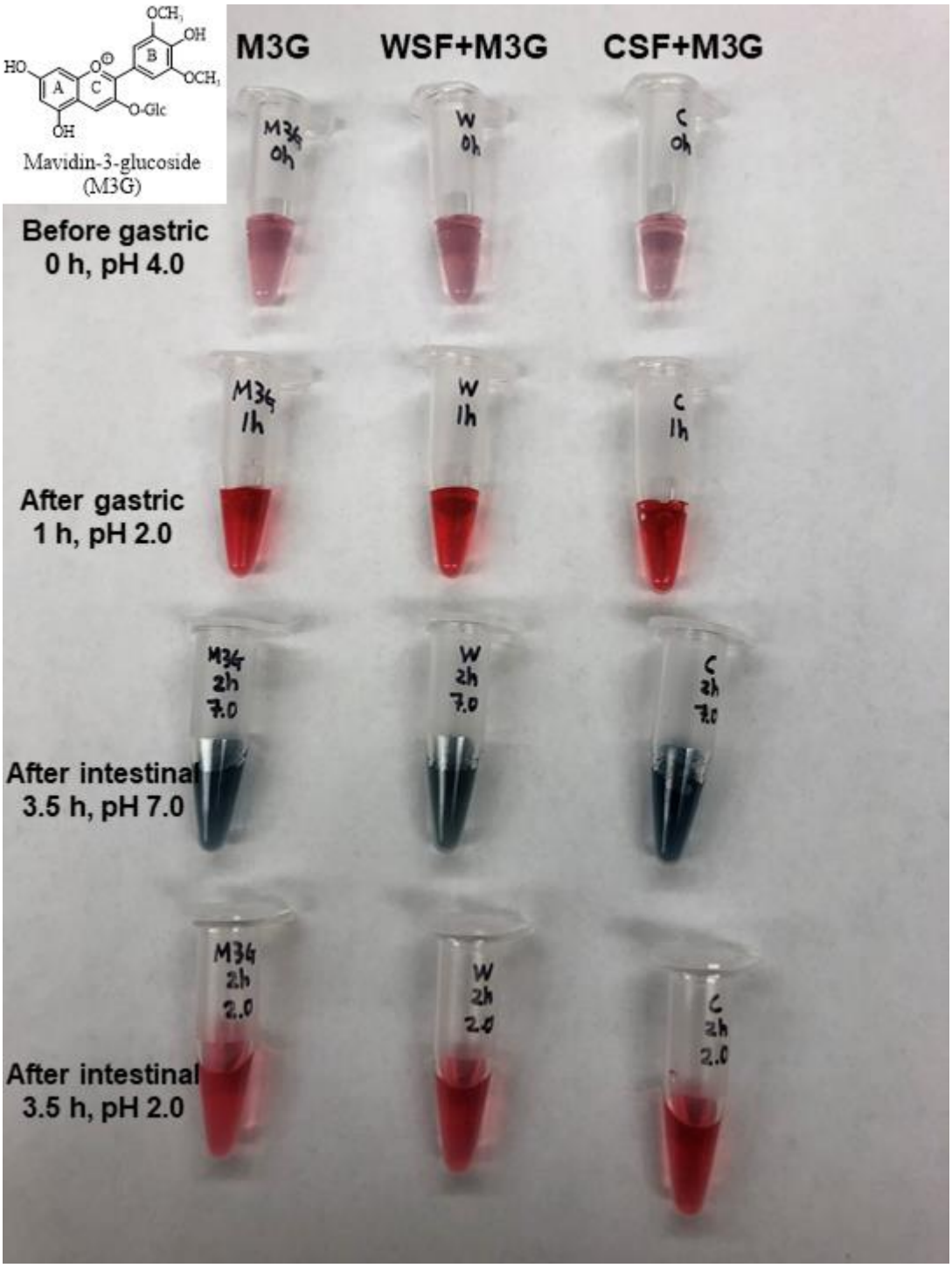

Figure 3.6. The color stability of free malvidin-3-glucoside (M3G) measured in filtrate from blueberry pectin-M3G complex after 18 h incubation and after sequential in vitro digestion.

HCl was added and the color remained red during storage at room temperature and under light.

Immediately after the small intestinal digestion at pH 7.0, the color of D3G was faint brown

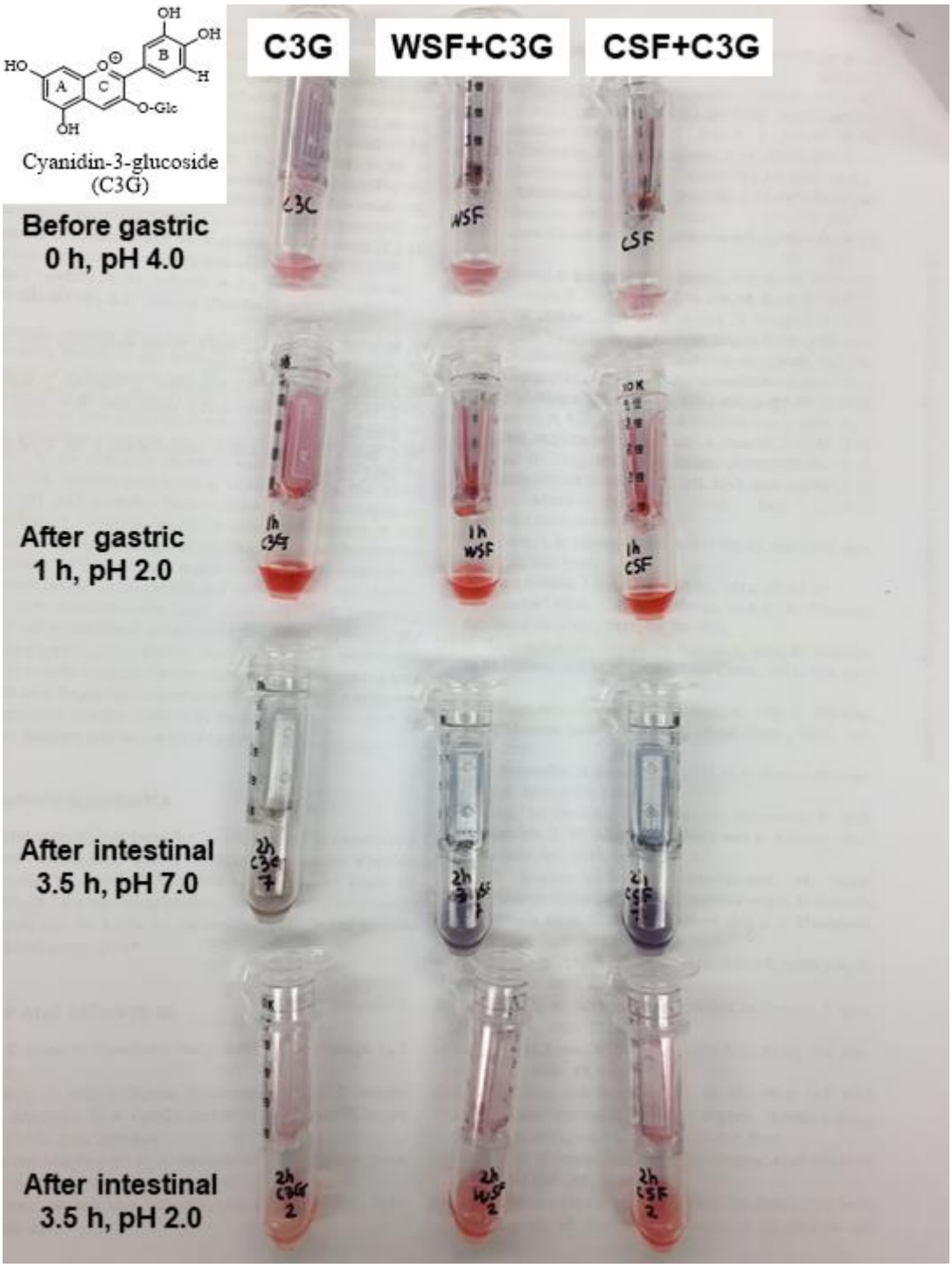

Figure 3.7. The color stability of free cyanidin-3-glucoside (C3G) measured in filtrate from blueberry pectin-C3G complex after 18 h incubation and after sequential in vitro digestion.

which confirmed D3G instability at high pH, temperature, and environment of the in vitro conditions (Figure 3.8). The D3G after small intestinal digestion was almost colorless when HCl

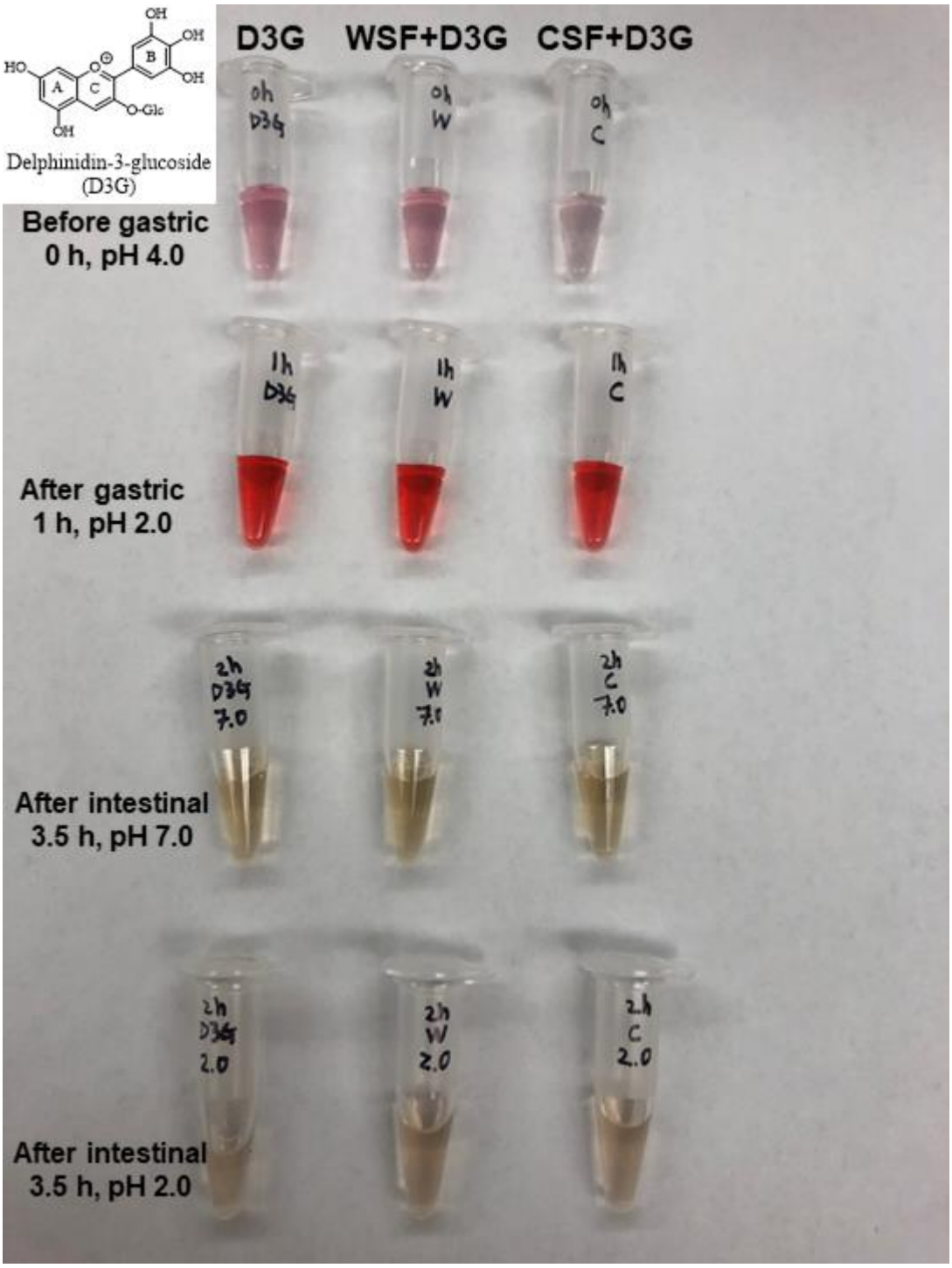

Figure 3.8. The color stability of free delphinidin-3-glucoside (D3G) measured in filtrate from blueberry pectin-D3G complex after 18 h incubation and after sequential in vitro digestion.

was added. Immediately after the small intestinal digestion at pH 7.0, BBE was brownish then it became faint pink as HCl was added at pH 2.0 (Figure 3.9). Citrus pectin improved color

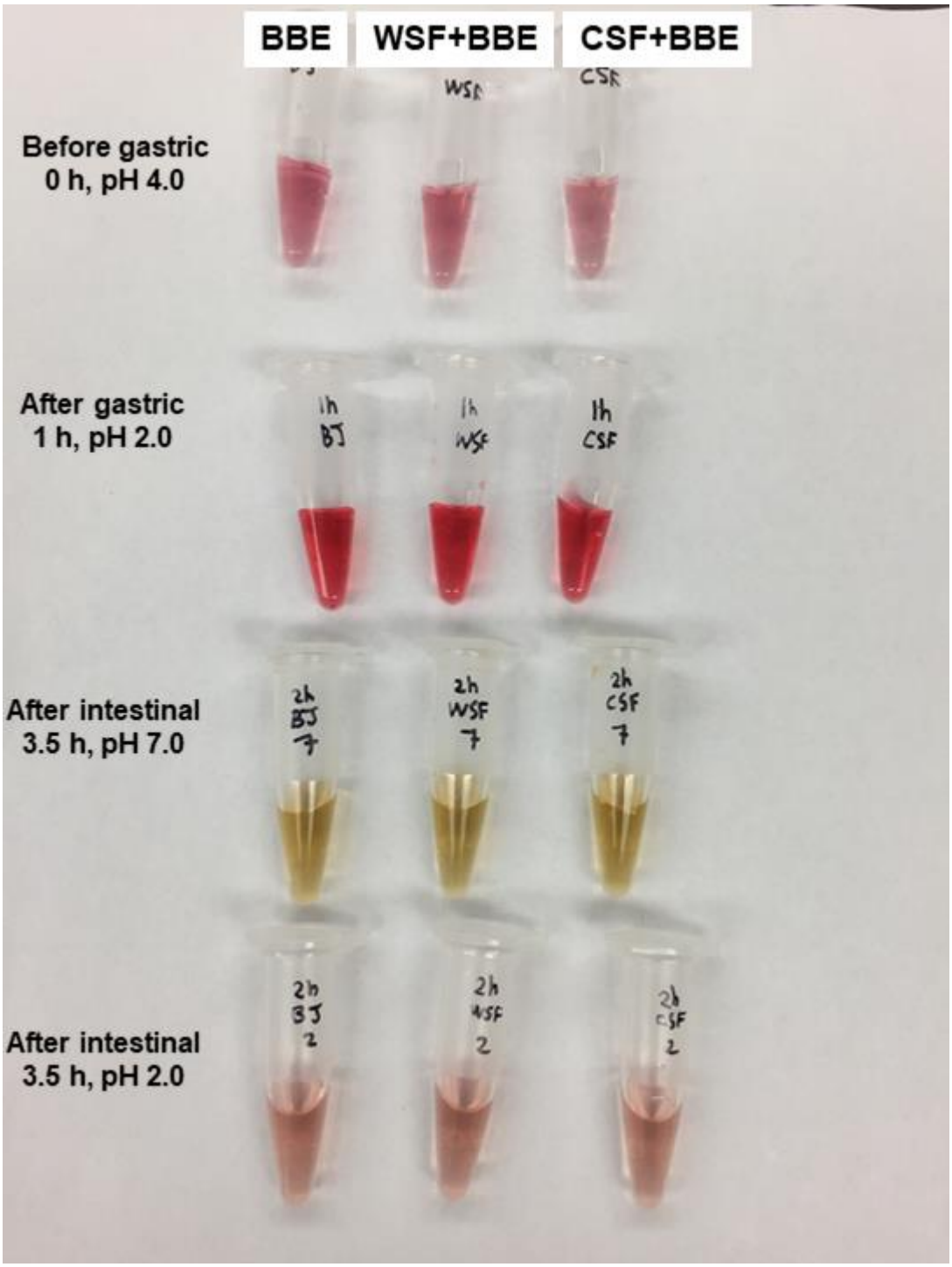

Figure 3.9. The color stability of free blueberry extract (BBE) measured in filtrate from blueberry pectin-BBE complex after 18 h incubation and after sequential in vitro digestion.

retention of delphinidin-glucoside more than cyanidin-glucoside at pH 3.0 (Buchweitz et al., 2013a). Degraded anthocyanins form insoluble brown products that do not return to red upon addition of HCl (Jackman et al., 1987).

Anthocyanins are unstable to heat, pH, light, oxygen, and enzymes (Castañeda-Ovando et al., 2009). An intrinsic factor in the instability of anthocyanin is the number of hydroxyl or methoxyl groups substituted on the B-ring. The more hydroxyl groups on the B-ring decreases anthocyanidin stability in neutral media (Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006). In the anthocyanins studied here, malvidin and cyanidin glycosides are more stable than delphinidin glycosides. Delphinidin is the least stable anthocyanin after blueberry juice processing due to the increased reactivity of three hydroxyl groups on the B-ring (Skrede, Wrolstad, & Durst, 2000). This stability ranking was in agreement with the measured M3G, C3G, and D3G contents in no pectin added control after gastrointestinal digestion, of 77, 31, and 0 μg/mL, respectively. M3G, containing two methoxyl groups and one hydroxyl group on B-ring, was the most stable during in vitro condition followed by C3G; the least stable was D3G. Also, blueberry pectin had no effect on free D3G content after small intestinal digestion, which indicated blueberry pectins did not prevent D3G degradation.

More negatively charged CSF was presumed to bind positively charged flavylium of M3G, C3G or D3G than less negatively charged WSF at stomach conditions. According to Z Lin et al. (2016), bound C3G content to WSF and CSF was about 30% and 80% at pH 2.0, respectively. However, there was no difference in free M3G or C3G content under gastric conditions between WSF or CSF, suggesting that the interaction between M3G or C3G and CSF or WSF are dependent on non-electrostatic, non-covalent interactions, such as hydrogen interaction. The 2-position of flavylium ion, which is poor in electrons is easily attacked by nucleophilic water and subsequent degradation (Castañeda-Ovando et al., 2009). CSF, rich in linear and negatively charged domains might interact with the 2-position of M3G or C3G during gastric digestion and protect from nucleophilic attack as pH increased under small intestinal

condition.

This study contributes to our understanding of the interaction of anthocyanin and pectin, both with charge and the complex inter- and intra- molecular reactions, under in vitro gastro-intestinal conditions. A pH of 4.0, the typical pH of fruits such as blueberries (A. Fernandes et al., 2014), is above the apparent pKa of pectin and pectin has a net negative charge. At pH 4.0, anthocyanin carries a positive charge and electrostatic interactions are presumed to be an initiating event in pectin-anthocyanin interaction. The non-planar nature of anthocyanin at pH 4.0 limits π-π, but hydrogen and hydrophobic interactions likely stabilize the complex. At pH 2.0, below the apparent pKa, pectin has minimal charge and any negative charge might be in localized micro-environment and be more prevalent in CSF than WSF. Although anthocyanin is in the positive flavylium cation form, minimal electrostatic interaction is expected. The more planar structure of anthocyanin at pH 2.0 promotes hydrogen and hydrophobic interactions. Some stacking of anthocyanin via π-π may be expected, but not a major contributor in the short time of the in vitro digestion. At pH 7.0, pectin is highly negative charged and CSF carries a greater charge than WSF. Anthocyanin exists in hemiketal/chalcone form and any interaction is likely to be hydrogen and hydrophobic interaction. Under the in vitro conditions of this study, bile was included in the simulated intestinal conditions. Pectin, especially high methoxyl pectin, readily binds bile (Dongowski, 1995) which may be by formation of a hydrophobic pocket (Pau-Roblot, Courtois, & Courtois, 2010). It is conceivable that bile acid and anthocyanin competed for binding sites on pectin at pH 7.0 and decrease the amount of pectin bound to anthocyanin. Both pectin and anthocyanin molecules show intra- and inter- molecular interactions that may result in aggregates, micro-gels, and other complex structures that result in physical adsorption, entrapment, self-assembly as a result of non-covalent interactions.

3.4. Conclusion

The effect of blueberry pectin on stability of anthocyanin standards and blueberry anthocyanin extract under in vitro digestion was demonstrated in this study. From the results, chelator soluble blueberry pectin prevented malvidin-3-glucoside, cyanidin-3-glucoside, and blueberry extract degradation during simulated gastrointestinal digestion, whereas water soluble blueberry pectin protected only cyanidin-3-glucoside. This is likely attributed to greater binding of chelator soluble pectin, rich in negatively charged, linear regions, to anthocyanins than water soluble pectin, rich in branched domains. Thus, pectin structure can be selectively extracted to optimize the anthocyanin interaction and functionality. Increased amount of protected anthocyanins with blueberry pectin will have a synergic effect on the colon to enhance and promote the gut microbiota fermentation. At pH 4.0, hydrogen bonding between blueberry pectin and anthocyanin likely contribute to binding in addition to ionic interaction. Binding kinetics between pectin and anthocyanin will be accessed in a future study.

3.5. References

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Table 3.1. Physico-chemical properties of blueberry pectins, water soluble fraction (WSF) and chelator soluble fraction (CSF)

	UA (μg/mg)	DE (%)	ζ (mV)	MW (kDa)	ACN (μg/mg)	Rha (μg/mg)	Ara (μg/mg)	Xyl (μg/mg)	Man (μg/mg)	Glc (μg/mg)	Gal (μg/mg)	TS (μg/mg)
WSF	$552^{a} \pm 25$	53^{a}	-21^{a}	$2452^{a} \pm 71$	0.4	6 ± 1	206 ± 24	8 ± 3	2 ± 0	12 ± 2	78 ± 1	$313^{a} \pm 21$
CSF	$567^{a} \pm 32$	21^{b}	-26^{b}	$2538^{a} \pm 145$	0.2	0.1 ± 0	25 ± 3	-	-	-	16 ± 1	$41^{b} \pm 4$

Average ± standard deviation. Averages of uronic acid (UA), degree of esterification (DE), ζ-potential, molecular weigth (MW), and total sugar are significantly different ($p < 0.05$) if they have different superscript in the same column. Abbreviation: bound anthocyanin (ACN), rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal).

Table 3.2. Linearity and degree of branching of blueberry pectins, water soluble fraction (WSF) and chelator soluble fraction (CSF)

	Linearity	Degree of branching
WSF	1.5	0.64
CSF	11.5	0.09

Linearity: UA/(Fuc+Rha+Ara+Gal+Xyl), degree of branching: (Ara+Gal)/UA

Chapter 4. Blueberry Pectin-Anthocyanin Interaction Influenced by Chemical Composition of Pectin, pH, and Ionic Strength

4.1. Introduction

Dietary guidelines for Americans emphasize benefit of consumption a variety of vegetables, fruits, and grains, more specifically vegetables and fruits from different colors and sources, and more vegetables, fruits, and grains as a whole (Health, Services, & Agriculture, December 2015). The key nutrients contributing to healthy diets are mostly likely dietary fibers and phytochemicals, such as polyphenols. Plant-based foods are essential not only contributing taste, color, and texture in food products but reducing the risk of various chronic diseases as well (Zhu, 2018). More interestingly, diets that combine dietary fibers and polyphenols had better health benefits than consuming nutrients in isolation such as improving glucose metabolism or lowering plasma and haptic lipid levels (Aprikian, Duclos, Guyot, Besson, Manach, Bernalier, et al., 2003; Awika, Rose, & Simsek, 2018; Gao, Wang, Wu, Ming, & Zhao, 2012). Due to the synergistic effects, the interaction between polysaccharides and polyphenols has gained much greater attention as functional ingredients.

In studies by various research groups, the polysaccharide-polyphenol interaction with a variety of materials show binding selectivity. Procyanidins extracted from apple, grape seed, or pear interact to various extent with polysaccharides from different type or modified apple cell walls (C Le Bourvellec, Bouchet, & Renard, 2005; C Le Bourvellec, Guyot, & Renard, 2009; Carine Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012). Cellulose-based composites interact with polyphenols of different charge (Padayachee, Netzel, Netzel, Day, Zabaras, Mikkelsen, et al., 2012; Phan, D'arcy, & Gidley, 2016; Phan, Flanagan, D'Arcy, & Gidley, 2017). Cell wall materials and proanthocyanidins extracted from grapes lose proanthocyanidin and binding capacity (Ruiz-Garcia, Smith, & Bindon, 2014). Throughout the studies, the highest

affinity is consistently observed with pectin or anthocyanin compared to other polysaccharides or phenolics.

Pectin is an anionic polysaccharide, which is mainly composed of galacturonic acid (GalA) (Voragen, Coenen, Verhoef, & Schols, 2009). The degree of esterification (DE) is estimated from the ratio of methyl-esterification relative to total sites (Ridley, O'Neill, & Mohnen, 2001). Depending on the composition, the structure of pectin is broadly categorized into linear region rich in GalA and branched region rich in neutral sugars (Thakur, Singh, Handa, & Rao, 1997). Anthocyanin is a natural color pigment composed of aromatic-heterocyclic ring connected to another aromatic ring with sugar attached (Castañeda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The distinct property of anthocyanin relative to other phenolic compounds is a structural transformation accompanied by charge change at different pH values; the flavylium cation at pH below 2 is positively charged and planar; hemiketal and chalcone forms exist at pH 4-5 without charge and non-planar structure; the quinoidal base mainly dominants in pH above 7 without charge but planar structure (Fernandes, Brás, Mateus, & de Freitas, 2014; Wrolstad, 2004).

Using saturation transfer difference NMR spectroscopy, greater binding is observed at pH 1.5 than pH 4.0 between citrus pectin and anthocyanins with two or three hydroxyl groups; the interaction is greater with 3-OH groups than 2-OH groups (Fernandes et al., 2014). From the same research group, anthocyanin-(+)-catechin association with/without citrus pectin, reveals that pectin might compete with phenolic association by decreasing the binding constant (Fernandes, Brás, Oliveira, Mateus, & de Freitas, 2016). Blueberry pectin-anthocyanin binding varies depending on the pectin fraction and pH; chelator soluble fraction binds anthocyanins more at pH 2.0-3.6, whereas water soluble fraction binds the least at all pH values (Lin, Fischer,

& Wicker, 2016).

The pectin-anthocyanin interaction is broadly used to increase the benefits of anthocyanins by improving stability. Citrus, apple, sugar beet pectins enhance the stability of anthocyanins from blackcurrant and strawberry, which favorably applies to anthocyanin containing foods and beverages (Buchweitz, Nagel, Carle, & Kammerer, 2012; Buchweitz, Speth, Kammerer, & Carle, 2013a, 2013b). Pectin is also used to encapsulate anthocyanin in hydrogels and control release (Mueller, Jung, Winter, Rogoll, Melcher, Kulozik, et al., 2018). The increased bioavailability of anthocyanin may modulate gut microbiota profile increasing the number of beneficial bacteria (Lee, Keirsey, Kirkland, Grunewald, Fischer, & de La Serre, 2018; Molan, Lila, Mawson, & De, 2009).

However, little is known about the binding site of anthocyanin onto pectin and the environment factors affecting the interaction. In this study, the extraction of two blueberry pectins, water- and chelator soluble fraction, and incubation with anthocyanins from freeze-dried blueberry powder is described. The objectives of the study are 1) to investigate the impact of blueberry pectin composition on anthocyanin adsorption and 2) to assess environment factors on the interaction between blueberry pectin and anthocyanin. From the result, bound or release conditions of pectin-anthocyanin complexes is suggested. It is important to better understand the pectin-anthocyanin interaction in food and pharmaceutical areas where pectin binding, anthocyanin delivery, or electrostatic-based technology are used.

4.2. Material and Methods

4.2.1. Material

Freeze-dried blueberry powder (Tifblue/Rubel 50/50 blend, lot# 30711) was donated by the U.S. Highbush Blueberry Council (Folsom, CA, USA) in 2016. Chemicals were obtained as

described: acetic acid (EMD Millipore Corp., Billerica, MA, USA), sodium acetate (Sigma-Aldrich Corp., St. Louis, MO, USA), citric acid and sodium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ, USA). All other chemicals and solvents used in this study were analytical grade.

4.2.2. Blueberry pectin extraction and characterization

Blueberry pectins, water soluble fraction (WSF) and chelator soluble fraction (CSF), were extracted from freeze-dried blueberry powder by modified method of (Koh, Xu, & Wicker, 2018). First, alcohol insoluble solid (AIS) was obtained from freeze-dried blueberry powder by 10 min boiling in ethanol, washing in ethanol four times and acetone once. Then, the AIS was sequentially fractionated by acetate citrate phosphate (50 mM, pH 5.2) to obtain water soluble fraction (WSF) and acetate buffer (50 mM, pH 5.2) containing 50 mM ethylenediaminetetraacetic acid (EDTA) to obtain chelator soluble fraction (CSF). Supernatants were collected, freeze-dried, and precipitated by ethanol. Then the precipitated pellets were dialyzed, freeze-dried, and stored at -20 ºC until analysis. The physicochemical properties of blueberry pectins were characterized: uronic acid (UA) by colorimetric assay, degree of esterification (DE) by FTIR, ζ-potential by dynamic light scattering, molecular weight (MW) by size exclusion with light scattering and concentration detectors, bound anthocyanins content (ACN) by liquid chromatography with photodiode detector, individual and total sugar content (TS) by gas chromatography, and centrifugal membrane separation. Linearity [= UA / (rhamnose + fucose + arabinose + xylose + galactose)] and degree of branching [= (arabinose + galactose) / UA] were calculated as previously described (Koh et al., 2018).

4.2.3. Adsorption studies influenced by endogenous factors (pectin composition and concentration)

4.2.3.1. Blueberry extract preparation

Blueberry extract (BBE) was prepared by modified method of Bener, Shen, Apak, Finley, and Xu (2013). Freeze-dried blueberry powder was dissolved in acetate buffer (25 mM, pH 4.0) for 10 min at room temperature and heated in a water bath at 100 ºC for 15 min. Then the mixture was centrifuged, and the supernatant was filtered through 5.0 µm syringe filter (Millipore Corporation, Billerica, MA, USA) and stored in a dark vial at 4 ºC. BBE was prepared fresh the day before analysis.

4.2.3.2. Adsorption experiment

For timed binding (adsorption kinetics), WSF or CSF were dissolved in 25 mM acetate buffer (pH 4.0) overnight to make concentration of 0.6 mg pectin/mL. BBE was diluted to make concentration of 0.4 mg anthocyanins/mL by 25 mM acetate buffer (pH 4.0). Then, equal volume of pectin dispersion and BBE were mixed in a dark vial with a Vortex Genie and incubated at 4 ºC for different time intervals from 1 min to 48 h. Control was mixed with equal volume of 25 mM acetate buffer (pH 4.0) and BBE.

For concentration binding (adsorption isotherm), an equal volume of 0.6 mg/mL WSF and CSF dispersion in 25 mM acetate buffer (pH 4.0) was mixed with 0.2-1.8 mg/mL BBE, incubated at 4 ºC for 6 h and 48 h. Control was mixed with equal volume of 25 mM acetate buffer (pH 4.0) and corresponding BBE. After incubation time, an aliquot of the mixture was transferred to centrifugal filter (Amicon ultra-0.5, NMWL: 30K, Millipore) and centrifuged at 14,000 x *g*, 4 ºC for 20 min. Free and bound anthocyanins were separated and the free anthocyanins content obtained in the filtrate was quantified by HPLC. Bound anthocyanins content was calculated by subtracting free anthocyanins content of experimental group (containing WSF or CSF) from free anthocyanins content of corresponding control group.

The Langmuir isotherm, a widely used adsorption isotherm, makes some assumptions: a

monolayer adsorption, uniform binding site of adsorbent and no interaction between adsorbates (Tu, Pan, & Saddler, 2009). The Langmuir isotherm was applied to fit the experimental data by following equation;

$$q_e = \frac{q_{max} \cdot K_L \cdot C}{(1 + K_L \cdot C)} \quad (1)$$

q_e is the amount of bound anthocyanins (μg/mg pectin), q_{max} is the total amount for available binding site or saturation level (μg/mg pectin), K_L is an affinity constant (mL/μg), C is free anthocyanins concentration (μg/mL).

4.2.4. Adsorption studies influenced by exogenous factors (pH and ionic strength)

4.2.4.1. Blueberry extract preparation passed through a C18 column

Blueberry extract passed through a C18 column (C18BBE) was prepared by modified method of Ronald E. Wrolstad (2005). Freeze-dried blueberry powder (1000 mg) was dissolved in 7 mL of Type I water (ASTM D1193-91) and mixed for 30 min at room temperature; 8 mL of acetone was added and mixed for another 30 min at room temperature. Insoluble freeze-dried blueberry powder was separated by filtering the mixture through Whatman no. 1 filter paper. One volume of chloroform was added into the filtrate and the upper portion of phase was separated by centrifugation. The collected upper portion was evaporated under nitrogen gas and re-dissolved in acidified Type I water. The extract was passed through a C18 cartridge (Waters, Milford, MA, USA) to remove sugars, acids, non-anthocyanin phenolics, evaporated under nitrogen gas, re-dissolved in acidified Type I water, and denoted as C18BBE. C18BBE was stored in a dark vial at 4 ºC and prepared fresh the day before analysis.

4.2.4.2. Adsorption experiment

Citrate-phosphate was used to prepare 0.6 mg pectin/mL CSF and hydrated overnight at room temperature. Citrate-phosphate at 25 mM, 100 mM, or 200 mM, was used to maintain

buffering capacity over the pH range of 2.6-4.0 and constant ionic strength (Segel & Segel, 1976). C18BBE was diluted to 0.4 mg/mL by corresponding buffers. An equal volume of CSF and C18BBE was mixed in a dark vial with vortex and incubated at 4 °C for different time intervals from 1 min to 24 h. A no pectin control was mixed with equal volume of corresponding citrate-phosphate buffer and C18BBE. After incubation time, and free and bound anthocyanins were estimated as above.

4.2.5. HPLC-PAD analysis

Anthocyanins content was quantified using a high-performance liquid chromatograph (HPLC) consisting of 2690 separation module (Waters, Torrance, CA, USA) and photodiode array detector (PAD), and Luna C18 column (i.d. 250 × 4.60 mm, 5 μm, Phenomenex, Torrance, CA, USA). The injection volume was 50 μL and the flow rate was 0.8 mL/min. Mobile phases were acetonitrile (A) and 10% (v/v) acetic acid (B). The elution gradients were as described: 0-15min, 0-15% A; 15-16 min, 15-100% A; 16-34 min, 100-100% A; 34-35 min, 100-0% A; 35-40 min, 0-0% A. The analysis was performed at room temperature and anthocyanins were monitored at 520 nm by Millenium32 chromatography manager (Waters). The concentration of anthocyanins was calculated using the calibration curve constructed from cyanidin chloride.

4.2.6. Statistical analysis

Experiments were conducted in triplicate and average ± standard deviation were reported. The data of adsorption isotherm was fitted by Langmuir equation (Eq. (1)) using curve fitting toolbox from Matlab R2018a version 9.4.0.813654 (Mathworks, Natick, MA, USA). Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Two sample t-test was conducted for blueberry pectin characterization and apparent isotherm parameters. One-way ANOVA was used for binding experiment. Statistical significance was

performed by Tukey at significance of 0.05 ($p < 0.05$).

4.3. Results and Discussion

Unique structural features of blueberry pectins from the physicochemical properties of blueberry pectins are summarized (Table 3.1 & 3.2). There are no significant differences in the uronic acid content and molecular weight between WSF and CSF ($p > 0.05$). However, considerable differences are observed in the degree of esterification, ζ-potential, and total sugar content of WSF and CSF ($p < 0.05$). WSF is high methoxyl pectin, less negatively charged, and has an abundance of neutral sugars whereas CSF is low methoxyl pectin, more negatively charged, and low in neutral sugars. Moreover, in aspect of structure, the high extent of branching in WSF (linearity: 1.5, degree of branching 0.64) and high extent of linearity in CSF (linearity: 11.5, degree of branching 0.09) possibly suggests that WSF is more highly branched, and CSF has a more linear structure. The number of theoretical binding sites of anthocyanins on the blueberry pectin was estimated from the mole ratio of pectin to anthocyanin. Considering the range of molecular weight for pectin of 2452 kDa (WSF) and 2538 kDa (CSF) and anthocyanins between 287 (cyanidin) and 331 (malvidin) Da, the binding sites of WSF and CSF for anthocyanins, theoretical number of binding sites was estimated at 7500-8700. This estimation is based on a number of assumptions including accessibility of sites, no aggregation of pectin or stacking of anthocyanin, no steric constraints from substitutions on the homogalacturon backbone. Clearly, theoretical and measured binding sites are different, so additional factors are involved in binding. From the theoretical calculation of binding sites of anthocyanins on the blueberry pectin, no difference of binding sites was expected between WSF and CSF. However, CSF clearly bound more anthocyanin than WSF. Increase in neutral sugar branches of pectin was reported to have compact conformation (Alba, Bingham, & Kontogiorgos, 2017). Therefore,

WSF is likely to be more compact than CSF and presumptively, has fewer accessible binding sites than CSF.

4.3.1. Adsorption studies influenced by endogenous factors (pectin composition and concentration)

4.3.1.1. Adsorption kinetics

Figure 4.1 shows the anthocyanins adsorption to blueberry pectin at fixed anthocyanins concentration for different time intervals from 1 min to 48 h, at pH 4.0, the typical pH value of fruit and vegetable (Fernandes, Rocha, Santos, Brás, Oliveira, Mateus, et al., 2018).

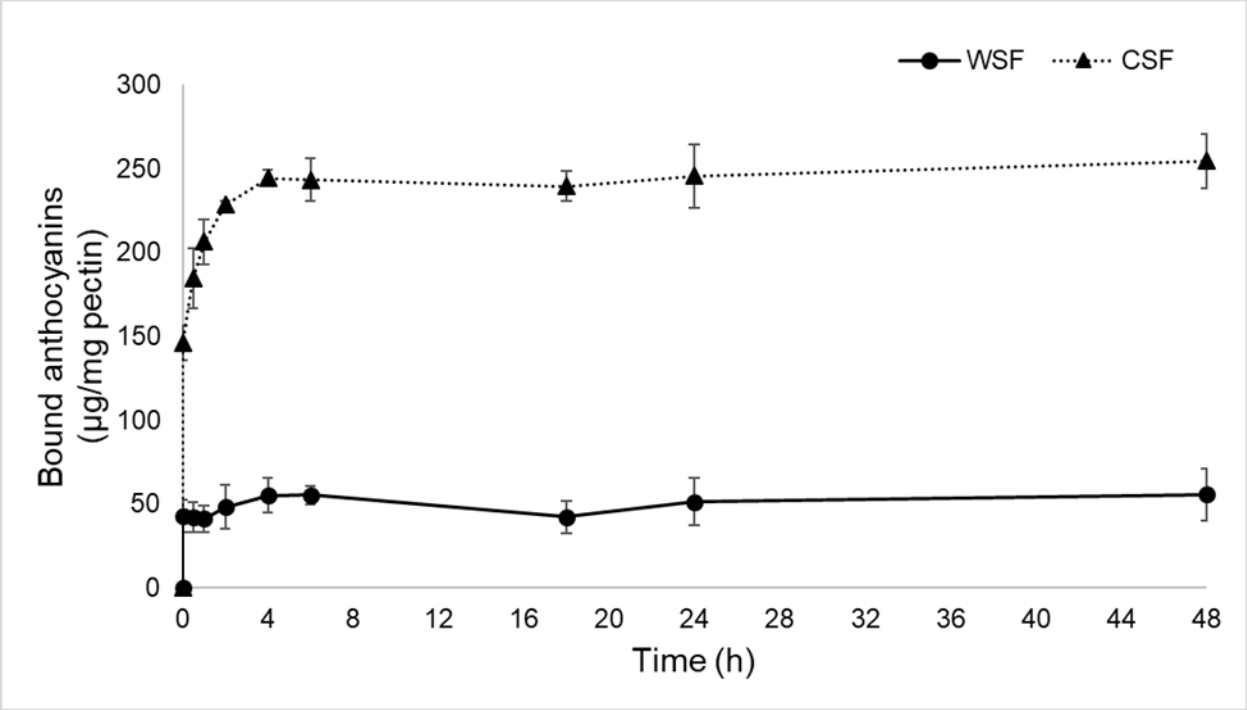


Figure 4.1. The adsorption of anthocyanins from blueberry extract to blueberry pectins, WSF and CSF, at pH 4.0 and different time intervals up to 48 h.

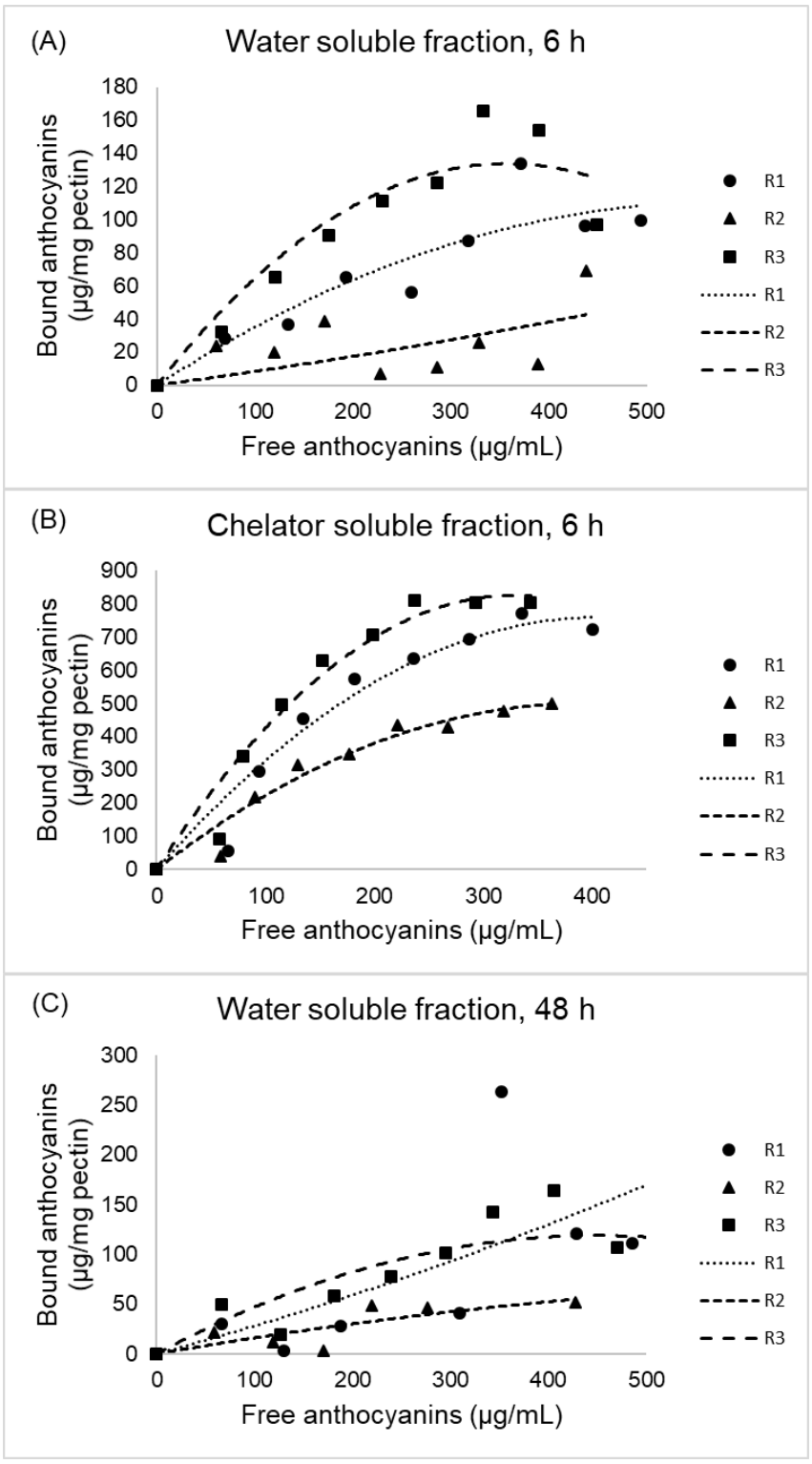
Bound anthocyanins are expressed per unit pectin mass. Different adsorption behavior is observed by blueberry pectin with different chemical composition. For WSF, the anthocyanins adsorption occurs rapidly and the binding plateaus at 1 min. While, anthocyanins binding to CSF occurs relatively slow until 4 h contact time with gradually increasing bound anthocyanins

content. Based on this result, the contact time of 6 h is used for further comparisons. While up to 83% of anthocyanin is depleted after 12 days (Padayachee et al. (2012), an apparent increase of bound anthocyanin content during 48 h extended incubation is not observed. Considering the similar pH values (4.0-4.3) conducted in both studies, most likely the incubation time of 48 h in this study is not enough to observe the anthocyanin stacking.

The pectin composition/structure influences considerably the behavior of anthocyanin adsorption. At 1 min of contact, WSF has 43 μg of bound anthocyanins per mg pectin whereas CSF has 146 μg of bound anthocyanins per mg pectin. At the beginning of equilibrium point, 6 h, bound anthocyanins to CSF, 243 μg/mg pectin, is four times higher than that of WSF, 55 μg/mg pectin ($p < 0.05$). Similar findings by (Lin et al. (2016) reports that CSF binds 30-60 % anthocyanin amount between pH values of 4.5-3.6. In this study, a similar percentage of 50% anthocyanins binds CSF at pH 4.0 (data not shown). WSF binds 30-40% anthocyanin (Lin et al. (2016) versus to 10% bound in this study. An earlier study shows that binding of anthocyanin by WSF is highly variable compared to CSF (Koh, in vitro study). The lower amount of anthocyanins bound to WSF is supported by the previous research who show that branched structure of pectin, rich in neutral sugar side chains, limited the interaction with procyanidins (Watrelot, Le Bourvellec, Imberty, & Renard, 2014). This result indicates that a linear structure of CSF with less neutral sugar side chains facilitates the interaction with anthocyanins and WSF, rich in branched regions, interacts less.

4.3.1.2. Adsorption isotherm

The adsorption of bound anthocyanins to blueberry pectins is plotted as a function of free anthocyanins concentration and the Langmuir isotherm is used to describe the interaction (Figure 4.2). Because of the difficulty defining data deviation, data of three replications is


(A)
Water soluble fraction, 6 h
Bound anthocyanins (µg/mg pectin)
Free anthocyanins (µg/mL)
R1
R2
R3
R1
R2
R3
(B)
Chelator soluble fraction, 6 h
Bound anthocyanins (µg/mg pectin)
Free anthocyanins (µg/mL)
R1
R2
R3
R1
R2
R3
(C)
Water soluble fraction, 48 h
Bound anthocyanins (µg/mg pectin)
Free anthocyanins (µg/mL)
R1
R2
R3
R1
R2
R3

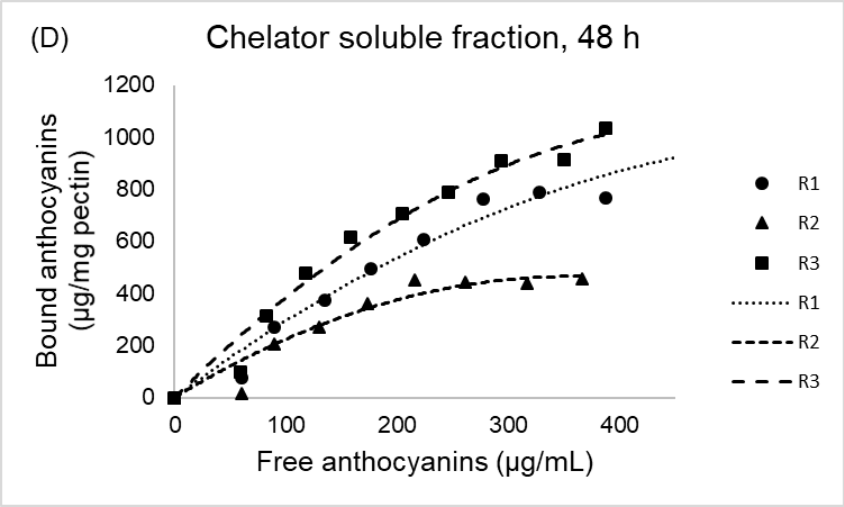


Figure 4.2. Langmuir binding isotherms for blueberry pectins and anthocyanins at pH 4.0 and 4 ºC as a function of free anthocyanins concentration. (A) Water soluble fraction at 6 h incubation time, (B) chelator soluble fraction at 6 h incubation time, (C) water soluble fraction at 48 h incubation time), and (D) chelator soluble fraction at 48 h incubation time.

presented in the figures. The amount of bound anthocyanins to blueberry pectins increases with increasing concentration of free anthocyanins. Considerably, different binding behavior is observed depending on the pectin composition and incubation time. At 6 h of incubation, WSF binds anthocyanins up to 166 µg/mg pectin whereas that of CSF binds anthocyanins to 810 µg/mg pectin. More anthocyanins interact with CSF than WSF and with longer time; the same pattern is also observed at 48 h of incubation. The amount of anthocyanins bound to WSF is up to 263 µg/mg pectin while that of CSF is up to 1033 µg/mg pectin. As incubation time increases from 6 h to 48 h, the adsorption behavior of anthocyanins interacting with WSF are more scattered and variable. Under these conditions, Langmuir isotherm analysis is weak. However, the adsorption between CSF and anthocyanins is relatively stable regardless of incubation time thus, Langmuir isotherm is fitted for the adsorption.

The calculations for the apparent isotherm parameters by Langmuir equation (Eq. (1)) are summarized in Table 4.1. As for the adsorption curves, all replications are shown. A good fitting of data is obtained from CSF ($R^2 > 0.94$) at both 6 h and 48 h of incubation whereas WSF

does not fit well to Langmuir isotherm ($R^2 > 0.62$). Because of the data deviation, no identifiable differences are observed on the binding site (q_{max}) and binding affinity (K_L) throughout the blueberry pectins and incubation time ($p > 0.05$). It should be mentioned that unknown causes of data deviation and unsatisfied assumptions which Langmuir equation requires, leads to the limited data interpretation on binding site and binding affinity between blueberry pectin and anthocyanins. Nonetheless, the data obtained this adsorption study still support the observation that CSF is more effective than WSF in blueberry pectin and anthocyanins interaction. The structure of blueberry pectin clearly has binding selectivity to anthocyanins. The linear-enriched CSF had a stable and greater interaction with anthocyanins, but the WSF-anthocyanins interaction was inconsistent.

Langmuir isotherm assumes monolayer adsorption with uniform binding site and no interaction among anthocyanins. As discussed before, the hypothetical binding sites of anthocyanins on the blueberry pectin ranged from 7500 to 8700. However, the binding sites of WSF and CSF obtained from Langmuir isotherm were much less. Considering most of anthocyanins bound on the galacturonic acid of CSF, it was estimated that every four or five galacturonic acid residues interact with one molecule of anthocyanin. The higher binding sites of anthocyanins obtained from hypothetical calculation are likely overestimated due to the pectin aggregation or steric hinderance between blueberry pectin and anthocyanins.

Electrostatic interaction of pectin and anthocyanin are proposed (Fernandes et al., 2014; Lin et al., 2016). At pH 4.0, anthocyanins are partially protonated (Jackman, Yada, Tung, & Speers, 1987) and pectins are negatively charged above the pKa value near 3.5 (Kim & Wicker, 2009; Sila, Van Buggenhout, Duvetter, Fraeye, De Roeck, Van Loey, et al., 2009). More negative charge of CSF and the greater amount of bound anthocyanins with CSF than WSF suggests that

ionic interaction occurs between protonated anthocyanin (flavylium cations) and free carboxylated pectin. The selection of CSF for further adsorption studies as influenced by environment factors yields further insight to pectin-anthocyanin interaction. Previous research reports that low DE pectin (30% DE) interacts with 83% of anthocyanin, double that of high DE pectin (64% DE), with 41% bound anthocyanins, at pH 4.3 and 12 days of contact time (Padayachee et al. (2012). Of several polysaccharides and polyphenols evaluated to compare binding, the greatest binding occurs between pectin and cyanidin-3-glucoside by ionic forces (Phan et al., 2017).

4.3.2. Adsorption studies influenced by exogenous factors (pH and ionic strength)

As mentioned before, several studies suggested that the electrostatic interaction are a major contributor to anionic pectin and cationic anthocyanin interaction (Lin et al., 2016; Padayachee et al., 2012; Phan et al., 2017). However, the charge of blueberry pectin and anthocyanin varies depending on the pH. Anionic pectin loses net charge below an apparent pKa of 3.5 (Voragen et al., 2009) whereas anthocyanin is completely deprotonated above pH 4.0 (Jackman et al., 1987). Also, from the empirical experience, blueberry pectin is partially precipitated between pH 2.6 to 3.0. If the electrostatic interaction plays an important role on blueberry pectin-anthocyanin association, the interaction is only valid within limited pH ranges. Figure 4.3 shows CSF-C18BBE adsorption behavior in citrate-phosphate buffer at different pH, ionic strength, and time. Most binding reaches a plateau after 6 h. The greatest amount of bound anthocyanins to CSF is observed at pH 3.0 and the least binding is observed at pH 4.0 regardless of citrate phosphate concentration. At pH 4.0, pectin is negatively charged, whereas only part of anthocyanin is protonated. Most anthocyanin exists in hemiketal or chalcone forms at pH 4.0. As pH changes from pH 4.0 to more acidic, the proportion of protonated anthocyanin (flavylium

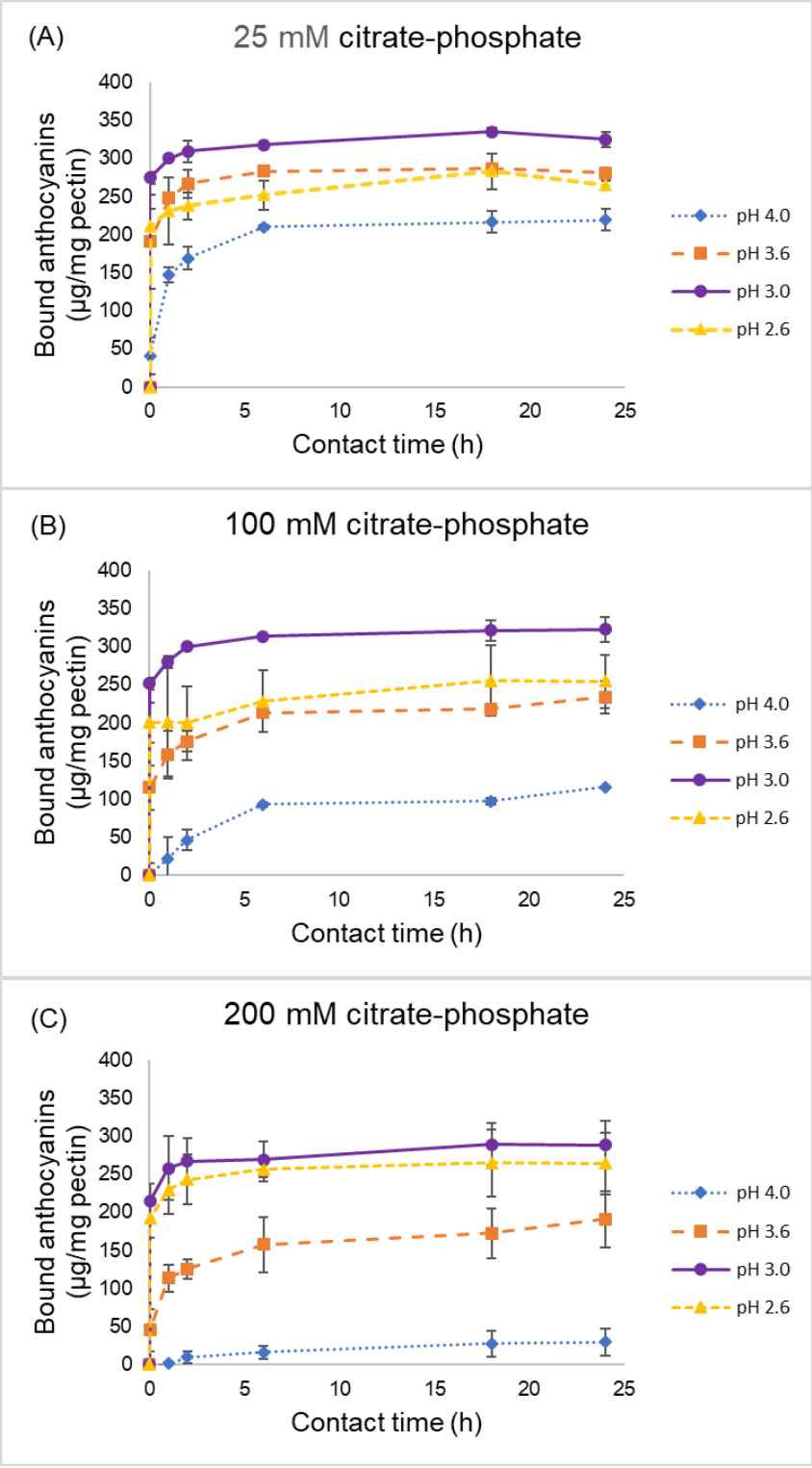


Figure 4.3. The adsorption of anthocyanins from blueberry extract passed through C18 column on blueberry pectin, CSF, in citrate-phosphate buffer at different pH, ionic strength, and time intervals up to 24 h. (A) 25 mM, (B) 100 mM, and (C) 200 mM citrate-phosphate.

cation) increases and that of hemiketal and chalcone decreases. Despite cationic anthocyanin is

more available at pH 2.6 for electrostatic interaction, the pectin likely has no charge. Thus, blueberry pectin and anthocyanins interact more at pH 3.0, presumably with localized negative charge on pectin.

In addition, the anionic charge of pectin varies by ionic strength of solution as well as pH (Schmidt, Schütz, & Schuchmann, 2017). Ionic strength also influences the positively charged anthocyanin. An adsorption study conducted by varying citrate-phosphate concentration from 25 mM to 200 mM between pH 2.6 and 4.0 (Figure 4.3). An apparent interaction change is observed between blueberry pectin and anthocyanins as the citrate-phosphate concentration increased. In 25 mM citrate-phosphate concentration, the amount of anthocyanins adsorption to CSF increases from pH 4.0 to 3.0, then decreases from pH 3.0 to 2.6. In 200 mM citrate-phosphate concentration, however, the amount of bound anthocyanins increases from pH 4.0 to pH 3.0 then stays constant as pH is decreased to 2.6. For more clear comparison, Figure 4.4 presents the extent of bound anthocyanins to CSF at 6 h of contact time. At pH 4.0, the amount

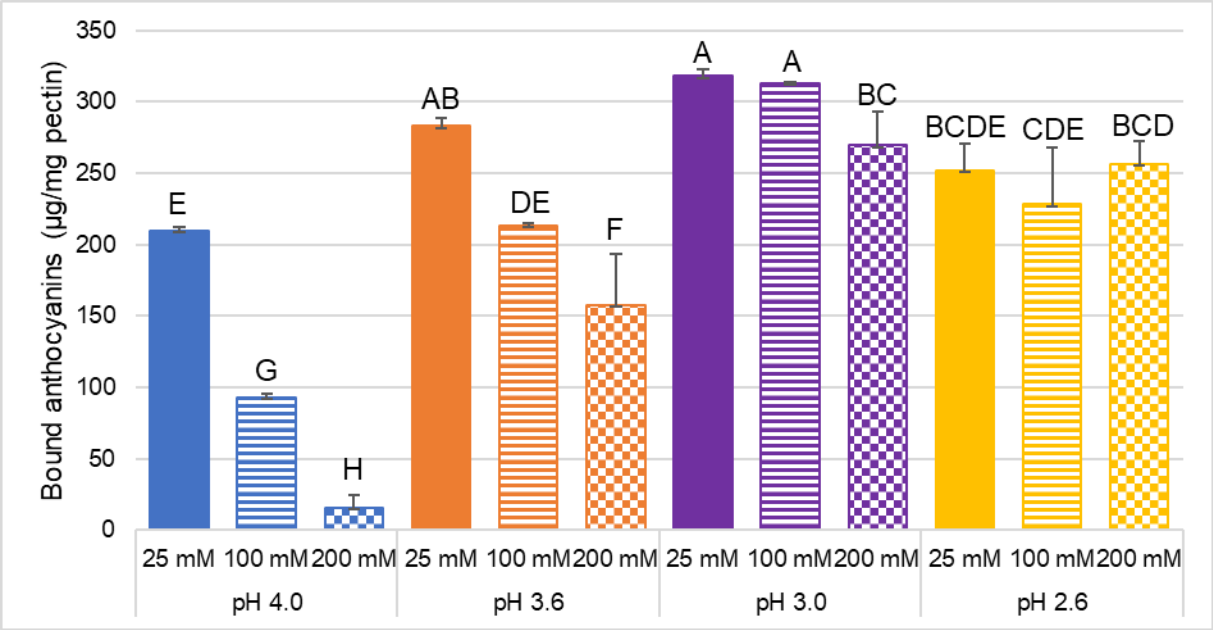


Figure 4.4. The extent of bound anthocyanins from blueberry extract passed through C18 column on blueberry pectin, chelator soluble fraction, in 25, 100, 200 mM citrate-phosphate buffers at contact time of 6 h. Letters denote significant difference if $p < 0.05$.

of bound anthocyanins to CSF is 210, 93, and 16 μg/mg pectin at citrate-phosphate concentration of 25, 100, and 200 mM, respectively. The extent of anthocyanins bound to CSF at pH 4.0 decrease substantially as citrate-phosphate concentration increases ($p < 0.05$). Possibly, the stronger ionic strength shields the pectin and anthocyanin charges. A significant finding of this result is that the ionic interaction between blueberry pectin and anthocyanins weakened as ionic strength increases, which agrees with the observation made for oppositely charged pectin and caseinate (Wang, Dumas, & Gharsallaoui, 2019). A similar, but smaller, decrease in bound anthocyanin with an increase in citrate phosphate concentration is observed at pH 3.6 and 3.0 ($p < 0.05$). Bound anthocyanins content decreases at pH 3.6 from 283, 214, to 158 μg/mg pectin and at pH 3.0 from 318, 313, to 269 μg/mg pectin as citrate phosphate concentration increased from 25, 100, to 200 mM, respectively. At pH 2.6, citrate phosphate concentration has no effect ($p>0.05$) on bound anthocyanin.

Interestingly, the magnitude of reduced bound anthocyanin with citrate phosphate concentration decreases as pH became more acidic. At pH 4.0, 56% (from 25 mM to 100 mM) and 92% (from 25 mM to 200 mM) less bound anthocyanin is observed as citrate phosphate concentration increases. Whilst, only 25% (from 25 mM to 100 mM) and 44% (from 25 mM to 200 mM) of reductions at pH 3.6, even none (from 25 mM to 100 mM) and 15% (from 25 mM to 200 mM) of decreases at pH 3.0 were quantified. The bound anthocyanins content at pH 2.6 was 252, 228, and 256 μg/mg at citrate phosphate concentrations of 25, 100, and 200 mM, respectively. No significant differences were detected at pH 2.6 even though the ionic strength increased ($p < 0.05$). These results indicate that electrostatic interaction as a major contributor between blueberry pectin and anthocyanin binding at pH 4.0 decreased as pH became more acidic.

Although the electrostatic interaction no longer predominates between blueberry pectin and anthocyanins, about 250 μg of anthocyanins still interacted with CSF at pH 2.6, which suggests the presence of other interactions. In 200 mM of citrate phosphate concentration, a significant increment of bound anthocyanins is observed as pH changes from 4.0, 3.6, to 3.0 ($p < 0.05$). As mentioned before, anthocyanin structure transforms from non-planar to planar at acidic condition and facilitation of anthocyanin self-association. Anthocyanin stacking is commonly observed in planar structure of flavylium cation which is driven by hydrophobic interaction to make stable π-π complexes (Fernandes et al., 2014). Moreover, the interaction between pectin and planar anthocyanin possibly forms intermolecular π-π stacking and hydrogen bonds from hydroxyl groups in both molecules (Fernandes et al., 2014). As the ratio of flavylium cation increases when pH changes from 4.0 to more acidic conditions, the possibility of forming π-π interaction from pectin-anthocyanin and anthocyanin-anthocyanin increases. Hence, the increase in bound anthocyanins in 200 mM of citrate phosphate concentration as pH changes from 4.0 to 3.0 might be attributed to the hydrophobic interaction. Interestingly, no significant difference is observed between pH 3.0 and 2.6 in 200 mM of citrate phosphate strength ($p < 0.05$), which conflicts with formation of intramolecular π-π complexes by anthocyanin. Considering 6 h of incubation time is insufficient for extensive anthocyanin stacking, π-π interaction is most likely to occur between pectin and anthocyanin.

Pectin aggregation is reported when the pH decreased from 3.5 to 2.0 with HCl (Sawayama, Kawabata, Nakahara, & Kamata, 1988). A similar aggregation was observed in okra pectin at pH 2.0 (Alba et al., 2017). When pH decreased, folded structures became more prominent which was mostly from folding chains of HG regions through hydrophobic interactions. It is possible that pectin molecules with shielded charge either by strong pH or by

strong ionic strength may have finite anthocyanin binding sites so that no significant increase or decrease of bound anthocyanins are observed at pH 3.0 (200 mM) and pH 2.6 (25-200 mM). The binding between no net charged pectin and anthocyanins might interact the same as the interaction between neutrally charged polysaccharide and anthocyanin. No significant effect of ionic strength occurs for cellulose and cyanidin-3-glucoside adsorption (Phan et al., 2016).

According to the previous study of anthocyanins stability in presence of blueberry pectins under in vitro digestion (Koh, this document) and Fernandes et al. (2014), high number of hydroxyl groups in anthocyanins form strong hydrogen bonds to pectin residues, thus facilitate pectin-anthocyanin binding. Considering the hydrogen bond is influenced by neither pectin charge nor anthocyanin charge, the pectin-anthocyanin interaction would have constant hydrogen bonds under conditions in this study. The bound anthocyanins amount at pH 4.0 and 200 mM citrate phosphate concentration, 16 μg/mg pectin, might result from hydrogen bonds because it occurred between shielded charge of pectin and non-planar anthocyanins.

4.4. Conclusion

Interaction between blueberry pectin and anthocyanins is influenced by pectin composition and environmental factors. Anthocyanins show different binding selectivity to blueberry pectin depending on the chemical composition of the pectin. Chelator soluble blueberry pectin has high and stable adsorption to anthocyanins which is attributed to the linear structure and low negative charge of the pectin. In contrast, water soluble blueberry pectin and anthocyanins interaction is variable and lower so that the Langmuir isotherm model cannot be applied. Further adsorption study reveals that blueberry pectin-anthocyanin interaction is highest at pH 3.0 and least at pH 4.0. Increasing ionic strength weakens the pectin-anthocyanin adsorption between pH values 3.0 and 4.0, however, no significant difference is observed at pH

2.6. As pH value change from 4.0 to more acidic, electrostatic interaction decreases while hydrophobic effect increases between blueberry pectin and anthocyanins. This blueberry pectin-anthocyanins study provides a valuable insight into polysaccharide-polyphenol interactions functionally and nutritionally.

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Table 4.1. Langmuir isotherm parameters for binding between blueberry pectins (water- and chelator soluble fractions) and anthocyanins at pH 4.0, 4 ºC, and incubation times of 6 and 48 h.

Blueberry pectin	Incubation time		q_{max}	K_L	R^2
WSF	6 h	Replication 1	250	0.0016	0.84
		Replication 2	35	0.0151	0.20
		Replication 3	223	0.0040	0.82
		Average ± SD	$169^a \pm 117$	$0.0069^a \pm 0.0017$	0.62 ± 0.36
	48 h	Replication 1	19790	0.0000	0.58
		Replication 2	245	0.0007	0.66
		Replication 3	229	0.0025	0.70
		Average ± SD	$6755^a \pm 11289$	$0.0011^a \pm 0.0013$	0.64 ± 0.06
CSF	6 h	Replication 1	1589	0.0026	0.93
		Replication 2	1034	0.0028	0.95
		Replication 3	1617	0.0035	0.93
		Average ± SD	$1413^a \pm 329$	$0.0029^a \pm 0.0005$	0.94 ± 0.01
	48 h	Replication 1	2314	0.0015	0.97
		Replication 2	957	0.0030	0.90
		Replication 3	2442	0.0019	0.97
		Average ± SD	$1904^a \pm 823$	$0.0021^a \pm 0.0008$	0.95 ± 0.04

WSF (water soluble fraction), CSF (chelator soluble fraction), and SD (standard deviation). Averages of q_{max} and K_L in the same blueberry pectin are not significantly different ($p > 0.05$) if they have same superscripts.

Chapter 5. Conclusion

To investigate the interaction between blueberry pectin and anthocyanin, blueberry pectins were extracted first by sequential buffers and characterized. Water soluble fraction (WSF) was high methoxyl pectin (degree of esterification: 53%), was rich in in neutral sugars (40 mol%), and had less negative charge (-21 mV). Chelator soluble fraction (CSF) was low methoxyl pectin (degree of esterification: 21%), was rich in uronic acids (92 mol%), and had lower negative charge (-26 mV).

The stability of blueberry pectin-anthocyanin complex was examined under in vitro digestion. CSF consistently bound anthocyanin more than WSF. Also, CSF prevented degradation of malvidin-3-glucoside and cyanidin-3-glucoside, and anthocyanins in blueberry powder extract. However, water soluble blueberry pectin only prevented cyanidin-3-glucoside degradation. This was attributed to greater binding of CSF, rich in negatively charged and linear regions, to anthocyanins than WSF, rich in branched domains.

Thus, pectin structure can be selectively extracted to optimize the anthocyanin interaction and functionality. Increased amount of protected anthocyanins with blueberry pectin may have a synergic effect on the colon to enhance and promote favorable gut microbiota fermentation. At pH 4.0, hydrogen bonding between blueberry pectin and anthocyanin likely contribute to binding in addition to ionic interaction.

The blueberry pectin-anthocyanins interaction was further determined by endogenous and exogenous factors. Anthocyanins showed a different binding selectivity to blueberry pectin. CSF had four time higher and stable adsorption ($R^2 > 0.94$) with anthocyanins, whereas WSF had less and more variable interaction with anthocyanins. The blueberry pectin-anthocyanin interaction was highest at pH 3.0 and lowest at pH 4.0. Increasing ionic strength weakened the

pectin-anthocyanin adsorption between pH values 3.0 and 4.0. However, no significant effect of ionic strength on binding was observed at pH 2.6. As pH value changed from 4.0 to lower pH values, electrostatic interactions were less dominant, while hydrophobic and hydrogen interactions contributed more to blueberry pectin and anthocyanin. The binding selectivity influenced by endogenous and exogenous factors will provide a valuable insight where anthocyanins is used such as extraction, loading or releasing efficiency, or targeted delivery.

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